Integrating Global Regulatory Input Into the *Salmonella* Pathogenicity Island 1 Type III Secretion System

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ABSTRACT *Salmonella enterica* serovar Typhimurium uses the *Salmonella* pathogenicity island 1 (SPI1) type III secretion system to induce inflammatory diarrhea and bacterial uptake into intestinal epithelial cells. The expression of *hilA*, encoding the transcriptional activator of the SPI1 structural genes, is directly controlled by three AraC-like regulators, HilD, HilC, and RtsA, each of which can activate the *hilD*, *hilC*, *rtsA*, and *hilA* genes, forming a complex feed-forward regulatory loop. A large number of factors and environmental signals have been implicated in SPI1 regulation. We have developed a series of genetic tests that allows us to determine where these factors feed into the SPI1 regulatory circuit. Using this approach, we have grouped 21 of the known SPI1 regulators and environmental signals into distinct classes on the basis of observed regulatory patterns, anchored by those few systems where the mechanism of regulation is best understood. Many of these factors are shown to work post-transcriptionally at the level of HilD, while others act at the *hilA* promoter or affect all SPI1 promoters. Analysis of the published transcriptomic data reveals apparent coregulation of the SPI1 and flagellar genes in various conditions. However, we show that in most cases, the factors that affect both systems control SPI1 independently of the flagellar protein FliZ, despite its role as an important SPI1 regulator and coordinator of the two systems. These results provide a comprehensive model for SPI1 regulation that serves as a framework for future molecular analyses of this complex regulatory network.

DURING infection, *Salmonella enterica* serovar Typhimurium induces inflammatory diarrhea and invades nonphagocytic epithelial cells using the type III secretion system (T3SS) encoded on *Salmonella* pathogenicity island 1 (SPI1) (Galan and Curtiss 1989; Watson *et al.* 1998; Tsolis *et al.* 1999; Wallis and Galyov 2000). The T3SS apparatus is a needle-like structure that injects bacterial effector proteins into the host cell cytosol. A subset of these proteins is required to promote actin cytoskeletal rearrangements leading to the engulfment of the bacterium (Zhou and Galan 2001). Structural genes for the assembly of the functional T3SS apparatus and several effector proteins are encoded in the SPI1 *prg/org*, *inv/spa*, and *sic/sip* operons, while other effectors are encoded elsewhere on the chromosome. The SPI1 locus also encodes several regulators of the system.

One goal of systems biology is a complete description of biological molecular networks, including the components, their interactions, and environmental inputs, with a hope of revealing emergent properties that are otherwise not apparent during studies of individual constituents. We strive for such an in-depth understanding of SPI1 regulation. On the basis of our genetic analyses and results from numerous other investigators, we have clarified the roles of a number of key regulators and effectively established the central regulatory framework of the SPI1 system (Ellermeier *et al.* 2005). SPI1-encoded HilA directly activates expression of the *prg/org* and the *inv/spa* operons, the latter encoding the AraC-like regulator InvF (Bajaj *et al.* 1995; Darwin and Miller 1999; Eichelberg and Galan 1999; Lostroh and Lee 2001). InvF, in complex with SicA, then activates expression of a number of genes encoding secreted effectors including the *sic/sip* operon, *sopE*, and *sigD* (Darwin and Miller 2000, 2001). Three AraC-like regulators, HilD, HilC, and RtsA,

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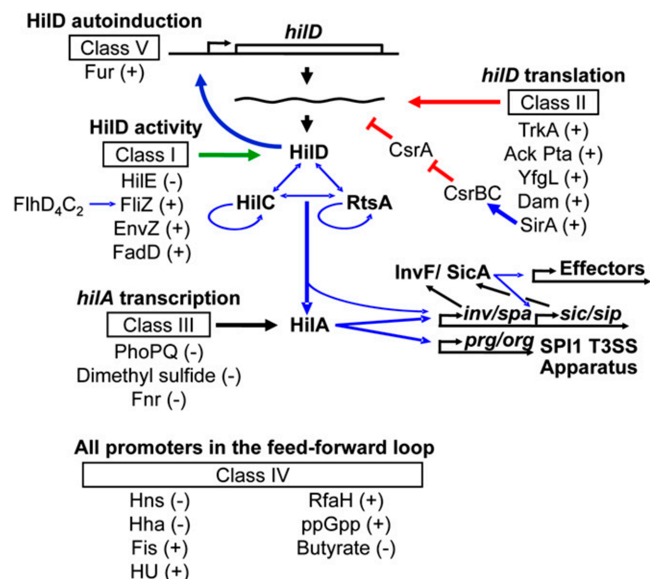

Figure 1 Working model for SPI1 regulation. Blue lines indicate transcriptional regulation. Red lines indicate post-transcriptional regulation. Green lines represent post-translational regulation. The effect of each regulator, positive (+) or negative (−) on *hilA* expression is indicated. For clarity, the genes encoding HilC, RtsA, and HilA are not shown.

control expression of *hilA*, and thereby induction of the SPI1 system. While HilD and HilC are encoded in the SPI1 locus (Mills *et al.* 1995), RtsA is encoded on a 15-kb island inserted in the *Salmonella* chromosome at $tRNA_{PheU}$ (Ellermeier and Slauch 2003). Each of these regulators is independently capable of inducing expression of the *hilD*, *hilC*, and *rtsA* genes, as well as *hilA*, forming a complex feed-forward regulatory loop to control SPI1 expression (Figure 1) (Ellermeier *et al.* 2005). Previous studies have shown that HilD, HilC, and RtsA bind to similar sites within the *hilD*, *hilC*, *rtsA*, and *hilA* promoter regions to counteract H-NS/Hha silencing (Olekhnovich and Kadner 2002, 2006, 2007; Schechter *et al.* 2003). HilD is the dominant regulator of the system, while HilC and RtsA work as amplifiers of the signal (Ellermeier *et al.* 2005; Saini *et al.* 2010a). The system works as a switch to turn on SPI1 (Song *et al.* 2004; Passerat *et al.* 2009; Bailly-Bechet *et al.* 2010). The switch is controlled primarily by affecting the threshold of HilD required for autoactivation (Saini *et al.* 2010a).

A substantial number of genes and environmental conditions have been implicated in regulation of SPI1 on the basis of genetic and transcriptomic data. Supporting information, Table S1 lists these factors along with the supporting references for each (File S1). These external regulatory inputs presumably ensure that SPI1 is only expressed at the appropriate time and place within the host. However, the complicated nature of the feed-forward loop has made it difficult to understand how these various systems feed into the regulatory circuit, and in most cases this question has never been addressed. We have recently studied the roles of three such regulatory factors in some detail, with the overall goal of understanding how input into the SPI1 system is integrated. HilE is a negative regulator of HilD activity that works by direct protein–protein interaction (Baxter *et al.* 2003; J. E. Chubiz and J. M. Slauch, unpublished data). FliZ is expressed as part of the flagellar regulon and presumably acts to coordinate flagellar expression with other systems in the cell, including SPI1 and the RpoS regulon (Saini *et al.* 2008; Pesavento *et al.* 2008; Chubiz *et al.* 2010; Saini *et al.* 2010c). FliZ also works at the level of HilD protein to positively control its activity (Chubiz *et al.* 2010). The action of Fur in SPI1 regulation is more complicated (Troxell *et al.* 2010; Teixido *et al.* 2011), but also requires HilD (Ellermeier and Slauch 2008).

Through our experiences characterizing the regulatory factors described above, we have developed a set of genetic assays that allow us to determine where any given factor feeds into the SPI1 regulatory circuit. Here, we utilize this system to characterize the role of ~20 regulatory factors and environmental conditions. Although the molecular details await further analysis, using our system and taking into account published data, we have grouped the known regulators of SPI1 into distinct classes. Our results provide increasing evidence for the feed-forward loop model of SPI1 regulation and give insights into the mechanism of action of individual regulators. Our data suggest that the majority of SPI1 regulators control HilD post-transcriptionally (classes I, II, and V), consistent with the idea that HilD acts as a major point of integration of regulatory signals. Class III and class IV regulators control the system at the level of *hilA* or affect promoters of all genes in the feed-forward loop, respectively.

Flagella are secreted and assembled via a distinct T3SS. The flagellar regulon contains >60 genes grouped into classes according to their transcriptional hierarchy (Frye *et al.* 2006). Class I genes encode the $FlhD_4C_2$ transcriptional activator, which activates class II genes encoding proteins required for the assembly of the flagellar hook-basal body, as well as the alternative σ-factor FliA and the anti–σ-factor FlgM. Upon completion of the hook-basal body, FlgM is secreted, freeing FliA to activate class III operons that encode flagellin subunits and motor proteins (Ohnishi *et al.* 1992; Hughes *et al.* 1993). FliZ, encoded in an operon with *fliA*, indirectly enhances class II flagellar gene expression by post-translationally affecting $FlhD_4C_2$ (Ikebe *et al.* 1999; Saini *et al.* 2008; Saini *et al.* 2010b; Wada *et al.* 2011). FliZ also positively regulates *hilA* expression (Eichelberg and Galan 2000; Lucas *et al.* 2000; Iyoda *et al.* 2001; Chubiz *et al.* 2010) thus serving as an important connection between the flagellar and SPI1 systems. Despite the coregulation of SPI1 and flagellar genes revealed by several microarray experiments, we show that a limited subset of the known SPI1 regulatory factors affect the system by controlling the flagellar regulon and hence function through FliZ. Overall, our results confirm that detailed genetic analyses are required to gain a full understanding of complex biological networks.

Materials and Methods

Bacterial strains and growth conditions

All *Salmonella* strains used in this study (Table S2) are isogenic derivatives of *Salmonella enterica* serovar Typhimurium 14028 (American Type Culture Collection) and were constructed using P22 HT105/1 *int*-201 (P22)-mediated transduction (Maloy *et al.* 1996). SOC medium was used for the recovery of transformants (Maloy *et al.* 1996). Luria-Bertani (LB) medium containing 10% tryptone, 5% yeast extract, and 5% NaCl was the standard medium used in experiments for growth of bacteria in aeration. Bacterial strains were grown at 37° except for the strains containing temperature-sensitive plasmids pCP20 and pKD46 (Cherepanov and Wackernagel 1995; Datsenko and Wanner 2000), which were grown at 30°. Antibiotics were used at the following concentrations: 100 μg/ml ampicillin; 20 μg/ml chloramphenicol; 50 μg/ml kanamycin; 25 μg/ml tetracycline (Tet); and 50 μg/ml apramycin. Enzymes were purchased from Invitrogen or New England Biolabs and used according to the manufacturer's recommendations. Primers were purchased from IDT.

Deletion of various genes and concomitant insertion of an antibiotic resistance cassette was carried out using λ-red–mediated recombination (Datsenko and Wanner 2000; Yu *et al.* 2000) as described in Ellermeier *et al.* (2002). The endpoints of each deletion/insertion are indicated in Table S2. The appropriate insertion of the antibiotic resistance marker was checked by P22 linkage to known markers and/or PCR analysis. In each case, the constructs resulting from this procedure were moved into a clean wild-type background (14028) by P22 transduction. In some cases, antibiotic resistance cassettes were removed using the temperature-sensitive plasmid pCP20 carrying the FLP recombinase (Cherepanov and Wackernagel 1995).

We have noted that the original *phoQ24* constitutive mutant strain (Miller and Mekalanos 1990; Gunn *et al.* 1996) has a secondary mutation(s) that affects *hilA* expression (data not shown). Using λ-red recombinase, we inserted a kanamycin resistance cassette in *ycfD*, just downstream of *phoQ*. This allowed us to transduce the *phoQ24* allele into various strains of interest. In each case, the resulting strains were carefully checked to make sure that *phoQ* was not duplicated. Such duplicated strains were common, suggesting that there is a selection against the *phoQ24* allele. The rebuilt *phoQ24* strains still showed decreased *hilA* expression, while having no apparent secondary background mutation(s).

Transcriptional and translational *lac* fusions to *hilD* were generated by FLP-mediated integration of fusion plasmids as described by Ellermeier *et al.* (2002). The integrated plasmid was tested by PCR to ensure that only a single copy was present. Standard recombinant DNA techniques were used for construction of plasmids (Sambrook *et al.* 1989). The *Salmonella rfaH* gene was amplified using primers carrying a site for either *Eco*RI or *Bam*HI restriction endonucleases (*rfaH* forward primer, ACGACTCGAGGCAACAGGACAG; *rfaH* reverse primer, ACGATCTAGAGTTGGCTCTTCG) and then cloned into vector pWKS30 (Wang and Kushner 1991).

β-Galactosidase assays

β-Galactosidase assays were performed using a microtiter plate assay as previously described (Slauch and Silhavy 1991) on strains grown under the indicated conditions. β-Galactosidase activity units are defined as [μmol of ortho-nitrophenol (ONP) formed $\min^{-1}$] $\times 10^6/(OD_{600} \times$ ml of cell suspension) and are reported as mean ± SD, where $n = 4$. Cultures grown in standard SPI1-inducing conditions were initially inoculated into LB (0.5% NaCl), grown for 8–12 hr, then subcultured 1/100, and grown statically for 18–22 hr in 3 ml LB with 1% NaCl (high salt LB, HSLB) in 13 × 100-mm tubes. LB or LB without NaCl (NSLB) were used where indicated.

Results

Rationale and approach

We have previously shown that HilE, FliZ, and Fur control *hilA* expression via HilD (Ellermeier and Slauch 2008; Lin *et al.* 2008; Chubiz *et al.* 2010). Indeed, the amassed data suggest that HilD is the primary point of integration of regulatory signals into SPI1, but only a fraction of the many systems that have been implicated in SPI1 regulation have been examined. We utilized a series of genetic tests that allowed us to group additional regulatory factors into distinct classes on the basis of how they feed into the SPI1 regulatory circuit. For each of the systems to be studied, we created a deletion in the regulatory gene of interest and transduced this deletion into a series of backgrounds. Alternatively, we tested the series of strains under a given environmental condition or in the presence of an added compound.

Initially, we tested whether a given regulatory factor affected *hilA* expression under the indicated growth conditions in both wild type and *hilD* null backgrounds using a *hilA–lacZ* transcriptional single-copy chromosomal fusion (Lin *et al.* 2008) (Figure 2A). The effect of a regulatory mutation on *hilA* expression in a *hilD* null background is difficult to accurately determine because *hilA* expression is greatly reduced in the absence of HilD; SPI1 is effectively shut off. Thus, to distinguish whether factors regulate *hilA* via HilD, or independently of HilD, we placed *rtsA* expression under the control of a tetracycline-inducible promoter (*tetRA–rtsA*). This allowed us to induce *rtsA*, with concomitant induction of *hilA* (and *hilC*) expression, independently of HilD (Figure 2B). Under these conditions we could clearly see whether a mutation of interest has an effect on *hilA* expression in the absence of HilD.

In addition, the effects of the regulatory mutations/conditions on *hilD* transcription and translation were studied using a *hilD–lac* transcriptional and *hilD'-'lac*

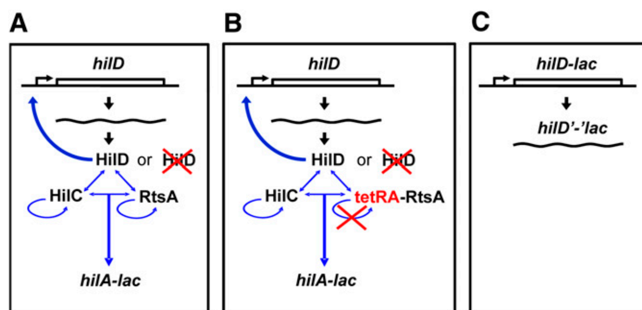

Figure 2 Rationale for interpretation of panels A, B, and C of the bar graphs in Figures 3–6 and Figure S1, Figure S2, Figure S3, Figure S4, Figure S5, Figure S6, Figure S7, Figure S8, Figure S9, Figure S11, Figure S12, Figure S13, Figure S14, Figure S15, Figure S16. (A) The transcriptional *hilA–lac* fusion serves as a major readout for SPI1 expression. First, test the effects of a regulatory factor on *hilA* expression in both *hilD*+ and *hilD*− backgrounds. (B) Second, test the effects of a regulatory factor on *hilA* expression in a background where the system can be induced via tetracycline control of *rtsA* with or without HilD. (C) Third, test the effects of a regulatory factor on *hilD–lac* transcriptional and *hilD'-'lac* translational locus fusions, which provide a readout of *hilD* transcription and translation, respectively, in the absence of HilD autoinduction. See detailed description of the experimental setup in *Rationale and approach* in *Results*.

translational fusion, respectively. Both *hilD* fusions have the same fusion joint at +67 from the start site of transcription, corresponding to 11 amino acids into the open reading frame. These fusions were constructed in the *hilD* locus in the *Salmonella* chromosome, and thus these strains are *hilD* nulls. This allows us to monitor transcription and translation of *hilD* without the complication of HilD autoinduction (Figure 2C).

The effects of various regulatory mutations and environmental conditions on SPI1, studied using the fusion constructs outlined above, are summarized in Table 1. On the basis of patterns of regulation observed, we have assigned these regulators to different classes. Described below are detailed results for each class of regulators using those examples where we generally understand the mechanism of action. Comparing these patterns to those obtained with unknown regulators provides a more comprehensive understanding of SPI1 regulation.

Class I: regulation via the post-translational control of HilD

Class I regulators function through HilD protein to affect *hilA* expression. HilE is a negative regulator of SPI1 that has been shown to directly interact with the HilD protein (Baxter *et al.* 2003; J. E. Chubiz and J. M. Slauch, unpublished data). Although the exact function of HilE remains to be determined, it clearly affects HilD protein activity (Ellermeier and Slauch 2008; Chubiz *et al.* 2010) and serves as an important example in the following experiments. In our series of assays, deletion of *hilE* resulted in a fourfold increase in β-galactosidase activity from the transcriptional *hilA–lac* fusion (Figure 3A), while it had no effect in a *hilD* null background. However, the absence of HilD resulted in a very low level of *hilA* expression. Therefore, it remained possible that HilE functions downstream of HilD, for example at the *hilA* promoter, but that this regulation is not evident in the *hilD* null background. To distinguish whether HilE regulates *hilA* via HilD, we induced *hilA* (and *hilC*) transcription in the presence or absence of HilD by the addition of increasing concentrations of tetracycline in a *tetRA–rtsA* background. If HilE controls *hilA* expression via HilD, we should no longer see the effect of a *hilE* deletion in the *hilD* null background. In the *hilD*+*tetRA–rtsA* strain, in the absence of tetracycline, loss of HilE caused a 7.5-fold increase in *hilA* transcription. Moreover, at higher Tet concentrations, HilE-dependent regulation was evident when HilD was present (Figure 3B), although regulation became less dramatic. This is consistent with the proposed interaction of the HilE and HilD proteins; there is not enough HilE available to bind the overproduced HilD. In the absence of HilD, at 1 μg/mL tetracycline, *hilA–lac* expression reached the level observed in the wild-type strain (compare with Figure 3A). But under these conditions, deletion of *hilE* had no effect on *tetRA–rtsA*-driven *hilA* expression in the absence of HilD. This result confirms that HilE works through HilD, which is consistent with previous data (Baxter *et al.* 2003; J. E. Chubiz and J. M. Slauch, unpublished data).

In theory, HilE could regulate *hilA* expression by controlling transcription or translation of *hilD*. If this were true, we would expect that loss of HilE would have an effect on the *hilD–lac* transcriptional and/or *hilD'-'lac* translational fusion. (Both are located at the *hilD* locus, and are *hilD* nulls.) However, the absence of HilE had no effect on the *hilD–lac* transcriptional and *hilD'-'lac* translational fusions, showing that the presence of the functional HilD protein is required for regulation (Figure 3C). These results again are consistent with the known mechanism of HilE acting at the level of HilD protein.

We have previously provided evidence that the flagellar protein FliZ positively regulates *hilA* expression via HilD protein and showed that FliZ, like HilE, had no effect on *hilA* expression in the absence of HilD (Chubiz *et al.* 2010). Results in Figure S1 show that the *hilD–lac* transcriptional and translational fusions were also not affected by the loss of FliZ, confirming that FliZ controls *hilA* expression via the post-translational control of HilD. In our previously published data, we also showed that both HilE and FliZ were able to regulate an ectopically expressed HilD protein (Chubiz *et al.* 2010). Thus, the results from this series of assays are consistent with these regulators controlling HilD protein activity.

This set of experiments was performed for all of the regulatory factors tested, and the resulting data are summarized in Table 1. Comparing the results for HilE and FliZ with those of other uncharacterized regulators shows that EnvZ and FadD also belong in class I (Figure S2 and Figure S3, respectively). It is possible that FliZ, EnvZ, and FadD affect *hilA* expression via HilE, but both published

Table 1 Integration of regulators and conditions that affect *hilA* expression into SPI1 regulatory circuit

Class	Regulator	Medium, growth conditions	Mode of regulation (positive or negative regulator)	Fold effect of null mutation or change in growth conditions	Regulation of *hilA* via HilD	Regulation of *hilD–lac* transcriptional fusion	Regulation of *hilD'-'lac* translational fusion
I	HilE	HSLB	−	4× ↑	Yes	No	No
	FliZ	HSLB	+	4× ↓			
	EnvZ	HSLB	+	3.5× ↓			
	FadD	HSLB	+	3× ↓			
II	SirA	HSLB	+	3× ↓	Yes	Yes/no	Yes
	Dam	HSLB	+	5× ↓			
	YfgL	HSLB	+	5× ↓			
	Ack Pta	HSLB, with MOPS pH 6.0	+	2× ↓			
	TrkA	HSLB	+	2.5× ↓			
III	PhoPQ (*phoQ24*)	HSLB	−	10× ↓	No	No	No
	Dimethyl sulfide	HSLB, 1.5% DMS	−	2.5× ↓			
	Fnr	HSLB	−	2× ↑			(↓)
IV	H-NS[a]	NSLB	−	↑	No	Yes	Yes
	Hha	HSLB	−	3.5× ↑			
	Fis	HSLB	+	15× ↓			
	HU	HSLB	+	4.7× ↓			
	RfaH	HSLB	+	4× ↓			
	Butyrate	HSLB, 10 mM butyrate	−	3× ↓			
	ppGpp (*relA spoT*)	HSLB	+	22× ↓			
V	Fur	HSLB	+	5× ↓	Yes	No	Yes

HSLB, high salt LB broth—standard SPI1 inducing conditions as described in *Materials and Methods*; NSLB, no salt LB broth.
[a] Based on published data (Schechter *et al.* 2003; Olekhnovich and Kadner 2006, 2007).

and unpublished data show that these regulators act independently of HilE; they regulate *hilA* expression in a *hilE* null background (Chubiz *et al.* 2010 and data not shown). Below we show that HilE, EnvZ, and FadD also act independently of FliZ. Note also that while data suggest that HilE regulates HilD activity via direct interaction, we can make no such conclusion about FliZ, EnvZ (a two-component sensor kinase), or FadD (encoding acyl-CoA synthetase). These factors could certainly act indirectly; we are concluding only that they ultimately affect SPI1 via control of HilD protein activity.

Class II: control of hilD mRNA stability/degradation or translation initiation

Class II regulators function through HilD to control *hilA* expression, but do so by controlling *hilD* mRNA stability or translation. The BarA/SirA two-component system is a known positive regulator of SPI1 (Johnston *et al.* 1996; Altier *et al.* 2000; Lawhon *et al.* 2002; Ellermeier *et al.* 2005; Ellermeier and Slauch 2007; Van *et al.* 2008; Martinez *et al.* 2011). SirA controls the transcription of two RNAs, CsrB and CsrC, which are antagonistic to the RNA-binding protein CsrA (Romeo 1998; Weilbacher *et al.* 2003; Fortune *et al.* 2006). We have previously shown that SirA-dependent regulation of *hilA* requires both HilD (Ellermeier *et al.* 2005) and CsrA (data not shown and Ellermeier and Slauch 2007), and Martinez *et al.* (2011) recently showed that CsrA directly binds the *hilD* mRNA near the ribosome binding site to block translation. In our system, deletion of *sirA* resulted in a 2.5-fold decrease in *hilA* transcription (Figure 4A). Figure 4B shows that while the *sirA* deletion decreased *hilA* expression in the presence of HilD, it had no effect on the *hilA* expression when *hilA* was being activated by the *tetRA–rtsA* construct at 1 μg/mL tetracycline in the absence of HilD. This result confirms that SirA controls *hilA* via HilD. However, in striking contrast to class I regulators, both the *hilD–lac* transcriptional and the *hilD'-'lac* translational fusions were regulated by SirA in the absence of HilD protein (Figure 4C), consistent with the RNA-binding protein CsrA acting at the level of the *hilD* mRNA to control stability or translatability. Thus, the pattern of expression observed in our system confirms that SirA functions through HilD, acting at the level of *hilD* mRNA.

Comparing the results for SirA with those of other regulators shows that Dam, YfgL, Ack Pta, and TrkA (Figure S4, Figure S5, Figure S6, and Figure S7, respectively) also belong to class II. Although regulation of the *hilD–lac* transcriptional fusion was evident in the case of SirA, Dam had only a small effect on expression of the *hilD* transcriptional fusion, but a significant effect on the translational fusion, in agreement with the recent study showing that Dam post-transcriptionally affects *hilD* mRNA stability (Lopez-Garrido and Casadesus 2010). Likewise, the loss of YfgL, Ack Pta, or TrkA primarily affected *hilD* translation. Accordingly, these regulators are all considered class II. We presume that the effect of these factors on *hilD* mRNA is

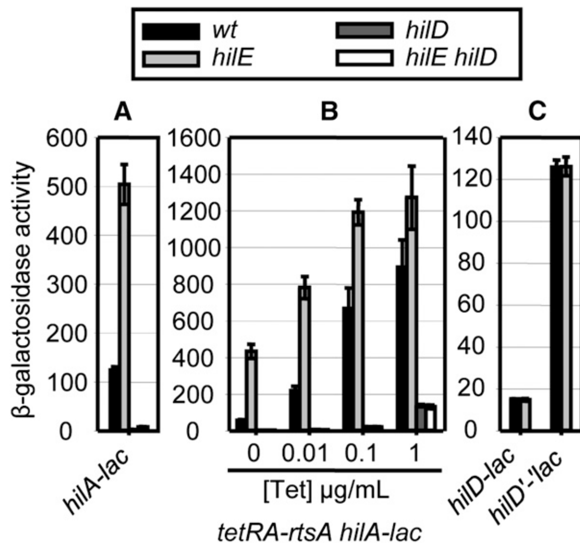

Figure 3 Class I, HilE regulates *hilA* expression via the post-translational control of HilD. (A) β-Galactosidase activity in strains containing a *hilA–lac* transcriptional fusion and the indicated mutations after growth under SPI1 inducing conditions. (B) β-Galactosidase activity of strains containing a *hilA–lac* transcriptional fusion and indicated mutations with *rtsA* under the control of a tetracycline-regulated promoter. Strains were grown under SPI1-inducing conditions with the indicated tetracycline concentrations. (C) β-Galactosidase activity in strains containing a *hilD–lac* transcriptional or a *hilD'-'lac* translational fusion and the indicated mutations after growth under 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 ± SD, where $n = 4$.

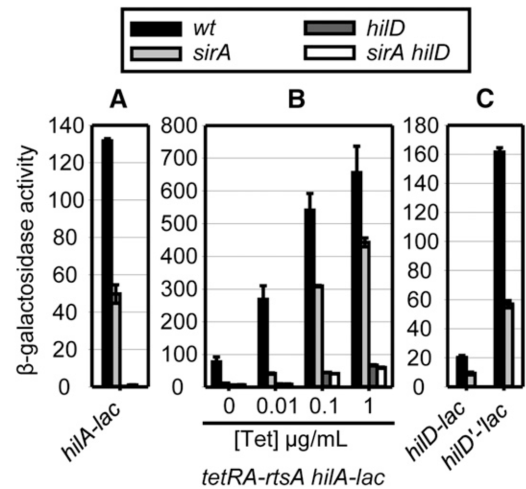

Figure 4 Class II, SirA activates *hilA* expression via the post-transcriptional control of *hilD*. See Figure 3 legend for details.

indirect, and, in the case of Dam, YfgL, Ack Pta, and TrkA, the mechanism of this regulation remains to be determined.

Class III: regulation at the level of the hilA promoter

The two-component regulatory system PhoPQ belongs to the class III regulators, which do not require the presence of the functional HilD protein and work at the level of the *hilA* promoter. Deletion of *phoP* does not have a significant effect on *hilA* expression in rich medium (HSLB). In these experiments, we are using the *phoQ24* constitutive mutation, which results in a hyperphosphorylation of the PhoP response regulator (Miller and Mekalanos 1990; Gunn *et al.* 1996). Introduction of the *phoQ24* constitutive allele caused a 10-fold reduction in *hilA* transcription (Figure 5A). In the *tetRA–rtsA* strain, the *phoQ24* mutation caused a decrease in *hilA* expression regardless of the presence or absence of HilD (Figure 5B). Thus, the *phoQ24* effect on *hilA* expression is independent of HilD. Results in Figure 5C showed that the *hilD–lac* transcriptional and the *hilD'-'lac* translational fusions were not regulated in *phoQ24* background, indicating that the PhoPQ system does not affect *hilD* transcription or translation. The simplest explanation for these results is that the PhoP response regulator acts directly or indirectly at the *hilA* promoter. However, it was possible that PhoPQ regulates *hilA* through HilC or RtsA. Therefore, we measured the effect of the *phoQ24* allele on *hilA* expression in *hilC* null or *rtsA* null backgrounds. Results in Figure S10A clearly showed that the PhoPQ effect on *hilA* expression was independent of either HilC or RtsA.

In addition to PhoPQ, the global regulator Fnr, as well as the effect of adding dimethyl sulfide (DMS) to the growth medium, belongs in Class III (Figure S8, Figure S9). Interestingly, despite the fact that Fnr is a negative regulator of *hilA* expression independent of HilD, the *hilD'-'lac* translational fusion showed a slight decrease in activity in the absence of Fnr. This phenomenon is likely attributed to the pleiotropic effects of the *fnr* deletion (Fink *et al.* 2007). Fnr controls a large number of genes in anaerobic conditions, so the loss of Fnr could potentially affect SPI1 through more than one mechanism. However, repression of SPI1 independently of HilD is a predominant mechanism of Fnr action on the basis of our data. We have also shown that both DMS and Fnr act independently of HilC or RtsA (Figure S10B and Figure S10C, respectively). These results confirm that DMS and Fnr act at the level of *hilA*. To determine whether DMS and Fnr acted via PhoPQ, their effect on *hilA* expression was tested in a *phoPQ* null background. The resulting data in Figure S10D showed that addition of DMS, as well as the loss of Fnr, still affected *hilA* expression in the absence of PhoPQ. Therefore, DMS and Fnr control *hilA* independently of the PhoPQ system. All of these systems could be acting indirectly and the exact mechanisms of action will require further analyses.

Class IV: regulation of all SPI1 regulatory promoters

Class IV is composed of a number of regulators and environmental conditions that apparently affect the promoters of all of the regulatory genes in SPI1. For example, the small nucleoid proteins H-NS and Hha have been shown to directly bind to the promoter regions and silence transcription of SPI1 genes including *hilD*, *hilC*, *rtsA*, and *hilA* (Olekhnovich and Kadner 2006, 2007; Banos *et al.* 2009). Deletion of *hha* caused a 3.5-fold increase in *hilA* transcription as expected (Figure 6A). This increase was also

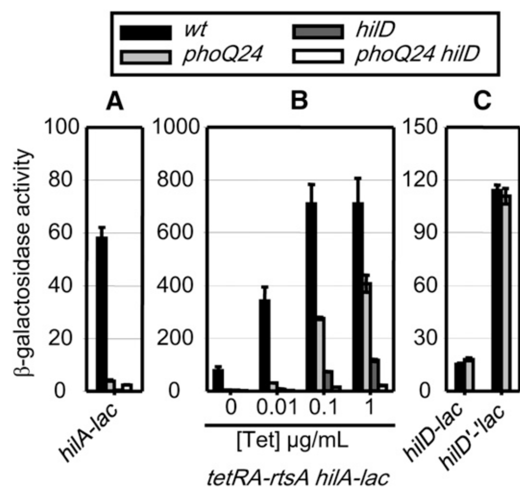

Figure 5 Class III, PhoPQ (PhoQ24) represses *hilA* expression independently of HilD. See Figure 3 legend for details.

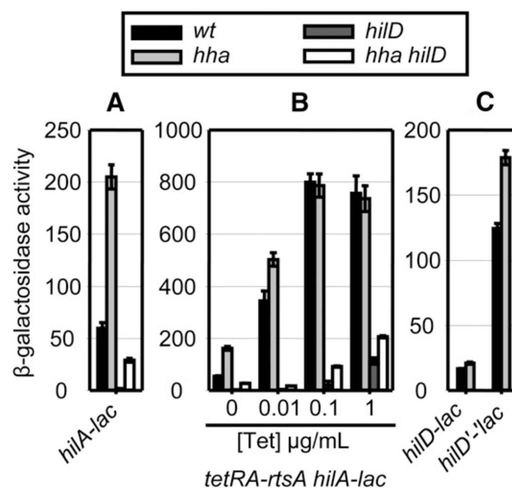

Figure 6 Class IV, Hha represses SPI1 expression independently of HilD (affects all regulators in the feed-forward loop). See Figure 3 legend for details.

evident in the *hilD* null strain, although the absolute level of expression was decreased. With *hilA* expression driven by increasing concentrations of tetracycline in the *tetRA–rtsA* strain, loss of Hha still resulted in *hilA* induction (Figure 6B). Deleting *hilD* in this strain did not abolish the Hha regulation showing that Hha acts independently of HilD. Not surprisingly, the *hilD–lac* transcriptional and translational fusions were also regulated by Hha (Figure 6C). Thus, we conclude that Hha does not require HilD protein, but rather regulates both *hilA* and *hilD* (as well as *hilC* and *rtsA*) transcription.

In Figure S11 and Figure S12, we showed that nucleoid proteins Fis and HU also independently control both *hilA* and *hilD* transcription. In addition to nucleoid proteins, the RfaH and RelA SpoT deletion mutations, as well as the presence of butyrate, resulted in similar expression profiles (Figure S13, Figure S14, Figure S15). Changes in temperature likely affect *hilA* independently of HilD, with H-NS implicated in this regulation (Ono *et al.* 2005).Whether some of these additional factors and conditions function through H-NS/Hha remains to be determined.

Class V: regulation by Fur

The global transcriptional regulator Fur has been placed in a separate class V (Figure S16) due to the fact that Fur requires both the HilD protein and *hilD* promoter to regulate *hilA* (Ellermeier and Slauch 2008). More recently, it was proposed that Fur activates *hilA* by repressing H-NS (Troxell *et al.* 2010). Together, these data suggest that Fur might activate SPI1 by reducing the H-NS–mediated silencing of the *hilD* promoter region and thereby lowering the threshold of HilD required to activate the *hilD* promoter, as we originally proposed (Ellermeier and Slauch 2008). However, Teixido *et al.* (2011) propose that Fur acts directly at the *hilD* promoter. Further analysis is required to determine the exact mechanism of Fur activation of SPI1, but in our hands, Fur behaves differently than other factors characterized here.

Other regulators not characterized here: We are not presenting results for some of the regulators listed in Table S1 (with references in File S1) due to the fact that the phenotypes conferred by these mutations/compounds (*fimZY*, *mlc*, *lrp*, *pmrM*, *cpxA*, *ygdP/apaH*, *ramA*, mitomycin, hydrogen peroxide) were not robust enough to draw conclusions under the conditions used in this study. Also, we have not characterized the effects of a number of regulators and conditions. We have included these so that Table S1 serves as a comprehensive list of factors previously implicated in SPI1 regulation.

Regulation via FliZ: Some of the factors that regulate SPI1 do so by affecting expression of the flagellar regulon in *Salmonella*. We have previously shown that DsbA and RcsCDB regulate *hilA* through HilD via FliZ (Lin *et al.* 2008; Chubiz *et al.* 2010). In addition, proteases ClpXP and Lon were suggested to affect *hilA* expression by indirectly or directly affecting FliZ levels (Kage *et al.* 2008; Chubiz *et al.* 2010). Published transcriptomic datasets reveal that SPI1 and flagellar genes are coregulated in response to a number of regulatory signals, including CsrA, YfgL, Fnr, Fis, and RfaH, as well as several environmental conditions (Table S3). These factors presumably affect expression or function of the flagellar master regulator $FlhD_4C_2$. Given that the flagellar protein FliZ, controlled by $FlhD_4C_2$, is a significant regulator of HilD activity (Chubiz *et al.* 2010), we originally hypothesized that these factors would regulate SPI1 through FliZ. We directly tested this hypothesis by performing tests of epistasis.

From published data we know that FliZ controls *hilA* expression independently of $FlhD_4C_2$ and other flagellar proteins, since ectopic expression of FliZ activates *hilA* in an *flhDC* null background (Chubiz *et al.* 2010). We characterized the effect of loss of a given SPI1 regulator on *hilA* expression in otherwise wild-type, *fliZ* null, and *hilD* null

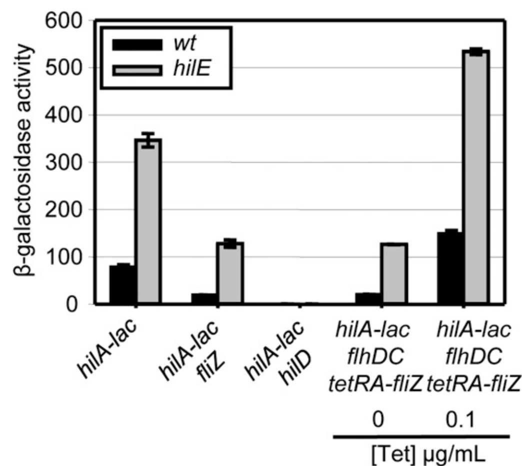

Figure 7 HilE and FliZ affect *hilA* expression independently of each other. β-Galactosidase activity in strains containing a *hilA–lac* transcriptional fusion and the indicated mutations after growth under SPI1 inducing conditions. β-Galactosidase activity units are defined as (μmol of ONP formed min^{-1}) × 10^6/(OD_{600} × ml of cell suspension) and are reported as mean ± SD, where $n = 4$.

backgrounds, as well as in a strain in which FliZ is expressed under the control of the *tetRA* promoter, and thus independently of $FlhD_4C_2$. If the regulator of interest controls *hilA* by affecting expression of FliZ, loss of this regulator would no longer affect *hilA* expression in the absence of FliZ or when FliZ was ectopically expressed.

First, we tested the class I factors. Since our data show that each functions at the level of HilD protein, it is possible that they do so via FliZ. As a control, we tested HilE, which we have previously shown acts independently of FliZ (Chubiz *et al.* 2010). As expected, deletion of *hilE* induced *hilA* transcription ~4.5 fold in both wild-type and *fliZ* null backgrounds (Figure 7). Moreover, loss of HilE still had an effect on *hilA* expression when *fliZ* production was controlled by tetracycline. These results confirmed that HilE functions independently of FliZ to control *hilA* expression, consistent with the previously published data (Chubiz *et al.* 2010). We also tested the other two class I regulators, EnvZ and FadD, and showed that both regulate *hilA* independently of FliZ (Figure S17A, Figure S17B).

The transcriptional regulator TdcA has been suggested to regulate SPI1 via FliZ (Kim *et al.* 2009). The authors reported that a deletion of *tdcA*, which resulted in less than a twofold decrease of *fliZ* transcription, also decreased expression of *hilA*. We saw a similar decrease of *fliZ* expression when the *tdcA* mutation was introduced (data not shown). However, loss of TdcA caused the same less-than-twofold decrease in *hilA* transcription in both wild-type and in the *fliZ* null background, as well as when *fliZ* production was controlled by tetracycline (Figure S17C). These results suggest that, while TdcA regulates *fliZ* transcription, its effect on *hilA* expression is independent of FliZ.

On the basis of the classification of SPI1 regulators above, we presumed that the class II–IV regulators cannot function through FliZ, given that these factors do not control SPI1 at the level of HilD protein activity. Using the set of experiments described above, we have confirmed that the class II–IV factors act independently of FliZ to control *hilA* expression, as expected (data not shown). Thus, a number of regulatory signals in Table S3, shown to affect expression of both flagellar and SPI1 genes, appear to control these two systems independently.

Discussion

Expression of the SPI1 T3SS is controlled by HilD, HilC, and RtsA, acting in a complex feed-forward loop to activate the *hilD*, *hilC*, and *rtsA* genes, as well as *hilA*, which encodes the transcriptional activator of the T3SS structural genes (Figure 1). HilD is the predominant regulator of the system, while HilC and RtsA act as amplifiers of activating signals (Ellermeier *et al.* 2005; Saini *et al.* 2010a). For years, numerous regulatory systems and conditions have been added to the growing list of factors that affect SPI1 expression. In this study, we determined where a number of these factors feed into the regulatory circuit. In agreement with the feed-forward loop model, we show that most of the known SPI1 regulators function via HilD. On the basis of previously published (Baxter *et al.* 2003; Ellermeier *et al.* 2005; Ellermeier and Slauch 2008; Lin *et al.* 2008) and unpublished data, we hypothesized that the majority of regulators would function post-translationally through HilD. However, our study shows that the regulation of SPI1 is more complex, with control exerted at multiple levels (Figure 1).

Class I regulators work post-translationally at HilD, controlling some aspect of HilD protein activity and/or stability. One of these, HilE, is a negative regulator of SPI1 that directly binds HilD protein (Baxter *et al.* 2003; J. E. Chubiz and J. M. Slauch, unpublished results). We recently reported that the positive regulator FliZ acts independently of HilE to control HilD protein activity (Chubiz *et al.* 2010). Although the exact mechanism of action of EnvZ and FadD has not been elucidated, they apparently affect factors independent of HilE and FliZ that work at the level of HilD protein and control some aspect of its function.

Class II regulators include those that affect *hilD* mRNA translation and/or stability. SirA activates expression of the CsrB and CsrC RNAs, which antagonize the action of CsrA (Romeo 1998; Weilbacher *et al.* 2003; Fortune *et al.* 2006). CsrA protein binding to *hilD* mRNA prevents translation of the *hilD* message (Martinez *et al.* 2011). Thus, SirA activates *hilD* expression post-transcriptionally. Data from our system are in agreement with this mechanism. In a recent study, the authors concluded that Dam affects *hilD* mRNA stability (Lopez-Garrido and Casadesus 2010), consistent with our results. Loss of YfgL, Ack Pta, or TrkA conferred similar patterns of expression in our fusion strains, suggesting that these regulators control *hilD* post-transcriptionally. The effects of Dam, YfgL, Ack Pta, and TrkA are most certainly indirect, and the details of this regulation remain to be elucidated.

SPI1 expression is activated when HilD reaches the threshold required to autoactivate the *hilD* promoter. HilE acts as a check to keep the system from inadvertently turning on (Saini *et al.* 2010a). We envision that the remaining class I, II, and V regulators, which act positively, are the primary systems responsible for precise induction of the system in conditions favorable for invasion. They act by increasing the level of HilD protein to overcome HilE, and controlling HilD activity, or in the case of Fur, lowering the threshold required at the promoter, such that HilD activates its own promoter as well as induces expression of HilC and RtsA, which then act to amplify and accelerate SPI1 expression (Saini *et al.* 2010a). Thus, the external signals that allow *Salmonella* to determine its location in the small intestine are integrated at HilD and only when the proper combination of signals is received is the system licensed for induction. This regulatory input gets amplified by the feed-forward regulatory loop to induce *hilA*, resulting in a full activation and timely production of the SPI1 T3SS.

After invasion has been accomplished, or when conditions are not favorable, the SPI1 system needs to be shut off. Factors in class III (and perhaps some in class IV) act at the level of *hilA* or affect all SPI1 promoters, respectively, providing a potentially fast turn-off mechanism that bypasses the feed-forward loop. PhoPQ, a two-component regulatory system known to negatively affect SPI1, and classified as class III, acts at the *hilA* promoter. A putative PhoP binding site in the *hilA* promoter region was predicted computationally by Zwir *et al.* (2005). However, direct repression by PhoP awaits experimental confirmation. The PhoPQ system is activated as *Salmonella* adapts to the intracellular environment of the macrophage (Groisman 2001) and SPI1 is no longer needed. This negative control by the PhoPQ system could allow for the fast turn off of SPI1 expression directly at the level of *hilA* during the systemic stage of infection.

The presence of DMSO reductases in intestinal bacteria, and the fact that dimethyl sulfide (the product of DMSO reduction) is found in the large intestine of mammals, suggest that this compound could serve as an environmental cue for *Salmonella* (Suarez *et al.* 1997, 1998; Antunes *et al.* 2010), although a direct role for dimethyl sulfide during *Salmonella* infection has not been demonstrated. Antunes *et al.* (2010) reported that dimethyl sulfide decreased expression of *hilA* and downstream SPI1 genes, but the mechanism of regulation was not characterized. Our data suggest that dimethyl sulfide inhibits SPI1 expression at the level of *hilA* independently of PhoPQ. Fnr, a global regulator of anaerobic metabolism, acts as a cytoplasmic oxygen sensor and regulates expression of target genes in response to oxygen availability. Previously, Fnr was suggested to activate SPI1 genes in anaerobic conditions (Fink *et al.* 2007). Subsequent studies (Van *et al.* 2008) and our results have shown that Fnr is a negative regulator of SPI1 gene expression. Fnr also represses SPI1 at the level of *hilA* independently of the PhoPQ system. Both of these systems could provide a mechanism to shut off the SPI1 system in the large intestine when the bacteria are beyond the point of optimal invasion or are being shed into the environment.

Nucleoid proteins H-NS and Hha have been implicated in silencing of horizontally acquired DNA (Lucchini *et al.* 2006; Navarre *et al.* 2006); activating signals must counteract this repression to turn on the respective genes. H-NS and Hha, members of class IV, repress transcription by binding to the promoter regions of all SPI1 genes (Schechter *et al.* 2003; Olekhnovich and Kadner 2006, 2007). Likewise, we show that nucleoid proteins Fis and HU fall into the same class with H-NS and Hha, acting independently of HilD by presumably affecting all promoters in the system. We do not envision that the overall levels of H-NS/Hha are changing significantly during normal colonization and invasion of the intestine. Rather, HilD, HilC, and RtsA are overcoming the effects of these proteins at the individual promoters and it is the regulation of HilD levels and action that is the key. Only a few other regulators and environmental conditions have been shown to belong to class IV, including RfaH, temperature, butyrate, and ppGpp. The effect of these regulatory mutations/conditions on SPI1 is likely indirect and further studies are warranted to determine whether they function through H-NS/Hha or Fis/HU.

Coregulation of the SPI1 and flagellar genes has been reported in a number of conditions (see Table S3), suggesting a regulatory overlap in the two systems. There is certainly a strong tie between the two; FliZ is a significant regulator of HilD activity and has been shown to play a role in *Salmonella* virulence only during oral infection, the observed virulence phenotype being largely dependent on SPI1 (Chubiz *et al.* 2010). A subset of SPI1 regulators enters the circuit via FliZ, including $FlhD_4C_2$, required for the activation of FliZ, and RcsCDB, which represses *flhDC* expression (Lin *et al.* 2008; Chubiz *et al.* 2010). We also recently published a study showing that Lon protease affects SPI1 expression primarily via FliZ (Chubiz *et al.* 2010). TdcA has also been suggested to regulate SPI1 via FliZ (Kim *et al.* 2009). However, our results suggest that while TdcA regulates *fliZ* transcription, its effect on *hilA* expression is independent of FliZ.

We have tested whether any of the other SPI1 regulators work through FliZ. On the basis of our classification of SPI1 regulators, we would expect that only the rest of class I factors can possibly function via FliZ. However, we showed that HilE, EnvZ, and FadD regulate SPI1 independently of FliZ. The class II–V regulators also work independently of FliZ, as expected. These results suggest that only a limited fraction of the overall regulatory input into SPI1 is FliZ dependent, despite the facts that FliZ is a significant regulator of HilD and many regulators affect both SPI1 and flagellar gene expression. The reason for coordination of expression of the flagellar genes and the SPI1 genes during infection in the host is not completely understood (Saini *et al.* 2010c). Induction of the flagellar regulon might help *Salmonella* to colonize the intestine of the host (Stecher *et al.* 2008). Additionally, flagellin-related inflammation is

beneficial for *Salmonella* during intestinal infection (Stecher *et al.* 2007).

Much work remains to understand the detailed mechanisms by which the various regulatory factors control this critical virulence machine, as well as the relative importance of each during infection. However, we are beginning to comprehend this biological network in some detail. Not surprisingly, the circuit is complex with regulation occurring at multiple levels. High throughput transcriptomic data reveal only the outlines of this regulation. Genetic analyses have been required to uncover the details. This more complete understanding of the regulatory inputs into the SPI1 T3SS provides an important foundation for future analysis.

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Integrating Global Regulatory Input Into the *Salmonella* Pathogenicity Island 1 Type III Secretion System

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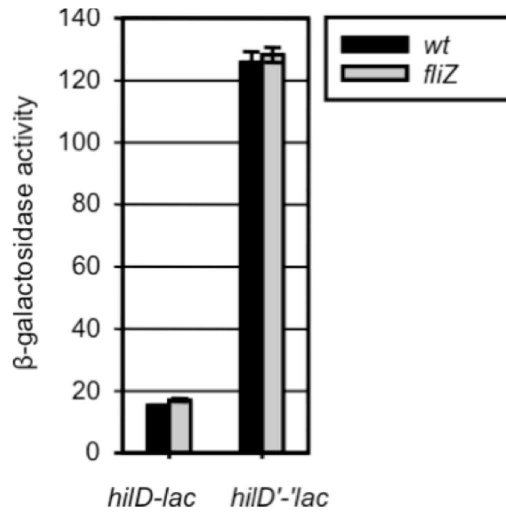

Figure S1 Class I, FliZ has no effect on HilD transcription or translation. For the equivalent of panels A and B (for example in Fig. S2) see FIGURE 3 in our previous publication (Chubiz et al., 2010). The panel above (equivalent to panel C in FIGURE S2) shows the β-galactosidase activity in strains containing a *hilD-lac* transcriptional or a *hilD'-'lac* translational fusion and the indicated mutations after growth under SPI1 inducing conditions. β-galactosidase activity units are defined as (μmol of ONP formed min^{-1}) x 10^3/(OD600 x ml of cell suspension) and are reported as mean ± standard deviation where n=4.

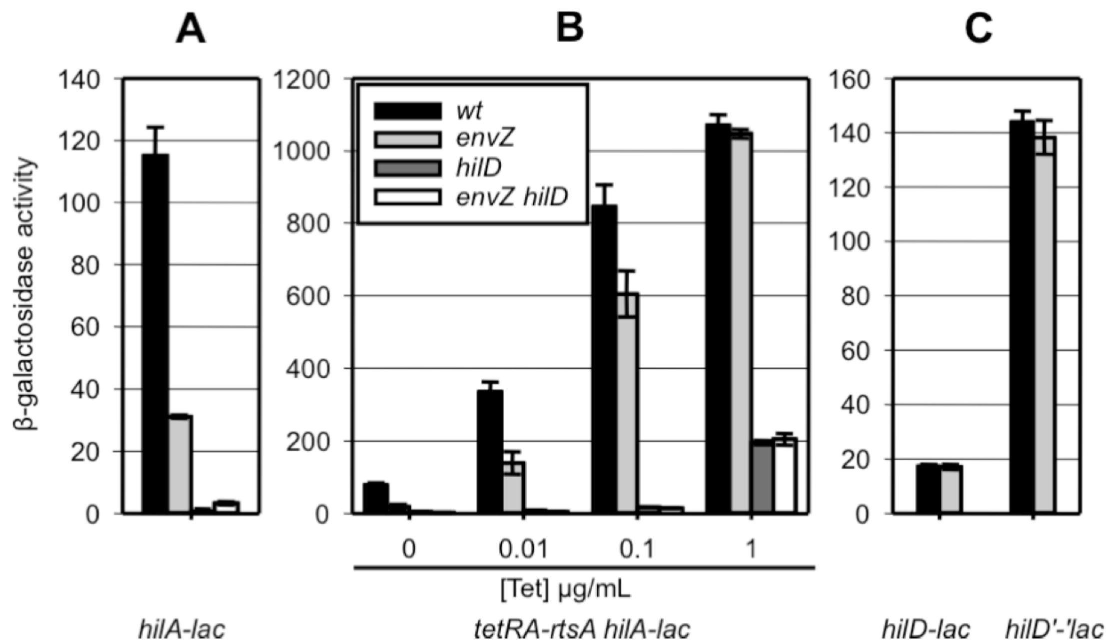

Figure S2 Class I, EnvZ activates *hilA* expression via post-translational control of HilD. (A) β-galactosidase activity in strains containing a *hilA-lac* transcriptional fusion and the indicated mutations after growth under SPI1 inducing conditions. (B) β-galactosidase activity of strains containing a *hilA-lac* transcriptional fusion and indicated mutations with *rtsA* under the control of a tetracycline regulated promoter. Strains were grown under SPI1-inducing conditions with the indicated tetracycline concentrations. (C) β-galactosidase activity in strains containing a *hilD-lac* transcriptional or a *hilD'-'lac* translational fusion and the indicated mutations after growth under SPI1 inducing conditions. β-galactosidase activity units are defined as (μmol of ONP formed min^{-1}) x 10^3/(OD600 x ml of cell suspension) and are reported as mean ± standard deviation where n=4.

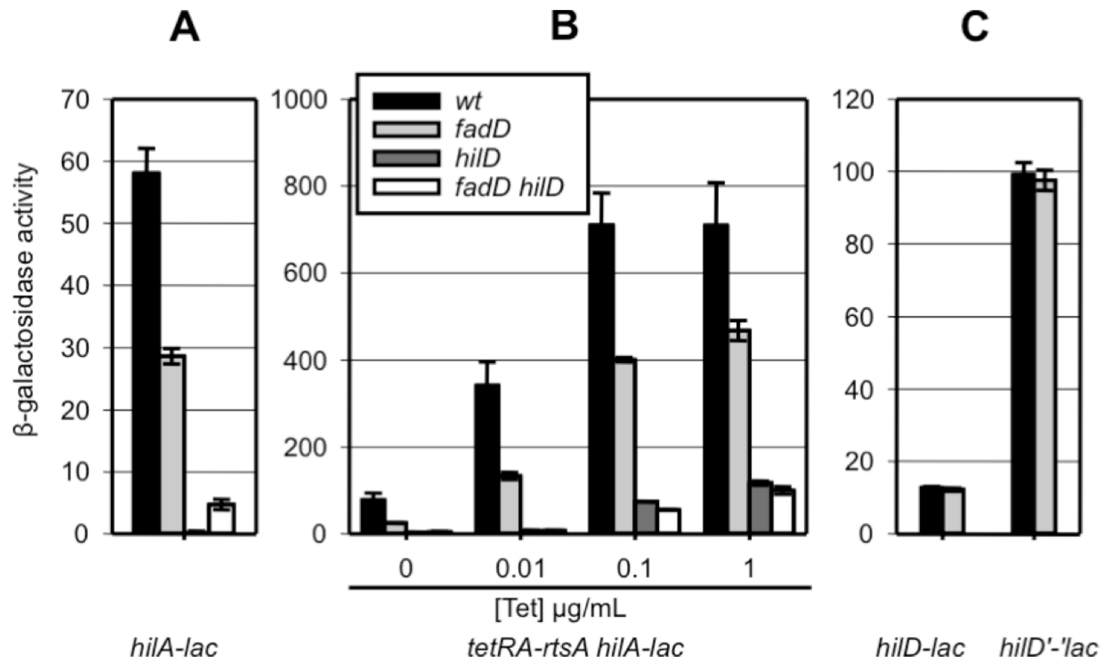

Figure S3 Class I, FadD activates *hilA* expression via the post-translational control of HilD. See FIGURE S2 legend for details.

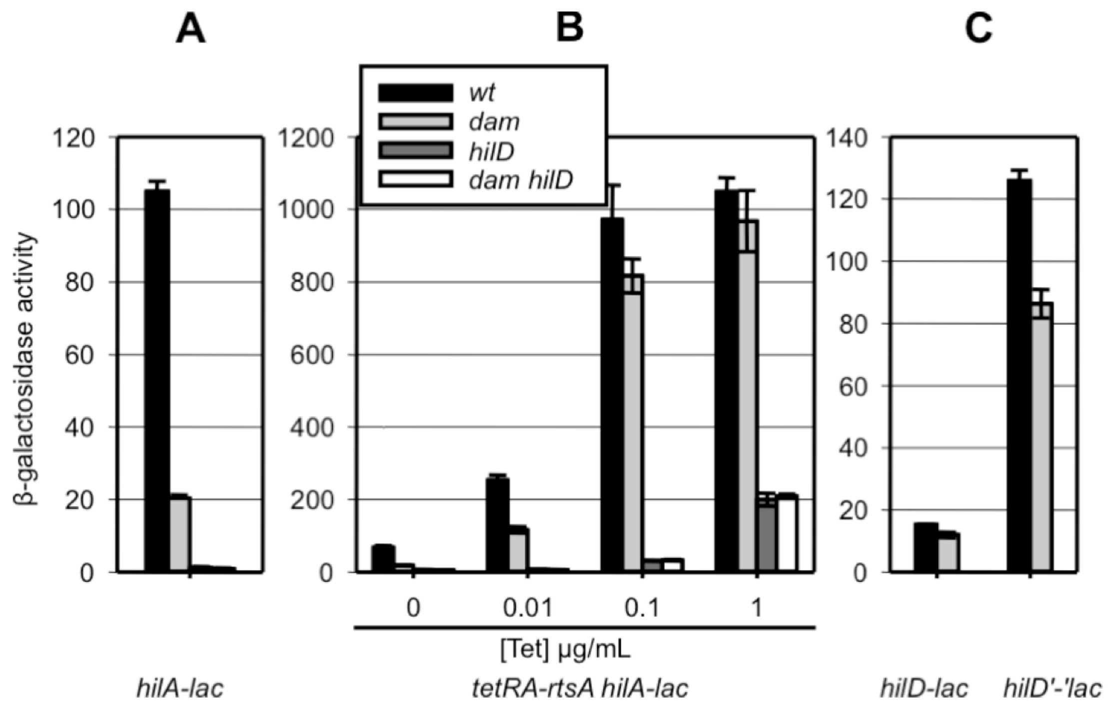

Figure S4 Class II, Dam activates *hilA* expression via the post-transcriptional control of *hilD*. See FIGURE S2 legend for details.

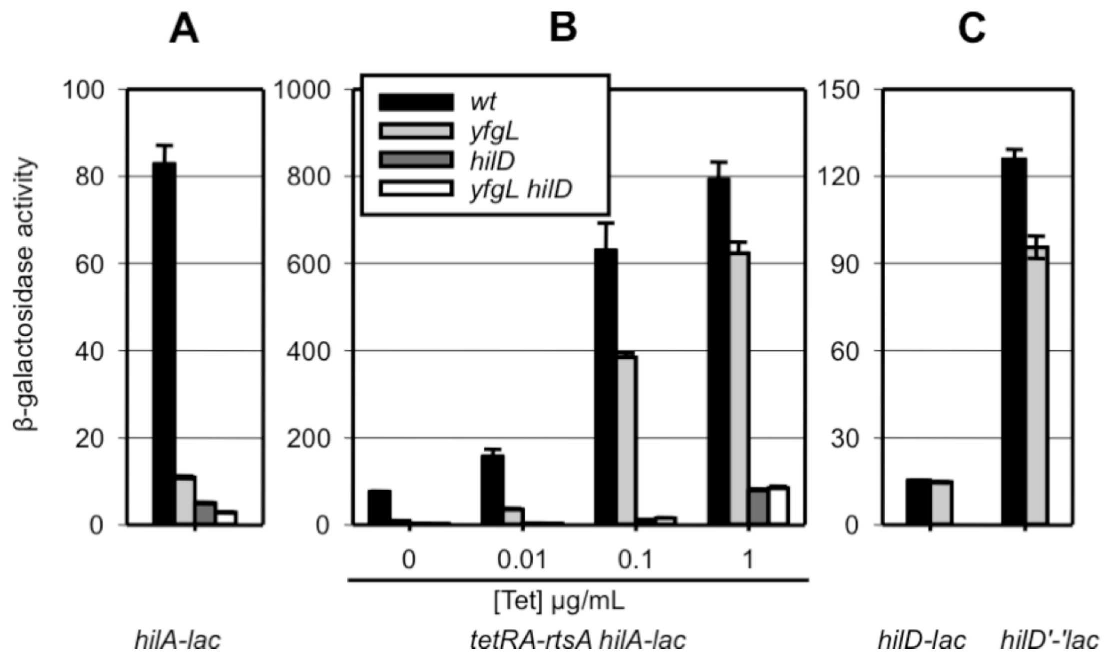

Figure S5 Class II, YfgL activates *hilA* expression via the post-transcriptional control of *hilD*. See FIGURE S2 legend for details.

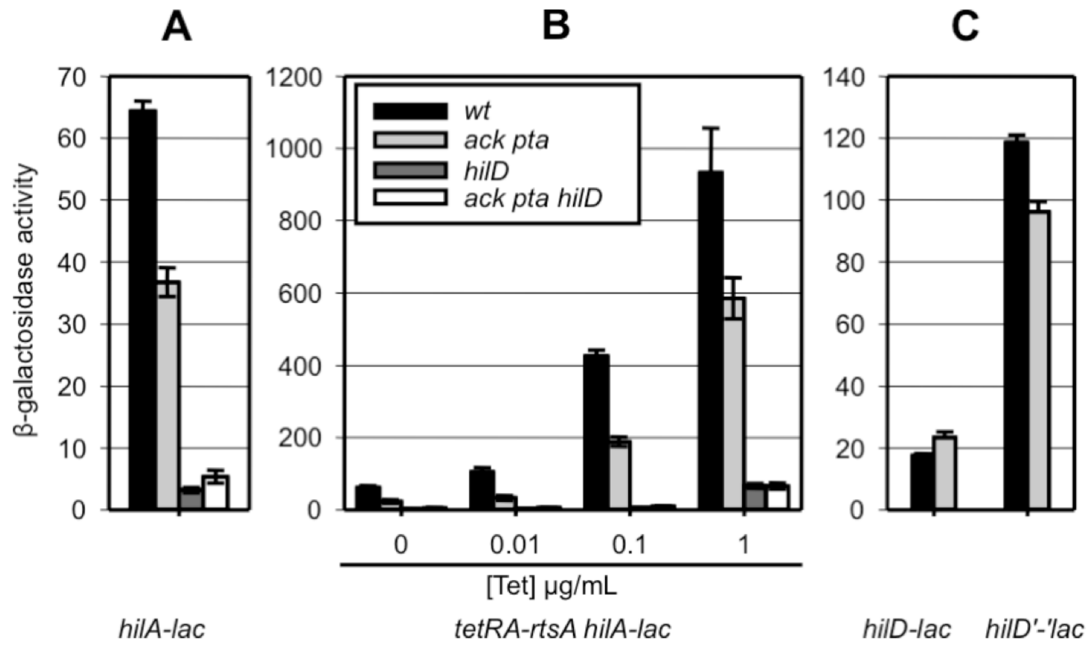

Figure S6 Class II, Ack Pta activates *hilA* expression via the post-transcriptional control of *hilD*. See FIGURE S2 legend for details.

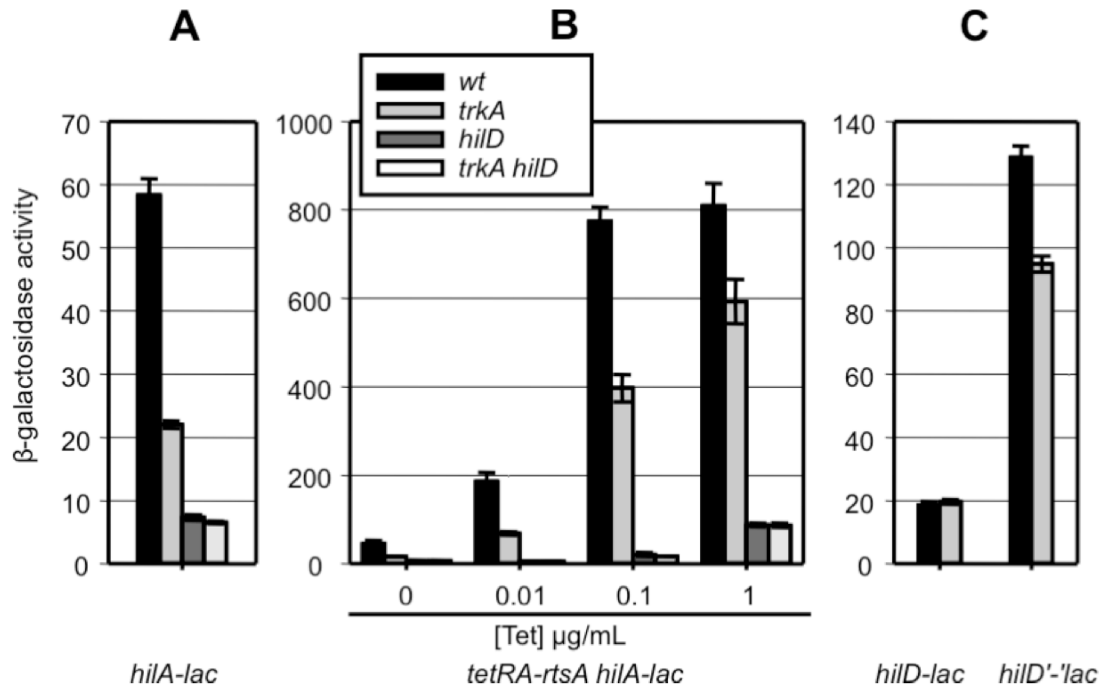

Figure S7 Class II, TrkA activates *hilA* expression via the post-transcriptional control of *hilD*. See FIGURE S2 legend for details.

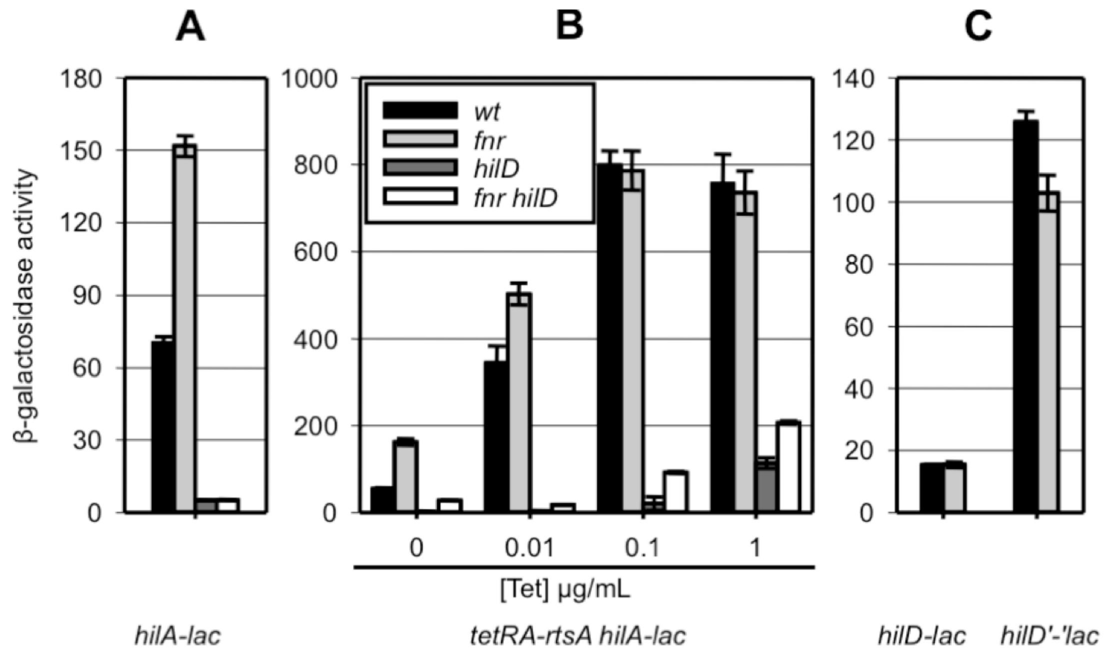

Figure S8 Class III, Fnr represses *hilA* expression independently of HilD. See FIGURE S2 legend for details.

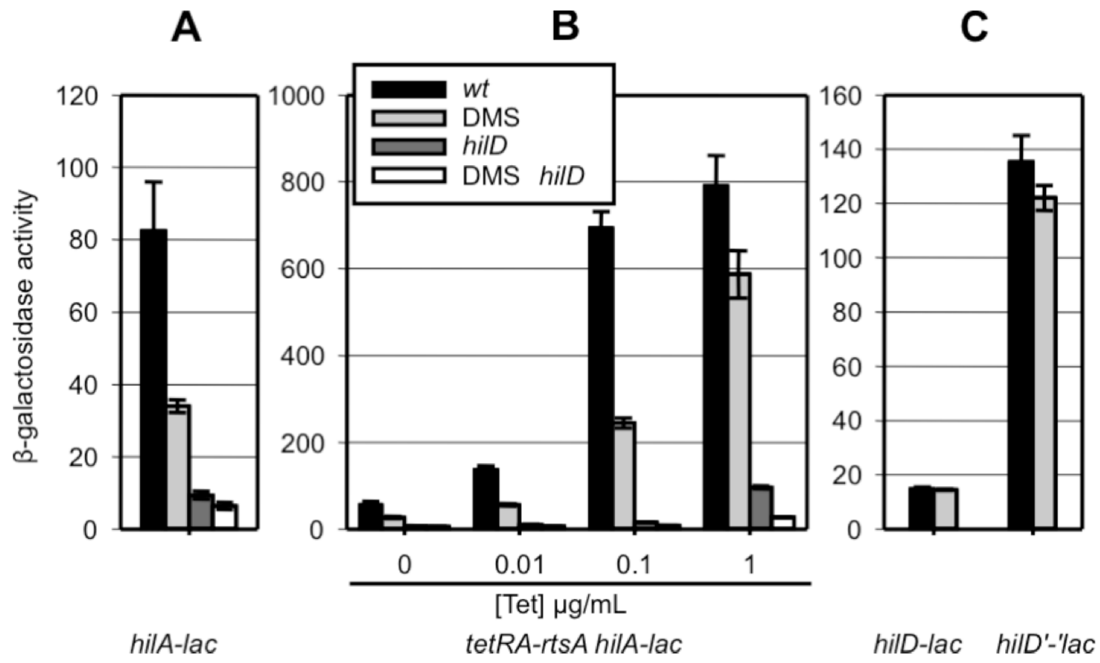

Figure S9 Class III, Dimethyl sulfide (DMS) represses *hilA* expression independently of HilD. See FIGURE S2 legend for details.

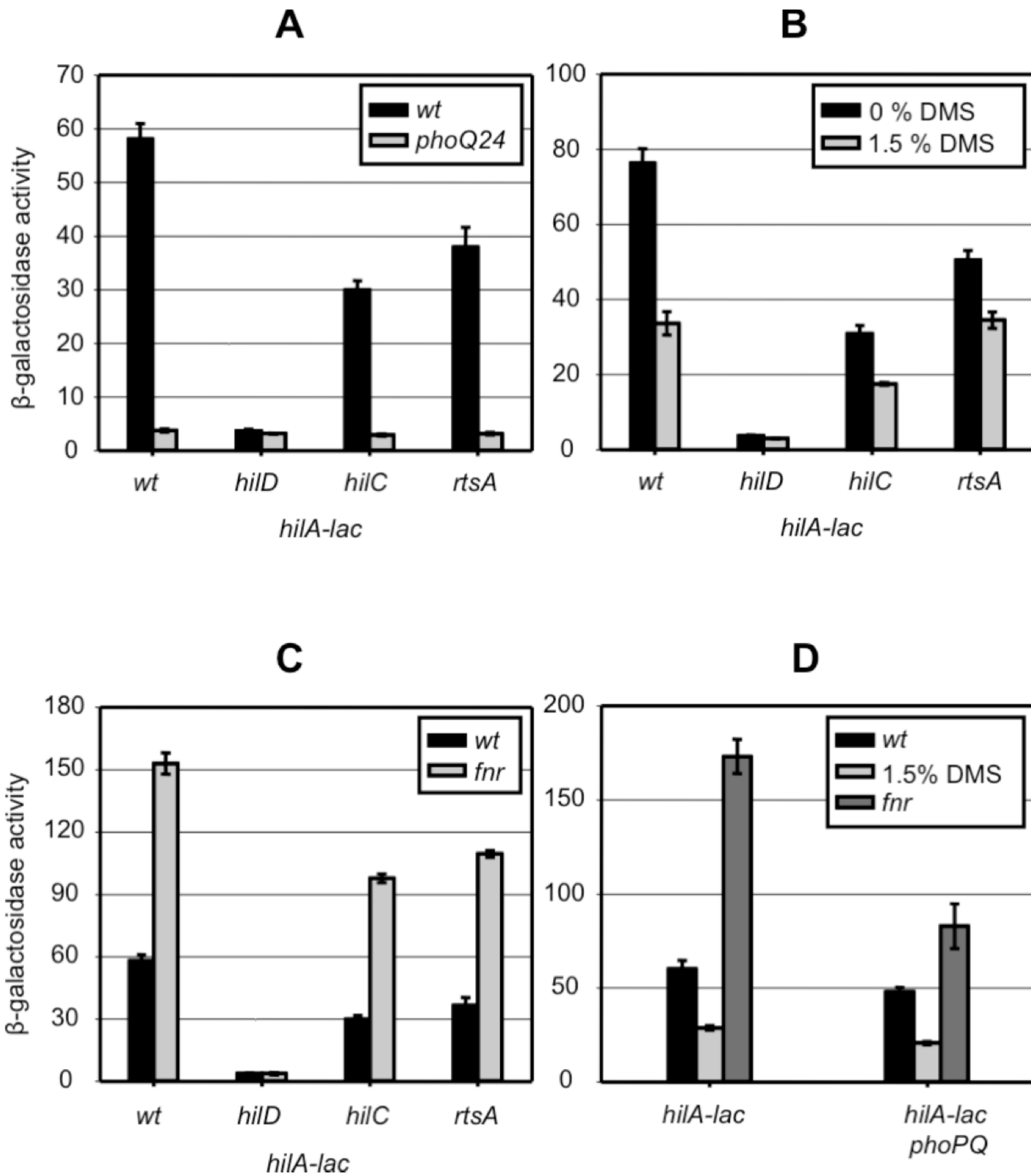

Figure S10 A) PhoPQ , (B) dimethyl sulfide (DMS), and (C) Fnr repress *hilA* expression independently of HilC and RtsA. (D) Dimethyl sulfide (DMS) and Fnr repress *hilA* expression independently of PhoPQ. β-galactosidase activity in strains containing a *hilA-lac* transcriptional fusion and the indicated mutations, or in the presence or absence of the 1.5% dimethyl sulfide, after growth under SPI1 inducing conditions. β-galactosidase activity units are defined as (μmol of ONP formed min^{-1}) x 10^3/(OD600 x ml of cell suspension) and are reported as mean ± standard deviation where n=4.

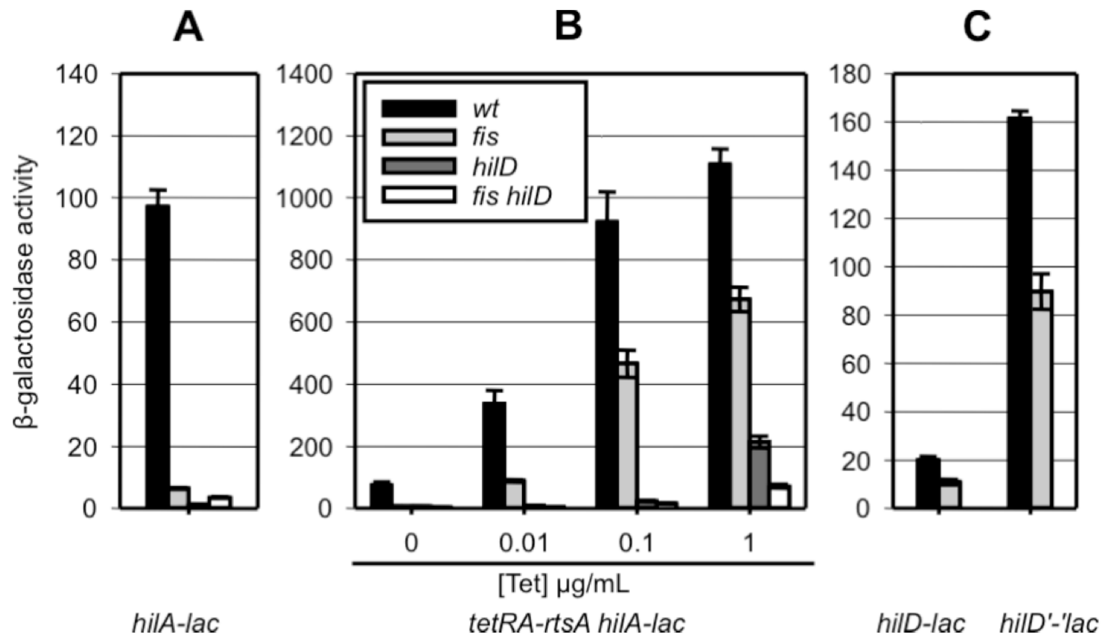

Figure S11 Class IV, Fis activates SPI 1 expression independently of HilD (affect all promoters in the feed-forward loop). See FIGURE S2 legend for details.

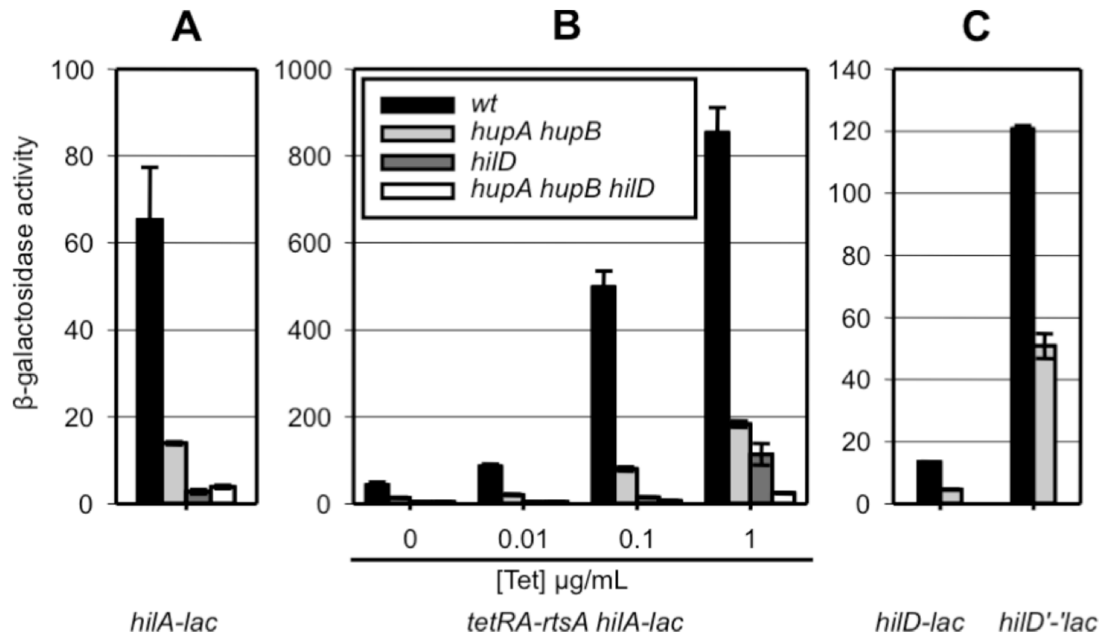

Figure S12 Class IV, HU (encoded by *hupA hupB*) activates SPI 1 expression independently of HilD (affect all promoters in the feed-forward loop). See FIGURE S2 legend for details.

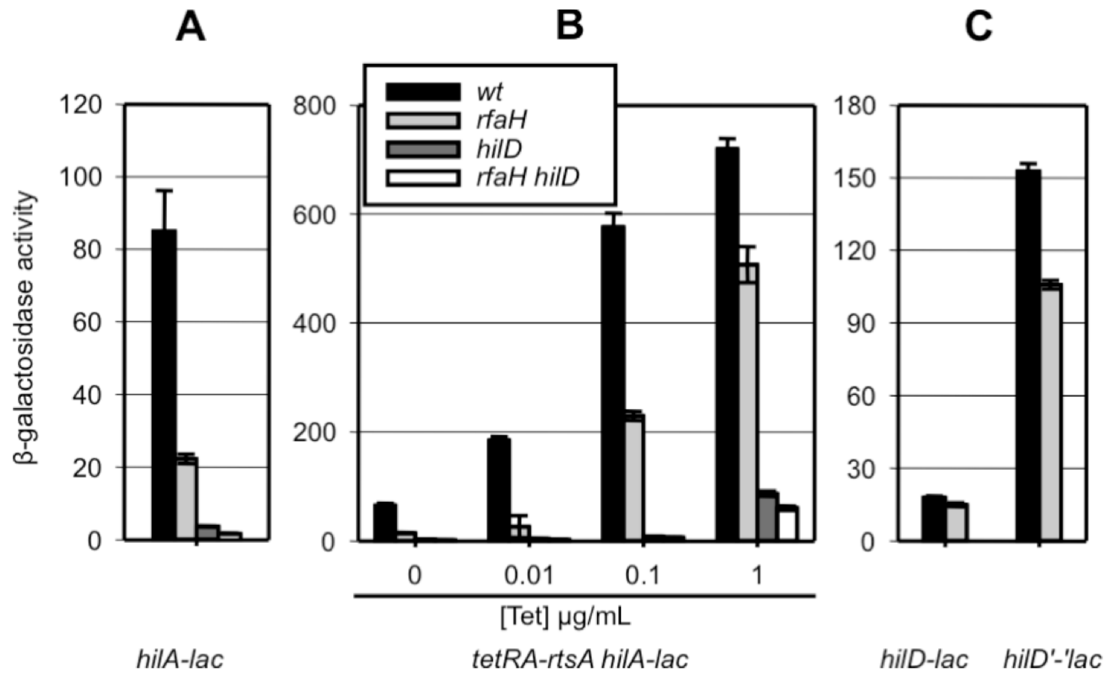

Figure S13 Class IV, RfaH activates SPI 1 expression independently of HilD (affect all promoters in the feed-forward loop). See FIGURE S2 legend for details.

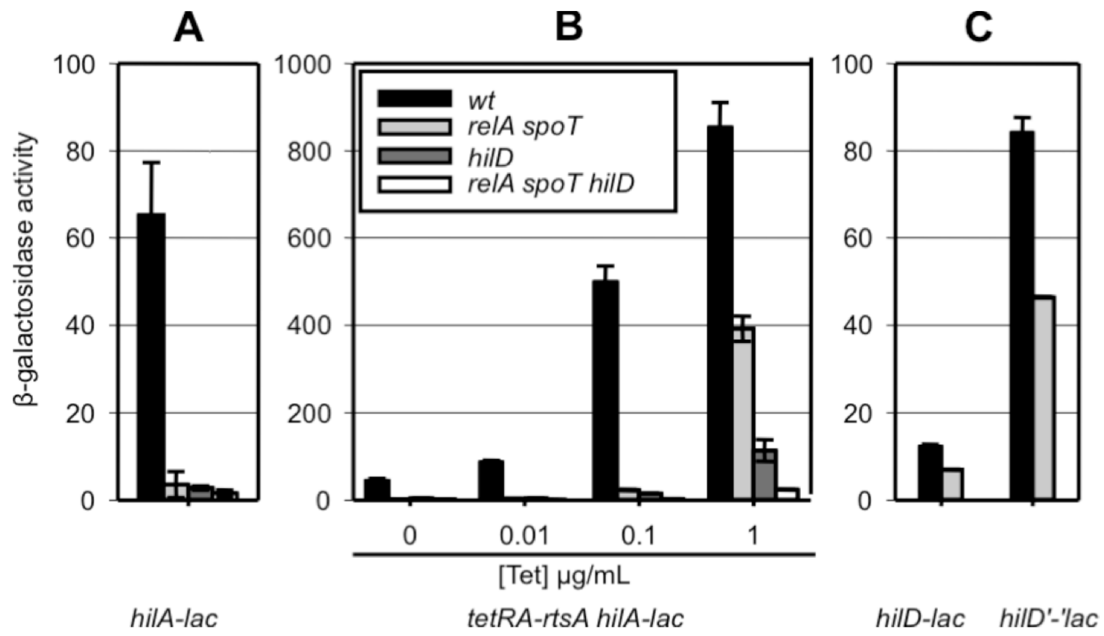

Figure S14 Class IV, ppGpp (produced by RelA and SpoT) activates SPI 1 expression independently of HilD (affect all promoters in the feed-forward loop). See FIGURE S2 legend for details.

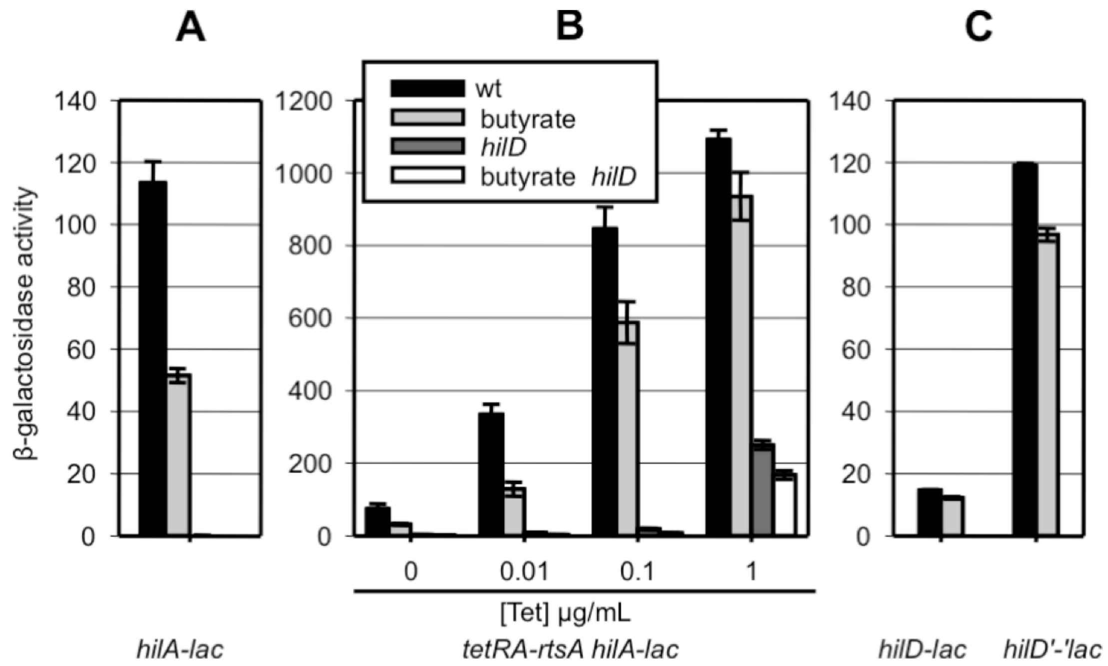

Figure S15 Class IV, Butyrate represses SPI1 expression independently of HilD (affect all promoters in the feed-forward loop). See FIGURE S2 legend for details.

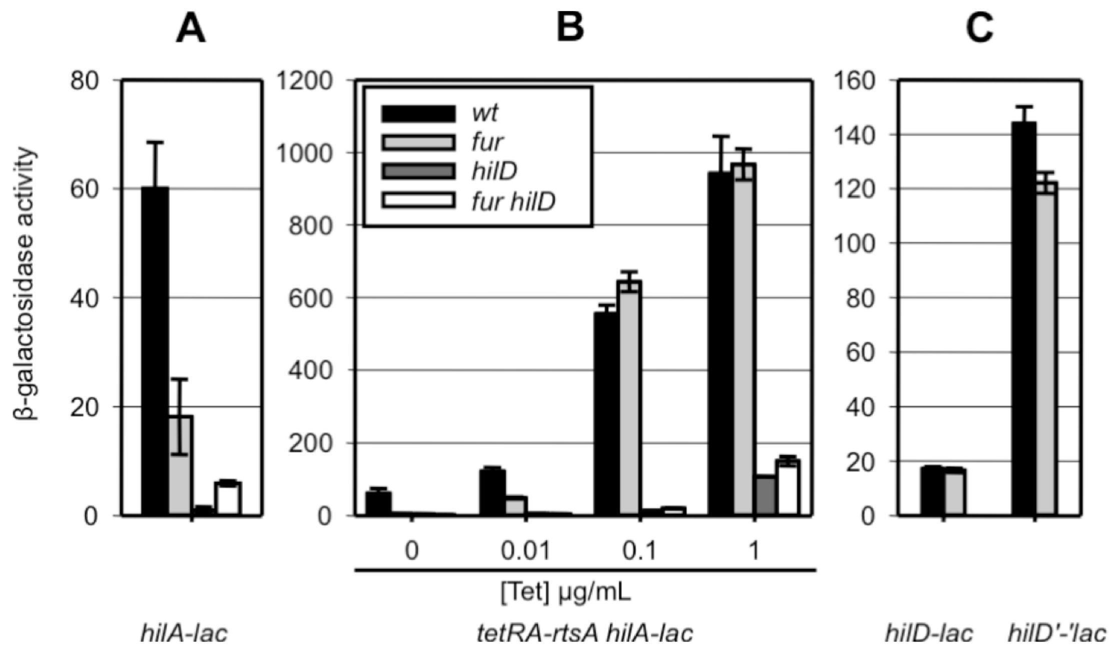

Figure S16 Class V, Fur activates *hilA* expression via HilD. See FIGURE S2 legend for details.

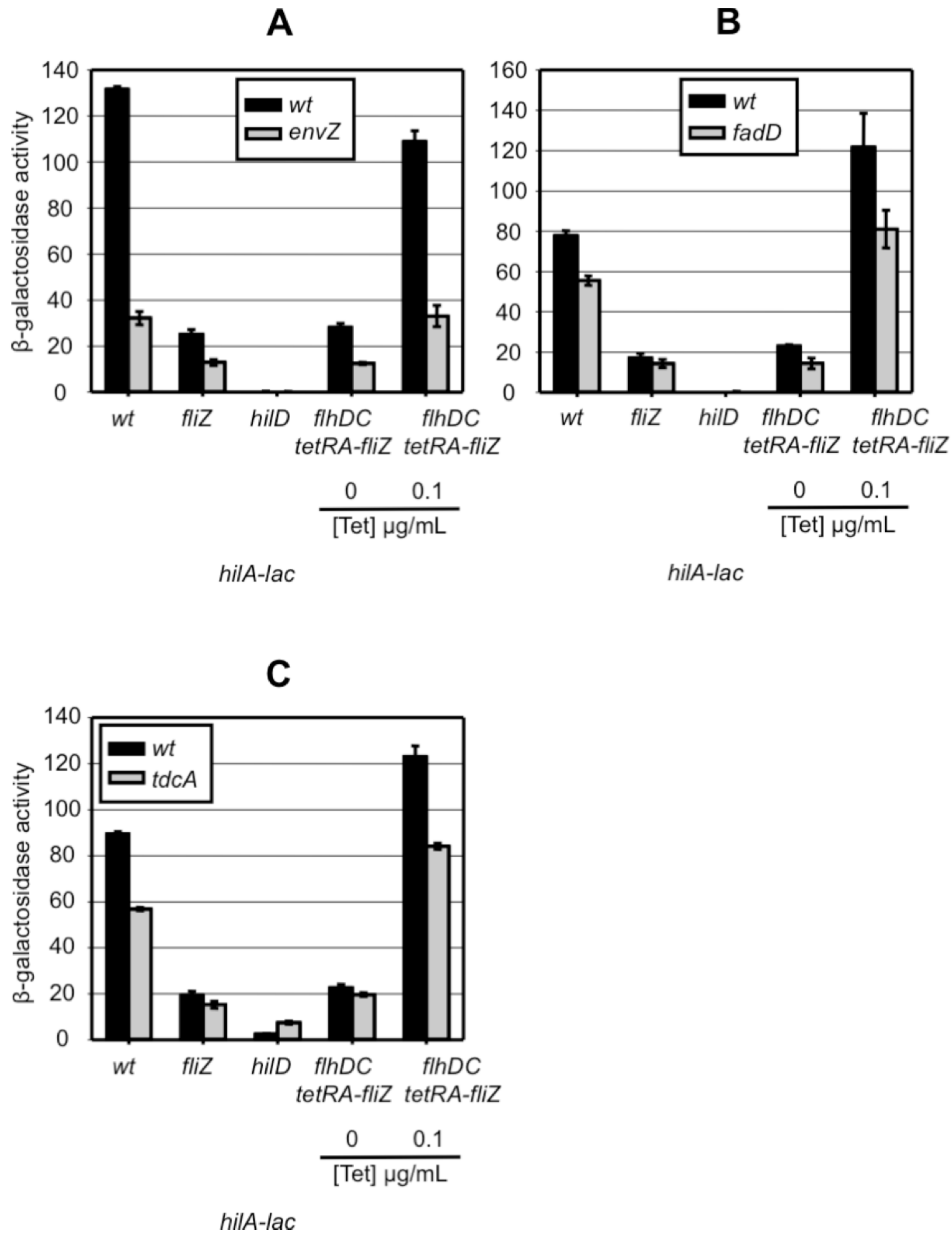

Figure S17 A) EnvZ, (B) FadD, and (C) TdcA affect *hilA* expression independently of FliZ. β-galactosidase activity in strains containing a *hilA-lac* transcriptional fusion and the indicated mutations after growth under SPI1 inducing conditions. β-galactosidase activity units are defined as (μmol of ONP formed min^{-1}) x 10^3/(OD600 x ml of cell suspension) and are reported as mean ± standard deviation where n=4.

TABLE S1 Regulatory factors/conditions affecting expression of SPI1

Regulator	Description	Mechanism of action/comments	References
HilA	Transcriptional activator, OmpR/ToxR family	Direct activation of *prg/org* and *inv/spa* operons	(8; 23; 27; 56; 57)
HilD	AraC-like transcriptional activator	Direct activation of *hilA*, *hilD*, *hilC*, and *rtsA*	(29; 88)
HilC	AraC-like transcriptional activator	Direct activation of *hilA*, *hilD*, *hilC*, and *rtsA*	(28; 29; 46; 88)
RtsA	AraC-like transcriptional activator	Direct activation of *hilA*, *hilD*, *hilC*, and *rtsA*	(29; 30)
HilE		Repression of *hilA* by binding to and preventing HilD function	(11); Chubiz JE (unpublished)
FliZ	Enhancer of class II flagellar genes expression	Activation of *hilA* via post-translational regulation of HilD	(20; 45; 54; 59; 85; 102)
EnvZ/OmpR	Two-component regulatory system; regulation of outer membrane porin genes, and virulence	Activation of *hilA* via HilD	(29; 58)
FadD	Acyl-CoA synthetase; degradation of long-chain fatty acids	Activation of *hilA* via an unknown mechanism	(59);Ellermeier JR (unpublished)
SirA	Transcriptional regulator; two-component regulatory system BarA/SirA; regulation of carbohydrate metabolism, motility, biofilm formation, and invasion	Activation of *hilA* via activation of *csrB/csrC* to block CsrA repression of *hilD*	(1; 29; 46; 52; 62; 99; 102);Ellermeier JR (unpublished)
Dam	DNA methylase	Activation of *hilA* via post-transcriptional regulation of *hilD*	(55)
Ack Pta	acetate kinase and phosphotransacetylase	Activation of *hilA* and *hilD* by formate via an unknown mechanism	(43)

TABLE S1 Regulatory factors/conditions affecting expression of SPI1

Regulator	Description	Mechanism of action/comments	References
YfgL	Outer membrane lipoprotein; assembly of the outer membrane β-barrel proteins in complex with YaeT, YfiO, and NlpB	Activation of SPI1genes via an unknown mechanism	(2; 37)
Trk (potassium)	Potassium transporter	Activation of SPI1 genes via an unknown mechanism	(94)
Fnr	Transcriptional regulator; cytoplasmic oxygen sensor	Repression of *hilA* via an unknown mechanism	(102);Ellermeier JR (unpublished)
PhoPQ (PhoQ24)	Two-component regulatory system; response to divalent cation limitation, pH and antimicrobial peptides	Repression of *hilA*, most likely direct	(9; 10; 14; 78; 111);Ellermeier JR (unpublished)
H-NS	Nucleoid protein	Direct repression of *hilA*, *hilC*, *hilD* and *rtsA*	(73; 74; 89)
Hha	Nucleoid protein	Direct repression of *hilA*, *hilC*, *hilD* and *rtsA*	(36; 73; 74; 102)
Fis	Nucleoid protein	Activation of SPI1 genes	(22; 48; 89; 110)}
HU	Nucleoid protein	Activation of SPI1 genes	(60; 89)
RfaH	Transcriptional anti-terminator; long operons for LPS core and O-antigen biosynthesis	Activation of SPI1genes via an unknown mechanism	(60; 67)
Fur	Transcriptional regulator; response to iron	Activation of *hilA* via an unknown regulation of HilD; repression of H-NS; direct binding of Fur to *hilD* promoter	(32; 98; 101)
TdcA	Transcriptional regulator of *tdc* operon; transport and metabolism of L-	Activation of *fliZ* and SPI1 genes expression	(50)

TABLE S1 Regulatory factors/conditions affecting expression of SPI1

Regulator	Description	Mechanism of action/comments	References
	threonine and L-serine		
FlhDC	Transcriptional regulator; activation of class II flagellar genes	Activation of *hilA* via activation of *fliZ*	(20; 54)
DsbA	Periplasmic disulfide bond oxidase	Activation of *hilA* via activation of FliZ, including repression of RcsCDB	(31; 54)
RcsCDB	phosphorelay system: sensor RcsC, response regulator RcsB, and phosphotransfer protein RcsD; regulation of capsule synthesis and biofilm formation	Repression of *hilA* via repression of FliZ and an independent unknown regulation of HilD	(54)
Lon	ATP-dependent protease	Repression of *hilA* via degradation of HilD, HilC, and FliZ	(16; 20; 95; 97)
ClpXP	ATP-dependent protease	Repression of SPI1 genes via FliZ	(47)
Formate	Short chain fatty acid	Activation of *hilA* and *hilD* (see Ack Pta)	(43)
Dimethyl sulfide/ DMSO		Repression of SPI1 genes via an unknown mechanism	(4)
Temperature		Activation of SPI1 genes when shifted from 25° to 37°C in H-NS-dependent manner	(75)
Butyrate	Short chain fatty acid	Repression of SPI1 genes via an unknown mechanism	(41)
ppGpp	Small signaling molecule; stringent response during starvation	Activation of *hilA* via an unknown mechanism	(79; 92; 93; 100);Ellermeier JR (unpublished)

TABLE S1 Regulatory factors/conditions affecting expression of SPI1

Regulator	Description	Mechanism of action/comments	References
FimZY	Transcriptional regulators; control of type 1 fimbriae gene expression	Repression of *hilA* via activation of *hilE*	(12; 87)
FimW	negative regulator of type 1 fimbriae	Activation of SPI1 genes via an unknown mechanism	(38)
Mlc	Transcriptional regulator; regulation of sugar uptake and metabolism	Repression of *hilA* via activation of *hilE*	(53)
CRP	cAMP-receptor protein	Activation of invasion via an unknown mechanism	(17)
CpxA	Sensor kinase, two-component regulatory system CpxRA; periplasmic stress responce	Activation of *hilA* via an unknown mechanism, apparently independent of CpxR	(68)
Lrp	Transcriptional regulator	Overproduction of Lrp represses SPI1 genes via an unknown mechanism	(6)
PmrM	Part of the *pmrHFIJKLM* operon	Activation of *hilA* via an unknown mechanism	(61; 65; 102)
ApaH/YgdP	Dinucleoside polyphosphate hydrolases	Activation of invasion via an unknown mechanism	(44)
PreAB (QseBC)	Two-component regulatory system; regulation of motility and virulence in response to quorum-sensing and hormonal signals	Activation of SPI1 genes via an unknown mechanism	(64; 66)
LuxS	Autoinducer 2 synthase	Activation of *invF*	(19)
PhoBR	Two-component regulatory system; phosphate limitation	Repression of *hilA* via PhoBR	(59)

TABLE S1 Regulatory factors/conditions affecting expression of SPI1

Regulator	Description	Mechanism of action/comments	References
SprB	Transcriptional regulator	Repression of *hilA* via repression of *hilD* transcription	(86)
RamA	AraC/XylS family transcriptional activator; regulation of multidrug resistance	Repression of SPI1 genes via an unknown mechanism	(7)
PPK	Polyphosphate kinase	Activation of invasion via an unknown mechanism	(49)
PNPase	Polynucleotide phosphorylase	Repression of SPI1 genes via an unknown mechanism	(21)
Hfq	RNA shaperone	Activation of SPI1 genes	(3; 90; 91)
SmpB	RNA-binding protein	Activation of SPI1 genes	(3)
IHF	Nucleoid protein	Activation of *hilA*; counteraction of H-NS mediated silencing	(35; 83)
RNAseE	5 -end-dependent endoribonuclease; part of degradosome complex	Repression of SPI1genes; exact mechanism unclear	(35)
Pag		Repression of SPI1 genes via an unknown mechanism	(35)
SirB		Activation of *hilA*; exact mechanism unclear	(84)
Sig32	Sigma factor; heat shock responce	Repression of *hilA* via degradation of HilD by Lon protease	(63)
TolC/AcrAB	Multidrug efflux pump	Activation of SPI1 genes via an unknown mechanism	(15; 103; 107)
AsmA	Outer membrane protein	Required for invasion; unknown mechanism	(80)
CorA	Mg^{2+} channel	Activation of SPI1 genes via an unknown mechanism	(76; 77)

TABLE S1 Regulatory factors/conditions affecting expression of SPI1

Regulator	Description	Mechanism of action/comments	References
PoxA	paralog of lysyl tRNA-synthetase	Repression of SPI1 genes via an unknown mechanism	(70)
YjeK	putative 2,3-β-lysine aminomutase	Repression of SPI1 genes via an unknown mechanism	(70)
Antimicrobial peptides		Repression of SPI1 genes via PhoPQ	(5)
Macrophages		Repression of SPI1 genes	(33; 96)
Epithelial cells		Activation of SPI1 genes	(42)
Bile	Role in lipid digestion	Repression of *hilA* via SirA	(81; 82)
Propanediol	Product of decomposition of rhamnose and fucose	Repression of *hilA* via an unknown mechanism	(69)
Microgravity	low-shear modeled microgravity	Repression of SPI1 genes	(109)
Lactobacillus supernatant; probiotics		Repression of SPI1 genes via an unknown mechanism	(25); (13)
Tetracycline		Activation of SPI1 genes	(108)
Nalidixic acid		Repression of SPI1 genes	(26)
Salicylidene acylhydrasides		Repression of SPI1 genes	(71)
Fluoroquinolone resistance		Repression of SPI1 genes	(34)

TABLE S2 Strains and plasmids

Name	Genotype [a]	Deletion endpoints [b]	Source or reference [c]
14028	Wild type		ATCC [d]
JS564	Δ*hilD138*::Kn		(32)
JS253	Δ*hilD114*::Cm		(30)
JS749	*attλ*::pDX1::*hilA'-lacZ*		(54)
JS951	Δ*hilD138*::Kn *attλ*::pDX1::*hilA'-lacZ*		(20)
JS576	Δ*hilD114*::Cm *attλ*::pDX1::*hilA'-lacZ*		(32)
JS953	*tetRA-rtsA attλ*::pDX1::*hilA'-lacZ*		(20)
JS955	Δ*hilD138*::Kn *tetRA-rtsA attλ*::pDX1::*hilA'-lacZ*		(20)
JS488	Φ(*hilD'*-lac$^+$)*114*		
JS892	Φ(*hilD'-'lacZ*)*hyb139*		(20)
JS996	Δ*hilE115*::Cm	4763527-4764108	
JS997	Δ*hilE115*::Cm *attλ*::pDX1::*hilA'-lacZ*		
JS998	Δ*hilE115*::Cm Δ*hilD138*::Kn *attλ*::pDX1::*hilA'-lacZ*		
JS999	Δ*hilE115*::Cm *tetRA-rtsA attλ*::pDX1::*hilA'-lacZ*		
JS1000	Δ*hilE115*::Cm Δ*hilD138*::Kn *tetRA-rtsA attλ*::pDX1::*hilA'-lacZ*		
JS1001	Φ(*hilD'*-lac$^+$)*114* Δ*hilE115*::Cm		
JS1002	Φ(*hilD'-'lacZ*)*hyb139* Δ*hilE115*::Cm		
JS950	Δ*fliZ8042*::Cm *attλ*::pDX1::*hilA'-lacZ*		(20)
JS1003	Φ(*hilD'*-lac$^+$)*114* Δ*fliZ8042*::Cm		
JS1004	Φ(*hilD'-'lacZ*)*hyb139* Δ*fliZ8042*::Cm		
JS1006	Δ*envZ182*::Cm *attλ*::pDX1::*hilA'-lacZ*		
JS1007	Δ*envZ182*::Cm Δ*hilD138*::Kn *attλ*::pDX1::*hilA'-lacZ*		
JS1008	Δ*envZ182*::Cm *tetRA-rtsA attλ*::pDX1::*hilA'-lacZ*		
JS1009	Δ*envZ182*::Cm Δ*hilD138*::Kn *tetRA-rtsA attλ*::pDX1::*hilA'-lacZ*		
JS1010	Φ(*hilD'*-lac$^+$)*114*Δ*envZ182*::Cm		
JS1011	Φ(*hilD'-'lacZ*)*hyb139* Δ*envZ182*::Cm		
JS1012	Δ*fadD21*::Kn	1915235-1916908	
JS1013	Δ*fadD21*::Kn *attλ*::pDX1::*hilA'-lacZ*		
JS1014	Δ*fadD21*::Kn Δ*hilD114*::Cm *attλ*::pDX1::*hilA'-lacZ*		
JS1015	Δ*fadD21*::Kn *tetRA-rtsA attλ*::pDX1::*hilA'-lacZ*		
JS1016	Δ*fadD21*::Kn Δ*hilD114*::Cm *tetRA-rtsA attλ*::pDX1::*hilA'-lacZ*		
JS1017	Φ(*hilD'*-lac$^+$)*114* Δ*fadD21*		
JS1018	Φ(*hilD'-'lacZ*)*hyb139* Δ*fadD21*		
JS1019	*sirA3*::Cm *attλ*::pDX1::*hilA'-lacZ*		

Name	Genotype [a]	Deletion endpoints [b]	Source or reference [c]
JS1020	*sirA3*::Cm Δ*hilD138*::Kn *attλ*::pDX1::*hilA'-lacZ*		
JS1021	*sirA3*::Cm *tetRA-rtsA attλ*::pDX1::*hilA'-lacZ*		
JS1022	*sirA3*::Cm Δ*hilD138*::Kn *tetRA-rtsA attλ*::pDX1::*hilA'-lacZ*		
JS1023	Φ(*hilD'*-lac$^+$)*114 sirA3*::Cm		
JS1024	Φ(*hilD'-'lacZ*)*hybb139 sirA3*::Cm		
JS1025	Δ*dam241*::Cm	3638689-3639527	
JS1026	Δ*dam241*::Cm *attλ*::pDX1::*hilA'-lacZ*		
JS1027	Δ*dam241*::Cm Δ*hilD138*::Kn *attλ*::pDX1::*hilA'-lacZ*		
JS1028	Δ*dam241*::Cm *tetRA-rtsA attλ*::pDX1::*hilA'-lacZ*		
JS1029	Δ*dam241*::Cm Δ*hilD138*::Kn *tetRA-rtsA attλ*::pDX1::*hilA'-lacZ*		
JS1030	Φ(*hilD'*-lac$^+$)*114* Δ*dam241*::Cm		
JS1031	Φ(*hilD'-'lacZ*)*hyb139* Δ*dam241*::Cm		
JS1032	Δ(*ack-pta)4202*::Cm	2447938-2451363	
JS1033	Δ(*ack-pta)4202*::Cm *attλ*::pDX1::*hilA'-lacZ*		
JS1034	Δ(*ack-pta)4202*::Cm Δ*hilD138*::Kn *attλ*::pDX1::*hilA'-lacZ*		
JS1035	Δ(*ack-pta)4202*::Cm *tetRA-rtsA attλ*::pDX1::*hilA'-lacZ*		
JS1036	Δ(*ack-pta)4202*::Cm Δ*hilD138*::Kn *tetRA-rtsA attλ*::pDX1::*hilA'-lacZ*		
JS1037	Φ(*hilD'*-lac$^+$)*114* Δ(*ack-pta)4202*::Cm		
JS1038	Φ(*hilD'-'lacZ*)*hyb139* Δ(*ack-pta)4202*::Cm		
JS1180	Δ*trkA*::Cm	3579771-3581196	
JS1181	Δ *trkA*::Cm *attλ*::pDX1::*hilA'-lacZ*		
JS1182	Δ *trkA*::Cm Δ*hilD138*::Kn *attλ*::pDX1::*hilA'-lacZ*		
JS1183	Δ *trkA*::Cm *tetRA-rtsA attλ*::pDX1::*hilA'-lacZ*		
JS1184	Δ *trkA*::Cm Δ*hilD138*::Kn *tetRA-rtsA attλ*::pDX1::*hilA'-lacZ*		
JS1185	Φ(*hilD'*-lac$^+$)*114* Δ*trkA*::Cm		
JS1186	Φ(*hilD'-'lacZ*)*hyb139* Δ*trkA*::Cm		
JS1039	Δ*yfgL611*::Cm	2653048-2654226	
JS1040	Δ*yfgL611*::Cm *attλ*::pDX1::*hilA'-lacZ*		
JS1041	Δ*yfgL611*::Cm Δ*hilD138*::Kn *attλ*::pDX1::*hilA'-lacZ*		
JS1042	Δ*yfgL611*::Cm *tetRA-rtsA attλ*::pDX1::*hilA'-lacZ*		
JS1043	Δ*yfgL611*::Cm Δ*hilD138*::Kn *tetRA-rtsA attλ*::pDX1::*hilA'-lacZ*		
JS1044	Φ(*hilD'*-lac$^+$)*114* Δ*yfgL611*::Cm		
JS1045	Φ(*hilD'-'lacZ*)*hyb139* Δ*yfgL611*::Cm		

Name	Genotype [a]	Deletion endpoints [b]	Source or reference [c]
JS1046	Δ*fnr1*::Cm	1754380-1755116	
JS1047	Δ*fnr1*::Cm *attλ*::pDX1::*hilA'-lacZ*		
JS1048	Δ*fnr1*::Cm Δ*hilD138*::Kn *attλ*::pDX1::*hilA'-lacZ*		
JS1049	Δ*fnr1*::Cm *tetRA-rtsA attλ*::pDX1::*hilA'-lacZ*		
JS1050	Δ*fnr1*::Cm Δ*hilD138*::Kn *tetRA-rtsA attλ*::pDX1::*hilA'-lacZ*		
JS1051	Φ(*hilD'*-lac$^+$)*114* Δ*fnr1*::Cm		
JS1052	Φ(*hilD'-'lacZ*)*hyb139* Δ*fnr1*::Cm		
JS1053	Δ*ycfD612*::Kn	1316880	
JS1054	*phoQ24* Δ*ycfD612*::Kn		
JS1055	*phoQ24* Δ*ycfD612*::Kn *attλ*::pDX1::*hilA'-lacZ*		
JS1056	*phoQ24* Δ*ycfD612*::Kn Δ*hilD114*::Cm *attλ*::pDX1::*hilA'-lacZ*		
JS1057	*phoQ24* Δ*ycfD612*::Kn *tetRA-rtsA attλ*::pDX1::*hilA'-lacZ*		
JS1058	*phoQ24* Δ*ycfD612*::Kn Δ*hilD114*::Cm *tetRA-rtsA attλ*::pDX1::*hilA'-lacZ*		
JS1059	Φ(*hilD'*-lac$^+$)*114 phoQ24* Δ*ycfD612*		
JS1060	Φ(*hilD'-'lacZ*)*hyb139 phoQ24* Δ*ycfD612*		
JS577	Δ*hilC113*::Cm *attλ*::pDX1::*hilA'-lacZ*		
JS579	Δ*rtsA5 attλ*::pDX1::*hilA'-lacZ*		
JS1061	*phoQ24* Δ*ycfD612*::Kn Δ*hilC113*::Cm *attλ*::pDX1::*hilA'-lacZ*		
JS1062	*phoQ24* Δ*ycfD612*::Kn Δ*rtsA5 attλ*::pDX1::*hilA'-lacZ*		
JS1063	Δ*fnr2*::Tet	1754321-1755129	
JS1064	Δ*fnr2*::Tet *attλ*::pDX1::*hilA'-lacZ*		
JS1065	Δ*fnr2*::Tet Δ*hilD114*::Cm *attλ*::pDX1::*hilA'-lacZ*		
JS1066	Δ*fnr2*::Tet Δ*hilC113*::Cm *attλ*::pDX1::*hilA'-lacZ*		
JS1067	Δ*fnr2*::Tet Δ*rtsA5 attλ*::pDX1::*hilA'-lacZ*		
JS1068	Δ*phoPQ*::Cm	1317242-1319310	
JS1069	Δ*phoPQ*::Cm *attλ*::pDX1::*hilA'-lacZ*		
JS1070	Δ*fnr2*::Tet Δ*phoPQ*::Cm *attλ*::pDX1::*hilA'-lacZ*		
JS1071	Δ*hha1*::Cm	528131-528349	
JS1072	Δ*hha1*::Cm *attλ*::pDX1::*hilA'-lacZ*		
JS1073	Δ*hha1*::Cm Δ*hilD138*::Kn *attλ*::pDX1::*hilA'-lacZ*		
JS1074	Δ*hha1*::Cm *tetRA-rtsA attλ*::pDX1::*hilA'-lacZ*		
JS1075	Δ*hha1*::Cm Δ*hilD138*::Kn *tetRA-rtsA attλ*::pDX1::*hilA'-lacZ*		
JS1076	Φ(*hilD'*-lac$^+$)*114* Δ*hha1*::Cm		
JS1077	Φ(*hilD'-'lacZ*)*hyb139* Δ*hha1*::Cm		

Name	Genotype [a]	Deletion endpoints [b]	Source or reference [c]
JS1078	*fis-3*::Cm		(72), listed as JG1160)
JS1079	*fis-3*::Cm *attλ*::pDX1::*hilA'-lacZ*		
JS1080	*fis-3*::Cm Δ*hilD138*::Kn *attλ*::pDX1::*hilA'-lacZ*		
JS1081	*fis-3*::Cm *tetRA-rtsA attλ*::pDX1::*hilA'-lacZ*		
JS1082	*fis-3*::Cm Δ*hilD138*::Kn *tetRA-rtsA attλ*::pDX1::*hilA'-lacZ*		
JS1083	Φ(*hilD'*-lac$^+$)*114 fis-3*::Cm		
JS1084	Φ(*hilD'-'lacZ*)*hyb139 fis-3*::Cm		
JS1085	Δ*hupA121*::Cm	4386709-4386981	
JS1086	Δ*hupB122*::Cm	508105-508378	
JS1087	Δ*hupA121*:: Δ*hupB122*::Cm *attλ*::pDX1::*hilA'-lacZ*		
JS1088	Δ*hupA121*:: Δ*hupB122*::Cm Δ*hilD138*::Kn *attλ*::pDX1::*hilA'-lacZ*		
JS1089	Δ*hupA121*:: Δ*hupB122*::Cm *tetRA-rtsA attλ*::pDX1::*hilA'-lacZ*		
JS1090	Δ*hupA121*:: Δ*hupB122*::Cm Δ*hilD138*::Kn *tetRA-rtsA attλ*::pDX1::*hilA'-lacZ*		
JS1091	Φ(*hilD'*-lac$^+$)*114* Δ*hupA121*:: Δ*hupB122*::Cm		
JS1092	Φ(*hilD'-'lacZ*)*hyb139* Δ*hupA121*:: Δ*hupB122*::Cm		
JS1093	Δ*rfaH4531*::Cm	4182923-4183411	
JS1094	Δ*rfaH4531*::Cm *attλ*::pDX1::*hilA'-lacZ*		
JS1095	Δ*rfaH4531*::Cm Δ*hilD138*::Kn *attλ*::pDX1::*hilA'-lacZ*		
JS1096	Δ*rfaH4531*::Cm *tetRA-rtsA attλ*::pDX1::*hilA'-lacZ*		
JS1097	Δ*rfaH4531*::Cm Δ*hilD138*::Kn *tetRA-rtsA attλ*::pDX1::*hilA'-lacZ*		
JS1098	Φ(*hilD'*-lac$^+$)*114* Δ*rfaH4531*::Cm		
JS1099	Φ(*hilD'-'lacZ*)*hyb139* Δ*rfaH4531*::Cm		
JS1100	Δ*relA81*::Kn	3102853-3105080	
JS1101	Δ*relA81* Δ*spoT292*::Cm *attλ*::pDX1::*hilA'-lacZ*		
JS1102	Δ*relA81* Δ*spoT292*::Cm Δ*hilD138*::Kn *attλ*::pDX1::*hilA'-lacZ*		
JS1103	Δ*relA81* Δ*spoT292*::Cm *tetRA-rtsA attλ*::pDX1::*hilA'-lacZ*		
JS1104	Δ*relA81* Δ*spoT292*::Cm Δ*hilD138*::Kn *tetRA-rtsA attλ*::pDX1::*hilA'-lacZ*		
JS1105	Φ(*hilD'*-lac$^+$)*114* Δ*relA81* Δ*spoT292*::Cm		
JS1106	Φ(*hilD'-'lacZ*)*hyb139* Δ*relA81* Δ*spoT292*::Cm		
JS1107	Δ*fur41*::Cm *attλ*::pDX1::*hilA'-lacZ*		
JS1108	Δ*fur41*::Cm Δ*hilD138*::Kn *attλ*::pDX1::*hilA'-lacZ*		
JS1109	Δ*fur41*::Cm *tetRA-rtsA attλ*::pDX1::*hilA'-lacZ*		

Name	Genotype [a]	Deletion endpoints [b]	Source or reference [c]
JS1110	Δ*fur41*::Cm Δ*hilD138*::Kn *tetRA-rtsA attλ*::pDX1::*hilA'-lacZ*		
JS1111	Φ(*hilD'*-lac$^+$)*114* Δ*fur41*::Cm		
JS1112	Φ(*hilD'-'lacZ*)*hyb139* Δ*fur41*::Cm		
JS1113	*attλ*::pDX1::*hilA'-lacZ hilD138*		
JS1114	*attλ*::pDX1::*hilA'-lacZ* Δ*fliZ8042*		
JS1115	*attλ*::pDX1::*hilA'-lacZ* Δ*flhDC8045 tetRA-fliZ*		
JS1116	*attλ*::pDX1::*hilA'-lacZ hilD138* Δ*hilE115*::Cm		
JS1117	*attλ*::pDX1::*hilA'-lacZ* Δ*fliZ8042* Δ*hilE115*::Cm		
JS1118	*attλ*::pDX1::*hilA'-lacZ* Δ*flhDC8045 tetRA-fliZ* Δ*hilE115* ::Cm		
JS1119	*attλ*::pDX1::*hilA'-lacZ hilD138* Δ*envZ182*::Cm		
JS1120	*attλ*::pDX1::*hilA'-lacZ* Δ*fliZ8042* Δ*envZ182*::Cm		
JS1121	*attλ*::pDX1::*hilA'-lacZ* Δ*flhDC8045 tetRA-fliZ* Δ*envZ182*::Cm		
JS1122	*attλ*::pDX1::*hilA'-lacZ hilD138* Δ*fadD21*::Kn		
JS1123	*attλ*::pDX1::*hilA'-lacZ* Δ*fliZ8042* Δ*fadD21*::Kn		
JS1124	*attλ*::pDX1::*hilA'-lacZ* Δ*flhDC8045 tetRA-fliZ* Δ*fadD21*::Kn		
JS1125	*attλ*::pDX1::*hilA'-lacZ hilD138 sirA3*::Cm		
JS1126	*attλ*::pDX1::*hilA'-lacZ* Δ*fliZ8042 sirA3*::Cm		
JS1127	*attλ*::pDX1::*hilA'-lacZ* Δ*flhDC8045 tetRA-fliZ sirA3*::Cm		
JS1128	*attλ*::pDX1::*hilA'-lacZ hilD138* Δ*dam241*::Cm		
JS1129	*attλ*::pDX1::*hilA'-lacZ* Δ*fliZ8042* Δ*dam241*::Cm		
JS1130	*attλ*::pDX1::*hilA'-lacZ* Δ*flhDC8045 tetRA-fliZ* Δ*dam241*::Cm		
JS1131	*attλ*::pDX1::*hilA'-lacZ hilD138* Δ(*ack-pta)4202*::Cm		
JS1132	*attλ*::pDX1::*hilA'-lacZ* Δ*fliZ8042* Δ(*ack-pta)4202*::Cm		
JS1133	*attλ*::pDX1::*hilA'-lacZ* Δ*flhDC8045 tetRA-fliZ* Δ(*ack-pta)4202*::Cm		
JS1134	*attλ*::pDX1::*hilA'-lacZ hilD138* Δ*yfgL611*::Cm		
JS1135	*attλ*::pDX1::*hilA'-lacZ* Δ*fliZ8042* Δ*yfgL611*::Cm		
JS1136	*attλ*::pDX1::*hilA'-lacZ* Δ*flhDC8045 tetRA-fliZ* Δ*yfgL611*::Cm		
JS1137	*attλ*::pDX1::*hilA'-lacZ hilD138* Δ*hha1*::Cm		
JS1138	*attλ*::pDX1::*hilA'-lacZ* Δ*fliZ8042* Δ*hha1*::Cm		
JS1139	*attλ*::pDX1::*hilA'-lacZ* Δ*flhDC8045 tetRA-fliZ* Δ*hha1*::Cm		
JS1140	*attλ*::pDX1::*hilA'-lacZ hilD138* fis-3::Cm		
JS1141	*attλ*::pDX1::*hilA'-lacZ* Δ*fliZ8042* Δ*fis*::Cm:		
JS1142	*attλ*::pDX1::*hilA'-lacZ* Δ*flhDC8045 tetRA-fliZ fis-3*::Cm		
JS1143	*attλ*::pDX1::*hilA'-lacZ hilD138* Δ*hupA121* Δ*hupB122*::Cm		
JS1144	*attλ*::pDX1::*hilA'-lacZ* Δ*fliZ8042* Δ*hupA121* Δ*hupB122*::Cm		
JS1145	*attλ*::pDX1::*hilA'-lacZ* Δ*flhDC8045 tetRA-fliZ* Δ*hupA121* Δ*hupB122*::Cm		
JS1146	*attλ*::pDX1::*hilA'-lacZ hilD138* Δ*rfaH4531*::Cm		

Name	Genotype [a]	Deletion endpoints [b]	Source or reference [c]
JS1147	*attλ*::pDX1::*hilA'-lacZ* Δ*fliZ8042* Δ*rfaH4531*::Cm		
JS1148	*attλ*::pDX1::*hilA'-lacZ* Δ*flhDC8045 tetRA-fliZ* Δ*rfaH4531*::Cm		
JS1149	*attλ*::pDX1::*hilA'-lacZ hilD138* Δ*relA81*::Kn Δ*spoT292*::Cm		
JS1150	*attλ*::pDX1::*hilA'-lacZ* Δ*fliZ8042* Δ*relA81*::Kn Δ*spoT292*::Cm		
JS1151	*attλ*::pDX1::*hilA'-lacZ* Δ*flhDC8045 tetRA-fliZ* Δ*relA81*::Kn Δ*spoT292*::Cm		
JS1152	*attλ*::pDX1::*hilA'-lacZ hilD138* Δ*fur41*::Cm		
JS1153	*attλ*::pDX1::*hilA'-lacZ* Δ*fliZ8042* Δ*fur41*::Cm		
JS1154	*attλ*::pDX1::*hilA'-lacZ* Δ*flhDC8045 tetRA-fliZ* Δ*fur41*::Cm		
JS1155	Δ*flhDC8045*::Cm	2022064-2021175	
JS1156	*attλ*::pDX1::*hilA'-lacZ* Δ*flhDC8045*::Cm		
JS1157	*attλ*::pDX1::*hilA'-lacZ hilD138* Δ*flhDC8045*::Cm		
JS1158	*attλ*::pDX1::*hilA'-lacZ* Δ*fliZ8042* Δ*flhDC8045*::Cm		
JS1159	Δ*tdcA51*::Cm	3412473-3413411	
JS1160	*attλ*::pDX1::*hilA'-lacZ* Δ*tdcA51*::Cm		
JS1161	*attλ*::pDX1::*hilA'-lacZ hilD138* Δ*tdcA51*::Cm		
JS1162	*attλ*::pDX1::*hilA'-lacZ* Δ*fliZ8042* Δ*tdcA51*::Cm		
JS1163	*attλ*::pDX1::*hilA'-lacZ* Δ*flhDC8045 tetRA-fliZ* Δ*tdcA51*::Cm		
Plasmids	**Relevant Characteristics**	**Cloned End Points**	
pKD46	*bla* P_{BAD} *gam bet exo* pSC101 oriTS		(24)
pCP20	*bla cat cI*857 λP_R *flp* pSC101 oriTS		(18)
pKD3	*bla* FRT *cat* FRT PS1 PS2 oriR6K		(24)
pKD4	*bla* FRT *aph* FRT PS1 PS2 oriR6K		(24)
pWKS30	pSC101 ori, Ap^r		(106)
pRfaH (pKG115)	pWKS30::*rfaH*$^+$	4182924-4183440	

[a] All strains are isogenic derivatives of ATCC 14028.
[b] The numbers indicate the base pairs that are deleted (strains) or cloned (plasmids) (inclusive) as defined in the *S. enterica* serovar Typhimurium LT2 genome sequence in the National Center for Biotechnology Information database.
[c] This study unless specified otherwise.
[d] ATCC, American Type Culture Collection.

TABLE S3 Transcriptomic datasets that reveal co-regulation of SPI 1 and flagellar genes in *Salmonella* Typhimurium

Global response to a regulatory system or specific environmental stimuli	Reference	Regulates *hilA* through FliZ?[a]	Class of SPI1 regulator
Macrophage	(33)	ND	
CsrA [b]	(51)	no	II
Antimicrobial peptides	(5)	ND	
Bile	(81)	no	
Swarming	(104; 105)	ND	
Fis	(48)	no	IV
RfaH	(67)	no	IV
YfgL	(37)	no	II
Fnr	(39)	no	III
Hydrogen peroxide	(40)	ND	

[a] No indicates that the factor regulates *hilA* in a *fliZ* null background and when FliZ is ectopically expressed. ND - Not Determined

[b] Effect of the loss of SirA was tested

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