Crosstalk between Virulence Loci: Regulation of *Salmonella enterica* Pathogenicity Island 1 (SPI-1) by Products of the *std* Fimbrial Operon

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Abstract

Invasion of intestinal epithelial cells is a critical step in *Salmonella* infection and requires the expression of genes located in *Salmonella* pathogenicity island 1 (SPI-1). A key factor for SPI-1 expression is DNA adenine (Dam) methylation, which activates synthesis of the SPI-1 transcriptional activator HilD. Dam-dependent regulation of *hilD* is posttranscriptional (and therefore indirect), indicating the involvement of unknown cell functions under Dam methylation control. A genetic screen has identified the *std* fimbrial operon as the missing link between Dam methylation and SPI-1. We show that all genes in the *std* operon are part of a single transcriptional unit, and describe three previously uncharacterized ORFs (renamed *stdD*, *stdE*, and *stdF*). We present evidence that two such loci (*stdE* and *stdF*) are involved in Dam-dependent control of *Salmonella SPI-1*: in a Dam⁻ background, deletion of *stdE* or *stdF* suppresses SPI-1 repression; in a Dam⁺ background, constitutive expression of *StdE* and/or *StdF* represses SPI-1. Repression of SPI-1 by products of *std* operon explains the invasion defect of *Salmonella Dam⁻* mutants, which constitutively express the *std* operon. Dam-dependent repression of *std* in the ileum may be required to permit invasion, as indicated by two observations: constitutive expression of *StdE* and *StdF* reduces invasion of epithelial cells *in vitro* (1,000 fold) and attenuates *Salmonella* virulence in the mouse model (≥60 fold). In turn, crosstalk between *std* and SPI-1 may play a role in intestinal infections by preventing expression of SPI-1 in the caecum, an intestinal compartment in which the *std* operon is known to be expressed.

Introduction

*Salmonella enterica* is a Gram-negative bacterium that causes intestinal and systemic diseases in a variety of animal hosts [1]. *Salmonella* is a typical foodborne pathogen, and infection usually starts by the ingestion of contaminated food or water [2]. *Salmonella* has the ability to penetrate epithelial cells in the small intestine, a process known as invasion [3,4]. After invasion, the infection can remain localized in the intestine, producing gastroenteritis. In specific serovar-host combinations, however, *Salmonella* can cross the intestinal epithelial barrier and disseminate inside the host, producing a systemic, life-threatening infection (e.g., typhoid fever in humans). It has been estimated that >90 million cases of *Salmonella*-associated gastroenteritis and >20 million cases of typhoid fever occur per year worldwide, resulting in 155,000 and 200,000 deaths respectively [5,6].

*Salmonella* and *Escherichia* are close relatives, and it has been estimated that genus divergence occurred 120–160 million years ago [7]. *Salmonella* pathogenicity has evolved by sequential acquisition of genetic elements, each contributing to distinct aspects of virulence [8,9]. Amongst those elements are the *Salmonella* pathogenicity islands (SPIs), which are clusters of virulence genes located in the chromosome. More than 10 SPIs have been described [10], although some of them are serotype-specific. These regions are absent in the chromosome of other enterics and usually have a G+C content different from that of the *Salmonella* chromosome, suggesting that they have been acquired by horizontal transfer [9,11]. This view is supported by two additional lines of evidence. First, SPIs are frequently inserted at tRNA genes [10], which are hotspots for the integration of foreign DNA elements [12,13]. Second, some SPIs contain putative homologs of genes encoding integrases or transposases, and are flanked by direct repeats [8,10].

*Salmonella* infection requires coordinated expression of virulence genes. During evolution, SPIs have been integrated into pre-existing regulatory networks, thus resulting in crosstalk between the *Salmonella* core genome and horizontally-acquired genetic elements [8]. One of the best characterized SPIs is the *Salmonella* pathogenicity island 1 (SPI-1), which is necessary for invasion of epithelial cells in the animal intestine. SPI-1 encodes a type 3 secretion system (TTSS) as well as effector proteins that are translocated into the eukaryotic cell cytoplasm [14–16]. SPI-1 expression is controlled by four SPI-1-encoded transcriptional activators: HilA, HilC, HilD, and InvF [14,17–19]. These regulators form a regulatory network that incorporates regulatory inputs from global regulators. For instance, the leucine-responsive regulatory protein, Lrp, reduces SPI-1 expression by repressing transcription of *hilA* and *invF* [20]. The nucleoid-associated...
proteins H-NS and Hha repress *hilA* expression by direct binding to regions located upstream and downstream the *hilA* promoter [21,22]. HilC and HilD are substrates for the ATP-dependent Lon protease [23], which contributes to SPI-1 repression after invasion of epithelial cells [24]. HilE is a negative regulator of SPI-1 [25] and may interfere with HilD function by direct protein-protein interaction [26]. Transcription of *hilE* is activated by the fimbrial regulator FimYZ [27], and is repressed by the PTS-dependent regulator Mlc [28], thus transmitting inputs to SPI-1 through HilD. In addition, the two-component systems PhoP/PhoQ and PhoB/PhoR may activate *hilE* expression [18,19].

SPI-1 is also regulated by the Csr system [29]. Overexpression of csrA represses SPI-1 expression [29,30]. CsrA binds to a region in *hilD* mRNA that overlaps with the ribosome-binding sequence, likely preventing translation and accelerating mRNA decay [30]. The two-component regulatory system BarA/SirA activates SPI-1 expression through the Csr pathway, activating transcription of the *csrB* and *csrC* genes, which encode CsrA antagonists [31]. Another activator of SPI-1 is the ferric uptake regulator, Fur, and the mechanism of regulation is controversial [32–34]. The EnvZ/OmpR two-component system activates SPI-1, likely by controlling *hilD* expression at the posttranscriptional level [18,35]. Furthermore, a recent report shows that FliZ, an RpoS inhibitor [36], activates SPI-1 expression by controlling HilD activity [37]. A diagram that summarizes SPI-1 regulation is shown in Figure 1.

In previous studies, we showed that DNA adenine (Dam) methylation is necessary to sustain a high level of SPI-1 expression [38–40]. Genetic analysis indicated that Dam-dependent regulation of SPI-1 is transmitted via HilD [39]. However, Dam-dependent regulation of *hilD* is not transcriptional but posttranscriptional [39], and several lines of evidence suggest that a posttranscriptional regulator whose synthesis is Dam-dependent may control *hilD* mRNA stability [39]. In this study, we show that Dam-dependent posttranscriptional regulation of *hilD* is exerted by products encoded on another horizontally-acquired genetic element, the *std* fimbrial cluster. Std fimbriae belong to the chaperone-usher-dependent assembly class [41,42]. StdA constitutes the major fimbrial subunit [43,44], while StdB and StdC are a putative outer membrane usher protein and a putative periplasmic chaperone, respectively [45]. We have characterized 5 additional genes of unknown function in the *std* cluster, and have renamed them *stdA*, *stdE*, and *stdF*. Products of two such genes, *stdE* and *stdF*, turn out to be the molecular link between Dam methylation and SPI-1: lack of Dam methylation permits expression of the *std* operon, and the *stdE* and *stdF* products directly or indirectly downregulate *hilD* mRNA. These findings contribute to understand the invasion defect of *Salmonella* Dam mutants [40,46] and the relief of virulence attenuation observed in Dam−*std* mutants [47]. Furthermore, we show that constitutive expression of StdE and StdF reduces invasion of epithelial cells and impairs *S. enterica* virulence in the mouse model. StdEF-mediated repression of SPI-1 may be an example of coordination in the control of virulence genes, preventing synthesis of the invasion machinery in environments which are not appropriate for invasion. One such compartment may be the caecum, an intestinal section in which Std fimbriae are known to be produced [48,49].

### Materials and Methods

**Bacterial strains, bacteriophages, and standard strain construction**

All the *Salmonella enterica* strains listed in Table S1 belong to serovar Typhimurium, and derive from the mouse virulent strain ATCC 14028. For simplicity, *Salmonella enterica* serovar Typhimurium is often abbreviated as *S. enterica*. Targeted gene disruption was achieved using pKD4 or pKD13 [50]. Antibiotic resistance

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**Figure 1. Diagram of SPI-1 regulation.** Boxes represent genes, and circles represent proteins. Grey boxes and grey circles indicate SPI-1-encoded regulators. White circles indicate regulators encoded outside SPI-1. doi:10.1371/journal.pone.0030499.g001
cassettes introduced during strain construction were excised by recombination with plasmid pCP20 [50]. The oligonucleotides used for disruption (labeled “UP” and “DO”) are listed in Table S2, together with the oligonucleotides (labeled “E”) used for allele verification by the polymerase chain reaction. For the construction of transcriptional and translational lac fusions in the Salmonella chromosome, FRT sites generated by excision of Km’ cassettes [50] were used to integrate either pCE37 or pCE40 [51]. Generation of a 3xFLAG epitope tag to protein-coding DNA sequences was carried out using plasmid pSUB11 (Km’, 3xFLAG) [52]. Transductional crosses using phage P22 HT 105/1 int201 [53] and G. Roberts, unpublished) were used for strain construction operations involving chromosomal markers. The transduction protocol was described elsewhere [54, 55]. To obtain phage-free isolates, transductants were purified by streaking on green plates. Phage sensitivity was tested by cross-streaking with the clear isolates, transductants were purified by streaking on green plates. Growth conditions

Luria-Bertani (LB) broth was used as standard liquid medium. Solid media contained 1.5% agar. When needed, kanamycin sulfate (Km), chloramphenicol (Cm), or ampicillin (Ap) was added to LB at a final concentration of 50 µg/ml, 20 µg/ml and 100 µg/ml, respectively. Green plates were prepared according to Chan and co-workers [53, 55], except that methyl blue (Sigma Chemical Co., St. Louis, MO) substituted for aniline blue. Plate tests for sensitive strains were grown at 37°C in LB until stationary phase (OD 600 = 2.0–2.5). Samples were taken when the cultures had reached the stationary phase (OD 600 = 2.0–2.5) using the 2 ml aliquot of DNA nase I-treated RNA was used for cDNA synthesis using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). One µl of retrotranscribed cDNA was used as template for PCR with primer pairs specific for contiguous std ORFs (Table S2). Co-retrotranscribed RNA and genomic DNA were used as negative and positive controls, respectively.

Protein extracts and Western blot analysis

Total protein extracts were prepared from bacterial cultures grown at 37°C in LB until stationary phase (final OD 600~2.5). Bacterial cells were collected by centrifugation (16,000 g, 2 min) and suspended in 100 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 (12%). Conditions for protein transfer have been described elsewhere [47]. Optimal dilutions of primary antibodies were as follows: anti-FLAG M2 monoclonal antibody (Sigma Chemical Co.), 1:5,000; anti-GroEL polyclonal antibody (Sigma Chemical Co.), 1:20,000. Goat anti-mouse horseradish peroxydase-conjugated antibody (1:5,000, BioRad, Hercules, CA) or Goat anti-rabbit horseradish peroxydase conjugated antibody (1:20,000, Santa Cruz Biotechnology, Heidelberg, Germany) were used as secondary antibodies. Proteins recognized by the antibodies were visualized by chemoluminescence using lucifer-in-luminol reagents in a LAS 3000 Mini Imaging System (Fujifilm, Tokyo, Japan). For quantification, the intensity of the bands was determined using MultiGauge software (Fujifilm). GroEL was used as loading control.

Co-transcriptional analysis of std genes

RNA used for retrotranscription was extracted from S. enterica cultures grown in LB to stationary phase (OD = 2.5) using the SV total RNA isolation system (Promega Co., Madison, WI) as described at http://www.ifr.ac.uk/safety/microarrays/protocols.html. The quality of the preparation and the concentration of RNA were determined using a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). To avoid genomic DNA contamination, the preparation was treated twice with DNase I (Turbol DNA free, Applied Biosystems/Ambion, Austin, TX), following manufacturer’s instructions. A 0.6 µl aliquot of DNase I-treated RNA was used for cDNA synthesis using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). One µl of retrotranscribed cDNA was used as template for PCR with primer pairs specific for contiguous std ORFs (Table S2). Non-retrotranscribed RNA and genomic DNA were used as negative and positive controls, respectively.

RNA extraction and Northern analysis

A 2 ml aliquot from a stationary culture (OD 600~2) was centrifuged at 16,000 g, 4°C, during 5 min. The pellet was resuspended in 100 µl of a solution of lysozyme (Sigma Chemical Co.), 3 mg/ml. Cell lysis was facilitated by three consecutive freeze-thaw cycles. After lysis, RNA was extracted using 1 ml of Trizol reagent (Invitrogen Co, Carlsbad, CA), according to manufacturer’s instructions. Lastly, total RNA was resuspended in 30 µl of RNase-free water. The quality of the preparation and the RNA concentration were determined using a ND-1000 spectrophotometer (NanoDrop Technologies). For Northern blot analysis, 10 µg of total RNA was loaded per well and electrophoresed in denaturing 1% agarose formaldehyde gels. Vacuum transfer and fixation to Hybond-N membranes (GE Healthcare, Little Chalfont, UK) were performed using 0.05 M NaOH. Filters were then hybridized using an internally labelled [(32P)UTP] riboprobe specific for the upstream (5’) 300 nucleotides of the stdD coding sequence. Hybridization was carried out at 65°C. As a control of RNA loading and transfer efficiency, the filters were hybridized with a riboprobe for the RNase P mRNA gene (npB). Images of
radioactive filters were obtained with a FLA-5100 imaging system (Fujifilm), and quantification was performed using MultiGauge software (Fujifilm).

β-galactosidase assays
Levels of β-galactosidase activity were assayed using the CHCl₃- sodium dodecyl sulfate permeabilization procedure [58]. β-galactosidase activity data are the averages and standard deviations from ≥3 independent experiments. The Student’s t test was used to determine if the differences in β-galactosidase activities were statistically significant.

Virulence assays in mice
Groups of 5 eight-weeks-old female BALB/c mice (Charles River Laboratories, Santa Perpetua de Mogoda, Spain) were inoculated with a 1:1 ratio of two strains, one carrying the P₅₅₆₅₀ stdEF construction and another carrying the P₅₅₆₅₋stdEF ΔstdEF construction. To differentiate between the two strains, a Mu recombination carrying a lacZ gene was inserted in the 3’ end locus in the chromosome of the P₅₅₆₅₀stdEF ΔstdEF strain. The βgal::lacZ allele has been shown to be neutral for Salmonella virulence ([59]; F. Ramos-Morales, personal communication). For oral inoculation, bacterial cultures were grown overnight at 37°C and shaken. Oral inoculation was performed by feeding the mice with 25 μl of saline containing 0.1% lactose and 10⁹ bacterial CFU. A competitive index (CI) for each mutant was calculated as the ratio between the mutant and the wild type strain in the output (bacteria recovered from the murine spleen after infection) divided by their ratio in the input (initial inoculum) [60,61]. The Student’s t test was used to determine whether the output ratio was or not significantly different from the input ratio.

Ethics statement
Animal research adhered to the principles mandatory in the European Union, as established in the Legislative Act 86/609 CEE (November 24, 1986), and followed the specific protocols established by the Royal Decree 1201/2005 of the Government of Spain (October 10, 2005). The protocols employed in the study were reviewed by the Comité Ético de Experimentación de la Universidad de Sevilla, and were approved on January 16, 2010 (permit number 59-A-2010). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Invasion assays
HeLa cells (ATCC CCL2) were cultured in tissue culture medium (Dulbecco's modified essential medium supplemented with 10% fetal calf serum and 2 mM L-glutamine). For routine cultivation, 60 μg/ml penicillin and 100 μg/ml streptomycin were added to the culture medium. The day before infection, approximately 1.5 x 10⁶ HeLa cells were seeded, using 24-well plates (Costar, Corning, New York, NY). Each well contained 1 ml of tissue culture medium without antibiotics. Cells were grown at 37°C, 5% CO₂ to obtain 80% confluency. One hour before infection, the culture medium was replaced and replaced by 0.5 ml fresh tissue culture medium without antibiotics. Bacteria were grown overnight at 37°C in LB with shaking, diluted into fresh medium (1:50), and incubated at 37°C without shaking up to an O.D.600 of 0.6–0.8 (overnight). Bacteria were added to reach a multiplicity of infection (MOI) of 30:1 bacteria/HeLa cell. HeLa cells were infected for 30 min, washed 3 times with PBS, incubated in fresh tissue culture medium containing 100 μg/ml gentamicin for 1.5 hours, and washed 3 times with PBS. Numbers of viable intracellular bacteria were obtained by lysing infected cells with 1% Triton X-100 (prepared in PBS) and subsequent plating. Invasion rates were determined as the ratio between viable intracellular bacteria and viable bacteria added to infect the HeLa cells. Data are averages and standard deviations of 3 independent experiments. The Student’s t test was used to determine the statistical significance of the differences observed.

Results
Genetic screen for regulators of hilD expression using a multiplicity plasmid library of Salmonella enterica
We previously reported that regulation by Dam methylation is transmitted to SPI-1 via HsdI [39]. However, Dam methylation was found to regulate hilD expression at the postranscriptional level, suggesting the involvement of additional, unknown regulators under Dam methylation control. The view that Dam-dependent regulation of hilD is indirect is further supported by the absence of GATC sites in the hilD promoter and upstream regulatory region, and by the observation that elimination of GATC sites in the hilD coding sequence does not abrogate Dam-dependent regulation (Figure S1). To search for Dam-dependent regulators of hilD, we considered two alternative possibilities: either Dam⁺ hosts might produce a factor that upregulates hilD or Dam⁻ hosts might produce a factor that downregulates hilD. We also considered the possibility that overexpression of the hypothetical regulator might render SPI-1 expression Dam-independent: namely, that overproduction of a repressor might downregulate SPI-1 in a Dam⁺ background, while overproduction of an activator might upregulate SPI-1 in Dam⁻ background. On these grounds, we devised a genetic screen for SPI-1 regulators in Dam⁺ and Dam⁻ backgrounds, using a pBR328-based multiplicity plasmid library of the Salmonella enterica genome. As a reporter, we used a fusion (hilD::lacZ930) that bears the lacZ gene inserted immediately downstream the hilD stop codon and shows Dam-dependent expression (Figure S2).

Dam⁺ and Dam⁻ isogenic strains carrying the hilD::lacZ930 fusion were transduced with 9 pools of the plasmid library, each containing around 1,000 independent clones. Transductants were selected on LB plates containing chloramphenicol and X-gal. Colonies with reduced β-galactosidase activity (white) were sought in the Dam⁺ background, and colonies with increased β-galactosidase activity (deep blue) were sought in the Dam⁻ background. Relevant results from these trials were as follows:

(i) Twelve independent candidates with increased β-galactosidase activity were chosen amongst blue colonies obtained in the Dam⁻ screen. The fragments contained in the library plasmids were sequenced using specific primers flanking the insertion site (Table S2). DNA sequencing revealed that all pBR328 derivatives carried the same cloned fragment, which contained the rtsA gene amongst other genes (Figure S3A). Because RtsA is known to activate hilD transcription [35], we concluded that increased hilD::lacZ930 expression was due to overproduction of RtsA. However, neither rts nor the other genes contained in the plasmid (STM4310, STM4312, STM4313, STM4316, STM4317 and STM4318) are regulated by Dam methylation [38]. Hence, we ruled out that RtsA might be the Dam-dependent factor that controls hilD expression.

(ii) In the Dam⁺ screen, five different plasmids were found to reduce hilD::lacZ930 activity (Figure S3B). One such plasmid contained the std fimbrial gene cluster, amongst other neighboring genes (Figure S3B). The latter finding, together
with transcriptomic data indicating that std mRNA is >100-fold more abundant in a Dam\(^{-}\) background [38], suggested that the std gene cluster might encode regulator(s) involved in SPI-1 repression in Dam\(^{-}\) mutants. None of the other loci present in the plasmids that reduced hilD::lac930 activity (Figure S3B) is known to be under Dam methylation control [38]. Hence, further work was centered upon std.

All genes in the std gene cluster are overexpressed in Dam\(^{-}\) mutants

Transcriptomic analysis had shown that stdA, stdB, stdC, and the uncharacterized putative genes STM3026 and STM3025 are all repressed by Dam methylation. Dam-dependent expression of stdA, stdB, and stdC had been confirmed by independent methods [38,47]. However, the effect of Dam methylation on STM3026 and STM3025 expression had not been further analyzed. On the other hand, DNA sequence analysis in silico had indicated the existence of a sixth, uncharacterized open reading frame (ORF), designated STM3025.1N, in the intergenic region between STM3026 and STM3025 [45]. To update and complete previous data on Dam-dependent control of std, we compared the expression of STM3026, STM3025.1N, and STM3025 in Dam\(^{+}\) and Dam\(^{-}\) backgrounds by two independent methods: (i) analysis of β-galactosidase activity using STM3026::lac, STM3025.1N::lac, and STM3025::lac translational fusions (Figure 2A); and (ii) determination of STM3026, STM3025.1N, and STM3025 protein levels in protein extracts from Dam\(^{+}\) and Dam\(^{-}\) hosts, using protein variants tagged with the 3xFLAG epitope (Figure 2B). β-galactosidase assays and Western blot analyses show that STM3026, STM3025.1N, and STM3025 are expressed in a Dam\(^{-}\) background. Detection of the proteins by Western blot indicates that the three ORFs encode proteins indeed. Detection of Std proteins in a Dam\(^{-}\) background is consistent with previous observations indicating that the std operon is repressed under laboratory conditions and becomes derepressed in Dam\(^{-}\) mutants [38,47]. To simplify and rationalize gene designations, STM3026, STM3025.1N, and STM3025 have been renamed stdD, stdE, and stdF, respectively.

**stdA, stdB, stdC, stdD, stdE, and stdF constitute a polycistronic transcriptional unit**

Expression of stdA is known to be driven by a promoter whose transcription is Dam-dependent [47]. The fact that expression of all the genes in the std cluster is upregulated in a Dam\(^{-}\) background suggested that the entire cluster might constitute a polycistronic unit transcribed from the stdA promoter. This hypothesis received experimental support from co-transcription analysis of contiguous ORFs by retrotranscription and PCR amplification. For this purpose, total RNA was extracted from a
Dam- mutant, and traces of DNA were removed by treatment with DNase I. The RNA sample was split in two fractions, one of which was retrotranscribed to cDNA using random primers; the other fraction underwent the same treatment but water was added instead of retrotranscriptase. Next, we performed PCR amplification with primer pairs specific for contiguous ORFs (Figure 3A, Table S2) in the presence of the following templates: Salmonella genomic DNA as positive control, nonretrotranscribed RNA as negative control, and cDNA as the experimental query. The PCR products were resolved in a 2% agarose gel with 0.5 mg/ml ethidium bromide, and visualized under UV light. As shown in Figure 3B, PCR products of the expected sizes were obtained using either genomic DNA or cDNA. However, no fragment was observed when RNA was used as template. These results indicate that the six genes in the std cluster constitute a polycistronic operon, transcribed from the promoter previously identified upstream stdA ([47]; Figure 3A). Our results, however, do not rule out the possibility that internal promoters may also exist.

The stdE and stdF gene products are functional links between Dam methylation and SPI-1

If overexpression of the std operon was the cause of SPI-1 repression in Dam- mutants (Figure S3B), we reasoned, SPI-1 repression in a Dam- background should be suppressed by deletion of the std operon. On these grounds, we compared the expression of invF::lac and sipB::lac fusions in isogenic Dam- and Dam+ strains that contained either an intact std operon or a complete deletion of std. As shown in Figure 4A, the β-galactosidase activities of the invF::lac and sipB::lac fusions were reduced in a Dam- background in the presence of a functional std operon. However, in a strain lacking the std operon, both fusions displayed similar β-galactosidase activities in Dam- and Dam+ backgrounds. These results support the hypothesis that one or more proteins encoded in the std operon are involved in the transmission of Dam-dependent regulation to SPI-1. In an attempt to identify such protein(s), Dam-dependent regulation of an invF::lac fusion was monitored in a set of mutants carrying in-frame, non-polar deletions in individual std genes. Figure 4B shows that invF::lac expression remains Dam-dependent in strains lacking stdA, stdB, stdC, and stdD, suggesting that these genes are not required for Dam-dependent control of SPI-1. However, repression of invF::lac in a Dam- background is suppressed in strains lacking either stdE or stdF, suggesting that the products of both genes are necessary for SPI-1 repression in Dam- mutants.

StdE and StdF independently repress SPI-1 expression

The above experiments provide a tentative explanation for the SPI-1 expression defect observed in the absence of Dam methylation [38,39]: in a Dam- background, the std operon is expressed, and the stdE and stdF gene products repress SPI-1. If this model was correct, we reasoned, constitutive expression of stdE and stdF should repress SPI-1 expression in a Dam- background. To test this hypothesis, we placed stdE and stdF under the control

Figure 3. Evidence that stdA, stdB, stdC, stdD, stdE, and stdF constitute a polycistronic transcriptional unit. A. Diagram of the std operon. Opposite arrows below the diagram represent primer pairs used to examine co-transcription of contiguous coding sequences. B. Co-transcription of contiguous coding sequences in the std operon. PCR fragments generated with primer pairs specific for contiguous coding sequences were resolved in a 2% agarose gel, stained with ethidium bromide, and visualized with UV light. The 1 kb+ DNA ladder (Innovaplex, Sugar Land, TX) was used as size marker.

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of the P_L promoter [56] to achieve moderate, Dam-independent expression of stdE and stdF. To avoid potential artefacts (e.g., caused by autogenous regulation of the std operon), the native stdA promoter and all the std genes upstream stdE were deleted. Two basic constructions were made: (i) P_LtetO-stdEF in which P_LtetO was placed upstream stdE on the chromosome, thus permitting constitutive expression of both stdE and stdF; (ii) P_LtetO-stdF, in which P_LtetO was inserted right upstream stdF, thus expressing stdF only. As controls, we used derivatives of the same strains which carry in-frame deletions in stdE, stdF, or in both genes (Figure 5A). Production of StdE and StdF in strains carrying the P_LtetO-stdEF or P_LtetO-stdF constructions was monitored by Western blotting, using protein variants tagged with the 3xFLAG epitope (Figure S4).

The effect of constitutive expression of stdE and stdF on the expression of SPI-1 genes hilA, invF, and sipB was monitored in strains carrying P_LtetO-stdEF, P_LtetO-stdF and the appropriate deletion controls. Two independent methods were used: (i) β-galactosidase activities were measured in strains carrying hilA::lac, invF::lac, and sipB::lac fusions; (ii) HilA, InvF, and SipB protein levels were monitored by Western blotting, using protein variants tagged with the 3xFLAG epitope. The results obtained with both methods were congruent (Figure 5, panels B–D), and can be summarized as follows:

(i) Expression of hilA, invF, and sipB is strongly downregulated when P_LtetO is inserted upstream stdE.
(ii) Downregulation is partially relieved when stdE is deleted, however, deletion of stdF alone does not restore SPI-1 expression. Deletion of both genes completely restores SPI-1 expression to wild type level, suggesting that SPI-1 downregulation is due to expression of both stdE and stdF.
(iii) Insertion of P_LtetO upstream stdF downregulates the expression of hilA, invF, and sipB, but less efficiently than P_LtetO insertion upstream stdE.
(iv) Deletion of stdF completely suppresses SPI-1 downregulation.

Altogether, these observations provide evidence that both StdE and StdF downregulate SPI-1 independently: each is able to downregulate SPI-1 in the absence of the other.

StdE and StdF regulate hilD expression at the posttranscriptional level

We previously reported that regulation of hilD by Dam methylation is posttranscriptional [39]. After finding that Dam-dependent hilD regulation depended on StdE and StdF, it seemed logical to expect that StdE and StdF would repress hilD expression at the posttranscriptional level. To test this hypothesis, we examined hilD expression using a transcriptional fusion (hilD::lac1) in which lacZ is inserted exactly at the hilD
transcription start site. The hilD::lac1 fusion faithfully reproduces transcriptional regulation of hilD, as indicated by the observation that the fusion is upregulated in the presence of a multicopy plasmid that expresses RtsA, a transcriptional activator of hilD [35] (Figure S5). The expression level of hilD::lac1 was determined in the wild type as well as in the P_LtetO-stdEF, P_LtetO-stdEF ΔstdE, and P_LtetO-stdEF ΔstdF backgrounds (Figure 5A, Figure 6A). The ß-galactosidase activities were similar in all strains, suggesting that StdE and StdF do not regulate hilD transcription initiation.

The possibility that StdE and StdF downregulate hilD expression at the postranscriptional level was examined by analyzing hilD mRNA levels by Northern blot in the following backgrounds: wild type, P_LtetO-stdEF, P_LtetO-stdEF ΔstdE, P_LtetO-stdEF ΔstdF, and P_LtetO-stdEF ΔstdF. As shown in Figure 6B, the level of hilD mRNA is reduced around 4 fold in the P_LtetO-stdEF strain. Deletion of stdE partially restores the hilD mRNA level, and simultaneous deletion of stdE and stdF completely restores hilD mRNA to wild type level. Furthermore, the amount of hilD mRNA is reduced twofold in the P_LtetO-stdF background, and this reduction

Figure 5. Downregulation of SPI-1 by StdE and StdF. A. Diagram representing P_LtetO-stdEF and P_LtetO-stdF constructions, and their respective deletion controls lacking stdE, stdF, or both. B–D. Expression of hilA, invF, and sipB in strains carrying a native std operon (control), P_LtetO-stdEF, P_LtetO-stdF, and their respective control constructs. Histograms represent ß-galactosidase activities of hilA::lac, invF::lac, and sipB::lac fusions. An asterisk indicates a statistically significant difference (P<0.005) compared to the control. HilA-3XFLAG, InvF-3xFLAG, and SipB-3xFLAG levels were determined by Western blotting using anti-FLAG antibodies. GroEL was used as loading control. For quantification, the ratio tagged protein/GroEL was relativized to 100 in control samples. The symbols ‘+’ and ‘−’ indicate the presence or the absence of StdE or StdF.
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is completely abolished by an stdF deletion. Altogether, these observations support the view that StdE and StdF downregulate hilD expression at the posttranscriptional level.

**Expression of stdE and stdF inhibits Salmonella invasion**

Because constitutive expression of stdE and stdF represses SPI-1 ([Figure 5; Figure 6] and SPI-1 is essential for Salmonella invasion, we examined the invasiveness of S. enterica ser. Typhimurium upon constitutive expression of stdE and stdF. For this purpose, we compared the ability of Salmonella strains carrying either the P_{Lmo}-stdEF or P_{Lmo}-stdF ΔstdF constructions to invade epithelial HeLa cells in vitro. As a positive control, we used the wild type strain. As a negative control, we used a strain with a deletion of SPI-1 (ΔSPI-1). As shown in **Figure 7**, the ΔSPI-1 strain is approximately 1,000 fold less invasive than the wild type. Constitutive expression of stdE and stdF (P_{Lmo}-stdEF) produces an invasion defect similar to deletion of SPI-1 (ΔSPI-1), and this defect is suppressed by mutation of stdE and stdF (P_{Lmo}-stdEF ΔstdF) ([Figure 7]). Hence, constitutive expression of stdE and stdF inhibits Salmonella invasion.

**Expression of stdE and stdF attenuates Salmonella virulence in mice**

The observation that expression of stdE and stdF inhibits Salmonella invasion in vitro led us to speculate that StdE and StdF might also inhibit invasion of epithelial cells in the animal intestine. If such was the case, we reasoned, a Salmonella strain which constitutively expresses stdE and stdF should be attenuated in mice infected by the oral route. To test this hypothesis, we compared the virulence of Salmonella strains that expressed or not stdE and stdF. Five BALB/c mice were orally inoculated with a 1:1 mixture of a strain that constitutively expressed stdE and stdF (P_{Lmo}-stdEF) and an isogenic strain carrying a deletion of both stdE and stdF (P_{Lmo}-stdEF ΔstdF). To distinguish the two strains on plates, we inserted a MuΔ transposon at the trg chromosomal locus of the P_{Lmo}-stdEF ΔstdF strain. The trg::MuΔ allele has been
shown to be neutral for Salmonella virulence ([59]; F. Ramos-Morales, personal communication). The competitive index of and a strain carrying the PL strain (expressing stdE and stdF) versus the PL strain (lacking stdE and stdF) was 0.016 (Table 1), indicating that constitutive expression of stdE and stdF attenuates Salmonella virulence by the oral route more than 60 fold.

Discussion

Evolutionary acquisition of genetic modules has provided Salmonella with new abilities to interact with eukaryotic cells and to exploit a variety of niches [8,11]. The std gene cluster is conserved amongst Salmonella serovars and absent in closely related species [11], suggesting horizontal acquisition. A critical requirement of modular evolution is to coordinate expression of the genetic modules, which in some cases carry regulatory genes that provide connections with the core genome [8]. In addition, examples of crosstalk between horizontally-acquired modules have been described: HilD, a regulator encoded by SPI-1, activates SPI-2 expression during late stationary phase [62]; expression of SPI-4 genes is activated by SprB, a transcriptional regulator encoded on SPI-4; expression of SPI-2 is repressed [38,43,44,47]. However, several observations indicate that std genes may be repressed whenever the std operon as the missing link between Dam methylation and SPI-1: (i) constitutive expression of stdE and stdF in Dam mutants of Salmonella enterica serovar Typhimurium leads to SPI-1 repression. Our observations may also explain the intriguing observation that the extreme attenuation of S. enterica Dam mutants upon oral infection [40,46] is partially suppressed by deletion of std [47].

Epistasis analysis indicates that Dam-dependent control of SPI-1 requires both StdE and StdF. This conclusion is further supported by the following observations: (i) constitutive expression of stdE and stdF in a Dam background represses SPI-1 expression (Figure 3); (ii) StdE and StdF are overproduced in Dam background (Figure 2); (iii) Dam methylation, StdE, and StdF regulate SPI-1 expression through HilD (Figure 6); and (iv) as in the case of Dam methylation, StdE and StdF do not regulate hild mRNA (Figure 6). However, it remains to be established whether StdE and StdF directly control the hild mRNA level or the regulatory mechanism involves intermediaries. A tentative model to explain SPI-1 regulation by Dam methylation is depicted in Figure 8: in a Dam background, GATC sites in the std regulatory region are methylated, thus preventing binding of HidR and subsequent activation of std expression. In the absence of Dam methylation, HidR activates transcription from Pstd and all the proteins encoded in the operon are produced. StdE and StdF then repress hild expression at the posttranscriptional level. As a consequence, the entire SPI-1 is downregulated.

A corollary from the above results is that Salmonella invasion can be expected to be inhibited whenever the std operon is expressed (Figure 8). This expectation was fulfilled by invasion assays in vitro showing that expression of StdE and StdF causes a >1,000 fold reduction in HeLa cell invasion (Figure 7). Furthermore, expression of StdE and StdF was found to attenuate Salmonella virulence about 60 fold upon infection of BALB/c mice (Table 1).

Under laboratory growth conditions, the std operon is tightly repressed [38,43,44,47]. However, several observations indicate that the six genes are co-transcribed (Figure 3). Transcription of std is driven by a promoter located upstream stdI [47]. Transcription from Pstd is activated by binding of HidR, a LysR-like factor, to a regulatory region upstream the promoter. However, methylation of two GATC sites in the regulatory region prevents binding of HidR, thus repressing std expression ([47], Jakomin et al., in preparation). Altogether, these observations suggest that all std genes may be coordinately regulated by Dam methylation upon transcription from Pstd. However, internal promoters may also exist.

| Strain number | Relevant genotype | CI (A/B) | P   |
|---------------|------------------|---------|-----|
| Strain A      | SV6503           | PstdtetOstdEF | 0.016 | <0.0001 |
| Strain B      | SV65901          | PstdtetOstdEFStdEF |      |        |

Table 1. Effect of constitutive expression of stdE and stdF in oral competition assays.

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Figure 2. A tentative model to explain SPI-1 regulation by Dam methylation upon transcription. In the absence of Dam methylation, HidR activates transcription from Pstd and all the proteins encoded in the operon are produced. StdE and StdF then repress hild expression at the posttranscriptional level. As a consequence, the entire SPI-1 is downregulated.

Figure 3. Transcription of std is driven by a promoter located upstream stdI [47]. Transcription from Pstd is activated by binding of HidR, a LysR-like factor, to a regulatory region upstream the promoter. However, methylation of two GATC sites in the regulatory region prevents binding of HidR, thus repressing std expression ([47], Jakomin et al., in preparation). Altogether, these observations suggest that all std genes may be coordinately regulated by Dam methylation upon transcription from Pstd. However, internal promoters may also exist.

Dam mutants of Salmonella enterica are attenuated in the mouse model and present a plethora of virulence-related defects both at the intestinal stage of the infection and during systemic infection [40,46,68]. A relevant defect during intestinal infection is reduced SPI-1 expression, which is a consequence of hild mRNA instability in Dam mutants [38,39]. Genetic screens and subsequent experiments described in this study have identified the std fimbrial operon as the missing link between Dam methylation and SPI-1: (i) a multicopy plasmid containing the std operon downregulates hild expression (Figure S3B); (ii) std genes are upregulated in Dam background (Figure 2); (iii) SPI-1 regulation by Dam methylation is completely suppressed in a strain lacking the std operon (Figure 4A). Altogether, these results suggest that expression of std in Dam mutants leads to SPI-1 repression. Our observations may also explain the intriguing observation that the extreme attenuation of S. enterica Dam mutants upon oral infection [40,46] is partially suppressed by deletion of std [47].
that Std fimbriae are produced in the animal intestine: (i) mice infected with serovar Typhimurium seroconvert to StdA, the major fimbrial component of Std fimbriae [43]; (ii) std deletion reduces the ability of Salmonella to colonize and persist in the caecum of infected mice, while it has no consequence for colonization of the small intestine [48]; and (iii) Std fimbriae bind α(1,2)fucose residues, which are abundant in the cecal mucosa [49]. In turn, Salmonella invasion takes place preferentially in the ileum [3] and is inhibited in the caecum [69]. Hence, it is tempting to speculate that StdE and StdF may play a role in SPI-1 expression inhibition in the caecum, an intestinal compartment which is not appropriate for invasion.

StdE shares 40–50% identity with the transcriptional activators GrlA and CaiF from E. coli and Enterobacter cloacae, respectively [70,71]. Interestingly, StdF is related to an uncharacterized protein encoded immediately downstream CaiF in the E. cloacae chromosome [70,71], which is part of a hypothetical fimbrial gene cluster whose genetic organization is reminiscent of the std operon [72]. StdF is also related to the SPI-1 Salmonella protein SprB, a transcriptional regulator that represses the hilD promoter and activates the siiA promoter [63]. Even though StdE and StdF are similar to known transcriptional regulators, they do not regulate hilD at the transcriptional level but at the postranscriptional level. Thus, these proteins may have either acquired the ability to control Salmonella gene expression at the postranscriptional level or may regulate transcription of a postranscriptional regulator of hilD. If the latter view is correct, crosstalk between std and SPI-1 may turn out to be more complex than described in this study, perhaps involving elements of the Salmonella core genome.

**Supporting Information**

**Figure S1** Elimination of the GATC sites in the hilD coding sequence does not alter the control of SPI-1 expression by Dam methylation. A. Diagram showing the distribution of GATCs within hilD; each CH2 represents a GATC. B. Site-directed mutagenesis of hilD GATCs. A single nucleotide exchange was introduced at each GATC site, generating a synonymous codon (shown in red). C. β-galactosidase activity of an invF::lac fusion in Dam+ (black histograms) and Dam− (white histograms) isogenic backgrounds. Measurements were performed in a strain that contained the 3 GATCs in the hilD coding sequence (control), and in a strain in which the 3 GATCs had been mutated (GATC*123). The differences observed between Dam+ and Dam− are statistically significant in both strains (P<0.005).

**Figure S2** β-galactosidase activity of the hilD::lac930 fusion in a Dam+ background (black histogram), and in a Dam− background (white histogram). The differences observed are statistically significant (P<0.005).

**Figure S3** β-galactosidase activity of the hilD::lac930 fusion in control strains (carrying pBR328), in candidates showing increased β-galactosidase activity in a Dam− background (A) and in candidates showing reduced β-galactosidase activity in a Dam+ background (B). Diagrams representing the fragments harbored by the plasmids present in the candidates are also shown.

**Figure S4** Production of StdE and StdF in strains carrying P_LtetO-stdEF and P_LtetO-stdF constructions A. Diagrams of strain construction and StdE and StdF tagging. B. Levels of StdE-3xFLAG and StdF-3xFLAG in protein extracts from the wild type, Dam−, P_LtetO-stdEF, and P_LtetO-stdF strains. 3xFLAG-tagged proteins were detected by Western blotting using a commercial anti-FLAG antibody. GroEL was used as loading control. For
quantification, the ratio tagged tag level was relativized to 100 in the Dm background.

**Figure S5** β-galactosidase activity of the hilD: lac fusion in a strain carrying pBR328 (black histogram), and in a strain carrying a pBR328 derivative that contains the tsd gene (white histogram). The differences observed are statistically significant (P<0.005).

**Table S1** Strain list.

**Table S2** Oligonucleotides used in this study (5'→3').

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**Author Contributions**

Conceived and designed the experiments: JLG JC. Performed the experiments: JLG. Analyzed the data: JLG JC. Contributed reagents/materials/analysis tools: JLG JC. Wrote the paper: JLG JC.
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