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FliZ Regulates Expression of the *Salmonella* Pathogenicity Island 1 Invasion Locus by Controlling HilD Protein Activity in *Salmonella enterica* Serovar Typhimurium▽†

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A prerequisite for *Salmonella enterica* to cause both intestinal and systemic disease is the direct injection of effector proteins into host intestinal epithelial cells via a type three secretion system (T3SS); the T3SS genes are carried on *Salmonella* pathogenicity island 1 (SPI1). These effector proteins induce inflammatory diarrhea and bacterial invasion. Expression of the SPI1 T3SS is tightly regulated in response to environmental signals through a variety of global regulatory systems. We have previously shown that three AraC-like regulators, HilD, HilC, and RtsA, act in a complex feed-forward regulatory loop to control the expression of the *hilA* gene, which encodes the direct regulator of the SPI1 structural genes. In this work, we characterize a major positive regulator of this system, the flagellar protein FliZ. Through genetic and biochemical analyses, we show that FliZ posttranslationally controls HilD to positively regulate *hilA* expression. This mechanism is independent of other flagellar components and is not mediated through the negative regulator HilE or through FliZ-mediated RpoS regulation. We demonstrate that FliZ controls HilD protein activity and not stability. FliZ regulates HilD in the absence of Lon protease, previously shown to degrade HilD. Indeed, it appears that FliZ, rather than HilD, is the most relevant target of Lon as it relates to SPI1 expression. Mutants lacking FliZ are significantly attenuated in their ability to colonize the intestine but are unaffected during systemic infection. The intestinal attenuation is partially dependent on SPI1, but FliZ has additional pleiotropic effects.

During the course of infection, *Salmonella enterica* serovar Typhimurium induces inflammatory diarrhea and invades intestinal epithelial cells using a type three secretion system (T3SS); the T3SS genes are carried on *Salmonella* pathogenicity island 1 (SPI1) (20, 51, 53, 55). The SPI1 locus is a 40-kb island and carries all of the genes for a functional T3SS apparatus, a number of secreted effectors, and the regulatory proteins HilA, HilC, and HilD (34). RtsA, homologous to HilD and HilC, is encoded on a 15-kb island inserted in the $tRNA^{PheU}$ gene (15). HilA is the master SPI1 regulator and directly binds to the promoters and activates expression of the *prg-org* and *inv-spa* operons, encoding the components of the apparatus (2, 8, 10, 11, 32). The expression of *hilA* is controlled by a complex feed-forward regulatory loop consisting of HilD, HilC, and RtsA, each of which can independently activate *hilA* expression (13) (Fig. 1). Of these three, HilD has the predominant role, but apparently, it alone cannot activate SPI1 sufficiently *in vivo* (13). HilC and RtsA act as amplifiers of the inducing signals. A number of additional regulatory systems also affect SPI1 regulation (1, 17). Most of these systems seem to act through HilD (18), suggesting that HilD is the integration point for signal transduction into the SPI1 system.

RtsA, HilD, and HilC directly activate *dsbA*, which encodes a periplasmic disulfide bond oxidoreductase (16). Loss of DsbA independently affects SPI1 regulation and SPI1 function (30). The *dsbA*-mediated regulation of SPI1 expression is indirect, caused by decreased expression of the flagellar protein FliZ, which had previously been implicated in SPI1 regulation (12, 25, 33). Our analysis indicates that the decrease in disulfide bond formation in the periplasm, caused by loss of DsbA, both prevents formation of a functional flagellar apparatus and activates the RcsCDB phosphorelay system, which represses the master flagellar regulatory genes *flhDC*. The combination of these effects results in a significant decrease in FliZ production, thereby affecting expression of the SPI1 system (30). Indeed, *hilA* expression largely mirrors *fliZ* expression under a variety of conditions (30).

FliZ is encoded by a gene in the *fliA* operon, and orthologs are found only in the flagellar regulons of members of the family *Enterobacteriaceae*. The mechanism of action of FliZ is unknown. It has been reported to enhance class II flagellar gene expression (24), with evidence suggesting that it posttranslationally regulates $FlhD_4C_2$ in *Salmonella* (39). In addition, *Escherichia coli* FliZ can act as a posttranslational inhibitor of RpoS, and as a consequence, interfere with curli fimbria formation (37). Despite its apparent posttranslational effects in *Salmonella* and *E. coli*, the FliZ protein contains a region that resembles the core DNA binding domain of phage integrases (49). Indeed, in *Xenorhabdus nematophila*, FliZ activates transcription of class II flagellar genes by binding directly to the *flhDC* promoter (29).

We previously showed that FliZ-mediated induction of *hilA* expression is through HilD (30). Kage and colleagues have

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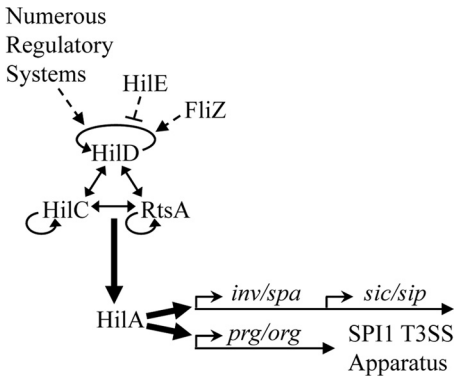

FIG. 1. Model for the *Salmonella* pathogenicity island 1 (SPI1) regulatory network. The expression of *hilA*, the master regulator for SPI1, is controlled by HilD, HilC, and RtsA, which act in a complex feed-forward loop. Each can independently activate expression of their own gene as well as each other and *hilA*. Signals are integrated by HilD; HilC and RtsA act as amplifiers of those signals. For clarity, the genes encoding HilD, HilC, RtsA, and HilA are not shown. The solid arrows indicate direct gene activation. T3SS, type three secretion system.

recently shown that FliZ controls SPI1 expression by affecting HilD at the posttranscriptional level (26). It was implied that FliZ somehow controls HilD translation, although no direct evidence was given. In this work, we further characterize the mechanism of FliZ regulation of HilD. Here we show that FliZ regulates HilD activity at the level of HilD protein. Our data also indicated that FliZ is critical *in vivo* for colonization or invasion of the intestine but is dispensable during systemic infection.

MATERIALS AND METHODS

Media, reagents, and enzymatic assays. Strains were routinely grown in Luria-Bertani (LB) medium at 37°C except for strains containing the temperature-sensitive plasmid pKD46 or pCP20, which were grown at 30°C. Strains were grown under *Salmonella* pathogenicity island 1 (SPI1)-inducing conditions, which are defined as high-osmolarity LB medium (1% NaCl) in standing cultures (low oxygen) for 18 to 22 h. Under non-SPI1-inducing conditions, cells were grown in low-salt LB medium (0.5% NaCl) at 37°C with shaking. The antibiotics and concentrations used were as follows: ampicillin, 50 μg/ml; chloramphenicol, 20 μg/ml; kanamycin, 50 μg/ml; tetracycline, 0.1, 0.4, and 0.8 μg/ml; streptomycin, 200 μg/ml; spectinomycin, 50 μg/ml; and rifampin, 100 μg/ml. Primers were ordered from IDT. Antibodies were purchased from Sigma (monoclonal anti-FLAG M2) or Biomeda (goat anti-mouse IgG conjugated to horseradish peroxidase [HRP]).

β-Galactosidase assays were performed using a microtiter plate assay as previously described (44). In most cases, cultures were grown aerobically overnight in low-salt LB medium (0.5% NaCl), subcultured 1:100 in 3 ml of high-salt LB medium (1% NaCl), and allowed to grow standing in 13-mm test tubes for 18 to 22 h. If needed, ampicillin (50 μg/ml) was added to the high-salt LB medium for plasmid maintenance, or the indicated amount of tetracycline was added for specific gene induction. β-Galactosidase activity units are defined as (micromoles of *ortho*-nitrophenol [ONP] formed per minute $\times 10^3$)/(OD_{600} $\times$ milliliter of cell suspension) (OD_{600} is the optical density at 600 nm) and are reported as means ± standard deviations.

Construction of strains and plasmids. All strains are isogenic derivatives of *Salmonella enterica* serovar Typhimurium strain 14028 and are listed in Table 1. Gene deletions and concomitant insertion of an antibiotic resistance cassette were constructed using lambda Red-mediated recombination as described previously (9, 14). All constructs were verified by PCR and moved into a clean background via P22HT*int*105 phage transduction. In some cases, the antibiotic resistance cassette was removed by FLP-mediated recombination with introduction of pCP20 (6). Transcriptional and translational *lac* fusions to *hilD* were constructed by FLP-mediated integration of fusion plasmids as described previously (14). FLAG-tagged HilD and FliZ alleles were constructed as described previously (52). To make plasmid pFliZ, the *fliZ* gene, corresponding to base pairs −14 to +571 relative to the start site of translation, was amplified using primers carrying EcoRI (5′) and BamHI (3′) sites and then cloned into vector pWKS30 (54).

HilD stability assays and Western blot analysis. To determine HilD levels in cells in stationary phase, bacteria were grown with shaking in low-salt LB medium (0.5% NaCl) until they reached stationary phase, then subcultured 1:100 in 90 ml of high-salt LB medium (1% NaCl) containing 100 μg/ml ampicillin and 0.4 μg/ml tetracycline (to induce *hilD*), and grown for 18 to 22 h standing at 37°C. One milliliter of each culture was used to determine the β-galactosidase activity produced from the *hilA-lacZ* fusion. The remaining cells were centrifuged, and the cell pellet was washed and suspended in 1 ml of phosphate-buffered saline (PBS). The OD_{600} of each sample was measured, and the volume was adjusted to ensure that equal concentrations of cells were used. The cells were disrupted by passing twice through a French pressure cell at 4°C. A 1/5 volume of 5× sodium dodecyl sulfate (SDS) loading buffer (43) and β-mercaptoethanol (final concentration of 5%) were added to an aliquot of each, and these samples were boiled for 5 min. Proteins were separated on 10% discontinuous SDS-polyacrylamide gels and transferred to Hybond ECL membranes (Amersham). The blots were blocked with 5% nonfat dried milk in PBS, exposed to monoclonal anti-FLAG M2 antibody, and HRP-conjugated goat anti-mouse IgG. The Western blot procedure and the detection of HRP-conjugated antibodies with a chemiluminescence system were performed according to the manufacturer's instructions (Amersham).

For log-phase assays, cells were grown in low-salt LB medium with shaking until the cells reached stationary phase, and then they were subcultured 1:100 in 10 ml of low-salt LB medium containing 0.8 μg/ml tetracycline. Cultures were grown shaking for 2.5 h at 37°C. After 2.5 h, the OD_{600} of each sample was measured, and the volume was adjusted to ensure equal concentrations of cells. One-milliliter samples were then removed to perform β-galactosidase assays. Concurrently, a transcription/translation inhibition cocktail containing 100 μg/ml rifampin, 200 μg/ml streptomycin, and 50 μg/ml spectinomycin were added to each culture (considered time zero). At appropriate intervals, 24-μl aliquots were removed from each culture and immediately mixed with 7.5 μl of 5× SDS loading buffer with β-mercaptoethanol and boiled for 5 min. SDS-PAGE and Western blot analysis were performed on the samples as described above. The intensity of bands was analyzed with ImageJ software (NIH), allowing half-lives to be determined. The mean half-life ± standard error of the mean (SEM) was calculated from 2 or 3 replicates for each strain.

To detect FLAG-tagged FliZ, 1-ml LB cultures of each strain were grown to saturation. Each was subcultured 1:100 in 40 ml of high-salt LB medium (1% NaCl) and grown standing at 37°C. The OD_{600} of each culture was determined at 18 h, and volumes were adjusted slightly to ensure equal concentrations of cells. Forty milliliters of each culture was centrifuged, and the resulting cell pellet was washed and suspended in 1 ml PBS with 0.5% Tween 20 (PBST). Cells were disrupted by passing twice through a French pressure cell. Each lysate was incubated with anti-FLAG Sepharose beads overnight. The beads were pelleted by centrifugation, washed six times with PBST, and then boiled in 50 μl of 1× SDS loading buffer with 5% β-mercaptoethanol. The samples were centrifuged to remove the beads. SDS-PAGE and Western blot analysis were performed as described above.

Virulence assays. BALB/c mice (Harlan) (6 to 8 weeks old) were inoculated either orally or intraperitoneally (i.p.) with 0.2 ml of a bacterial suspension. For oral infections, the cells were washed and suspended at 5×10^7 or 5×10^9 cells per ml in sterile 0.1 M sodium phosphate buffer, pH 8.0. For intraperitoneal infections, the cells were diluted to 5×10^3 cells per ml in sterile 0.15 M NaCl. Between 3 and 5 days after infection, the mice were sacrificed by CO_2 asphyxiation, and the ileal small intestines and spleens were harvested from mice inoculated orally and mice inoculated i.p., respectively. These organs were homogenized, and serial dilutions 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). Each mutant strain was reconstructed at least once to ensure that the virulence phenotype was the result of the designated mutation(s). The Student *t* test was used to determine whether the output ratio was significantly different from the input ratio. All animal work was reviewed and approved by the

TABLE 1. *Salmonella* strains and plasmids used in this study

Salmonella strain[a] or plasmid	Relevant genotype or phenotype	Deletion endpoints[b]	Source or reference
Salmonella strains			
14028	Wild type		ATCC[c]
JS135	*zii8104*::Tn*10*dTc		45
JS481	Δ(*invH-avrA*)*2916*::Kn		13
JS531	Φ(*sodCII′-lac*$^+$)*110*		21
JS541	Δ*rpoS1191*::Tc Φ(*sodCII′-lac*$^+$)*110*		21
JS576	Δ*hilD114*::Cm *att*λ::pDX1::*hilA′-lacZ*		18
JS577	Δ*hilC113*::Cm *att*λ::pDX1::*hilA′-lacZ*		18
JS579	Δ*rtsA5 att*λ::pDX1::*hilA′-lacZ*		18
JS633	Δ*hilE115*::*aadA att*λ::pDX1::*hilA′-lacZ*		18
JS634	Δ*hilD114*::Cm Δ*hilE115*::*aadA att*λ::pDX1::*hilA′-lacZ*		18
JS635	Δ*hilC113*::Cm Δ*hilE115*::*aadA att*λ::pDX1::*hilA′-lacZ*		18
JS636	Δ*rtsA5* Δ*hilE115*::*aadA att*λ::pDX1::*hilA′-lacZ*		18
JS749	*att*λ::pDX1::*hilA′-lacZ*		30
JS778	Δ*fliZ8042*::Tc *att*λ::pDX1::*hilA′-lacZ*		30
JS798	*att*λ::pDX1::*hilA′-lacZ*/pWKS30		30
JS799	*att*λ::pDX1::*hilA′-lacZ*/pFliZ		30
JS800	*fliZ8042*::Tc *att*λ::pDX1::*hilA′-lacZ*/pWKS30		30
JS801	*hilD114*::Km *att*λ::pDX1::*hilA′-lacZ*/pWKS30		30
JS802	*rtsA5*::Km *att*λ::pDX1::*hilA′-lacZ*/pWKS30		30
JS803	*hilC113*::Cm *att*λ::pDX1::*hilA′-lacZ*/pWKS30		30
JS804	*fliZ8042*::Tc *att*λ::pDX1::*hilA′-lacZ*/pFliZ		30
JS805	*hilD114*::Km *att*λ::pDX1::*hilA′-lacZ*/pFliZ		30
JS806	*rtsA5*::Km *att*λ::pDX1::*hilA′-lacZ*/pFliZ		30
JS807	*hilC113*::Cm *att*λ::pDX1::*hilA′-lacZ*/pFliZ		30
JS883	Φ(*hilD′-lac*$^+$)*139*	3017867–3018727	This study
JS892	Φ(*hilD′-′lacZ*)*hyb139*	3017867–3018727	This study
JS902	Δ*katE11*::Cm	1397114–1399406	27
JS909	Φ(*katE′-lac*$^+$)*11*		27
JS910	Δ*rpoS1191*::Tc Φ(*katE′-lac*$^+$)*11*		27
JS940	Δ(*wza-wcaM*)*4201*::Kn	2202646–2180030	This study
JS941	Δ*fliZ8042*::Cm	2044136–2044684	This study
JS942	Δ*flhDC8044*::Kn	2022215–2021141	This study
JS943	*hilD*-3×FLAG-Kn	3018761–3018773	This study
JS944	*tetRA-hilD*	3017792–3017800	This study
JS945	*tetRA-rtsA*	4561798–4561830	This study
JS946	Δ*flhDC8044*::Kn *att*λ::pDX1::*hilA′-lacZ*		This study
JS947	Δ*flhDC8044*::Kn *att*λ::pDX1::*hilA′-lacZ*/pWKS30		This study
JS948	Δ*flhDC8044*::Kn *att*λ::pDX1::*hilA′-lacZ*/pFliZ		This study
JS950	Δ*fliZ8042*::Cm *att*λ::pDX1::*hilA′-lacZ*		This study
JS951	Δ*hilD138*::Kn *att*λ::pDX1::*hilA′-lacZ*		This study
JS952	Δ*fliZ8042*::Cm Δ*hilD138*::Kn *att*λ::pDX1::*hilA′-lacZ*		This study
JS953	*tetRA-rtsA att*λ::pDX1::*hilA′-lacZ*		This study
JS954	Δ*fliZ8042*::Cm *tetRA-rtsA att*λ::pDX1::*hilA′-lacZ*		This study
JS955	Δ*hilD138*::Kn *tetRA-rtsA att*λ::pDX1::*hilA′-lacZ*		This study
JS956	Δ*fliZ8042*::Cm Δ*hilD138*::Kn *tetRA-rtsA att*λ::pDX1::*hilA′-lacZ*		This study
JS957	Φ(*hilD′-lac*$^+$)*139*/pFliZ		This study
JS958	Φ(*hilD′-lac*$^+$)*139*/pHilC		This study
JS959	Φ(*hilD′-′lacZ*)*hyb139*/pFliZ		This study
JS960	Φ(*hilD′-′lacZ*)*hyb139*/pHilC		This study
JS961	*tetRA-hilD att*λ::pDX1::*hilA′-lacZ*		This study
JS962	Δ*fliZ8042*::Kn *tetRA-hilD att*λ::pDX1::*hilA′-lacZ*		This study
JS963	Δ*hilE115*::*aadA tetRA-hilD att*λ::pDX1::*hilA′-lacZ*		This study
JS964	Δ*fliZ8042*::Tc Δ*hilE115*::*aadA att*λ::pDX1::*hilA′-lacZ*		This study
JS965	Δ*hilD114*::Cm Δ*fliZ8042*::Tc Δ*hilE115*::*aadA att*λ::pDX1::*hilA′*		This study
JS966	Δ*hilC113*::Cm Δ*fliZ8042*::Tc Δ*hilE115*::*aadA att*λ::pDX1::*hilA′*		This study
JS967	Δ*rtsA5* Δ*fliZ8042*::Tc Δ*hilE115*::*aadA att*λ::pDX1::*hilA′*		This study
JS968	Δ*rpoS*::Cm *att*λ::pDX1::*hilA′-lacZ*		This study
JS969	*att*λ::pDX1::*hilA′-lacZ* /pFliZ		This study
JS970	Δ*rpoS*::Cm *att*λ::pDX1::*hilA′-lacZ*/pFliZ		This study
JS971	Φ(*katE′-lac*$^+$)*11*/pFliZ		This study
JS972	Δ*rpoS1191*::Tc Φ(*katE′-lac*$^+$)*11*/pFliZ		This study
JS973	Φ(*sodCII′-lac*$^+$)*110*/pFliZ		This study
JS974	Δ*rpoS1191*::Tc Φ(*sodCII′-lac*$^+$)*110*/pFliZ		This study
JS975	*tetRA-hilD*-3×FLAG *att*λ::pDX1::*hilA′-lacZ*/pWKS30		This study
JS976	Δ*fliZ8042 tetRA-hilD*-3×FLAG *att*λ::pDX1::*hilA′-lacZ*::Kn/pWKS30		This study
JS977	Δ*fliZ8042 tetRA-hilD*-3×FLAG *att*λ::pDX1::*hilA′-lacZ*::Kn/pFliZ		This study

Continued on following page

TABLE 1—*Continued*

Salmonella strain[a] or plasmid	Relevant genotype or phenotype	Deletion endpoints[b]	Source or reference
JS978	Δ*hilE115*::*aadA tetRA-hilD*-3×FLAG *att*λ::pDX1::*hilA′-lacZ*/pWKS30		This study
JS979	Δ*hilE115*::*aadA tetRA-hilD*-3×FLAG *att*λ::pDX1::*hilA′-lacZ*/pHilE		This study
JS980	Δ*lon*::Kn Δ(*wza-wcaM*)*4201 tetRA-hilD*-3×FLAG attλ::pDX1::*hilA-′lacZ*/pWKS30		This study
JS981	Δ*lon*::Kn Δ(*wza-wcaM*)*4201 tetRA-hilD*-3×FLAG attλ::pDX1::*hilA-′lacZ*/pFliZ		This study
JS982	Δ(*wza-wcaM*)*4201 att*λ::pDX1::*hilA′-lacZ*		This study
JS983	Δ*lon*::Kn Δ(*wza-wcaM*)*4201 att*λ::pDX1::*hilA′-lacZ*		This study
JS984	Δ*fliZ8042*::Cm Δ*lon*::Kn Δ(*wza-wcaM*)*4201 att*λ::pDX1::*hilA′-lacZ*		This study
JS985	Δ*lon*::Kn Δ(*wza-wcaM*)*4201 att*λ::pDX1::*hilA′-lacZ*/pFliZ		This study
JS987	*fliZ*-3×FLAG	2044137–2044139	This study
JS988	Δ*lon*::Kn	505552–507860	This study
JS989	Δ*lon*::Kn *fliZ*-3×FLAG		This study
JS990	Δ*fliZ8042*::Cm *zii8104*::Tn*10*dTc		This study
JS991	Δ*fliZ8042*::Cm Δ(*invH-avrA*)*2916*::Kn		This study
Plasmids			
pWKS30	pSC101 origin; Ap^r		54
pFliZ (pDX2)	pWKS30::*fliZ*$^+$		30
pHilC (pLS119)	*bla* P_{BAD} *hilC-myc*-His pACYC184 origin		41

[a] All *Salmonella* strains are isogenic derivatives of *S. enterica* serovar Typhimurium strain 14028.
[b] Numbers indicate the base pairs that are deleted (inclusive) as defined in the *S. enterica* serovar Typhimurium LT2 genome sequence (National Center for Biotechnology Information).
[c] ATCC, American Type Culture Collection.

University of Illinois Institutional Animal Care and Use Committee (IACUC) and performed under protocol 07070 or 10050.

RESULTS

FliZ activates *hilA* through HilD, independent of FlhDC. We have previously shown that FliZ is a positive regulator of *hilA* expression (30). To further understand the mechanism of this regulation, we examined expression of a *hilA-lacZ* transcriptional fusion in various mutant backgrounds. Deletion of *fliZ* or *flhDC*, encoding the master transcriptional activator of the flagellar regulon, including *fliZ*, caused a 4-fold decrease in *hilA* expression (Fig. 2A). Overproduction of FliZ from a plasmid led to a 5-fold increase in *hilA* transcription. Importantly, ectopic expression of FliZ activates *hilA* expression in strains with *flhDC* deleted (Fig. 2B). Therefore, the SPI1 regulatory circuit is responding to FliZ, not flagella *per se*, and FliZ acts independently of any other flagellar protein.

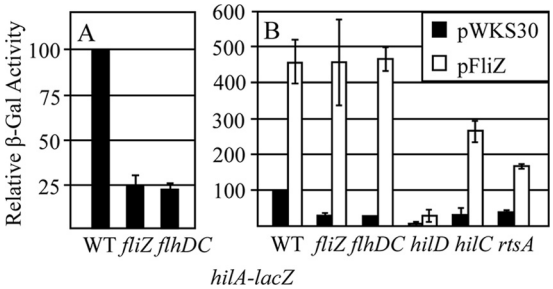

FIG. 2. FliZ activates *hilA* through *hilD*. β-Galactosidase (β-Gal) activity was examined in strains containing *hilA-lacZ* transcriptional fusions and the indicated plasmids and/or mutations. The strains were grown under SPI1-inducing conditions. β-Galactosidase activity units are defined as (micromoles of ONP formed per minute × 10^3)/(OD_{600} × milliliter of cell suspension) and are reported as means ± standard deviations (error bars) for four replicate samples relative to the results for the wild-type (WT) strain. The strains used were JS749, JS778, JS946, JS798 to JS807, JS947, and JS948.

To determine where FliZ fits into our model of SPI1 regulation, we also reexamined the effects of deleting *hilD*, *hilC*, and *rtsA* on expression of a *hilA-lacZ* fusion while overexpressing FliZ (Fig. 2B). Introduction of a *hilD* deletion into the *hilA-lacZ* fusion strain resulted in the expected decrease in *hilA* expression. Moreover, overproduction of FliZ no longer activated *hilA* in the *hilD* background. In contrast, neither RtsA nor HilC was required for FliZ induction of *hilA* expression, although the absolute level of expression was reduced in both mutants, consistent with our model of SPI1 regulation (Fig. 1). These data are consistent with our previous results (30) and provide genetic evidence that HilD is required for FliZ-mediated regulation of *hilA*; RtsA and HilC act as amplifiers of the signal.

Further analysis showed that loss of FliZ also had no effect in a *hilD* null background (Fig. 3A). These data also suggest that FliZ functions through HilD. However, the level of *hilA* expression is low in the *hilD* background. Therefore, it remained possible that FliZ functions at some step downstream of HilD action, for example at the *hilA* promoter, but that this regulation is not evident in the *hilD* null background. To distinguish whether FliZ regulates *hilA* via HilD or independently of HilD, we put *rtsA* under the control of a tetracycline-inducible promoter (*tetRA-rtsA*). This construct allows us to turn on *hilA* (and *hilC*) even in the absence of HilD (13). RtsA, HilC, and HilD each activate *hilA* by binding to the same sites in the promoter region (13, 35, 36). If FliZ affects *hilA* expression solely through HilD, then there should be no *fliZ* phenotype in a Δ*hilD* background when the system is activated by other means. In *hilD*$^+$ strains in which *rtsA* is controlled by a tetracycline-inducible promoter (Fig. 3B), FliZ-dependent regulation was evident in the absence of tetracycline. This condition is equivalent to an *rtsA* null mutation. The loss of HilD in this

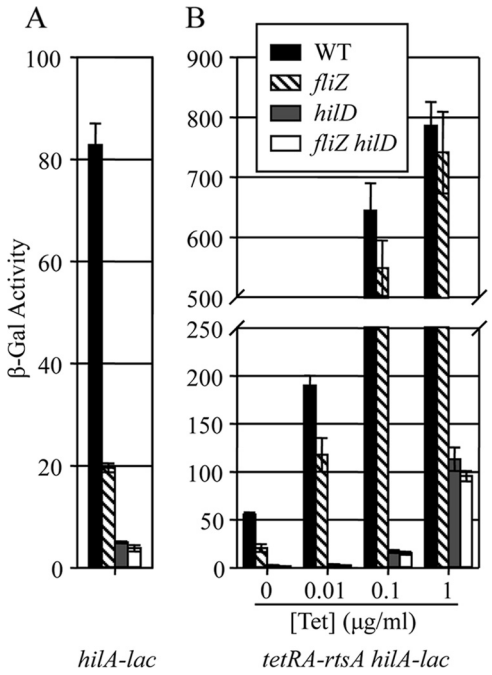

FIG. 3. FliZ activation of *hilA* is dependent on HilD. (A) β-Galactosidase activity in strains containing a *hilA-lacZ* transcriptional fusion and the indicated mutations after growth under SPI1-inducing conditions. (B) β-Galactosidase activity of strains containing a *hilA-lacZ* transcriptional fusion and indicated mutations with *rtsA* under the control of a tetracycline-regulated promoter. The strains were grown under SPI1-inducing conditions with the indicated concentrations of tetracycline (Tet). The strains used were JS749 and JS950 to JS956. β-Galactosidase activity units are defined as (μmol of ONP formed per min × 10^3)/(OD_{600} × ml of cell suspension) and are reported as means ± standard deviations (n = 4).

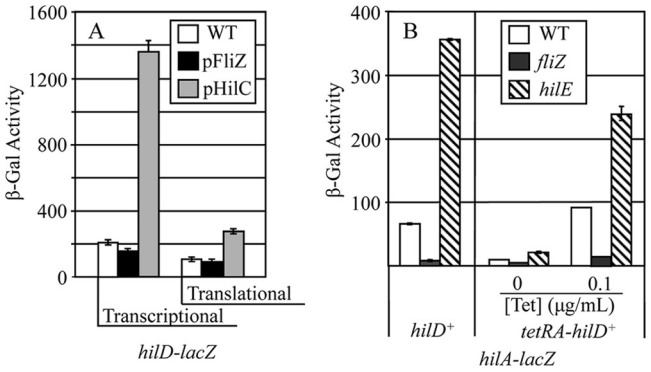

FIG. 4. FliZ acts at the level of HilD protein. (A) β-Galactosidase activity in strains containing either a *hilD-lacZ* transcriptional or translational fusion and the indicated plasmids. The fusion joints of the two constructs are identical (14). The strains were grown under SPI1-inducing conditions with 10 mM arabinose. Arabinose is required for induction of pHilC but was included in all cultures. The strains used were JS883, JS957, JS958, JS892, JS959, and JS960. (B) β-Galactosidase activity in strains containing a *hilA-lacZ* transcriptional fusion and the indicated mutations. The strains were grown under SPI1-inducing conditions (left panel) or in LB medium (0.5% NaCl) with the indicated tetracycline concentrations and with shaking (right panel). The strains used were JS749, JS778, JS633, JS961, JS962, and JS963. β-Galactosidase activity units are defined as (μmol of ONP formed per min × 10^3)/(OD_{600} × ml of cell suspension) and are reported as means ± standard deviations (n = 4).

background conferred low-level expression. The addition of tetracycline induced *hilA* expression in this *hilD* null background. Indeed, with 1 μg/ml tetracycline, *hilA* expression was similar to that observed in a wild-type background under SPI1-inducing conditions (compare Fig. 3B to Fig. 3A). However, the loss of FliZ had no significant effect under these conditions. These results are consistent with our model that FliZ is functioning through HilD to control *hilA* expression. Note that in the *hilD*$^+$ background, the addition of tetracycline led to a significant increase in *hilA* expression, as expected. Moreover, there was an apparent loss of FliZ-dependent regulation at increasing concentrations of tetracycline. Thus, overproduction of HilD confers FliZ-independent activation of *hilA*. This loss of regulation is also evident when HilD is overproduced by other methods (not shown).

FliZ regulates HilD posttranslationally. Given that HilD is required for FliZ regulation of *hilA*, it seemed possible that FliZ could regulate *hilD* transcription or translation. To test this hypothesis, we examined the effects of FliZ on expression of *hilD-lacZ* fusions. Importantly, these *hilD* fusion constructs are *hilD* null so that we can examine regulation in the absence of autoactivation by HilD. Figure 4A shows that overproduction of FliZ had no effect on either a transcriptional or translational *lacZ* fusion to *hilD*. In contrast, overproduction of HilC activates transcription of these fusions as expected; HilC is known to independently activate *hilD* transcription (13). These data show that FliZ does not act by controlling *hilD* transcription. Moreover, the fact that a *hilD* translational fusion was not affected by FliZ shows that FliZ regulation is not mediated at the level of translation initiation. Thus, FliZ-mediated regulation of HilD is posttranslational.

FliZ acts at the level of HilD protein. The data above suggest that FliZ regulates HilD function posttranslationally. The system is complicated by the fact that HilD is autoregulated and *hilD* transcription is normally induced upon activation of the regulatory circuit (13). Thus, in order to determine whether regulation works at the level of HilD protein, we had to place *hilD* transcription under a different promoter. To accomplish this, we inserted a tetracycline resistance cassette in place of the normal *hilD* promoter, placing transcription of *hilD* under tetracycline control. This construct was introduced into a strain containing a *hilA-lacZ* transcriptional fusion. Starting with this background, we deleted *fliZ* or *hilE* and examined expression of the *hilA-lacZ* fusion.

In the control strains with *hilD* under its own promoter, deletion of *fliZ* resulted in a 6- to 7-fold decrease in *hilA* expression (Fig. 4B). HilE inhibits HilD function by direct protein-protein interaction as suggested by two-hybrid analysis (3) and coimmunoprecipitation (J. E. C. Chubiz and J. M. Slauch, unpublished data). As expected, deletion of HilE resulted in a 5-fold increase in expression. In the TetR-controlled *hilD* background, without the addition of tetracycline, *hilA* expression was low and deletion of *fliZ* or *hilE* had little effect; these strains behave essentially as *hilD* null mutants. The addition of 0.1 μg/ml tetracycline resulted in *hilA* expression comparable to expression in the *hilD*$^+$ background. Deletion of *hilE* under these conditions conferred a 2.6-fold in-

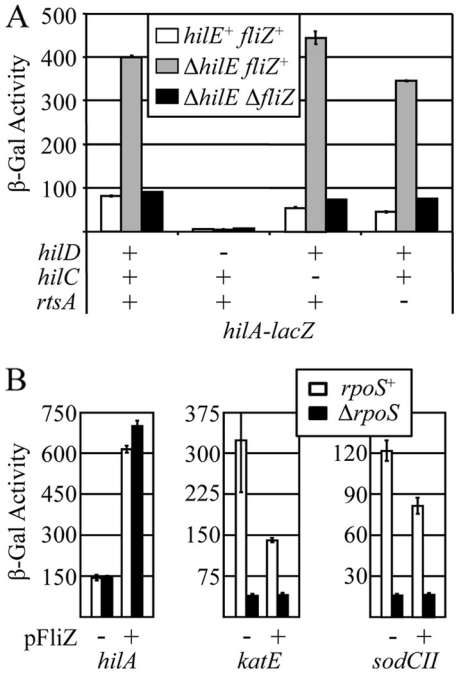

FIG. 5. FliZ regulates *hilA* independently of HilE and RpoS. (A) β-Galactosidase activity in strains containing a *hilA-lacZ* transcriptional fusion and the indicated mutations after growth under SPI1-inducing conditions. The strains used were JS749, JS576, JS577, JS579, JS633 to JS636, and JS964 to JS967. (B) β-Galactosidase activity of strains containing *sodCII*, *katE*, or *hilA* transcriptional fusions in otherwise wild-type or *rpoS* backgrounds with or without pFliZ. The strains were grown under SPI1-inducing conditions. The strains used were JS749, JS968, JS969, JS970, JS909, JS910, JS971, JS972, JS531, JS541, JS973, and JS974. β-Galactosidase activity units are defined as (μmol of ONP formed min^{-1}) $\times$ 10^3/(OD_{600} $\times$ ml of cell suspension) and are reported as means $\pm$ standard deviations ($n = 4$).

crease in *hilA* expression. Deletion of *fliZ* resulted in a 4.7-fold decrease in *hilA* expression. The fact that these proteins control an ectopically expressed HilD suggests that both HilE and FliZ control HilD activity at the level of HilD protein. Note that the level of regulation was not as robust as that seen in the wild-type background. This is as expected, given that these signals are no longer amplified by transcriptional activation of the *hilD* promoter.

FliZ acts independently of HilE. One model that would explain FliZ posttranslational regulation of HilD is that FliZ could negatively regulate HilE expression or function. To test this hypothesis, we examined the effects of *hilE* and *fliZ* deletions on *hilA* expression. If FliZ works through *hilE*, a deletion of *fliZ* would have no effect on the expression of *hilA* in the Δ*hilE* background. Figure 5A shows that while a deletion of *hilE* increased expression of *hilA* as expected, a deletion of *fliZ* in the Δ*hilE* background was still able to decrease *hilA* expression about 4.5-fold. As expected, deletion of *hilD* completely blocked both HilE- and FliZ-mediated regulation. In contrast, deletion of *hilC* or *rtsA* has little effect on regulation by HilE or FliZ. Thus, while both HilE and FliZ act through HilD, the effects of FliZ are the same in wild-type and *hilE* backgrounds, indicating that these two factors regulate HilD independently of each other.

FliZ independently regulates RpoS and HilD. It has recently been shown that in *E. coli*, FliZ negatively regulates RpoS activity posttranslationally, independent of other flagellar proteins (37). While RpoS has never been shown to affect SPI1 expression in *Salmonella*, these results raised the possibility that FliZ could be working through RpoS to control HilD activity. This hypothesis was tested, and the results are shown in Fig. 5B. Deletion of *rpoS* had no effect on expression of a *hilA-lacZ* fusion under SPI1-inducing conditions. Moreover, overexpression of FliZ activated *hilA* even in the *rpoS* deletion strain. For controls for this experiment, we examined the expression of two known RpoS-regulated genes, *katE* (23) and *sodCII* (21). In both cases, deletion of *rpoS* significantly decreased expression of these genes as expected. Interestingly, overproduction of FliZ also decreased expression of both genes. This FliZ-mediated decrease was not seen in the *rpoS* deletion background. These data show that FliZ does affect RpoS-mediated activation in *Salmonella* but that FliZ-mediated regulation of SPI1 is independent of RpoS.

FliZ affects the function of HilD; its effects on HilD stability are secondary. We next examined HilD protein levels in response to FliZ- or HilE-mediated regulation. We constructed a 3×FLAG-tagged version of HilD and proved that this tagged version was functional (see Fig. S1 in the supplemental material). We then placed FLAG-tagged *hilD* under the control of the tetracycline-inducible promoter as described above. The strain also contained a *hilA-lacZ* transcriptional fusion. Starting with this background, we deleted *fliZ* and then introduced a vector control or pFliZ (*fliZ* null versus FliZ overproduction), or we deleted *hilE* and introduced a vector control or pHilE (*hilE* null versus HilE overproduction). The *fliZ*$^+$ *hilE*$^+$ strain containing the vector was included as a control. We grew the strains in SPI1-inducing conditions with the addition of tetracycline. We used part of the culture to determine the β-galactosidase activity produced from the *hilA-lacZ* fusion. We monitored the levels of the FLAG-tagged HilD protein in the remaining cells by Western blot analysis. Our results are shown in Fig. 6A. The β-galactosidase activity produced from the *hilA-lacZ* fusion showed clear regulation by FliZ and HilE. However, there was no strict correlation between the level of HilD protein and the level of *hilA* expression; HilD protein levels in the Δ*fliZ* and pFliZ backgrounds differed by only 2-fold, whereas *hilA* transcription differed by 10-fold. HilD levels were altered significantly in the *hilE* null background versus pHilE background. However, while the levels of HilD in the strain carrying pHilE were essentially equal to those in the wild-type strain, activity was markedly reduced. The effects of HilE on HilD levels will require further analysis. However, these results do suggest that FliZ controls some aspect of HilD activity *per se* and suggest that subtle differences in steady-state levels are a secondary effect.

To better understand the action of FliZ on HilD, we monitored HilD stability during log-phase growth. Using the same strains as in the stationary-phase experiment above, we induced *hilD* transcription with tetracycline and aerated the cells until they reached late log phase. Then, transcription and translation were stopped by the addition of rifampin, streptomycin, and spectinomycin, and samples were removed every 15 min to monitor the level of HilD over time via Western blot analysis. An aliquot of each sample was also removed at time

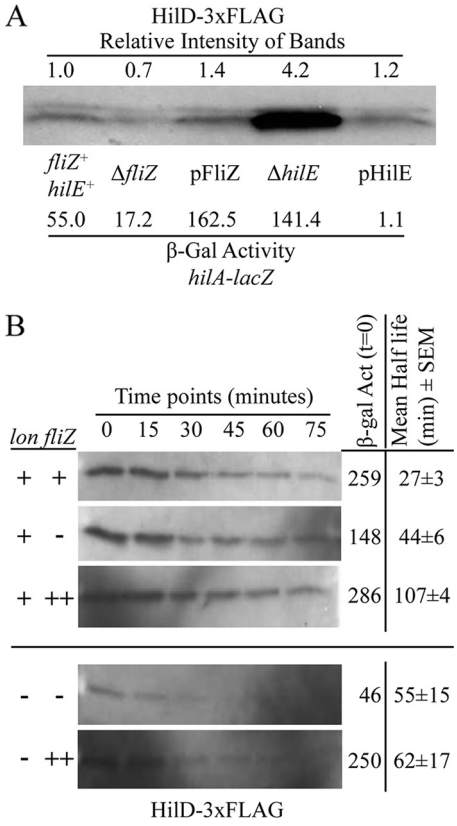

FIG. 6. HilD protein levels in relation to FliZ and HilE. The *hilD*-3×FLAG construct is under *tetRA* control, and all strains contained a *hilA-lacZ* transcriptional fusion and the indicated mutations or plasmids. (A) HilD protein levels in stationary-phase cells. The strains were grown under SPI1-inducing conditions with 0.4 μg/ml tetracycline. The cultures were divided to determine β-galactosidase activity and to perform the Western blot analysis to detect FLAG-tagged HilD. Extracts from equal concentrations of cells were loaded on the gel. The intensities of the bands were quantified using ImageJ and are presented above the gel relative to the wild-type strain (set at 1). Note that the doublets seen are artifacts of this particular gel. The strains used were JS975 to JS979. (B) HilD protein stability in cells in late log phase. The genotypes for *lon* and *fliZ* strains are indicated to the left of the gels (++ indicates overproduction [pFliZ]). The cells were induced with 0.8 μg/ml tetracycline and grown in LB medium (0.5% NaCl) with shaking to late log phase, and antibiotics were added to stop transcription and translation. β-Galactosidase activity produced from the *hilA-lacZ* fusion in the samples shown on these gels was determined from each sample taken at time zero. ImageJ was used for half-life analysis. The half-life was calculated from 2 (*lon*) or 3 replicates of the experiments. The mean half-life ± SEM is listed for each background. The strains used were JS975, JS976, JS977, JS980, and JS981.

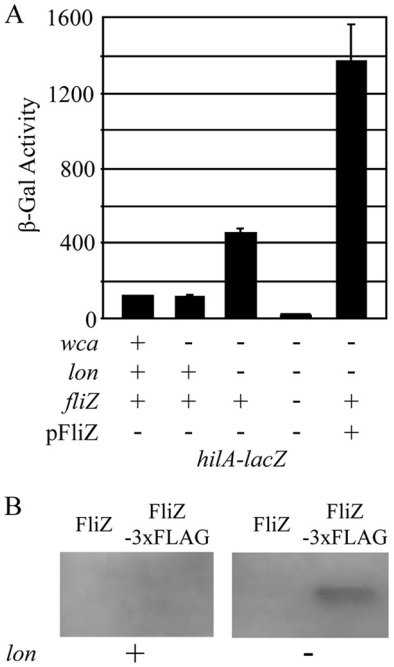

FIG. 7. FliZ regulates HilD in the absence of Lon protease. (A) β-Galactosidase activity in strains containing a *hilA-lacZ* transcriptional fusion and various mutations or pFliZ as indicated. The strains were grown under SPI1-inducing conditions. The strains used were JS749 and JS982 to JS985. (B) Immunoprecipitation of FliZ-3×FLAG. Strains produced either wild-type FliZ or 3×FLAG-tagged FliZ as indicated in *lon*$^+$ or *lon* mutant backgrounds. The cultures were grown under SPI1-inducing conditions. FLAG-tagged protein was immunoprecipitated from lysates from equal concentrations and numbers of cells. The proteins were separated by SDS-PAGE and subjected to Western blot analysis to detect FLAG-tagged protein. The strains used were 14028, JS987, JS988, and JS989.

zero to perform a β-galactosidase assay to monitor *hilA* transcription. The results can be seen in Fig. 6B (top three gels). Regulation by FliZ was evident in this experiment, although it was not as robust as in the stationary-phase cells above in Fig. 6A. The amounts of HilD present at time zero were nearly identical in each case. The half-life of HilD protein in a wild-type background was approximately 30 min. Loss of FliZ actually increased HilD half-life slightly. Overproduction of FliZ increased HilD half-life even further. In all cases, the half-life of HilD is longer than the doubling time of the cells under these conditions. Thus, although the half-life of HilD is slightly affected by changes in FliZ levels, this does not explain the mechanism of regulation.

FliZ regulates HilD independently of Lon. HilC and HilD were reported to be degraded by the Lon protease, and this presumably contributes to shutting down SPI1 expression after invasion (4, 50). If FliZ acts by controlling stability, it would likely do so by affecting the accessibility of HilD to Lon. To test this hypothesis, we performed β-galactosidase assays to monitor *hilA-lacZ* activity in *lon* mutant backgrounds. In these experiments, *hilD* was under the control of its native promoter and the cells were grown under SPI1-inducing conditions. The results are shown in Fig. 7A. Mutants defective in Lon are mucoid due to the production of colanic acid (22). Therefore, the capsule production genes in the *wca* operon were deleted in order to make the strains easier to work with. The *wca* mutation had no effect on *hilA* expression. As expected, *hilA* expression was increased in the *lon* background. However, *hilA* expression in this background was even more significantly affected by FliZ than in the wild type; activity was decreased about 20-fold in the absence of FliZ and increased 3-fold when FliZ was overexpressed.

We then measured the half-life of HilD in a *lon* background. This experiment was performed as described above using strains in which *hilD* expression was under *tet* control. We

TABLE 2. Role of FliZ during infection

Infection route	Inoculum (CFU)	Genotype of strain[a]: A	Genotype of strain[a]: B	Median CI[b]	*P* value[c]	No. of mice
i.p.[d]	10^3	Δ*fliZ*	WT	1.08	NS	9
Oral[e]	10^7	Δ*fliZ*	WT	0.016	<0.005	11
	10^9	Δ*fliZ* Δ*spi1*	Δ*spi1*	0.2	<0.005	16

[a] The strains used were JS135, JS990, JS481, and JS991.
[b] The competitive index (CI) was calculated as described in Materials and Methods.
[c] The Student *t* test was used to compare the CIs to the inocula. NS, not significant.
[d] Bacteria were recovered from the spleen in the case of intraperitoneal (i.p.) competition assays.
[e] Bacteria were recovered from the distal portion of the small intestine in oral competition assays.

compared a strain lacking FliZ to a strain in which FliZ was being overproduced from the plasmid. Again, regulation by FliZ was even more robust in the *lon* mutants (Fig. 6B, bottom two gels). The concentrations of HilD at time zero were nearly identical in both cases. Surprisingly, the half-lives of HilD were nearly identical in the two strains and were only slightly longer than the half-life in strains with Lon. Clearly, FliZ controls HilD activity by a mechanism independent of degradation.

The results above suggest that proteases in addition to Lon are responsible for degradation of HilD. Nevertheless, in the absence of Lon, *hilA* expression is increased 4-fold. This increase seems to be dependent on FliZ. We had constructed a 3×Flag-tagged version of FliZ (FliZ-3×FLAG) and shown it to be functional (albeit less active than the wild-type protein; see Fig. S1 in the supplemental material). However, we never detected FLAG-tagged FliZ in a Western blot. Given our results, we compared the levels of FliZ in *lon*$^+$ and *lon* mutant strains. Figure 7B shows that we could immunoprecipitate FliZ-3×FLAG from *lon* cells, but the protein was not detected in a wild-type background. Thus, FliZ appears to be stabilized in the absence of Lon. Taken together, our results suggest that the increased *hilA* expression seen in the absence of Lon is not due to increased stability of HilD but to increased stability of FliZ.

FliZ contributes to the invasion mechanism of virulence. The data above show that FliZ significantly affects SPI1 regulation *in vitro*, but is FliZ-mediated regulation important during intestinal invasion? To test this, we performed oral and intraperitoneal (i.p.) competition assays, infecting mice with a 1:1 mix of wild-type and Δ*fliZ* strains and determining the ratio of the two strains after 3 to 5 days of infection in either the ileal small intestine (oral) or spleen (i.p.). As shown in Table 2, the *fliZ* mutant was significantly attenuated compared to the wild type (63-fold) when recovered from the small intestine after oral infection. However, the *fliZ* mutant competed evenly with the wild type after an i.p. infection, suggesting that FliZ has a role primarily during intestinal colonization and/or invasion. A *fliZ* mutant was previously shown to be attenuated after oral infection as measured in a time-to-death assay (25), and our data are consistent with these findings. To determine whether the attenuation of the *fliZ* mutant was due to decreased expression of *hilA*, we tested the effects of deleting *fliZ* in a strain in which the entire SPI1 locus was deleted (13). In this background, FliZ still contributed to virulence, but the mutant was attenuated only 5-fold compared to the isogenic strain. These data suggest that FliZ plays a pleiotropic role during intestinal colonization and invasion, but at least part of this effect is via SPI1.

DISCUSSION

Salmonella enterica has evolved a complex regulatory network to control the expression of the SPI1 T3SS, critical for initiating both inflammatory diarrhea and systemic infection. Although this system has been intensely studied for decades and is continuously uncovered as a major target of global *Salmonella* gene expression, we are just beginning to understand the details of its regulation. We have used a variety of genetic and biochemical techniques to unravel this very complex network, allowing us to develop a new model for the SPI1 regulatory circuit (13). This model provides insight into many previous studies and explains all of the data amassed on the system thus far.

Here we focus on FliZ, a regulator with a dramatic effect on SPI1 regulation. Null mutations in *fliZ* decrease *hilA* expression approximately 5-fold, while overproduction of FliZ from a plasmid increases *hilA* expression about 5-fold above that of the wild-type strain. This regulation is clearly mediated through HilD, but FliZ controls neither *hilD* transcription nor translation initiation. Moreover, FliZ can affect regulation when HilD protein is expressed ectopically. These results show that FliZ acts at the level of HilD protein.

Kage et al. (26) showed that ClpXP protease affects SPI1 expression indirectly by increasing transcription of *fliZ*. These investigators showed that FliZ controls HilD protein levels even when *hilD* is transcribed from a different promoter. On the basis of this experiment, they implied that FliZ regulates HilD at the level of translation. Their data are consistent with ours, but we have investigated FliZ-dependent regulation of HilD in more detail and can, therefore, make more precise conclusions.

The stability of HilD protein is affected slightly by the presence or absence of FliZ. Importantly, this confirms that FliZ is acting at the level of HilD protein, as it is difficult to imagine a mechanism whereby action that does not directly involve the protein could affect stability. However, it is clear that regulation by FliZ is apparent in the Δ*lon* background. There are data suggesting that Lon degrades HilD and HilC (4, 50). However, our data suggest that the half-life of HilD is only slightly increased in the absence of Lon. In contrast, we could detect FliZ only in the *lon* background. This result and the fact that the increase in *hilA* expression seen in the *lon* mutant is

dependent on FliZ suggest that the primary consequence of losing Lon is increased stability of FliZ, which in turn activates HilD. Taken together, these results suggest that FliZ regulation works at the level of HilD function and that stability is a secondary effect. It is not clear what step in HilD function is affected by FliZ. One possibility is that FliZ could facilitate HilD binding to promoter regions.

FliZ positively regulates HilD function at the level of HilD protein. This regulation is independent of HilE, which clearly functions by direct protein-protein interaction. However, like HilE, regulation is affected by both loss and overproduction of FliZ protein. Moreover, overproduction of HilD overcomes FliZ regulation. The FliZ protein shows significant homology only with other FliZ proteins from members of the family *Enterobacteriaceae*. However, interestingly, a search of the "conserved domains" database shows that the C-terminal half of FliZ has a sterile alpha motif (SAM)-like fold. SAM domains are known as versatile protein-protein interaction domains found in a large variety of proteins (38). Taking all of our data into account, we hypothesize that FliZ controls the activity of HilD by direct interaction, but we cannot currently rule out indirect effects. The instability of FliZ has made it difficult to provide biochemical data to prove or disprove direct interaction, and confirmation of our hypothesis requires further investigation.

FliZ plays a major role in the regulation of SPI1. Under our *in vitro* conditions, it is striking how regulation of *hilA* mirrors regulation of *fliZ* (data in reference 30). On the other hand, FliZ is also pleiotropic. Its apparent role is to indicate to other regulatory circuits in the cell the status of the flagellar system. To date, in *Salmonella*, FliZ is known to independently regulate three very different transcriptional regulators, HilD, RpoS, and $FlhD_4C_2$. Here we show that a *fliZ* mutant is attenuated during intestinal colonization/invasion. Our data suggest that part of this attenuation is via SPI1, but the loss of FliZ has an effect in the absence of SPI1. Flagella are implicated in intestinal colonization (46), and flagellin has a role in inducing intestinal inflammation (48), which is apparently beneficial for *Salmonella* (47). The loss of FliZ decreases flagellar gene expression 2- to 3-fold (28, 39) and has a detectable but modest effect in a motility assay (30), but whether the SPI1-independent attenuation in the *fliZ* mutant is due to changes in flagellar expression or other effects is not clear. FliZ plays no apparent role during systemic infection. This is not surprising, since neither flagella nor SPI1 are transcriptionally active (5, 7, 19) or required during extraintestinal infection (13, 31, 42). It is not clear why this regulatory relationship exists between flagellar and invasion systems. It is likely that the two must be coordinated during intestinal colonization leading to invasion. Indeed, there is coordinate regulation of induction of flagella and SPI1 *in vitro* that is dependent on FliZ (40).

Numerous regulatory systems are known to affect SPI1 expression. Our studies show that, in most cases, these signals feed into the SPI1 regulatory circuit through HilD (13, 18, 30). On the basis of our results, we hypothesize that regulation of SPI1 is mediated predominantly at the level of HilD protein. Therefore, understanding how environmental parameters filtered through multiple signal transduction systems are integrated at HilD is the key to SPI1 regulation.

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