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Regulation of *Salmonella enterica* Pathogenicity Island 1 by DNA Adenine Methylation

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ABSTRACT

DNA adenine methylase (Dam$^-$) mutants of *Salmonella enterica* are attenuated in the mouse model and present multiple virulence-related defects. Impaired interaction of Salmonella Dam$^-$ mutants with the intestinal epithelium has been tentatively correlated with reduced secretion of pathogenicity island 1 (SPI-1) effectors. In this study, we show that *S. enterica* Dam$^-$ mutants contain lowered levels of the SPI-1 transcriptional regulators HilA, HilC, HilD, and InvF. Epistasis analysis indicates that Dam-dependent regulation of SPI-1 requires HilD, while HilA, HilC, and InvF are dispensable. A transcriptional *hilD::lac* fusion is expressed at similar levels in Dam$^+$ and Dam$^-$ hosts. However, lower levels of *hilD* mRNA are found in a Dam$^-$ background, thus providing unsuspected evidence that Dam methylation might exert post-transcriptional regulation of *hilD* expression. This hypothesis is supported by the following lines of evidence: (i) lowered levels of *hilD* mRNA are found in Salmonella Dam$^-$ mutants when *hilD* is transcribed from a heterologous promoter; (ii) increased *hilD* mRNA turnover is observed in Dam$^-$ mutants; (iii) lack of the Hfq RNA chaperone enhances *hilD* mRNA instability in Dam$^-$ mutants; and (iv) lack of the RNA degradosome components polynucleotide phosphorylase and ribonuclease E suppresses *hilD* mRNA instability in a Dam$^-$ background. Our report of Dam-dependent control of *hilD* mRNA stability suggests that DNA adenine methylation plays hitherto unknown roles in post-transcriptional control of gene expression.

DEOXYADENOSYL methyltransferases are common in bacteria, and most of them are part of restriction/modification systems (Marinus 1996; Wion and Casadesus 2006). In addition, many bacterial genomes contain solitary DNA adenine methylases, not involved in protecting DNA from a restriction enzyme companion. Two of these enzymes, the Dam methylase of gamma-proteobacteria and the CcrM methylase of alpha-proteobacteria, are paradigms of evolutionary processes that have turned DNA adenine methylation into an epigenetic signal for DNA–protein interactions (Reisenauer *et al.* 1999; Løbner-Olesen *et al.* 2005; Casadesus and Low 2006; Wion and Casadesus 2006).

In *Escherichia coli* and *Salmonella enterica*, Dam methylation controls chromosome replication, nucleoid organization, chromosome segregation, mismatch repair, and expression of certain genes (Marinus 1996; Løbner-Olesen *et al.* 2005; Wion and Casadesus 2006; Heusipp *et al.* 2007; Low and Casadesus 2008). Because of its multiple roles in bacterial physiology, loss of Dam methylation causes pleiotropic defects in certain species (*e.g.*, *E. coli* and *S. enterica*) and inviability in others (*e.g.*, *Vibrio cholerae* and certain strains of *Yersinia enterocolitica*) (Wion and Casadesus 2006).

DNA adenine methylase (Dam$^-$) mutants of *S. enterica* are severely attenuated in the mouse model and present a plethora of virulence-related defects, both at the intestinal stage of infection and during systemic infection (Garcia-Del Portillo *et al.* 1999; Heithoff *et al.* 1999). Lack of Dam-dependent mismatch repair sensitizes Dam$^-$ mutants to the DNA-damaging action of bile salts (Prieto *et al.* 2004). Envelope instability may also contribute to bile sensitivity in Salmonella Dam$^-$ mutants (Pucciarelli *et al.* 2002). Lack of Dam methylation perturbs also the interaction of Salmonella with the intestinal epithelium. Impaired invasion of epithelial cells by Dam$^-$ mutants has been confirmed in tissue cultures and has been tentatively correlated with reduced secretion of invasion effectors encoded on Salmonella pathogenicity island 1 (SPI-1) (Garcia-Del Portillo *et al.* 1999). High-throughput analysis of gene expression has confirmed that SPI-1 is transcribed at lowered levels in Dam$^-$ mutants (Balbontin *et al.* 2006).

SPI-1 is an ~40-kb gene cluster containing at least 37 genes (Lostroh and Lee 2001; Altier 2005; Jones 2005), located at centisome 63 on the *S. enterica* chro-

Supporting information is available online at http://www.genetics.org/cgi/content/full/genetics.109.108985/DC1.

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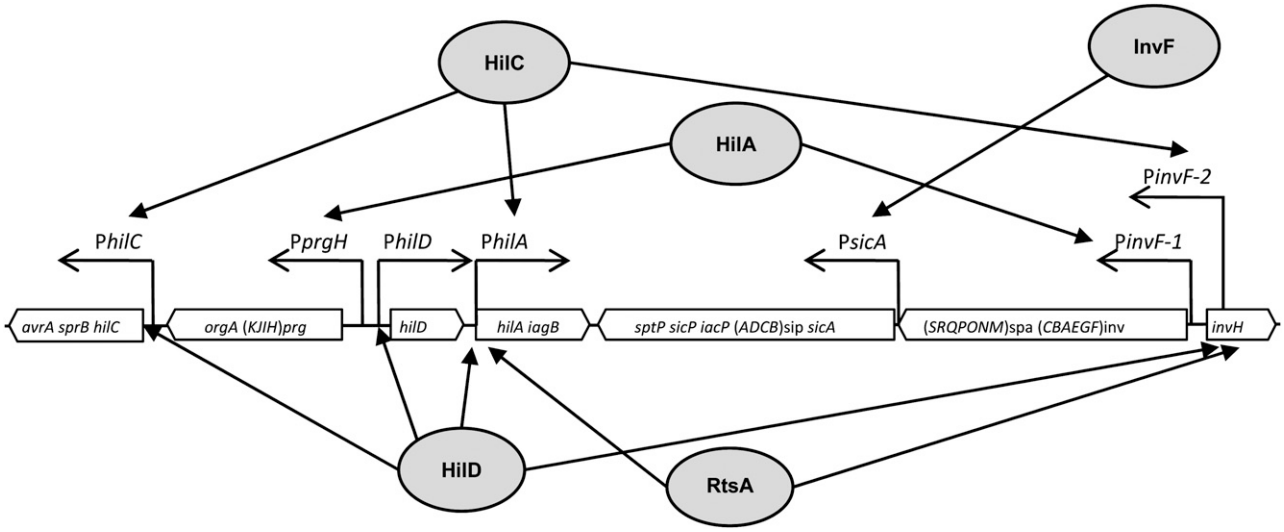

Figure 1.—Diagram showing the transcriptional units of *Salmonella enterica* SPI-1 and the regulatory circuits under the control of transcription factors HilA, HilD, HilC, RtsA, and InvF (adapted from Lostroh and Lee 2001; Ellermeier and Slauch 2003; Altier 2005; Jones 2005).

mosome (McClelland *et al.* 2001). SPI-1 encodes a type 3 secretion system and secreted effectors that interact with proteins inside epithelial cells in the animal intestine (Galan and Curtiss 1989). SPI-1 genes are organized in seven or more transcriptional units, whose expression is under the control of four SPI-encoded transcription factors: HilA, HilC, HilD, and InvF (Lostroh and Lee 2001). HilA, a member of the OmpR/ToxR family (Lee *et al.* 1992; Bajaj *et al.* 1995), activates transcription of SPI genes that encode components of the secretion apparatus as well as the gene for the InvF transcriptional regulator (Bajaj *et al.* 1996). In association with SicA, InvF is necessary to boost transcription of the *sicA* and s*ipBCDA* transcriptional units (Darwin and Miller 1999; Eichelberg and Galan 1999). HilC and HilD are members of the AraC/XylS family and activate transcription from the p_{invF} and p_{sicA} promoters in an apparently redundant manner (Akbar *et al.* 2003). Transcriptional activation by HilC and HilD relieves repression of the *hilA* promoter by the nucleoid proteins H-NS and Hha (Olekhnovich and Kadner 2006). Furthermore, HilC and HilD can activate *inv/sicA* transcription in the absence of HilA (Rakeman *et al.* 1999; Akbar *et al.* 2003). A transcription factor located outside SPI-1, RtsA, is also involved in transcriptional control of SPI-1 (Ellermeier and Slauch 2003). A diagram of SPI-1 transcriptional regulation is presented in Figure 1. Besides the regulatory actions described above, positive feedback loops are involved in the control of *hilD*, *hilC*, and *rtsA* transcription (Ellermeier *et al.* 2005).

SPI-1 expression is under the control of additional regulators located outside the island. The ferric uptake regulatory protein, Fur, and the BarA/SirA two-component system are SPI-1 activators (Fortune *et al.* 2006; Ellermeier and Slauch 2008). In turn, HilE (Fahlen *et al.* 2000) and Lon (Takaya *et al.* 2003; Boddicker and Jones 2004) are negative regulators of SPI-1.

In this study, we show that Dam-dependent regulation of SPI-1 has a single target, the *hilD* gene. However, we present evidence that Dam methylation regulates *hilD* expression at the post-transcriptional level. Because Dam methylase is not known to have functions other than GATC methylation, a reasonable interpretation is that Dam methylation may control transcription of a post-transcriptional regulator of *hilD* expression.

MATERIALS AND METHODS

Bacterial strains, plasmids, bacteriophages, and strain construction: The *S. enterica* strains listed in Table 1 belong to serovar Typhimurium and derive from ATCC 14028. For simplicity, *S. enterica* serovar Typhimurium is often abbreviated as *S. enterica*. Luria–Bertani (LB) broth was used as liquid medium. Solid LB broth contained agar at 1.5% final concentration. Green plates (Chan *et al.* 1972) contained methyl blue (Sigma, St. Louis) instead of aniline blue. The indicator for monitoring β-galactosidase activity in plate tests was 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside ("X-gal"; Sigma, 40 μg/ml). Antibiotics were used at the concentrations described previously (Torreblanca *et al.* 1999). Targeted gene disruption was achieved using plasmid pKD13 (Datsenko and Wanner 2000). Antibiotic resistance cassettes introduced during strain construction were excised by recombination with plasmid pCP20 (Datsenko and Wanner 2000). The oligonucleotides used for disruption (labeled "UP" and "DO") are listed in supporting information, Table S1, together with the oligonucleotides (labeled "E") used for allele verification by the polymerase chain reaction. Disruption of the *rne* gene, which encodes ribonuclease E, was performed with primers that eliminate the C-terminal region (Viegas *et al.* 2007). For the construction of transcriptional and translational *lac* fusions in the Salmonella chromosome, FRT sites generated by excision of Km^r cassettes (Datsenko

TABLE 1

Strains of *Salmonella enterica* serovar Typhimurium

Strain designation	Genotype	Reference or source
14028	Wild type	ATCC
SV5264	Δ*dam-231*	This study
SV5278	Φ(*sicA′-lacZ*$^+$) (Hyb)	This study
SV5279	Δ*dam-231* Φ(*sicA′-lacZ*$^+$) (Hyb)	This study
SV5284	Φ(*hilA′-lacZ*$^+$) (Hyb)	This study
SV5285	Δ*dam-231* Φ(*hilA′-lacZ*$^+$) (Hyb)	This study
SV5286	Φ(*hilD-lacZ*)	This study
SV5288	Δ*dam-231* Φ(*hilD-lacZ*)	This study
SV5293	Φ(*sipC′-lacZ*$^+$) (Hyb)	This study
SV5294	Δ*dam-231* Φ(*sipC′-lacZ*$^+$) (Hyb)	This study
SV5297	Φ(*invF′-lacZ*$^+$) (Hyb)	This study
SV5298	Δ*dam-231* Φ(*invF′-lacZ*$^+$) (Hyb)	This study
SV5301	Φ(*invH′-lacZ*$^+$) (Hyb)	This study
SV5302	Δ*dam-231* Φ(*invH′-lacZ*$^+$) (Hyb)	This study
SV5308	Δ*dam-231* Δ*hilA* Φ(*sipB′-lacZ*$^+$) (Hyb)	This study
SV5310	Δ*dam-231* Δ*hilC* Φ(*sipB′-lacZ*$^+$) (Hyb)	This study
SV5312	Δ*dam-231* Δ*hilD* Φ(*sipB′-lacZ*$^+$) (Hyb)	This study
SV5314	Δ*invF* Δ*dam-231* Φ(*sipB′-lacZ*$^+$) (Hyb)	This study
SV5316	Δ*hilA* Φ(*sipB′-lacZ*$^+$) (Hyb)	This study
SV5318	Δ*hilC* Φ(*sipB′-lacZ*$^+$) (Hyb)	This study
SV5320	Δ*hilD* Φ(*sipB′-lacZ*$^+$) (Hyb)	This study
SV5322	Δ*invF* Φ(*sipB′-lacZ*$^+$) (Hyb)	This study
SV5335	P*tetA-hilD* Φ(*invF′-lacZ*$^+$) (Hyb)	This study
SV5336	Δ*dam-231* P*tetA-hilD* Φ(*invF′-lacZ*$^+$) (Hyb)	This study
SV5382	Φ(*sipB′-lacZ*$^+$) (Hyb)	This study
SV5383	Δ*dam-231* Φ(*sipB′-lacZ*$^+$) (Hyb)	This study
SV5384	Φ(*hilC′-lacZ*$^+$) (Hyb)	This study
SV5385	Δ*dam-231* Φ(*hilC′-lacZ*$^+$) (Hyb)	This study
SV5386	Δ*hilD* Φ(*hilC′-lacZ*$^+$) (Hyb)	This study
SV5387	Δ*dam-231* Δ*hilD* Φ(*hilC′-lacZ*$^+$) (Hyb)	This study
SV5399	Δ*hilD* Φ(*hilA′-lacZ*$^+$) (Hyb)	This study
SV5400	Δ*dam-231* Δ*hilD* Φ(*hilA′-lacZ*$^+$) (Hyb)	This study
SV5401	Δ*hilC* Φ(*hilA′-lacZ*$^+$) (Hyb)	This study
SV5402	Δ*dam-231* Δ*hilC* Φ(*hilA′-lacZ*$^+$) (Hyb)	This study
SV5403	Δ*hilA* Φ(*invF′-lacZ*$^+$) (Hyb)	This study
SV5404	Δ*dam-231* Δ*hilA* Φ(*invF′-lacZ*$^+$) (Hyb)	This study
SV5405	Δ*hilC* Φ(*invF′-lacZ*$^+$) (Hyb)	This study
SV5406	Δ*dam-231* Δ*hilC* Φ(*invF′-lacZ*$^+$) (Hyb)	This study
SV5407	Δ*hilD* Φ(*invF′-lacZ*$^+$) (Hyb)	This study
SV5408	Δ*dam-231* Δ*hilD* Φ(*invF′-lacZ*$^+$) (Hyb)	This study
SV5415	Δ*hilD* Φ(*invH′-lacZ*$^+$) (Hyb)	This study
SV5416	Δ*dam-231* Δ*hilD* Φ(*invH′-lacZ*$^+$) (Hyb)	This study
SV5417	Δ*hilC* Φ(*invH′-lacZ*$^+$) (Hyb)	This study
SV5418	Δ*dam-231* Δ*hilC* Φ(*invH′-lacZ*$^+$) (Hyb)	This study
SV5419	Δ*hilA* Φ(*invH′-lacZ*$^+$) (Hyb)	This study
SV5420	Δ*dam-231* Δ*hilA* Φ(*invH′-lacZ*$^+$) (Hyb)	This study
SV5455	*hilC*::3× FLAG	This study
SV5456	*hilA*::3× FLAG	This study
SV5457	*invF*::3× FLAG	This study
SV5540	Δ*rtsA* Φ(*sipB′-lacZ*$^+$) (Hyb)	This study
SV5541	Δ*dam-231* Δ*rtsA* Φ(*sipB′-lacZ*$^+$) (Hyb)	This study
SV5542	Δ*rtsA* Φ(*invF′-lacZ*$^+$) (Hyb)	This study
SV5543	Δ*dam-231* Δ*rtsA* Φ(*invF′-lacZ*$^+$) (Hyb)	This study
SV5592	DUP[(*purG*)*MudP*(*argG*)] Φ(*hilD-lacZ*)	This study
SV5594	DUP[(*purG*)*MudP*(*argG*)] Δ*hilD* Φ(*hilD-lacZ*)	This study
SV5596	Δ*dam-231* DUP[(*purG*)*MudP*(*argG*)] Φ(*hilD-lacZ*)	This study
SV5598	Δ*dam-231* DUP[(*purG*)*MudP*(*argG*)] Δ*hilD* Φ(*hilD-lacZ*)	This study
SV5624	*hilD*::HA	This study

(*continued*)

TABLE 1

(Continued)

Strain designation	Genotype	Reference or source
SV5625	Δ*dam-231 hilD*::HA	This study
SV5646	Δ*hfq*::*cat*	M. Jakomin
SV5826	P*tetA-hilD* Φ(*sipB′-lacZ*$^+$) (Hyb)	This study
SV5827	Δ*dam-231* P*tetA-hilD* Φ(*sipB′-lacZ*$^+$) (Hyb)	This study
SV5828	P*tetA-hilD*	This study
SV5829	Δ*dam-231* P*tetA-hilD*	This study
SV5847	Δ*dam-231* Δ*hfq*::*cat*	This study
SV5848	Δ*hfq*::*cat* Φ(*hilA′-lacZ*$^+$) (Hyb)	This study
SV5849	Δ*dam-231* Δ*hfq*::*cat* Φ(*hilA′-lacZ*$^+$) (Hyb)	This study
SV5850	Δ*hfq*::*cat* Φ(*invF′-lacZ*$^+$) (Hyb)	This study
SV5851	Δ*dam-231* Δ*hfq*::*cat* Φ(*invF′-lacZ*$^+$) (Hyb)	This study
SV5852	Δ*hfq*::*cat* Φ(*sipB′-lacZ*$^+$) (Hyb)	This study
SV5853	Δ*dam-231* Δ*hfq*::*cat* Φ(*sipB′-lacZ*$^+$) (Hyb)	This study
SV5854	Δ*hfq*::*cat* Φ(*sipC′-lacZ*$^+$) (Hyb)	This study
SV5855	Δ*dam-231* Δ*hfq*::*cat* Φ(*sipC′-lacZ*$^+$) (Hyb)	This study
SV5856	Δ*hfq*::*cat* Φ(*sicA′-lacZ*$^+$) (Hyb)	This study
SV5857	Δ*dam-231* Δ*hfq*::*cat* Φ(*sicA′-lacZ*$^+$) (Hyb)	This study
SV5873	Δ*dam-231 hilC*::3× FLAG	This study
SV5874	Δ*dam-231 hilA*::3× FLAG	This study
SV5875	Δ*dam-231 invF*::3× FLAG	This study
SV5876	Δ*hfq*::*cat* P*tetA-hilD* Φ(*sipB′-lacZ*$^+$) (Hyb)	This study
SV5877	Δ*dam-231* Δ*hfq*::*cat* P*tetA-hilD* Φ(*sipB′-lacZ*$^+$) (Hyb)	This study
SV5878	Δ*hfq*::*cat* P*tetA-hilD* Φ(*invF′-lacZ*$^+$) (Hyb)	This study
SV5879	Δ*dam-231* Δ*hfq*::*cat* P*tetA-hilD* Φ(*invF′-lacZ*$^+$) (Hyb)	This study
SV5961	Δ*rne*::*cat*	This study
SV5962	Δ*dam-231* Δ*rne*::*cat*	This study
SV5963	Δ*pnp*::*cat*	This study
SV5964	Δ*dam-231* Δ*pnp*::*cat*	This study
SV5965	Δ*rne*::*cat* Φ(*hilA′-lacZ*$^+$) (Hyb)	This study
SV5966	Δ*dam-231* Δ*rne*::*cat* Φ(*hilA′-lacZ*$^+$) (Hyb)	This study
SV5967	Δ*rne*::*cat* Φ(*sicA′-lacZ*$^+$) (Hyb)	This study
SV5968	Δ*dam-231* Δ*rne*::*cat* Φ(*sicA′-lacZ*$^+$) (Hyb)	This study
SV5969	Δ*rne*::*cat* Φ(*invF′-lacZ*$^+$) (Hyb)	This study
SV5970	Δ*dam-231* Δ*rne*::*cat* Φ(*invF′-lacZ*$^+$) (Hyb)	This study
SV5971	Δ*rne*::*cat* Φ(*sipB′-lacZ*$^+$) (Hyb)	This study
SV5972	Δ*dam-231* Δ*rne*::*cat* Φ(*sipB′-lacZ*$^+$) (Hyb)	This study
SV5973	Δ*rne*::*cat* Φ(*sipC′-lacZ*$^+$) (Hyb)	This study
SV5974	Δ*dam-231* Δ*rne*::*cat* Φ(*sipC′-lacZ*$^+$) (Hyb)	This study
SV5975	Δ*pnp*::*cat* Φ(*hilA′-lacZ*$^+$) (Hyb)	This study
SV5976	Δ*dam-231* Δ*pnp*::*cat* Φ(*hilA′-lacZ*$^+$) (Hyb)	This study
SV5977	Δ*pnp*::*cat* Φ(*sicA′-lacZ*$^+$) (Hyb)	This study
SV5978	Δ*dam-231* Δ*pnp*::*cat* Φ(*sicA′-lacZ*$^+$) (Hyb)	This study
SV5979	Δ*pnp*::*cat* Φ(*invF′-lacZ*$^+$) (Hyb)	This study
SV5980	Δ*dam-231* Δ*pnp*::*cat* Φ(*invF′-lacZ*$^+$) (Hyb)	This study
SV5981	Δ*pnp*::*cat* Φ(*sipB′-lacZ*$^+$) (Hyb)	This study
SV5982	Δ*dam-231* Δ*pnp*::*cat* Φ(*sipB′-lacZ*$^+$) (Hyb)	This study
SV5983	Δ*pnp*::*cat* Φ(*sipC′-lacZ*$^+$) (Hyb)	This study
SV5984	Δ*dam-231* Δ*pnp*::*cat* Φ(*sipC′-lacZ*$^+$) (Hyb)	This study

and Wanner 2000) were used to integrate either plasmid pCE37 or pCE40 (Ellermeier *et al.* 2002). Unless specified otherwise, all *lac* fusions used in this study are translational. Addition of 3× FLAG and HA epitope tags to protein-coding DNA sequences was carried out using plasmids pSUB11 (Km^r, 3× FLAG) and pSU314 (Cm^r, HA) as templates (Uzzau *et al.* 2001). Transductional crosses using phage P22 HT 105/1 *int201* (Schmieger 1972; G. Roberts, unpublished data) were used for strain construction operations involving chromosomal markers. The transduction protocol was described elsewhere (Garzon *et al.* 1995). To obtain phage-free isolates, transductants were purified by streaking on green plates. Phage sensitivity was tested by cross-streaking with the clear-plaque mutant P22 H5. Reconstruction of chromosomal duplications by P22 HT transduction was performed as previously described (Camacho and Casadesus 2001).

Construction of strain SV5828: Strain SV5298 was transduced with a Tn*10d*Tc pool prepared as previously described (Cano *et al.* 2002). Transductants were selected on LB plates supplemented with tetracycline and X-gal. Independent Lac$^+$

transductants were sought and purified on green plates. Individual isolates were then patched on LB broth with X-gal and LB broth with X-gal and tetracycline. An isolate that was Lac$^+$ in LB broth + X-gal + tetracycline and Lac$^-$ in LB broth + X-gal was used as donor in a P22 HT transductional cross to introduce the Tn*10d*Tc insertion in a wild-type background. A transductant of this kind was propagated as SV5828. Two-strand DNA sequencing of the Tn*10d*Tc element of SV5828 revealed that insertion had occurred in a GGG/GCT motif upstream of *hilD*, with the *tetA* promoter pointing out toward *hilD*. The insertion had thus generated a conditional, tetracycline-dependent *hilD* allele. Additional details about this allele are given in Figure S1 and Figure S2.

Protein extracts and Western blot analysis: Total protein extracts were prepared from bacterial cultures grown at 37° in LB medium until stationary phase (final $OD_{600} \sim 2.5$). Bacterial cells contained in 0.2 ml of culture were collected by centrifugation (16,000 × *g*, 2 min, 4°) and suspended in 50 μl of Laemmli sample buffer [1.3% SDS, 10% (v/v) glycerol, 50 mM Tris-HCl, 1.8% β-mercaptoethanol, 0.02% bromophenol blue, pH 6.8]. Proteins were resolved by Tris-Tricine-PAGE, using 12% gels. Conditions for protein transfer have been described elsewere (Jakomin *et al.* 2008). Primary antibodies were anti-Flag M2 monoclonal antibody (1:5000, Sigma), anti-HA HA.11 monoclonal antibody (1:1000; Covance, Princeton, NJ), and anti-GroEL polyclonal antibody (1:20,000, Sigma). Goat anti-mouse horseradish peroxidase-conjugated antibody (1:5000; Bio-Rad, Hercules, CA) or goat anti-rabbit horseradish peroxidase-conjugated antibody (1:20,000; Santa Cruz Biotechnology, Heildelberg, Germany) was used as secondary antibody. Proteins recognized by the antibodies were visualized by chemoluminescence using the luciferin–luminol reagents.

Quantitative reverse transcriptase PCR and calculation of relative expression levels: RNA was extracted from *S. enterica* stationary phase cultures ($OD_{600} \sim 2.5$), using the SV total RNA isolation system (Promega, Madison, WI) as described at http://www.ifr.ac.uk/safety/microarrays/protocols.html. The quantity and quality of the extracted RNA were determined using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). To diminish genomic DNA contamination, the preparation was treated with DNase I (Turbo DNA free; Applied Biosystems/Ambion, Austin, TX). An aliquot of 0.6 μg of DNase I-treated RNA was used for cDNA synthesis using the High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). Quantitative reverse transcriptase (RT)–PCR reactions were performed in an Applied Biosystems 7500 Fast Real-Time PCR System. Each reaction was carried out in a total volume of 25 μl on a 96-well optical reaction plate (Applied Biosystems) containing 12.5 μl Power SYBR Green PCR Master Mix (Applied Biosystems), 11.5 μl cDNA (1/10 dilution), and two gene-specific primers at a final concentration of 0.2 μM each. Real-time cycling conditions were as follows: (i) 95° for 10 min and (ii) 40 cycles at 95° for 15 sec and 60° for 1 min. No-template control was included for each primer set. Melting curve analysis verified that each reaction contained a single PCR product. Gene expression levels were normalized to transcripts of *ompA* or *gmk*, two housekeeping genes that served as internal controls. Gene-specific primers, designed with PRIMER3 software (http://primer3.sourceforge.net), are listed in Table S1.

Analysis of *hilD* mRNA decay: Use of quantitative RT–PCR to monitor mRNA decay has been previously described (Baker *et al.* 2007). An overnight LB culture of the strain under study was diluted 50-fold and incubated at 37° with shaking until an $OD_{600} \sim 2.5$. Transcription initiation was stopped by adding 500 μg/ml rifampicin and shaking vigorously during 10 sec. Cultures were kept at 37°. Aliquots were extracted at 1-min intervals and treated with a phenol (5%)–ethanol (95%) mixture. Each aliquot was immediately immersed in liquid N_2 and kept frozen until RNA extraction. RNA was extracted using the standard protocol described above. Four independent quantitative (q)RT–PCR reactions, all using primers for the 5′ region of *hilD* mRNA, were used.

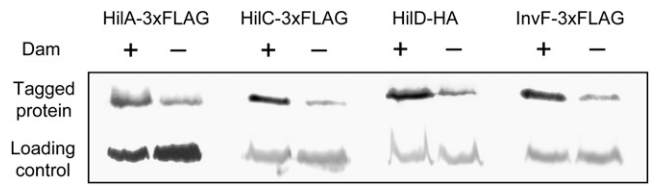

Figure 2.—Levels of HilA, HilC, HilD, and InvF in protein extracts from Dam$^+$ and Dam$^-$ isogenic strains. Epitope-tagged proteins were detected by Western blotting with either anti-FLAG or anti-HA commercial antibodies, as appropriate. The charge control was GroEL in all cases. Strains were SV5456 (*hilA*::3× FLAG), SV5874 (*hilA*::3× FLAG Dam$^-$), SV5455 (*hilC*::3× FLAG), SV5873 (*hilC*::3× FLAG Dam$^-$), SV5624 (*hilD*::HA), SV5625 (*hilD*::HA Dam$^-$), SV5457 (*invF*::3× FLAG), and SV5875 (*invF*::3× FLAG Dam$^-$).

β-Galactosidase assays: Levels of β-galactosidase activity were assayed using the $CHCl_3$-sodium dodecyl sulfate permeabilization procedure (Miller 1972).

RESULTS

Levels of the SPI-1 transcription factors HilA, HilC, HilD, and InvF in Dam$^+$ and Dam$^-$ hosts: We examined the effect of Dam methylation on the levels of the main SPI-1 regulatory proteins: HilA, HilC, HilD, and InvF. For this purpose, we used HilA, HilC, and InvF protein variants tagged with the 3× FLAG epitope and a HilD variant tagged with the HA epitope. Western blot analysis in extracts from isogenic Dam$^+$ and Dam$^-$ strains indicated that all four regulators were less abundant in Dam$^-$ hosts (Figure 2). This observation confirmed that SPI-1 expression is entirely under Dam methylation control as previously proposed (Balbontin *et al.* 2006), but did not provide any hint about the target(s) of Dam-dependent regulation. *In silico* examination of GATC site distribution in or near the *hilA*, *hilC*, *hilD*, and *invF* genes was likewise uninformative (data not shown).

Dam-dependent regulation of SPI-1 is transmitted via HilD: In an attempt to identify the SPI-1 regulator(s), if any, involved in transmission of Dam-dependent control to SPI-1, we examined the involvement of the SPI-1 "general" transcription factors HilA, HilC, and HilD and the *sip*-specific transcription factor InvF (Darwin and Miller 1999; Eichelberg and Galan 1999). RtsA, a general SPI-1 transcription factor encoded outside SPI-1 (Ellermeier and Slauch 2003), was also included in the survey. SPI-1 expression was monitored in a set of mutants, each lacking one SPI-1 transcription factor. Epistasis analysis took advantage of two well known traits of SPI-1 expression. One is

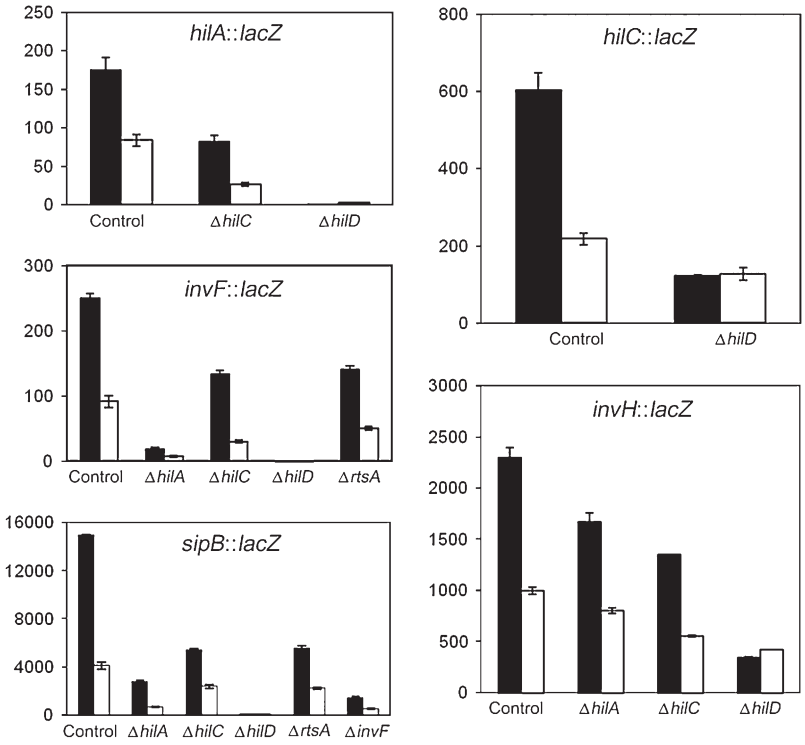

Figure 3.—β-Galactosidase activities of *hilA::lac*, *invF::lac*, *sipB::lac*, *hilC::lac*, and *invH::lac* fusions in the presence and in the absence of individual transcription factors involved in SPI-1 control. Solid histograms represent β-galactosidase activities measured in a Dam$^+$ background. Open histograms represent β-galactosidase activities measured in a Dam$^-$ background. Strains were SV5284 (*hilA::lac*), SV5285 (*hilA::lac* Dam$^-$), SV5401 (*hilA::lac* HilC$^-$), SV5402 (*hilA::lac* HilC$^-$ Dam$^-$), SV5399 (*hilA::lac* HilD$^-$), SV5400 (*hilA::lac* HilD$^-$ Dam$^-$), SV5297 (*invF::lac*), SV5298 (*invF::lac* Dam$^-$), SV5403 (*invF::lac* HilA$^-$), SV5404 (*invF::lac* HilA$^-$ Dam$^-$), SV5405 (*invF::lac* HilC$^-$), SV5406 (*invF::lac* HilC$^-$ Dam$^-$), SV5407 (*invF::lac* HilD$^-$), SV5408 (*invF::lac* HilD$^-$ Dam$^-$), SV5542 (*invF::lac* RtsA$^-$), SV5543 (*invF::lac* RtsA$^-$ Dam$^-$), SV5382 (*sipB::lac*), SV5383 (*sipB::lac* Dam$^-$), SV5316 (*sipB::lac* HilA$^-$), SV5308 (*sipB::lac* HilA$^-$ Dam$^-$), SV5318 (*sipB::lac* HilC$^-$), SV5310 (*sipB::lac* HilC$^-$ Dam$^-$), SV5320 (*sipB::lac* HilD$^-$), SV5312 (*sipB::lac* HilD$^-$ Dam$^-$), SV5540 (*sipB::lac* RtsA$^-$), SV5541 (*sipB::lac* RtsA$^-$ Dam$^-$), SV5322 (*sipB::lac* InvF$^-$), SV5314 (*sipB::lac* InvF$^-$ Dam$^-$), SV5384 (*hilC::lac*), SV5385 (*hilC::lac* Dam$^-$), SV5386 (*hilC::lac* HilD$^-$), SV5387 (*hilC::lac* HilD$^-$ Dam$^-$), SV5301 (*invH::lac*), SV5302 (*invH::lac* Dam$^-$), SV5419 (*invH::lac* HilA$^-$), SV5420 (*invH::lac* HilA$^-$ Dam$^-$), SV5417 (*invH::lac* HilC$^-$), SV5418 (*invH::lac* HilC$^-$ Dam$^-$), SV5415 (*invH::lac* HilD$^-$), and SV5416 (*invH::lac* HilD$^-$ Dam$^-$). Data are averages and standard deviations from three experiments.

regulatory redundancy by certain transcription factors (*e.g.*, HilC and HilD) (Altier 2005; Jones 2005). The other is that lack of a single transcription factor does not completely abolish expression in certain transcriptional units (Ellermeier *et al.* 2005). Expression of SPI-1 transcriptional units was monitored by measuring β-galactosidase activities of *lac* fusions in representative genes. Only those regulators that are known to control a specific SPI-1 transcriptional unit were included in the analysis. For instance, expression of *hilC* in the absence of HilA was not tested because *hilC* is not regulated by *hilA* (Rakeman *et al.* 1999; Lostroh *et al.* 2000). In turn, expression of the *hilA* in the absence of InvF was omitted because InvF is downstream from HilA in the SPI-1 regulatory cascade (Eichelberg *et al.* 1999) (Figure 1). The results of these surveys are shown in Figure 3 and can be summarized as follows:

i. Dam-dependent regulation of *hilA* was not abolished in the absence of HilC. No information was obtained, however, on the potential involvement of HilD on Dam-dependent *hilA* regulation, since a *hilD* mutation completely abolished expression of the *hilA::lac* fusion (Figure 3). In an analogous fashion, Dam-dependent regulation of *invF* was still observed in HilA$^-$, HilC$^-$, and RtsA$^-$ backgrounds, and no information was obtained in a HilD$^-$ background (Figure 3). Similar observations were made for *sipB*, which remained under Dam methylation control in HilA$^-$, HilC$^-$, RtsA$^-$, and InvF$^-$ backgrounds. As above, absence of *sipB* expression in both HilD$^-$ Dam$^+$ and HilD$^-$ Dam$^-$ hosts prevented any conclusion about Dam methylation dependence (Figure 3). However, these experiments provided evidence that none of the HilA, HilC, RtsA, and InvF transcription factors is involved in Dam-dependent control of SPI-1.

ii. Expression of a *hilC::lac* fusion was not completely abolished in a HilD$^-$ background (Figure 3), and similar levels of β-galactosidase activity were detected in cultures of HilD$^-$ Dam$^+$ and HilD$^-$ Dam$^-$ hosts. Similar results were obtained for an *invH::lac* fusion, which remained under Dam methylation control in HilA$^-$ and HilC$^-$ hosts, but not in a HilD$^-$ background (Figure 3). The epistatic effect of a *hilD* mutation over a *dam* mutation thus provided evidence that Dam-dependent regulation of SPI-1 requires a functional *hilD* gene.

Dam methylation regulates the level of *hilD* mRNA: In an attempt to confirm that Dam methylation regulates *hilD* expression, the activity of a *hilD::lac* transcriptional fusion was monitored in Dam$^+$ and Dam$^-$ hosts. To our surprise, no difference was found (Figure 4). However, these experiments left one possibility open. Transcription of *hilD* is under the control of an autogenous, positive feedback loop by the HilD product (Ellermeier *et al.* 2005; Ellermeier and Slauch

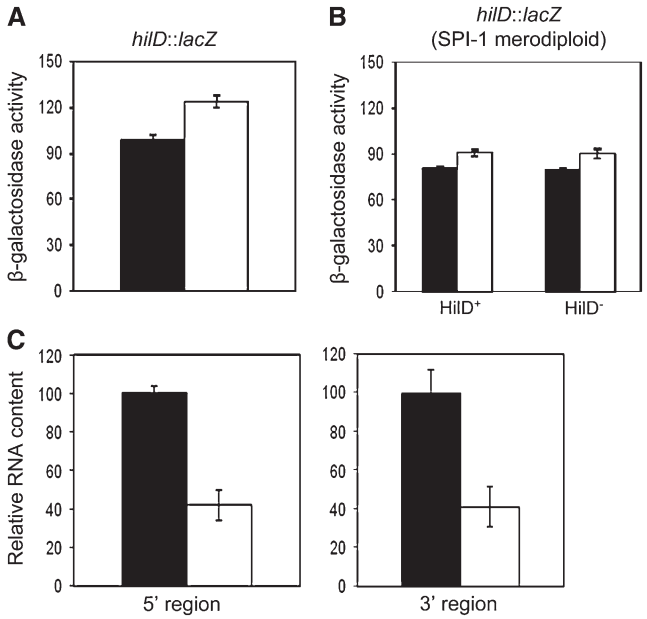

FIGURE 4.—(A) β-Galactosidase activity of a *hilD::lac* transcriptional fusion in Dam$^+$ (SV5286) and Dam$^-$ (SV5288) isogenic hosts. Data are averages and standard deviations from three experiments. (*B*) β-Galactosidase activity of the same *hilD::lac* transcriptional fusion in Dam$^+$ HilD$^+$ (SV5592), Dam$^+$ HilD$^-$ (SV5594), Dam$^-$ HilD$^+$ (SV5596), and Dam$^-$ HilD$^-$ (SV5598) isogenic merodiploids (averages of three experiments). (C) Relative amounts of *hilD* mRNA in Dam$^+$ (ATCC 14028) and Dam$^-$ (SV5264) strains, normalized to *ompA* mRNA. Two primer pairs, complementary to 5′ and 3′ *hilD* regions, were used. Histograms represent the averages from three independent experiments.

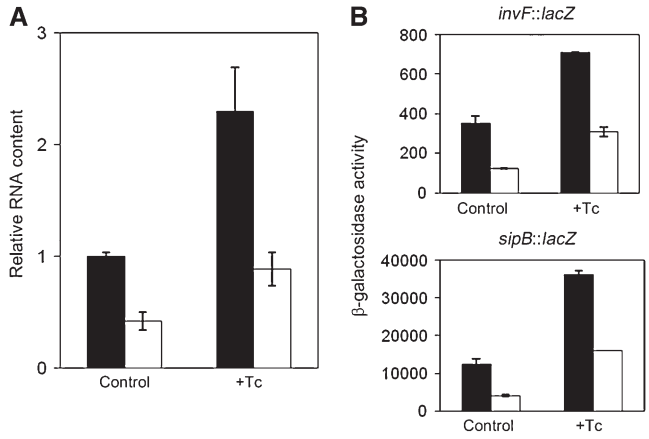

FIGURE 5.—(A) Relative amounts of *hilD* mRNA in Dam$^+$ (solid histograms) and Dam$^-$ (open histograms) isogenic strains expressing *hilD* from a heterologous, tetracycline-dependent promoter. Levels of *hilD* mRNA were normalized to *ompA* mRNA, as above. Strains were SV5828 (P_{tetA}-*hilD*), and SV5829 (*dam* P_{tetA}-*hilD*). Data are averages and standard deviations from three independent experiments. (B) Transcription levels of two SPI-1 genes under HilD control (*invF* and *sipB*) in Dam$^+$ (solid histograms) and Dam$^-$ (open histograms) strains that express *hilD* from a heterologous, tetracycline-dependent promoter. Strains were SV5297 (*invF*::*lac*), SV5298 (*invF*::*lac* Dam$^-$), SV5335 (P_{tetA}-*hilD invF*::*lac*), SV5336 (P_{tetA}-*hilD invF*::*lac* Dam$^-$), SV5382 (*sipB*::*lac*), SV5383 (*sipB*::*lac* Dam$^-$), SV5826 (P_{tetA}-*hilD sipB*::*lac*), and SV5827 (P_{tetA}-*hilD sipB*::*lac* Dam$^-$). Data are averages and standard deviations from three independent experiments.

2008). Hence, use of a *hilD::lac* fusion might prevent the observation of differences, if any, between Dam$^+$ and Dam$^-$ hosts, simply because the *hilD::lac* strain is HilD$^-$. To circumvent this potential problem, the *hilD::lac* fusion was transduced to isogenic Dam$^+$ and Dam$^-$ strains carrying a chromosomal duplication that includes SPI-1 (CAMACHO and CASADESUS 2001). β-Galactosidase activities were then monitored in Dam$^+$ HilD$^+$/*hilD::lac* and Dam$^-$ HilD$^+$/*hilD::lac* merodiploids. No difference was found (Figure 4), thus ruling out the possibility that similar levels of *hilD* expression in Dam$^+$ and Dam$^-$ hosts resulted from disruption of the HilD feedback loop. Evidence that transcription of the *hilD* gene is not under Dam methylation control (Figure 4) was in stark contrast with Western blot experiments showing different levels of HilD protein in Dam$^+$ and Dam$^-$ hosts (Figure 2).

Analysis of *hilD* mRNA content in Dam$^+$ and Dam$^-$ hosts (ATCC 14028 and SV5264, respectively) was performed by quantitative reverse transcriptase PCR, using primer pairs complementary to both the 5′ and the 3′ regions of *hilD*. A lower level of *hilD* mRNA was found in the Dam$^-$ background (Figure 4). Hence, decreased levels of both *hilD* mRNA and HilD protein were found in Salmonella Dam$^-$ hosts (Figures 2 and 4), even though a *hilD::lac* transcriptional fusion did not show Dam-dependent control (Figure 4).

Expression of *hilD* from a heterologous promoter is Dam dependent: The failure of a *hilD::lac* transcriptional fusion to show Dam-dependent regulation admits a number of explanations, artifactual or not. Hence, we considered the possibility that *hilD* regulation by Dam methylation might be in fact transcriptional. If such was the case, we reasoned, Dam-dependent *hilD* regulation should be no longer observed when *hilD* expression was driven from a heterologous promoter. In contrast, Dam dependence in a *hilD* gene driven from a heterologous promoter would provide evidence for post-transcriptional control. On these grounds, we examined whether *hilD* expression remained Dam dependent in strain SV5828. This strain, whose construction is described in MATERIALS AND METHODS, carries a conditional *hilD* mutation that renders the strain HilD$^-$ in the absence of tetracycline and HilD$^+$ in the presence of either tetracycline or autoclaved chlortetracycline. Using this strain and its isogenic Dam$^-$ derivative SV5829, we compared *hilD* mRNA levels in Dam$^+$ and Dam$^-$ hosts in the presence and in the absence of tetracycline. Expression of *hilD* was Dam dependent in the presence of tetracycline (Figure 5), thus indicating that a *hilD* transcript driven by the *tetA* promoter remained under Dam methylation control like wild-type *hilD* mRNA. As a validation for

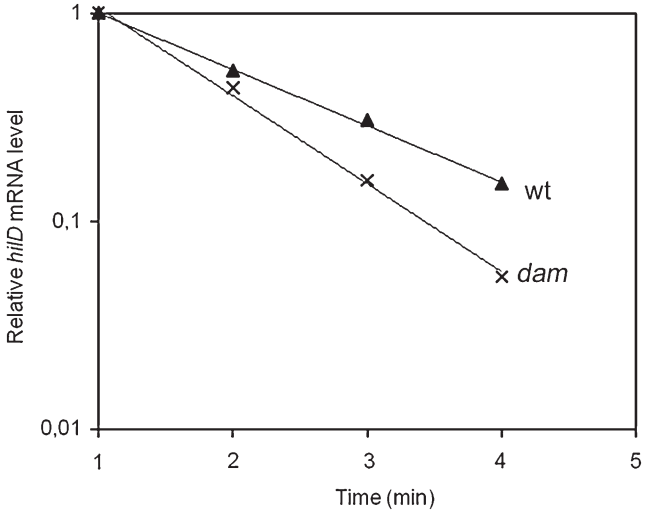

Figure 6.—Stability of *hilD* mRNA in Dam$^+$ (ATCC 14028) and Dam$^-$ (SV5264) isogenic hosts. Values are averages from four independent qRT–PCR reactions. Error bars are not shown because the standard deviations were extremely small.

this conclusion, we observed that expression of *invF::lac* and *sipB::lac* fusions remained under Dam methylation control when *hilD* expression was tetracycline dependent (Figure 5). These results supported the view that Dam methylation might not regulate *hilD* transcription but might regulate *hilD* mRNA stability. This possibility was puzzling, because Dam methylation is a DNA modification function, not known to interact with nucleic acid molecules other than double-stranded DNA (Marinus 1996; Wion and Casadesus 2006).

Dam methylation regulates *hilD* mRNA stability: To compare *hilD* mRNA stability in Dam$^+$ and Dam$^-$ hosts, stationary cultures (OD_{600} = 2.5) were treated with rifampicin to stop transcription. RNA samples were extracted at 1-min intervals and subjected to quantitative RT–PCR primed by two oligonucleotides of the 5′ region of *hilD*. In all RNA preparations, *hilD* mRNA was found to decay in a linear manner from 1 to 4 min after rifampicin addition, and a substantial difference in the decay rate was observed between the RNA preparations from a Dam$^+$ strain and those from a Dam$^-$ mutant (Figure 6). The half lives of *hilD* mRNA were calculated as 67 sec in a Dam$^+$ host and 47 sec in a Dam$^-$ host. These experiments provided direct evidence that *hilD* mRNA is less stable in the absence of Dam methylation. Because increased turnover of RNA is not a trait of Salmonella Dam$^-$ mutants (Balbontin *et al.* 2006), we interpret that *hilD* mRNA may undergo different post-transcriptional regulation in Dam$^+$ and Dam$^-$ hosts.

Lack of Hfq enhances *hilD* mRNA instability in Salmonella Dam$^-$ mutants: The evidence that *hilD* mRNA undergoes post-transcriptional control led us to test the involvement of Hfq, an RNA chaperone that is known to interact with multiple RNA molecules including *hilD* mRNA (Sittka *et al.* 2008). To investigate whether lack of Hfq affected *hilD* mRNA stability, analysis of *hilD* mRNA content was performed in isogenic Dam$^+$ Hfq$^+$, Dam$^-$ Hfq$^+$, Dam$^+$ Hfq$^-$, and Dam$^-$ Hfq$^-$ isogenic strains. Oligonucleotides complementary to both the 5′ and the 3′ regions of *hilD* were used to prime quantitative RT–PCR. In a Dam$^-$ background, the *hilD* mRNA level decreased 2.5-fold in the presence of Hfq and >10-fold in the absence of Hfq (Figure 7). Hence, lack of Hfq enhances the *hilD* mRNA instability caused by a *dam* mutation. A recent study has suggested that binding of Hfq to the AU-rich *hilD* mRNA might be peculiar, in the sense that Hfq might not bind one or more specific RNA regions but the entire mRNA molecule (Sittka *et al.* 2008). This binding pattern might contribute to the Hfq protective effect.

Lack of Hfq enhances the SPI-1 expression defect of Salmonella Dam$^-$ mutants: The effect of an *hfq* null mutation on Dam-dependent SPI-1 expression was examined in five SPI-1 genes, selected on the basis of their strong HilD dependence. β-Galactosidase activities were measured in Dam$^+$ Hfq$^+$, Dam$^-$ Hfq$^+$, Dam$^+$ Hfq$^-$, and Dam$^-$ Hfq$^-$ isogenic strains carrying *hilA::lac*, *sicA::lac*, *invF::lac*, *sipB::lac*, and *sipC::lac* fusions. Raw data are shown in Table S2. Figure 8 is an elaboration of Table S2 data that outlines the differences between Dam$^-$ Hfq$^+$ and Dam$^-$ Hfq$^-$ mutants. Because *lac* fusions in individual SPI-1 genes have disparate β-galactosidase activities, the activity of each fusion has been normalized to 100 in the Dam$^+$ background. Lack of Hfq caused a decrease in SPI-1 expression (Table S2), as previously described (Sittka *et al.* 2007). For the purpose of our study, however, the noteworthy result was that an *hfq* mutation enhanced the SPI-1 expression defect of Dam$^-$ mutants (Figure 7).

Dam-dependent expression of SPI-1 was also affected by an *hfq* mutation when *hilD* was expressed from a heterologous promoter. In the experiments summarized in Figure S3, we compared the expression of *lac* fusions in two SPI-1 genes, *invF* and *sipB*, in isogenic Hfq$^+$ Dam$^+$, Hfq$^+$ Dam$^-$, Hfq$^-$ Dam$^+$, and Hfq$^-$ Dam$^-$ hosts, all expressing *hilD* under the control of the *tetA* promoter. Lack of Hfq enhanced the SPI-1 expression defect of Salmonella Dam$^-$ mutants (Figure S3). Hence, an *hfq* mutation enhances the *hilD* mRNA instability associated to lack of Dam methylation, irrespective of the promoter that drives *hilD* expression.

Lack of degradosome components polyribonucleotide phosphorylase and ribonuclease E suppresses *hilD* mRNA instability in Salmonella Dam$^-$ mutants: If lack of Dam methylation decreases *hilD* mRNA stability, we reasoned, mutations that reduce RNA turnover might suppress the SPI-1 expression defect of Dam$^-$ mutants. On these grounds, we constructed mutants lacking either ribonuclease E (Rne) or polynucleotide phosphorylase (Pnp), two components of the bacterial degradosome (Carpousis 2002). Ribonuclease E had

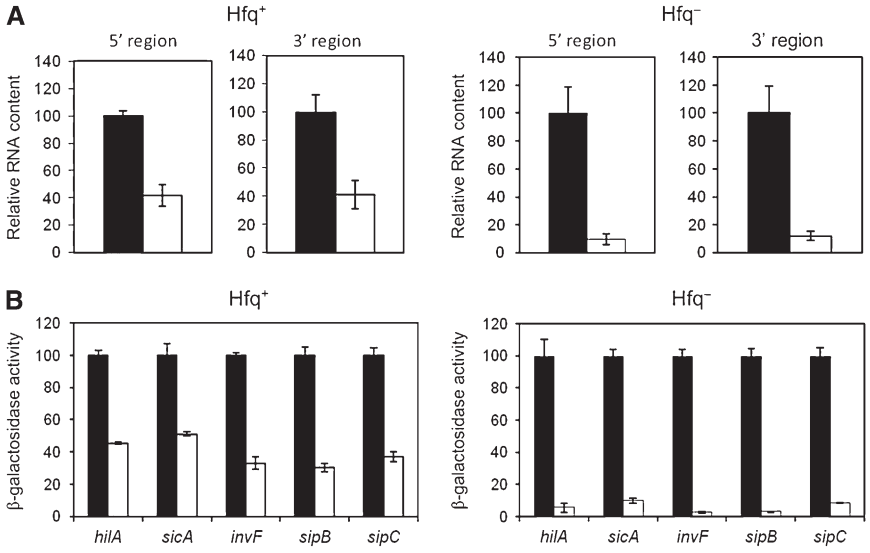

FIGURE 7.—(A) Enhancement of *hilD* mRNA instability in the absence of Hfq. Solid histograms are for Dam$^+$ strains, and open histograms are for their Dam$^-$ derivatives. RNA levels were normalized to either *ompA* mRNA or *gmk* mRNA. Strains were ATCC 14208 (wild type), SV5264 (Dam$^-$), SV5646 (Hfq$^-$), and SV5847 (Hfq$^-$ Dam$^-$). Values are averages and standard deviations from three independent experiments. (B) Enhancement of the SPI-1 expression defect of *S. enterica* Dam$^-$ mutants by *hfq* null mutations. Solid histograms are for Dam$^+$ strains, and open histograms are for their Dam$^-$ derivatives. To facilitate visual perception of differences, the β-galactosidase activities of individual *lac* fusions in Dam$^+$ hosts have been normalized to 100. Strains were as follows: SV5284 (*hilA*::*lac*), SV5285 (*hilA*::*lac* Dam$^-$), SV5278 (*sicA*::*lac*), SV5279 (*sicA*::*lac* Dam$^-$), SV5297 (*invF*::*lac*), SV5298 (*invF*::*lac* Dam$^-$), SV5382 (*sipB*::*lac*), SV5383 (*sipB*::*lac* Dam$^-$), SV5293 (*sipC*::*lac*), SV5294 (*sipC*::*lac* Dam$^-$), SV5848 (*hilA*::*lac* Hfq$^-$), SV5849 (*hilA*::*lac* Hfq$^-$ Dam$^-$), SV5856 (*sicA*::*lac* Hfq$^-$), SV5857 (*sicA*::*lac* Hfq$^-$ Dam$^-$), SV5850 (*invF*::*lac* Hfq$^-$), SV5851 (*invF*::*lac* Hfq$^-$ Dam$^-$), SV5852 (*sipB*::*lac* Hfq$^-$), SV5853 (*sipB*::*lac* Hfq$^-$ Dam$^-$), SV5854 (*sipC*::*lac* Hfq$^-$), and SV5855 (*sipC*::*lac* Hfq$^-$ Dam$^-$). Data are averages and standard deviations from three experiments.

been previously described as a SPI-1 regulator (FAHLEN *et al.* 2000). For construction of an Rne$^-$ mutant, only a portion at the 3′ end of the *rne* coding sequence was eliminated (VIEGAS *et al.* 2007). Analysis of *hilD* mRNA content was performed in two sets of experiments. In the first set, Dam$^+$ Rne$^+$, Dam$^-$ Rne$^+$, Dam$^+$ Rne$^-$, and Dam$^-$ Rne$^-$ isogenic strains were used. In the second set, we employed Dam$^+$ Pnp$^+$, Dam$^-$ Pnp$^+$, Dam$^+$ Pnp$^-$, and Dam$^-$ Pnp$^-$ isogenic strains. Oligonucleotides complementary to both the 5′ and the 3′ regions of *hilD* (Table S1) were used to prime quantitative RT–PCR. Both *rne* and *pnp* mutations restored the *hilD* mRNA level of Salmonella Dam$^-$ mutants to levels similar to those found in a Dam$^+$ strain (Figure 8A). Hence, lack of either Rne or Pnp suppresses the *hilD* mRNA instability caused by a *dam* mutation.

Lack of degradosome components Rne and Pnp suppresses the SPI-1 expression defect of Salmonella Dam$^-$ mutants: The effect of *rne* and *pnp* mutations on Dam-dependent SPI-1 expression was examined in five SPI-1 genes strongly dependent on HilD (as above). β-Galactosidase activities were measured in two sets of isogenic strains. One set carried *hilA*::*lac*, *sicA*::*lac*, *invF*::*lac*, *sipB*::*lac*, and *sipC*::*lac* fusions in Dam$^+$/Dam$^-$ Rne$^+$/Rne$^-$ backgrounds. The second set carried the same fusions in Dam$^+$/Dam$^-$ Pnp$^+$/Pnp$^-$ backgrounds. Raw data are shown in Table S2. Figure 8B is a normalized presentation of Table S2 data that outlines the differences between Dam$^-$ Rne$^+$ and Dam$^-$ Rne$^-$ mutants, as well as those found between Dam$^-$ Pnp$^+$ and Dam$^-$ Pnp$^-$ mutants. In the Dam$^-$ background, lack of ribonuclease E increased expression of all SPI *lac* fusions about twofold (Figure 8B). In turn, lack of polyribonucleotide phosphorylase completely restored the wild-type level of expression in the five *lac* fusions used to monitor SPI-1 expression (Figure 8B). Partial suppression by an *rne* mutation and complete supression by a *pnp* mutation further strengthen the evidence that the SPI-1 expression defect of Salmonella Dam$^-$ mutants is post-transcriptional.

DISCUSSION

Lowered levels of all SPI-1-encoded transcriptional regulators (HilA, HilC, HilD, and InvF) are found in Salmonella Dam$^-$ mutants (Figure 2), thereby confirming that the entire SPI-1 is under Dam-dependent control. Epistasis analysis indicates that SPI-1 activation by Dam methylation requires HilD, while the remaining SPI-1 transcriptional activators (HilA, HilC, RtsA, and InvF) are dispensable for Dam-dependent control (Figure 3). Hence, the first conclusion of this study is that Dam methylation activates SPI-1 expression by sustaining high levels of the HilD transcription factor. In the absence of Dam methylation, the HilD level is lower, and SPI expression decreases. This defect may contribute to the reduced capacity of Salmonella Dam$^-$ mutants to invade epithelial cells (GARCIA-DEL PORTILLO *et al.* 1999).

Because the methylation state of critical GATC sites can control binding of RNA polymerase and transcription factors, differences in gene expression between Dam$^+$ and Dam$^-$ hosts usually provide evidence for transcriptional regulation (ROBERTS *et al.* 1985; KÜCHERER *et al.* 1986; BLYN *et al.* 1989; TORREBLANCA and CASADESUS 1996; HAAGMANS and VAN DER WOUDE

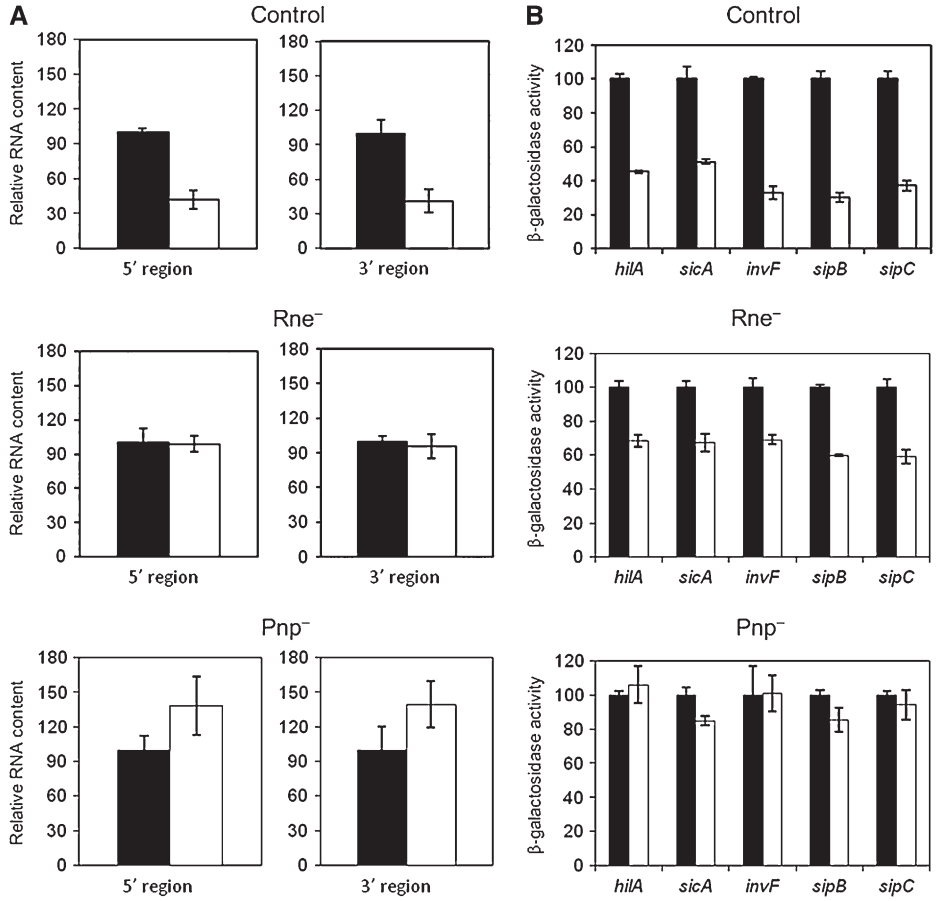

Figure 8.—(A) Suppression of *hilD* mRNA instability in the absence of degradosome components ribonuclease E (Rne) and polynucleotide phosphorylase (Pnp). Solid histograms are for Dam$^+$ strains, and open histograms are for their Dam$^-$ derivatives. RNA levels were normalized to either *ompA* mRNA or *gmk* mRNA. Strains were ATCC 14028 (wild type), SV5264 (Dam$^-$), SV5961 (Rne$^-$), SV5962 (Rne$^-$ Dam$^-$), SV5963 (Pnp$^-$), and SV5964 (Pnp$^-$ Dam$^-$). Values are averages and standard deviations from three independent experiments. (B) Suppression of the SPI-1 expression defect of *S. enterica* Dam$^-$ mutants by *rne* and *pnp* mutations. Solid histograms are for Dam$^+$ strains, and open histograms are for their Dam$^-$ derivatives. To facilitate visual perception of differences, the β-galactosidase activities of *lac* fusions in individual SPI-1 genes in Dam$^+$ hosts have been normalized to 100. Strains were as follows: SV5284 (*hilA*::*lac*), SV5285 (*hilA*::*lac* Dam$^-$), SV5278 (*sicA*::*lac*), SV5279 (*sicA*::*lac* Dam$^-$), SV5297 (*invF*::*lac*), SV5298 (*invF*::*lac* Dam$^-$), SV5382 (*sipB*::*lac*), SV5383 (*sipB*::*lac* Dam$^-$), SV5293 (*sipC*::*lac*), SV5294 (*sipC*::*lac* Dam$^-$), SV5965 (*hilA*::*lac* Rne$^-$), SV5966 (*hilA*::*lac* Rne$^-$ Dam$^-$), SV5967 (*sicA*::*lac* Rne$^-$), SV5968 (*sicA*::*lac* Rne$^-$ Dam$^-$), SV5969 (*invF*::*lac* Rne$^-$), SV5970 (*invF*::*lac* Rne$^-$ Dam$^-$), SV5971 (*sipB*::*lac* Rne$^-$), SV5972 (*sipB*::*lac* Rne$^-$ Dam$^-$), SV5973 (*sipC*::*lac* Rne$^-$), SV5974 (*sipC*::*lac* Rne$^-$ Dam$^-$), SV5975 (*hilA*::*lac* Pnp$^-$), SV5976 (*hilA*::*lac* Pnp$^-$ Dam$^-$), SV5977 (*sicA*::*lac* Pnp$^-$), SV5978 (*sicA*::*lac* Pnp$^-$ Dam$^-$), SV5979 (*invF*::*lac* Pnp$^-$), SV5980 (*invF*::*lac* Pnp$^-$ Dam$^-$), SV5981 (*sipB*::*lac* Pnp$^-$), SV5982 (*sipB*::*lac* Pnp$^-$ Dam$^-$), SV5983 (*sipC*::*lac* Pnp$^-$), and SV5984 (*sipC*::*lac* Pnp$^-$ Dam$^-$). Data are averages and standard deviations from three experiments.

2000; Camacho and Casadesus 2002; Waldron *et al.* 2002; Balbontin *et al.* 2006; Jakomin *et al.* 2008). However, several lines of evidence suggest that Dam-dependent regulation of *hilD* expression is not transcriptional: (i) a transcriptional *hilD*::*lac* fusion is expressed at similar levels in Dam$^+$ and Dam$^-$ hosts (Figure 4); (ii) reduced levels of both *hilD* mRNA and HilD protein are, however, found in Dam$^-$ mutants (Figures 2 and 4); (iii) reduced amounts of *hilD* mRNA are found in a Dam$^-$ mutants when the *hilD* gene is expressed from a heterologous promoter (Figure 5); (iv) SPI-1 remains under Dam-dependent control when *hilD* transcription is activated by tetracycline (Figure 5); and (v) lack of DNA adenine methylation results in *hilD* mRNA instability (Figure 6). Therefore, the second, unsuspected conclusion from this study is that Dam methylation does not regulate *hilD* transcription but does regulate *hilD* mRNA turnover.

The hypothesis, at first sight odd, that Dam methylation is a post-transcriptional regulator of SPI-1, receives further support from the nature of mutations that act either as enhancers or as suppressors of *hilD* mRNA instability. Lack of the Hfq RNA chaperone enhances the SPI-1 expression defect of Salmonella Dam$^-$ mutants (Figure 7) and increases *hilD* mRNA instability (Figure 7). In turn, lack of degradosome components ribonuclease E or polynucleotide phosphorylase (Carpousis 2002) suppresses the SPI-1 expression defect of Salmonella Dam$^-$ mutants (Figure 8). Hfq has been previously shown to stabilize *hilD* mRNA (Sittka *et al.* 2008), and our observations indicate that absence of Hfq results in increased *hilD* mRNA degradation in a Dam$^-$ background (Figure 7). Binding of Hfq to *hilD* mRNA is unusual, and a tentative explanation is that Hfq may "coat" the entire *hilD* transcript (Sittka *et al.* 2008). Hence, Hfq binding might slow down *hilD* mRNA turnover. This possibility is supported by a previous study in *E. coli*, indicating that Hfq protects AU-rich RNA molecules from degradation by ribonuclease E and polynucleotide phosphorylase (Folichon *et al.* 2003).

The occurrence of Dam-dependent post-transcriptional control of *hilD* stability fits well in the current view that *hilD* mRNA may be the target for integration of multiple signals that regulate SPI-1 expression (Lucas and Lee

2001; Ellermeier and Slauch 2008; Kage *et al.* 2008). However, with the potential exception of FliZ (Kage *et al.* 2008) and CsrA (Altier *et al.* 2000; Ellermeier and Slauch 2007), post-transcriptional regulators of *hilD* seem to affect either the HilD protein level (Takaya *et al.* 2005; Matsui *et al.* 2008) or HilD protein activity (Baxter *et al.* 2003; Ellermeier and Slauch 2008). In contrast, Dam methylation regulates *hilD* mRNA turnover.

Because no evidence exists that Dam methylase can interact with RNA molecules, conceivable models to explain Dam-dependent control of *hilD* mRNA stability are either that Dam^+ hosts produce a factor that stabilizes *hilD* mRNA or that Dam^- mutants produce a *hilD* mRNA destabilizing factor. Such hypothetical factor(s) might be, for instance, an Hfq-independent sRNA or an RNA-binding protein. None of the RNA metabolism proteins investigated in this study (Hfq, ribonuclease E, and polynucleotide phosphorylase) is under transcriptional control by Dam methylation, as indicated by qRT–PCR experiments shown in Figure S4.

Additional cases in which Dam methylation appears to exert post-transcriptional control of gene expression are found in the literature. Dam^- mutants of enterohemorrhagic *E. coli* (EHEC) synthesize elevated levels of three virulence proteins (intimin, Tir, and $EspF_U$). However, the corresponding mRNA levels remain unaltered (Campellone *et al.* 2007), suggesting the possibility that Dam-dependent regulation is translational. In *Y. enterocolitica*, overproduction of Dam methylase alters the composition of the O antigen, increasing the amount of lipid A core. However, the transcript levels in the O antigen cluster remain unaltered in Dam-overproducing strains, thus raising the possibility that Dam-dependent regulation is post-transcriptional (Falker *et al.* 2007). Another intriguing case involves the *E. coli* DNA repair endonuclease Vsr. The *vsr* gene is cotranscribed with the DNA cytosine methylase gene, *dcm* (Bell and Cupples 2001). In stationary cultures of *E. coli* Dam^- mutants, Vsr synthesis is reduced while Dcm synthesis is not (Bell and Cupples 2001). Hence, differential mRNA translation and/or differential degradation of the *dcm-vsr* transcript may occur in Dam^- hosts. Like the *hilD* mRNA stability control presented in this study, those cases from the literature remain to be deciphered at the molecular level. However, their very existence is interesting since it indicates that Dam methylation has additional, hitherto unsuspected physiological functions. Their identification is therefore a challenge for future studies.

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TABLE S1

Oligonucleotides used in this study (5'→3')

Oligonucleotide	Sequence
hilCUP	agggcatattgatttttcttcactggaagtttcctatgacattccggggatccgtcgacc
hilCDO	attgtacgcataaagctaagcggtgtaatcttaaaatgccgtgtaggctggagctgcttc
hilDUP	aaatgtaacctttgtaagtaatagtcatcagcgtcctgccattccggggatccgtcgacc
hilDDO	ttcattcttgccgataagtagatgtcgctaaagctggtacgtgtaggctggagctgcttc
hilAUP	atccgagagtctgcattactctatcgtgaagggattatcgattccggggatccgtcgacc
hilADO	gcttcgccgtgggcaaccagcactaacggtaataatcccggtgtaggctggagctgcttc
invFUP	aggattagtggacacgacatatgctgaatccgataaatggattccggggatccgtcgacc
invFDO	aaatgtgaaggcgatgagtaaccatgattaacggctaattgtgtaggctggagctgcttc
sipBUP	cctcgctgaggcggcttttgaaggcgttcgtaagaacacgattccggggatccgtcgacc
sipBDO	cgcgaagcatccgcattttgctgtaccgcagaagacatgggtgtaggctggagctgcttc
sipCUP	tagcagcagtaaagtcagtgacctggggttgagtcctacaattccggggatccgtcgacc
sipCDO	tcctgaatcaggctggtcgatttacgtgaactttcacggggtgtaggctggagctgcttc
sicAUP	ggaaatgatttgggatgccgttagtgaaggcgccacgctaattccggggatccgtcgacc
sicADO	tccttttcttgttcactgtgctgctctgtctccgccgtttgtgtaggctggagctgcttc
invHUP	tcctgtctttttactgatcggctgtgctcaggtgcccctcattccggggatccgtcgacc
invHDO	gcttgcagtctttcatgggcagcaagtaacgtctgatatagtgtaggctggagctgcttc
rtsAUP	aaatttactgcagtccgtactcatcaagctcaccacgggtattccggggatccgtcgacc
rtsADO	ttaacatattgatgacgagaggaagataaaaacgctaaaagtgtaggctggagctgcttc
hilD-HAUP	taaaactacgccatcgacattcataaaaatggcgaaccattatccgtatgatgttcctga
hilD-HADO	ttaataaaaatctttacttaagtgacagatacaaaaaatgcatatgaatatcctccttag
hilC-3xFLAGUP	taagattacaccgcttagctttatgcgtacaatgaaccatgactacaaagaccatgacgg
hilC-3xFLAGDO	taacgcaaacagatagtaacgtttaaaataatttcacaaacatatgaatatcctccttag
hilA-3xFLAGUP	caaaagatggaaacaggatccccgcttgattaaattacgggactacaaagaccatgacgg
hilA-3xFLAGDO	acgatgataaaaaaaataatgcatatctcctctctcagattcatatgaatatcctccttag
invF-3xFLAGUP	gccgcggaaattatcaaatattattcaattggcagacaaagactacaaagaccatgacgg
invF3xFLAGDO	gcggcacatgccagcactctggccaaaagaatatgtgtctcatatgaatatcctccttag
RT-hilD5'-UP	agtttgctttcggagcggta
RT-hilD5'-DO	agcaccaacatcccaggttc
RT-hilD3'-UP	agcttacggatgttgccgatc
RT-hilD3'-DO	gcctgattcattcttgccgata
RT-ompA-UP	tgtaagcgtcagaaccgatacg
RT-ompA-DO	gagcaacctggatccgaaag
RT-gmk-UP	ttggcagggaggcgttt
RT-gmk-DO	gcgcgaagtgccgtagtaat
hilC-E1	acgaaatgaacgcgcgttgg
hilC-E2	tcactggtgtagcgatactg

hilD-E1	agaccattgccaacacacgc
hilD-E2	gcgtgttaatgcgcagtctg
hilA-E1	tactcaacatggacggctcc
hilA-E2	aagccagcaatcagcccatg
invF-E1	accagtatcaggagacctgg
invF-E2	tgtaaccagaacaagcgcgg
sipB-E1	gcgttggtctatctggaggc
sipB-E2	tttatgcgcgactctggcgc
sipC-E1	gcttcgcaatccgttagcgc
sipC-E2	atagcagcgagtgcggatgc
sicA-E1	tgttcactaaccaccgtcgg
sicA-E2	gctttcgttgccaccacatc
invH-E1	gtcagataacgttctgacgg
invH-E2	gatgagttcagccaacggtg
rtsA-E1	gttgtatgcctttcctggcc
rtsA-E2	Tccagagttgccttgcctac
rneUP	gaaacgaaaaccgtcgaaacagccgcgccgaaagcggaagcatatgaatatcctccttag
rneDO	aaaagccgacctggcggtcggctttgtatcagcatttacatgtaggctggagctgcttcg
pnpUP	gcgcgtcaggccactgccgctgttatggtaagcatggatgcatatgaatatcctccttag
pnpDO	agccgcaggttgagactgctcggttgcttctttaatgctctgtaggctggagctgcttcg
rne-E1	gacattcgctatgccagatg
rne-E2	tcataaacgcctggagtgac
pnp-E1	cttccgttgcagaggttcgc
pnp-E2	tcaacaaggcgtccagccag
RT-hfq-UP	cgatttctactgttgtcccgtc
RT-hfq-DO	ccgtgatggtagttattgctgg
RT-rne-UP	aagagacaaaagcggaagcg
RT-rne-DO	acttttccaccacctgggc
RT-pnp-UP	tcccggttaaggttctggaa
RT-pnp-DO	caggttgagactgctcggttg

TABLE S2

Effect of *hfq*, *rne*, and *pnp* mutations on SPI expression

	Background							
Fusion	wt	*dam*	*hfq*	*hfq dam*	*rne*	*rne dam*	*pnp*	*pnp dam*
hilA::*lacZ*	288,1	144,5	114,7	6,3	1401,3	959,7	725,3	769,5
sicA::*lacZ*	1945,3	990,9	766,8	74,3	4639,0	3115,2	5168,8	4385,7
invF::*lacZ*	401,5	132,9	87,4	2,2	516,6	356,7	530,3	535,7
sipB::*lacZ*	17795,8	5373,7	2055,5	61,1	39031,0	23418,0	20235,7	17282,3
sipC::*lacZ*	7940,8	2949,6	1950,5	167,0	29602,9	17447,1	28290,5	26667,3

β-galactosidase activities are averages of 3 independent experiments. Standard deviations are omitted for simplicity.

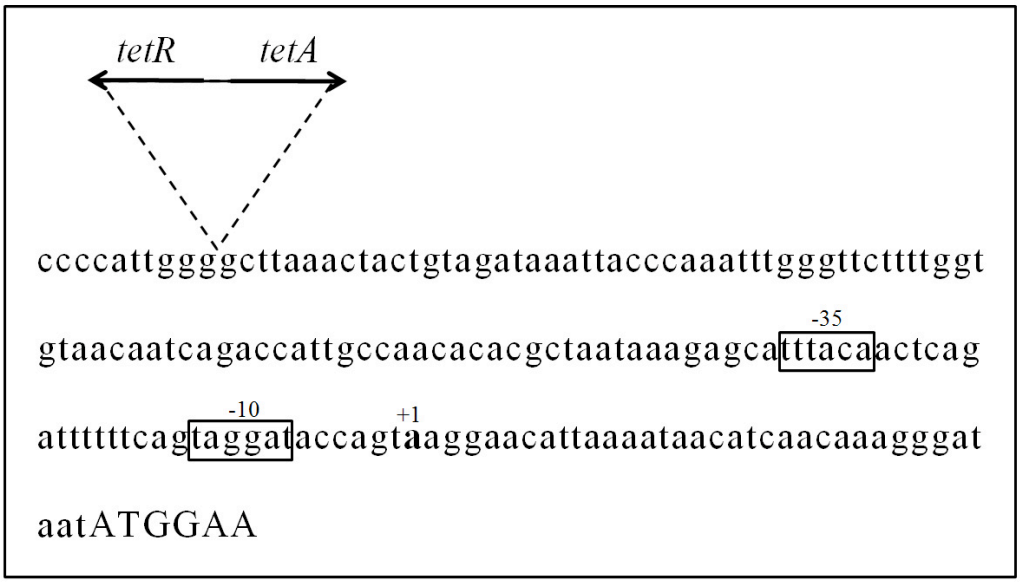

FIGURE S1.—Diagram of the *hilD* promoter region in strain SV5828. A Tn*10d*Tc insertion upstream the *hilD* promoter, with the *tetA* promoter in the proper orientation to transcribe *hilD*, was obtained with the genetic screen for Lac$^+$ derivatives of a Dam$^-$ strain carying an *invF::lac* translational fusion (SV5298). The diagram shows the Tn*10d*Tc insertion site, the -35 and -10 modules of the *hilD* promoter, the start site of the *hilD* transcript, and the first two codons of the *hilD* coding sequence.

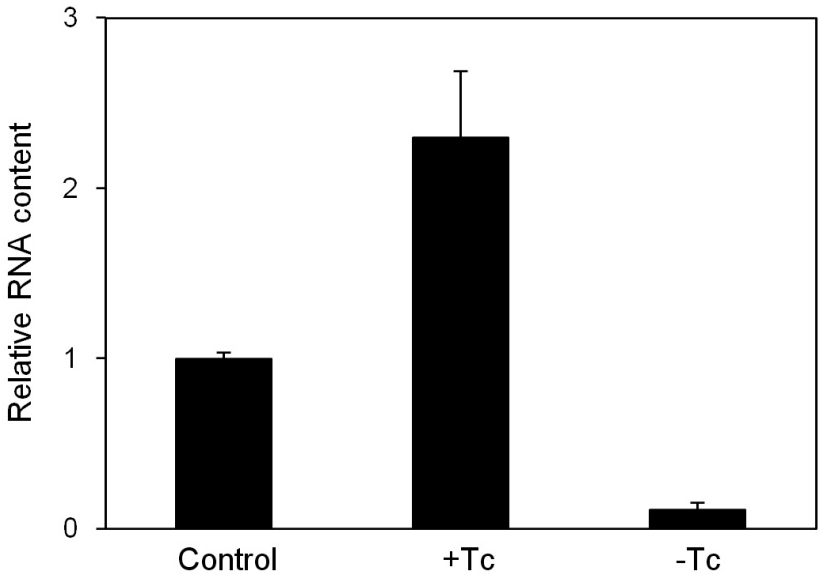

FIGURE S2.—Tetracycline dependence of *hilD mRNA* synthesis in strain SV5828. Levels of *hilD mRNA* were measured by quantitative RT-PCR using the RT-hilD5'-UP and RT-hilD5'-DO oligonucleotides (Table S1). The control was strain ATCC 14028. Tetracycline was used at the final concentration of 10 mg/ml. Transcription of *hilD* in strain SV5828 is not completely abolished in the absence of tetracycline. However, insertion of the Tn*10d*Tc element upstream the *hilD* promoter decreases *hilD* transcription around 9 fold. As a consequence, SV5828 is HilD$^-$ (leaky) in the absence of tetracycline, and HilD$^+$ in the presence of tetracycline.

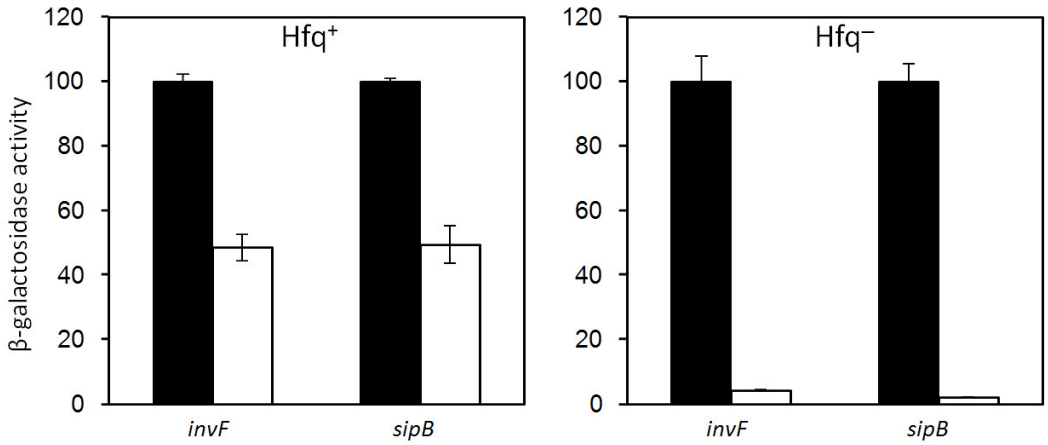

FIGURE S3.—Effect of *dam* and *hfq* mutations on the expression of SPI-1 genes *invF* and *sipB* when *hilD* is expressed from an heterologous, tetracycline-dependent promoter. β-galactosidase activities are averages of 3 independent experiments.

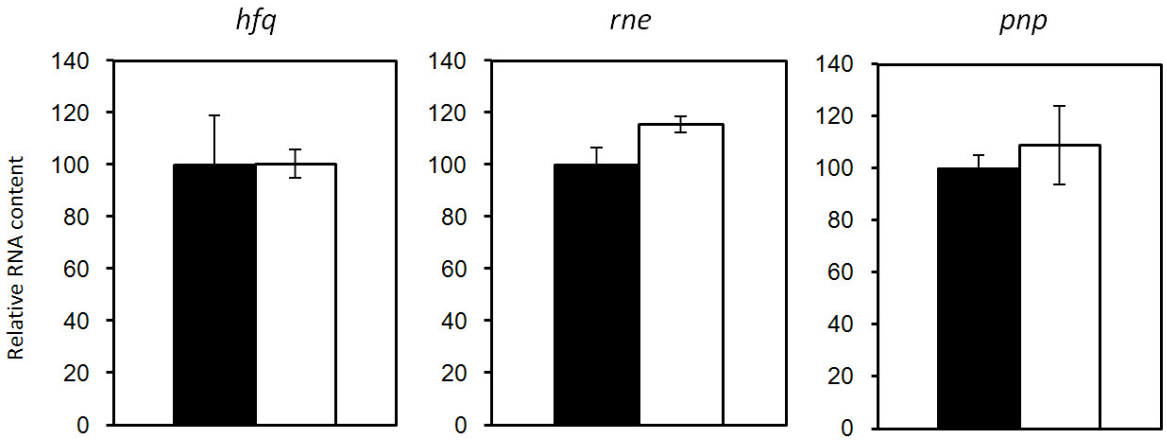

FIGURE S4.—Levels of the *hfq*, *rne* and *pnp* transcripts in Dam$^+$ and Dam$^-$ strains of *S. enterica*. Relative amounts of *hfq, rne, and pnp* mRNAs in Dam$^+$ (black histograms) and Dam$^-$ (white histograms) strains. RNA levels were normalized to *ompA* mRNA. Data are averages and standard deviations from 4 independent experiments.