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Postranscriptional Regulation of *Salmonella* Pathogenicity Island 1

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INTRODUCTION

1. The genus *Salmonella*

The genus *Salmonella* includes facultative anaerobic Gram-negative, rod-shaped bacteria, able to infect a variety of animal hosts, including mammals, birds, reptiles, and amphibians. Most Salmonellae are motile and produce peritrichous flagella. The *Salmonella* are members of the family *Enterobacteriaceae* in the γ-proteobacteria subdivision and are close relative to the genera *Escherichia*, *Shigella* and *Citrobacter.*

Currently, the genus *Salmonella* is divided into 2 species, called *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* can be further subdivided into 6 subspecies: *Salmonella enterica* subsp. *enterica* (subsp. I), *Salmonella enterica* subsp. *salamae* (subsp. II), *Salmonella enterica* subsp. *arizonae* (subsp. IIIa), *Salmonella enterica* subsp. *diarizonae* (subsp. IIIb), *Salmonella enterica* subsp. *enterica* (subsp. I), Salmonella *enterica* subsp. *houtenae* (subsp. IV), and *Salmonella enterica* subsp. *indica* (subsp. VI).

Every *Salmonella* subspecies are classified in serovars. *Salmonella* serovars are distinguished by antisera to two highly variable surface antigens, the O antigen and the H antigen, reflecting variation in the exposed part of lipopolysaccharide and in flagellin, respectively (Grimont, 2007; McQuiston *et al.*, 2004). There are more than 2500 serovars belonging to the different subspecies (Grimont, 2007).

Only serovars of subsp. *enterica* have the ability to colonize warm-blooded vertebrates, and account for 99 % of all human infections by *Salmonella*, while members of *Salmonella bongori* and the rest of *Salmonella enterica* subspecies rarely infect mammals and birds. More than 1,500 serovars belonging to subsp. *enterica* have been identified so far (Popoff et al., 2004), and they differ in host specificity and the disease conditions they promote. Some of them are host-restricted, while others can infect a broad variety of animal hosts. The diseases produced by subsp. *enterica* range from self-limiting gastroenteritis to life-threatening systemic infection, and the outcome of the infection depends on the specific serovar-host combination. For example, the human-restricted serovar Typhi produces typhoid fever. However, serovar Typhimurium produces mild gastroenteritis in humans, but causes a systemic disease similar to human typhoid fever when infecting mice. For that reason, the combination serovar Typhimurium-mouse has been extensively used as a model for typhoid fever in humans, and most of the work done with *Salmonella* has been carried out with that

serovar. In this work, we have used the virulent strain *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC14028 (see Jarvik et al., 2010, for a deep description of that strain). For simplicity, it will be abbreviated as *Salmonella typhimurium* 14028, or simply *Salmonella typhimurium.*

4. Evolution of *Salmonella* pathogenicity

Salmonella and *Escherichia coli* are close relatives, and diverged from 120 to 160 million years ago (Ochman and Wilson, 1987). Thus, the study of *Salmonella* has taken advantage of all the information available for *Escherichia coli*, and basic metabolism is relatively well characterized. What makes *Salmonella* unique from standard *Escherichia coli*, of course, is pathogenesis.

Almost 25 % of *Salmonella* genome consists of material that is absent in *Escherichia coli* (McClelland et al., 2001; Prowllic andMcClelland, 2003). The evolution of *Salmonella* pathogenicity has involved the sequential acquisition of genetic elements, each one contributing to different aspects of *Salmonella* virulence (Ochman and Groisman, 1997; Kelly et al., 2009). Amongst those elements are the *Salmonella* pathogenicity islands (SPIs), which are clusters of virulence genes in *Salmonella* chromosome. More than 10 SPIs has been described so far (Hensel, 2004), but some of them are serotype-specific. Since those regions are absent in *Escherichia coli* chromosome, many of them have a different G-C content than the average *Salmonella* chromosome, and some are flanked by insertion sequences, it is thought that SPIs have been acquired by horizontal gene transfer (Kelly et al., 2009; Prowollik and McClelland, 2003).

The 2 better characterized SPIs are *Salmonella* pathogenicity island 1 (SPI-1), involved in the invasion of intestinal epithelial cells, and *Salmonella* pathogenicity island 2 (SPI-2), that allow *Salmonella* to survive in macrophages and colonize deeper tissues (Ochman et al., 1996).

SPI-1 was acquired around 25-40 million years ago by the common *Salmonella* ancestor, that got then the ability to invade eukaryotic cells and became an intracellular pathogen presumably associated with cold-blooded vertebrates. The next major event was the acquisition of SPI-2. SPI-2 is a defining characteristic of *Salmonella enterica*,

and its acquisition resulted in the split between the 2 *Salmonella* species. Hence, only members of *Salmonella enterica* have the ability to reach deeper tissues and produce systemic infections.

The subsp. *enterica* ancestor acquired the capacity to infect warm-blooded vertebrates, and different strains subsequently evolved to colonize a variety of hosts. Even though the mechanism of host specificity is not fully understood, it has been speculated that presence of a virulence plasmid in some subsp. *enterica* serovars may contribute to that. Another factor that can be involved in host specificity is the presence of different sets of fimbrial operons in different serovars (Townsend et al., 2001).

A tentative phylogeny of the *Salmonella* pointing out the acquisition of main virulence traits is depicted in **Figure I1**

3. Overview of *Salmonella* infection

As mentioned above, depending on the serovar and the host, *Salmonella* infections have different outcomes: (i) gastroenteritis; (ii) systemic infection; and (iii) asymptomatic chronic carriage.

Salmonella lives primarily in the intestine of animal hosts, and is usually transmitted by the fecal-oral route. Infection normally starts via ingestion of contaminated water or food. Along the digestive track, *Salmonella* must endure some adverse conditions, such as the acidic pH in the stomach and the presence of bile in the duodenum. pH of the stomach is approximately 3.0, and this acidic environment destroys the majority of bacteria that enter the stomach (McGowan *et al.*, 1996; Tennant *et al.*, 2008). However, *S.* Typhimurium, which prefers to live and grow at a pH near neutrality, responds to acidic challenges through a complex adaptive system called the acid tolerance response, in which adaptation to mild (pH 5.8) or moderate (pH 4.4) acid conditions enables the cell to endure periods of severe acid stress (pH 3) (Foster & Hall, 1990; Lee *et al.*, 1994; Lee *et al.*, 1995). In the small intestine, *Salmonella* finds high concentrations bile, secreted in the duodenum during digestion. Bile salts have at least two distinct antibacterial activities: as detergents that disrupt the cell envelope (Gunn, 2000) and as DNA-damaging agents that cause DNA rearrangements and point mutations (Prieto *et*

al., 2004). Nevertheless, enteric bacteria are intrinsically resistant to high concentrations of bile and individual bile salts (Gunn, 2000).

When *Salmonella* reaches the distal small intestine, the pathogen has the ability to penetrate inside epithelial cells in a bacterial-induced phagocytosis-like process known as invasion. Invasion is a critical step for *Salmonella* infection in both, independently if the final outcome is gastroenteritis or systemic infection.

In the case of gastroenteritis, the infection is localized in the intestine, and invasion of intestinal epithelial cells triggers an inflammatory reaction in the intestinal mucosa, what leads to liquid accumulation in the intestinal lumen producing diarrhea. The inflammatory response creates a novel luminal niche, which favors growth of *Salmonella* over the resident microbiota of the intestine. Remarkably, the cascade of events that takes place as consequence of inflammation produces the accumulation of tetrathionate ($S_4O_6^{2-}$) in the intestinal lumen (Winter et al., 2010). Since *Salmonella* can use tetrathionate as terminal electron acceptor (Muller, 1923; Hensel et al., 1999), that series of events enables the pathogen to use tetrathionate respiration to obtain energy for growth in the inflamed gut lumen (Winter et al., 2010), taking advantage over the resident microbiota of the intestine.

In the case of systemic infection, the strategy used by *Salmonella* is different. After invasion, the pathogen crosses the epithelial barrier, and can survive inside macrophages and disseminates through the lymphatic system reaching deeper tissues. *Salmonella* can colonize target organs, particularly the spleen, liver, gall bladder and bone marrow, where bacteria can proliferate, and eventually causing death.

A fraction of individuals recovering from systemic infection become asymptomatic, life-long carriers of *Salmonella*, acting as reservoirs for future infections. In humans, serovar Typhi can establish chronic carriage in the gall bladder.

4. Type 3 secretion and *Salmonella* pathogenicity

The interaction between *Salmonella* and host cells involves the delivery of bacterial proteins into host cells cytoplasm through a specialized organelle called type 3 secretion

systems (TTSS) (Galan, 1999; Galan and Collmer, 1999; Galan, 2001). These systems are evolutionarily related to the flagellar export apparatus and are present not only in several species of bacteria pathogenic for animals but also in bacteria pathogenic for plants or in symbionts for plants or insects (Cornelis and Van Gijsegem, 2000; Galan, 2001).
TTSS are typically composed of approximately 25 proteins (Cornelis and Van Gijsegem, 2000) forming a needle-like complex that spans both the inner and outer bacterial membranes (Kubori et al., 1998).

As mentioned above, SPI-1 and SPI-2 encode complete type 3 secretion systems (TTSS), and some proteins, called effectors, that are delivered to the eukaryotic cell cytoplasm through the corresponding TTSS.

5. *Salmonella* invasion

Salmonella has the ability to invade epithelial cells in the animal intestine. That process is induced by the bacteria and requires the expression of genes encoded in SPI-1. *Salmonella* preferentially invades M cells of Peyer's patches in the ileum (Carter and Collins, 1974; Jones et al., 1994).
Mechanistically, the process is similar to macropinocytosis (Swanson and Watts, 1995): *Salmonella* induces the formation of membrane ruffles in the epithelial cells. The ruffles are localized in the site of bacterium-host cell interaction (Francis et al., 1999), and its formation involves actin polymerization (Goshima et al., 1984; Yahara et al., 1982; Finlay and Falkow, 1988). *Salmonella* promotes actin filaments rearrangements by delivering effectors into the target cell cytoplasm through SPI-1 TTSS (Guiney and Lesnick, 2005).
Invasion is a critical step in *Salmonella* infection, independently of the final outcome.

6. Reguation of *Salmonella* pathogenicity island 1 (SPI-1)

Since *Salmonella* pathogenicity islands have been acquired by horizontal transfer, a critical aspect of *Salmonella* pathogenesis is achieving a coordinated regulation of virulence genes. In some cases, the islands encode transcriptional regulators of their own expression that serve as link between the genes in the island and ancestral regulatory systems. That is illustrated in the case of SPI-1 and SPI-2 regulation:

(i) SPI-2 encodes a two-component regulatory system called SsrA (SpiR)/SsrB, which is responsible for SPI-2 genes expression. SsrA is the predicted integral membrane sensor, and SsrB is the cognate response regulator. SsrB binds to the promoter of all SPI-2 functional gene clusters and is essential for expression of the SPI-2-encoded TTSS and its effectors (Walthers at al., 2007). SsrA/SsrB integrates regulatory inputs by the nucleoid-associated protein H-NS (Bustamante et al., 2008), and the two component systems EnvZ/OmpR (Feng et al., 2003; Lee et al., 2000) and PhoP/PhoQ (Bijlsma and Groisman, 2005), thus contributing to connect SPI-1 expression with global regulators encoded in the core genome.

(ii) SPI-1 encodes 4 transcriptional activators of its own expression: HilA, HilC, HilD, and InvF. Those activators form a regulatory network that controls the expression of genes encoding TTSS components and effector proteins. HilA, a member of the OmpR/ToxR family (BAJAJ *et al.* 1995; LEE *et al.* 1992) 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 (AKBAR *et al.* 2003; RAKEMAN *et al.* 1999). 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).

The regulatory network formed by HilA, HilC, HilD and InvF serves to incorporate regulatory inputs coming from global regulators: the leucine-responsive regulatory protein, Lrp, reduces SPI-1 expression by directly repressing transcription of *hilA* and *invF* (Baek et al., 2009). HilC and HilD are substrates for the ATP-dependent Lon protease (Takaya et al., 2005), what contributes to turn down SPI-1 expression after invasion of epithelial cells (Boddicker and jones, 2004). The cytosolic protein HilE is a negative regulator of SPI-1, (Fahlen et al., 2000), and it likely interferes with HilD function by direct protein-protein interaction (Baxter and Jones, 2003). *hilE* transcription is directly activated by the fimbrial regulator FimYZ (Baxter and Jones, 2005), and repressed by the PTS-dependent regulator Mlc (Lim et al., 2007), thus transmitting those inputs to SPI-1 through HilD. In addition, it has been proposed that the two-component systems PhoP/PhoQ and PhoB/PhoR also activate *hilE* expression (Ellermeier and Slauch, 2007; Jones, 2005). In the case of PhoB/PhoR, the activation of *hilE* may me mediated by FimYZ (Jones, 2005). The Csr system also regulates SPI-1 (Altier et al., 2000). Overexpression of *csrA* represses SPI-1 expression (Altier et al., 2000; Martinez et al., 2011), and it has been shown that CsrA binds to a region in *hilD* mRNA that overlaps with the SD sequence, likely preventing translation and accelerating mRNA decay (Martinez et al., 2011). Genetic evidence suggests that BarA/SirA two-component regulatory system induces SPI-1 expression through Csr pathway, activating transcription of the CsrA antagonists CsrB and CsrC (Fortune et al., 2006). Fur (ferric uptake regulator) activates SPI-1 expression, and a functional HilD protein is necessary for that activation (Ellermeier and Slauch, 2008). EnvZ/OmpR two component system also activates SPI-1, likely controlling *hilD* expression at posttranscriptional level (Ellermeier et al., 2005; Ellermeier and Slauch, 2007). It has been recently reported that FliZ activates SPI-1 expression by controlling HilD activity (Chubiz et al., 2010).

Most of the regulatory systems known to control SPI-1 primarily target HilD expression, and then regulation is transmitted to the rest of SPI-1 genes (Ellermeier and Slauch, 2007). Surprisingly, those regulatory systems seem to control *hilD* expression at postranscriptional or postranslational level, rather than at the level of transcription initiation (Ellermeier and Slauch, 2007). In such context, it has been shown that HilD protein is the target for several regulatory systems: HilD is degraded by the ATP-dependent Lon protease (Takaya et al., 2005); HilE, a negative SPI-1 regulator (Fahlen

et al., 2000), physically interacts with HilD (Baxter and Jones, 2003), likely interfering with its function; it has been recently proposed that FliZ activates SPI-1 expression by somehow controlling HilD activity (Chubitz et al., 2010). Regulation of *hilD* expression at mRNA level has also been proposed: overproduction of the RNA binding protein CsrA represses SPI-1 expression (Altier et al., 2000; Martinez et al., 2011), and it has been shown that CsrA binds to a region in *hilD* mRNA that overlaps with the SD sequence, likely preventing translation and accelerating mRNA decay (Martinez et al., 2011); DNA adenine (Dam) methylation contribute to sustain high levels of SPI-1 expression by decreasing *hilD* mRNA turnover (López-Garrido and Casadesus, 2010). Hence, postranscriptional control of *hilD* expression seems to be a key event for SPI-1 regulation.

Postranscriptional control of *hilD* expression is essential for SPI-1 regulation by different regulatory systems (Ellermeier and Slauch, 2007). However, despite its importance in SPI-1 regulation, the mechanisms of postranscriptional control of *hilD* are poorly understood. In this work we study postranscriptional control of *hilD* expression and its impact on SPI-1 expression.

Regulation of *Salmonella enterica* pathogenicity island 1 (SPI-1) by DNA adenine methylation

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Regulation of SPI-1 by Dam 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 postranscriptional 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 an 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 unkown roles in postranscriptional control of gene expression.

INTRODUCTION

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 (CASADESUS and LOW 2006; LØBNER-OLESEN *et al.* 2005; REISENAUER *et al.* 1999; 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 (HEUSIPP *et al.* 2007; LØBNER-OLESEN *et al.* 2005; LOW and CASADESUS 2008; MARINUS 1996; WION and CASADESUS 2006). Because of its multiple roles in bacterial physiology, loss of Dam methylation causes pleiotropic defects in certain species (e. g., *Escherichia coli* and *Salmonella enterica*) and inviability in others (e. g. *Vibrio cholerae* and certain strains of *Yersinia enterocolitica*) (WION and CASADESUS 2006).

Dam⁻ mutants of *Salmonella 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 a ~40 kb gene cluster containing at least 37 genes (ALTIER 2005; JONES 2005; LOSTROH and LEE 2001), located at centisome 63 on the *Salmonella enterica* chromosome (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 7 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 (BAJAJ *et al.* 1995; LEE *et al.* 1992) 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 (AKBAR *et al.* 2003; RAKEMAN *et al.* 1999). 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 (ELLERMEIER and SLAUCH 2008; FORTUNE *et al.* 2006). In turn, HilE (FAHLEN *et al.* 2000) and Lon (BODDICKER and JONES 2004; TAKAYA *et al.* 2003) are negative regulators of SPI-1.

Figure 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 postranscriptional 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 postranscriptional regulator of *hilD* expression.

MATERIALS AND METHODS

Bacterial strains, plasmids, bacteriophages, and strain construction: The *Salmonella enterica* strains listed in Table 1 belong to serovar Typhimurium, and derive from ATCC 14028. For simplicity, *Salmonella enterica* serovar Typhimurium is often abbreviated as *S. enterica*. Luria-Bertani (LB) broth was used as liquid medium. Solid LB contained agar at 1.5% final concentration. Green plates (Chan *et al.* 1972) contained methyl blue (Sigma Chemical Co, St. Louis, MO) 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 Chemical Co., 40 mg/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 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 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 3xFLAG and HA epitope tags to protein-coding DNA sequences was carried out using plasmids pSUB11 (Km^{r}, 3xFLAG) and pSU314 (Cm^{r}, HA) as templates (Uzzau *et al.* 2001). Transductional crosses using phage P22 HT 105/1 *int201* [(Schmieger 1972) and G. Roberts, unpublished] 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. Re-construction of chromosomal duplications by P22 HT transduction was performed as previously described (Camacho and Casadesus 2001).

Table 1

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 with X-gal and LB with X-gal and tetracycline. An isolate which was Lac^+ in LB + X-gal + tetracycline and Lac^- in LB + 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 towards *hilD*. The insertion had thus generated a conditional, tetracycline-dependent *hilD* allele. Additional details about this allele are given in Figures S1 and S2.

Protein extracts and Western blot analysis. Total protein extracts were prepared from bacterial cultures grown at 37°C in LB medium until stationary phase (final $O.D._{600}$ ~2.5). Bacterial cells contained in 0.2 ml of culture were collected by centrifugation (16,000 g, 2 min, 4°C) and suspended in 50 ml 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 (1:5,000, Sigma Chemical Co, St. Louis, MO), anti-HA HA.11 monoclonal antibody (1:1,000, Covance, Princeton, NJ), and anti-GroEL polyclonal antibody (1:20,000, Sigma). Goat anti-mouse horseradish peroxidase-conjugated antibody (1:5,000, BioRad, Hercules, CA) or Goat anti-rabbit horseradish peroxidase-conjugated antibody (1:20,000, Santa Cruz Biotechnology, Heildelberg, Germany) were used as secondary antibodies. Proteins recognized by the antibodies were visualized by chemoluminescence using the luciferin-luminol reagents.

Quantitative reverse transcriptase PCR (quantitative RT-PCR) and calculation of relative expression levels. RNA was extracted from *S. enterica* stationary phase cultures ($O.D._{600}$ ~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 quantity and quality

of the extracted RNA were determined using a 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 g of DNase I-treated RNA was used for cDNA synthesis using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Quantitative 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 mM each. Real-time cycling conditions were as follows: (i) 95°C for 10 min; (ii) 40 cycles at 95°C for 15 s, 60°C 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°C with shaking until an OD_{600} around 2.5. Transcription initiation was stopped by adding 500 mg/ml rifampicin, and shaking vigorously during 10 s. Cultures were kept at 37°C. 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 qRT-PCR reactions, all using primers for the 5' region of *hilD* mRNA, were used.

-galactosidase assays: Levels of -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 3xFLAG 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).

Figure 2

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 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 b-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* (LOSTROH *et al.* 2000; RAKEMAN *et al.* 1999). 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 b-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.

Figure 3

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 suprise, 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 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). b-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 (Figs. 2 and 4), even though a *hilD::lac* transcriptional fusion did not show Dam-dependent control (Figure 4).

Figure 4

Expression of *hilD* from an heterologous promoter is Dam-dependent. The failure of a *hilD::lac* transcriptional fusion to show Dam-dependent regulation admits a number of explanations, artefactual 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 not be longer observed when *hilD* expression was driven from an heterologous promoter. In contrast, Dam dependence in a *hilD* gene driven from an heterologous promoter would provide evidence for postranscriptional 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 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 *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).

Figure 5

Dam methylation regulates *hilD* mRNA stability. To compare *hilD* mRNA stability in Dam^+ and Dam^- hosts, stationary cultures ($O.D._{600} = 2.5$) 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 min 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 s in a Dam^+ host, and 47 s 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 postranscriptional regulation in Dam^+ and Dam^- hosts.

Figure 6

Lack of Hfq enhances *hilD* mRNA instability in *Salmonella* Dam^- mutants. The evidence that *hilD* mRNA undergoes postranscriptional 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.

Figure 7

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. b-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 which outlines the differences between Dam$^-$ Hfq$^+$ and Dam$^-$ Hfq$^-$ mutants. Because *lac* fusions in individual SPI-1 genes have disparate b-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 an 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 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 8, panel A). Hence, lack of either Rne or Pnp suppresses the *hilD* mRNA instability caused by a *dam* mutation.

Figure 8

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). b-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 8, panel B is a normalized presentation of Table S2 data which 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 two fold (Figure 8, panel B). 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 8, panel B). Partial suppression by an *rne* mutation and complete supression by a *pnp* mutation further strengthens the evidence that the SPI-1 expression defect of *Salmonella* Dam$^-$ mutants is postranscriptional.

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 (BALBONTIN *et al.* 2006; BLYN *et al.* 1989; CAMACHO and CASADESUS 2002; HAAGMANS and VAN DER WOUDE 2000; JAKOMIN *et al.* 2008; KÜCHERER *et al.* 1986; ROBERTS *et al.* 1985; TORREBLANCA and CASADESUS 1996; WALDRON *et al.* 2002). 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 an 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 *hilD* mRNA turnover.

The hypothesis, at first sight odd, that Dam methylation is a postranscriptional 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 postranscriptional 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 (ELLERMEIER and SLAUCH 2008; KAGE *et al.* 2008; LUCAS and LEE 2001). However, with the potential exception of FliZ (KAGE *et al.* 2008) and CsrA (ALTIER *et al.* 2000; ELLERMEIER and SLAUCH 2007), postranscriptional regulators of *hilD* seem to affect either the HilD protein level (MATSUI *et al.* 2008; TAKAYA *et al.* 2005) 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 postranscriptional 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 *Yersinia 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 postranscriptional (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 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*::3xFLAG	This study
SV5456	*hilA*::3xFLAG	This study
SV5457	*invF*::3xFLAG	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
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*::3xFLAG	This study
SV5874	Δ*dam-231 hilA*::3xFLAG	This study
SV5875	Δ*dam-231 invF*::3xFLAG	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

LEGENDS TO FIGURES

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 ALTIER 2005; ELLERMEIER and SLAUCH 2003; JONES 2005; and LOSTROH and LEE 2001).

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*::3xFLAG), SV5874 (*hilA*::3xFLAG Dam^{-}), SV5455 (*hilC*::3xFLAG), SV5873 (*hilC*::3xFLAG Dam^{-}), SV5624 (*hilD*::HA), SV5625 (*hilD*::HA Dam^{-}), SV5457 (*invF*::3xFLAG), and SV5875 (*invF*::3xFLAG Dam^{-}).

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. Black histograms represent β-galactosidase activities measured in a Dam^{+} background. White 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 3 experiments.

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 3 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 3 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 3 independent experiments.

FIGURE 5.– A. Relative amounts of *hilD* mRNA in Dam$^+$ (black histograms) and Dam$^-$ (white histograms) isogenic strains expressing *hilD* from an 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 3 independent experiments. B. Transcription levels of two SPI-1 genes under HilD control (*invF* and *sipB*) in Dam$^+$ (black histograms) and Dam$^-$ (white histograms) strains that express *hilD* from an 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 3 independent experiments.

FIGURE 6.– Stability of *hilD* mRNA in Dam$^+$ (ATCC 14028) and Dam$^-$ (SV5264) isogenic hosts. Values are averages from 4 independent qRT-PCR reactions. Error bars are not shown because the standard deviations were extremely small.

FIGURE 7.– A. Enhancement of *hilD* mRNA instability in the absence of Hfq. Black histograms are for Dam$^+$ strains, and white histograms 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 3 independent experiments. B. Enhancement of the SPI-1 expression defect of *S. enterica* Dam$^-$ mutants by *hfq* null mutations. Black histograms are for Dam$^+$

strains, and white histograms 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 3 experiments.

FIGURE 8.– A. Suppression of *hilD* mRNA instability in the absence of degradosome components ribonuclease E (Rne) and polynucleotide phosphorylase (Pnp). Black histograms are for Dam^{+} strains, and white histograms 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 3 independent experiments. B. Suppression of the SPI-1 expression defect of *S. enterica* Dam^{-} mutants by *rne* and *pnp* mutations. Black histograms are for Dam^{+} strains, and white histograms 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 3 experiments.

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Figure 1

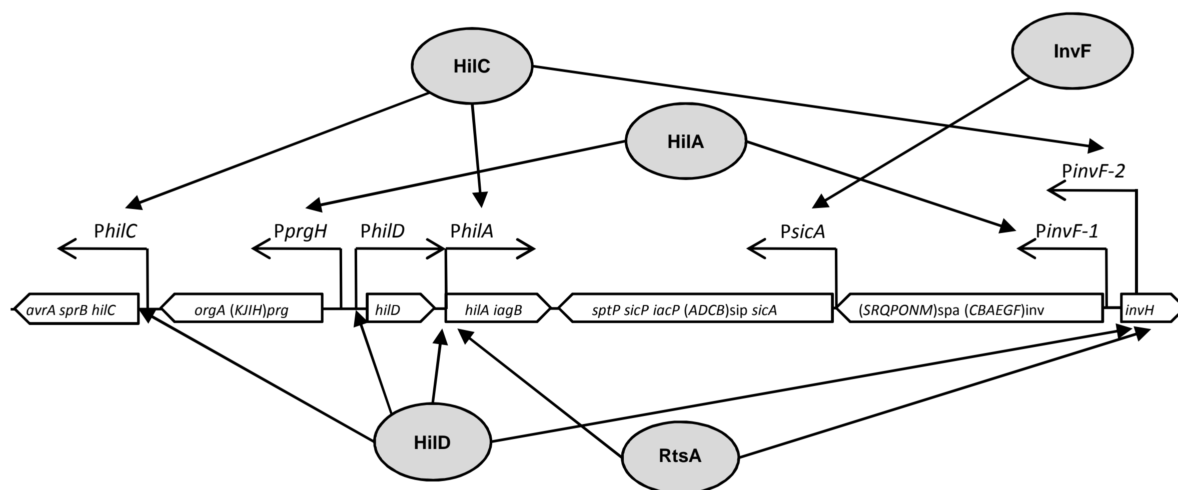


Figure 2

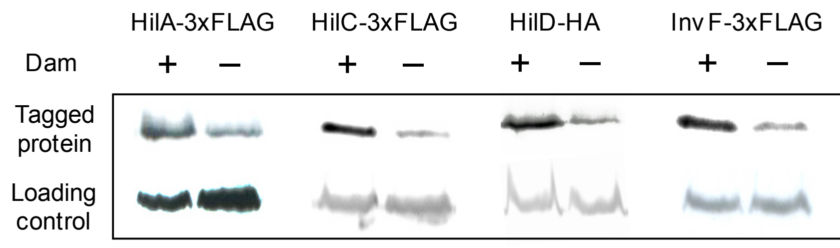


Figure 3

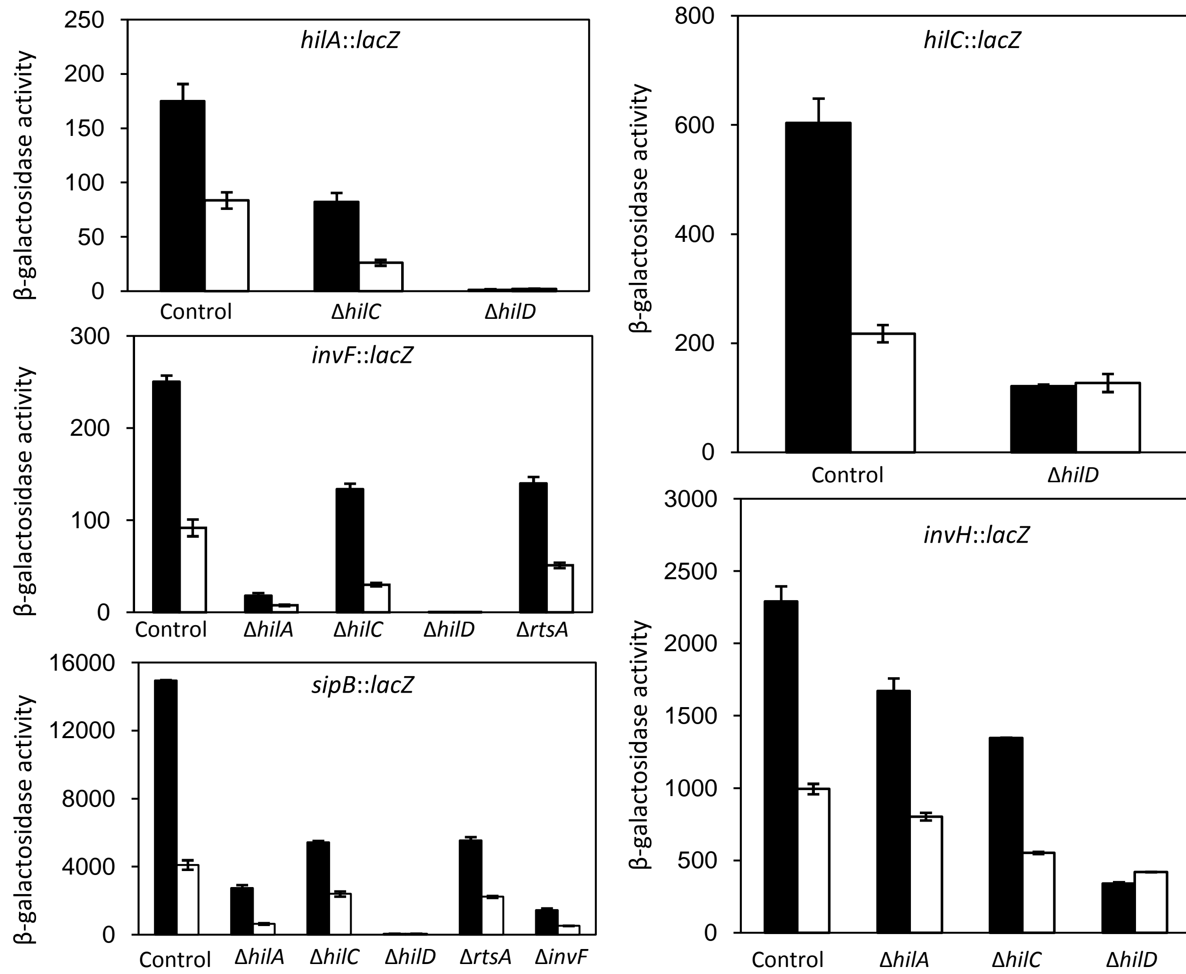


Figure 4

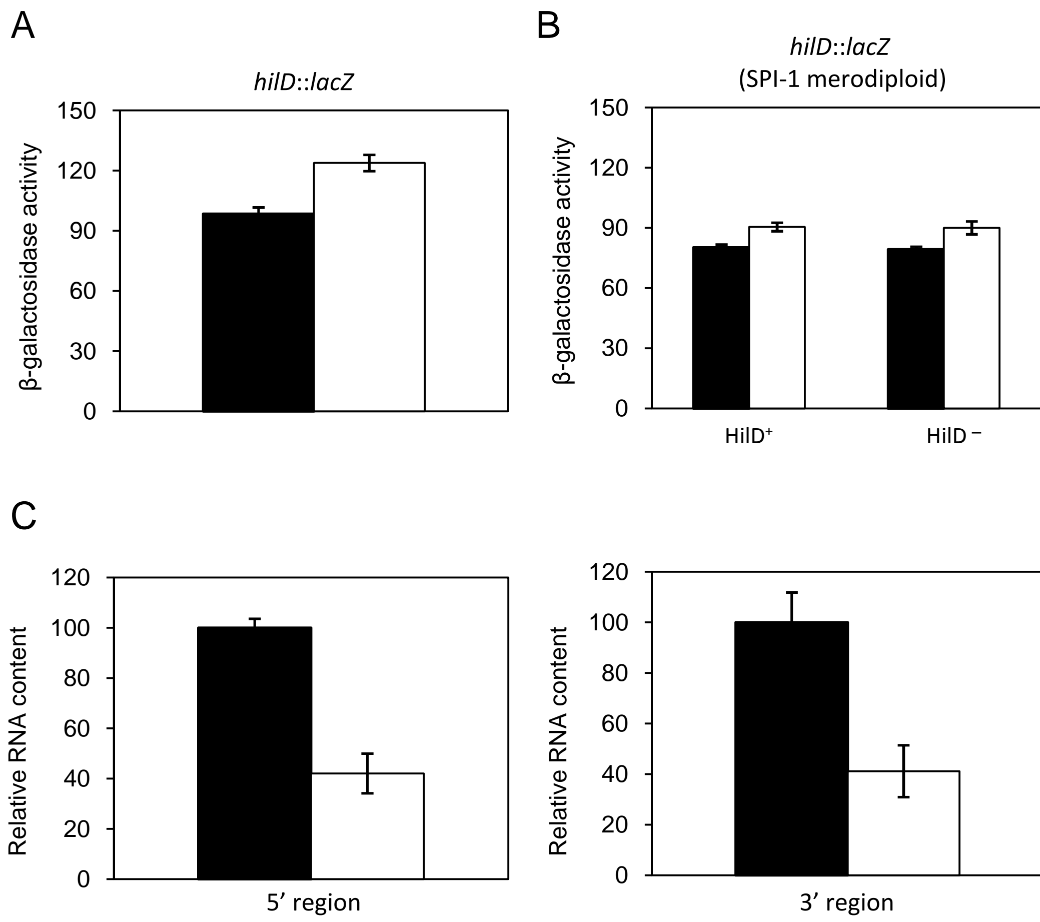


Figure 5

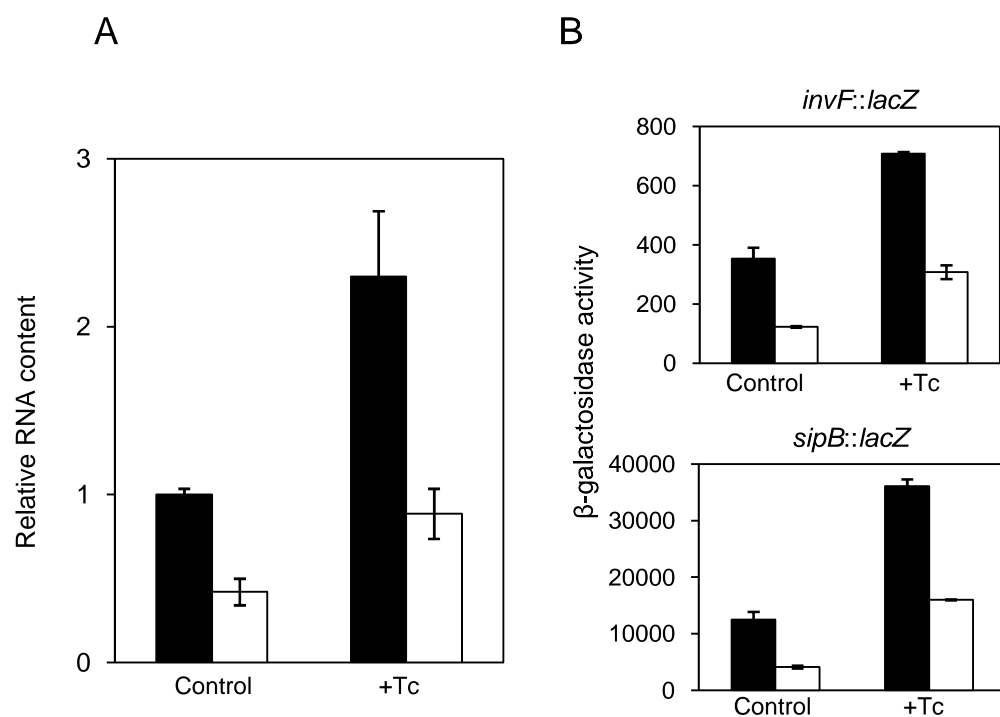


Figure 6

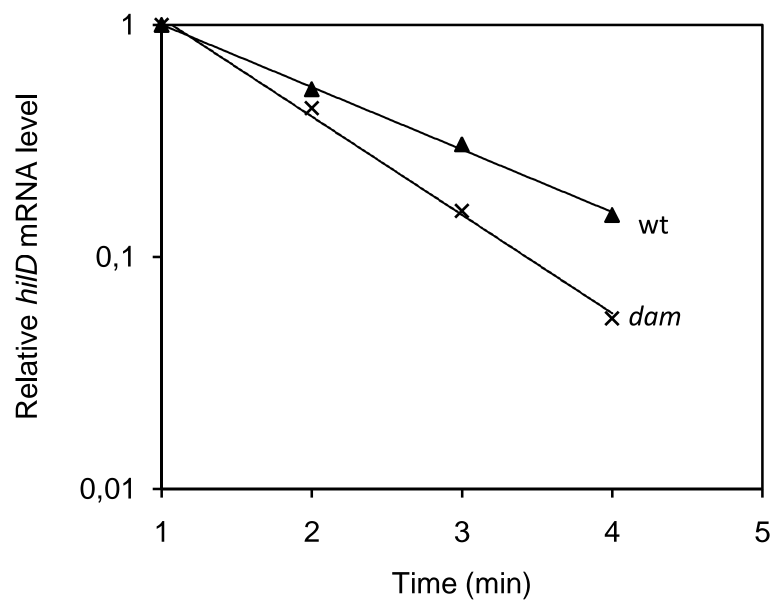


Figure 7

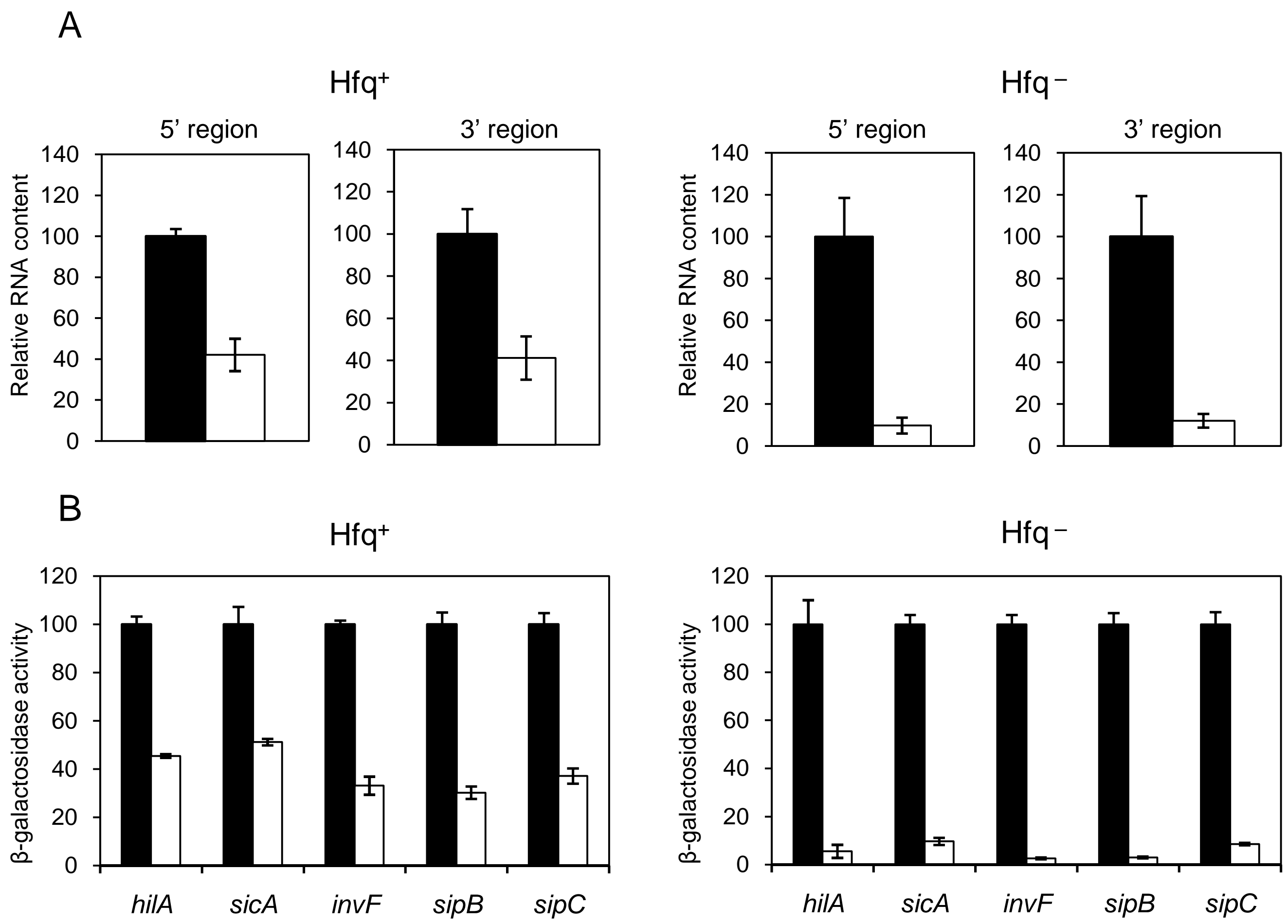


Figure 8

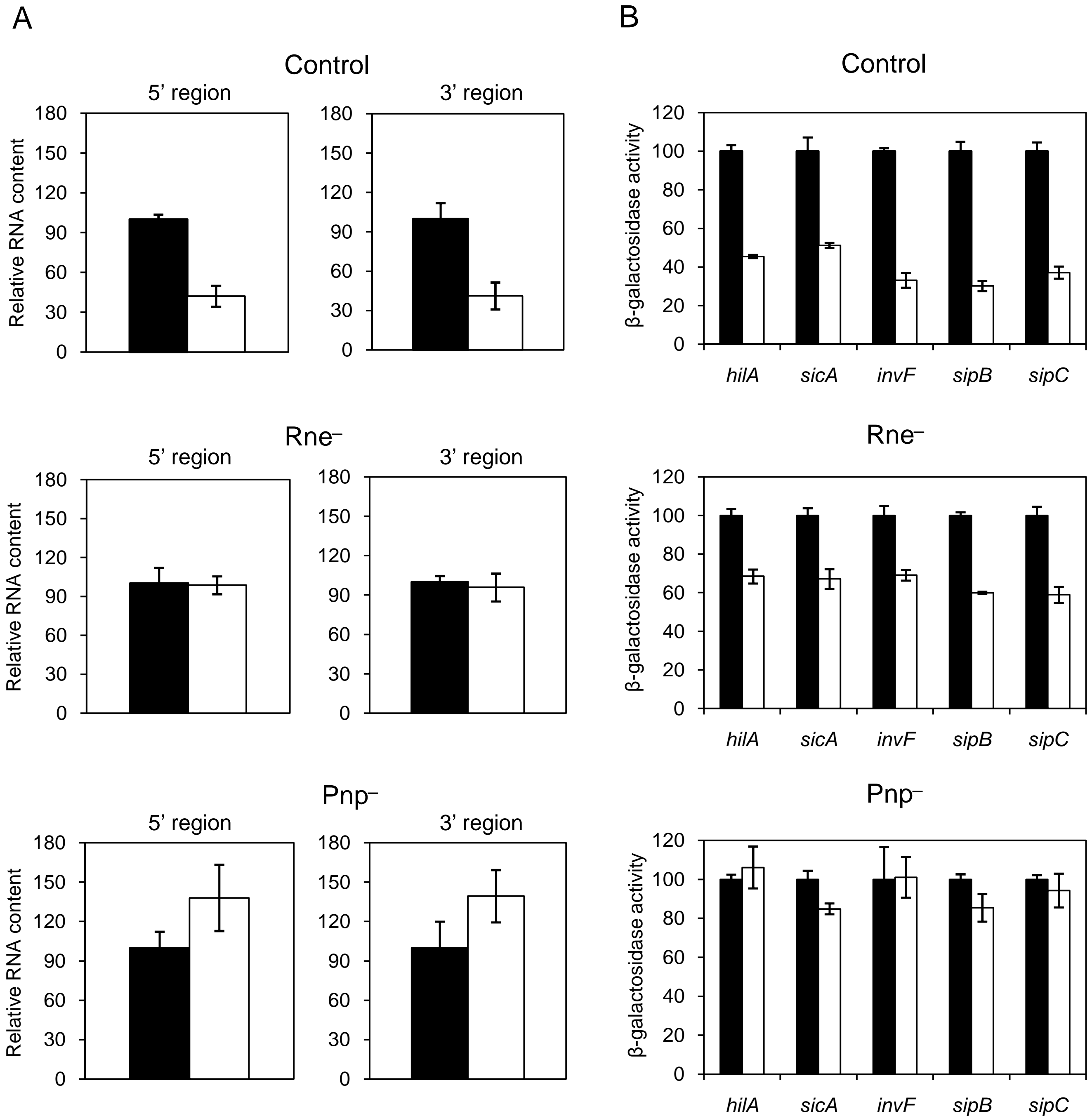


Table S1. Oligonucleotides used in this study (5'→3')

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	acgatgataaaaaaataatgcatatctcctctctcagattcatatgaatatcctccttag
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

Genetic cross-talk between Std fimbriae, SPI-1 and flagellum in *Salmonella enterica* serovar Typhimurium

Javier López-Garrido, Elena Puerta-Fernández and Josep Casadesús

ABSTRACT

Invasion of intestinal epithelial cells is a critical step in *Salmonella* infection and requires the expression of genes located in the *Salmonella* pathogenicity island 1 (SPI-1). We previously reported that methylation of adenines in the DNA (Dam methylation) is necessary to sustain a high level of SPI-1 expression. Dam methylation controls the expression of the SPI-1 transcriptional activator HilD at postranscriptional level, suggesting that the regulation is indirect. A genetic screen using a multicopy plasmid library of *Salmonella* genome has shown that *std* fimbrial operon is the link between Dam methylation and SPI-1. We have characterized 3 new ORFs belonging to *std* operon (*stdD*, *stdE*, and *stdF*), and have shown that all of them are upregulated in Dam$^-$ background. Deletion of *stdE* or *stdF* suppresses SPI-1 repression in *dam* mutants, and their overproduction in Dam$^+$ hosts leads to SPI-1 repression. Overexpression of StdE and StdF fail to regulate a *hilD::lac* transcriptional fusion, but reduce the level of *hilD* mRNA, suggesting that they control *hilD* expression at postranscriptional level. In addition to repress SPI, ectopic expression of StdE and StdF inhibits motility and represses flagellar gene expression. The regulatory corss-talk mediated by StdE and StdF may contribute to coordinate Std fimbriae-dependent adhesion, invasion and motility *in vivo*.

INTRODUCTION

Salmonella enterica is a Gram-negative bacterium that can cause gastric and systemic diseases is a variety of animal hosts. *Salmonella* is a typical foodborne pathogen, and infection usually starts by the ingestion of contaminated food or water. *Salmonella* has the ability to penetrate inside epithelial cells in the small intestine, in a process known as invasion. After invasion, the infection can remain localized in the intestine, usually producing self-limiting gastroenteritis. Depending on the specific strain-host combination, in some cases *Salmonella* can cross the intestinal epithelial barrier, and disseminates inside the host producing a systemic life-threatening infection, such as typhoid fever in humans. It has been estimated that 94 million of cases of gastroenteritis due to *Salmonella* species and 21 million of cases of typhoid fever occurs around the world every year, resulting in 155,000 and 200,000 deads respectively (Majowicz et al., 2010; Crumpet al., 2004).

Salmonella and *E. coli* are close relatives, and it has been estimated that both species diverged from 120 to 160 million years ago (Ochman and Wilson, 1987). The evolution of *Salmonella* pathogenicity has involved the sequential acquisition of genetic elements, each one contributing to different aspects of *Salmonella* virulence (Ochman and Groisman, 1997; Kelly et al., 2009). Amongst those elements are the *Salmonella* pathogenicity islands (SPIs), which are clusters of virulence genes in *Salmonella* chromosome. More than 10 SPIs has been described so far (Hensel, 2004), but some of them are serotype-specific. Since those regions are absent in *E. coli* chromosome, many of them have a different G-C content than the average *Salmonella* chromosome, and some are flanked by insertion sequences, it is thought that SPIs have been acquired by horizontal gene transfer (Kelly et al., 2009; Prowollik and McClelland, 2003). A key point of *Salmonella* pathogenesis success is the coordinated expression of virulence genes. That is achieved because SPI gene expression is integrated into preexisting regulatory networks, what generates a cross-talk between the core genome and horizontally-acquired elements (Ochman and Groisman, 1997).

One of the better characterized SPIs is the *Salmonella* pathogenicity island 1 (SPI-1), necessary for invasion of epithelial cells in the animal intestine. SPI-1 encodes a whole type 3 secretion system (TTSS), and some effector proteins that are translocated into the eukaryotic cell cytoplasm (Lostroh and Lee, 2001; Darwin and Miller, 1999). SPI-1

expression is directly controlled by four SPI-1-encoded transcriptional activators called HilA, HilC, HilD, and InvF (Altier, 2005; Ellermeier and Slauch, 2007; Jones, 2005; Lostroh and Lee, 2001). Those regulators form a regulatory network that serves to incorporate regulatory inputs coming from global regulators: the leucine-responsive regulatory protein, Lrp, reduces SPI-1 expression by directly repressing transcription of *hilA* and *invF* (Baek et al., 2009). HilC and HilD are substrates for the ATP-dependent Lon protease (Takaya et al., 2005), what contributes to turn down SPI-1 expression after invasion of epithelial cells (Boddicker and Jones, 2004). The cytosolic protein HilE is a negative regulator of SPI-1, (Fahlen et al., 2000), and it likely interferes with HilD function by direct protein-protein interaction (Baxter and Jones, 2003). *hilE* transcription is directly activated by the fimbrial regulator FimYZ (Baxter and Jones, 2005), and repressed by the PTS-dependent regulator Mlc (Lim et al., 2007), thus transmitting those inputs to SPI-1 through HilD. In addition, it has been proposed that the two-component systems PhoP/PhoQ and PhoB/PhoR also activate *hilE* expression (Ellermeier and Slauch, 2007; Jones, 2005). In the case of PhoB/PhoR, the activation of *hilE* may me mediated by FimYZ (Jones, 2005). The Csr system also regulates SPI-1 (Altier et al., 2000). Overexpression of *csrA* represses SPI-1 expression (Altier et al., 2000; Martinez et al., 2011), and it has been shown that CsrA binds to a region in *hilD* mRNA that overlaps with the SD sequence, likely preventing translation and accelerating mRNA decay (Martinez et al., 2011). Genetic evidence suggests that BarA/SirA two-component regulatory system induces SPI-1 expression through Csr pathway, activating transcription of the CsrA antagonists CsrB and CsrC (Fortune et al., 2006). Fur (ferric uptake regulator) activates SPI-1 expression, and a functional HilD protein is necessary for that activation (Ellermeier and Slauch, 2008). EnvZ/OmpR two component system also activates SPI-1, likely controlling *hilD* expression at posttranscriptional level (Ellermeier et al., 2005; Ellermeier and Slauch, 2007). It has been recently reported that FliZ activates SPI-1 expression by controlling HilD activity (Chubiz et al., 2010). A tentative diagram of SPI-1 regulation by global regulators is shown in **Figure 1**.

We have shown that DNA adenine (Dam) methylation is necessary to sustain a high level of SPI-1 expression (Balbontin et al., 2006; Lopez-Garrido et al., 2010). Dam methylase catalyzes postreplicative methylation of adenosines located in the palindromic sequence 5’-GATC-3’ (Casadesus and Low, 2006; Lobner-Olenes et al.,

2005; Wion and Casadesus, 2006). Genetic analysis have indicated that Dam-dependent regulation of SPI-1 is transmitted via HilD (Lopez-Garrido et al., 2010). As methylation state of specific GATC sites in promoter and regulatory regions can interfere with protein-DNA interactions, differential gene expression in Dam^+ and Dam^- backgrounds usually provides evidence for transcriptional regulation (Low and Casadesus, 2008; Marinus and Casadesus, 2009). However, our results indicate that Dam-dependent regulation of *hilD* is not transcriptional, but postranscriptional (Lopez-Garrido and Casadesus, 2010). In addition *hilD* region lacks GATC sites potentially involved in Dam-dependent regulation, what suggests that Dam-methylation controls *hilD* expression indirectly (see below).

The present study provides evidence that Dam-dependent regulation of *hilD* is transmitted through the horizontally-acquired *std* fimbrial gene cluster. Expression of *std* genes is directly repressed by Dam methylation (Balbontin et al., 2006; Jakomin et al., 2008; Jakomin et al., in preparation), and they are not expressed in Dam^+ background under laboratory growth conditions (Humphries et al., 2003; Humphries et al., 2005; Jakomin et al., 2008; Jakomin et al., in preparation). However, there are evidences that *std* fimbriae is produced in the animal intestine (Humphries et al., 2005, Weening et al., 2005). We have characterized 3 new genes belonging to the *std* gene cluster, and have renamed them *stdD*, *stdE*, and *stdF*. Below, we provide evidence that StdE and StdF are the molecular link between Dam methylation and SPI-1. In addition, we show that StdE and StdF inhibit motility by repressing flagellar gene expression. Those results underline the importance of cross-talk between horizontally-acquired elements and the core genome for *Salmonella* virulence.

MATHERIALS AND METHODS

Bacterial strains, plasmids, bacteriophages, and standard strain construction: All the *Salmonella enterica* strains listed in **Table 1** 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 plasmid pKD4 or 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 **Table S1**, together with the oligonucleotides (labeled "E") used for allele verification by the polymerase chain reaction. For the construction transcriptional and translational *lac* fusions in the *Salmonella* chromosome, FRT sites generated by excision of Kmr cassettes (DATSENKO and WANNER 2000) were used to integrate either plasmid pCE37 or pCE40 (ELLERMEIER *et al.* 2002). Addition of 3xFLAG epitope tag to protein-coding DNA sequences was carried out using plasmid pSUB11 (Kmr, 3xFLAG) (UZZAU *et al.* 2001). Transductional crosses using phage P22 HT 105/1 *int201* [(SCHMIEGER 1972) and G. Roberts, unpublished] 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.

Growth conditions

Luria-Bertani (LB) broth was used as standard liquid medium. Solid media were prepared by the addition of 1.5 % agar. When needed, kanamycin sulfate or chloramphenicol were added to LB at a final concentration of 50 μg/ml and 20 μg/ml respectively. For determination of expression of SPI-1 genes by β-galactosidase assay, western blot, or northern blot, saturated cultures were diluted 1:50 in LB and incubated at 37 ºC with shaking (200 rpm). Samples were taken when the cultures had reached stationary phase (O.D. 2-2.5). Green plates were prepared according to Chan and co-workers (CHAN *et al.* 1972), except that methyl blue (Sigma Chemical Co, St. Louis, MO) substituted for aniline blue. Plate tests for monitoring β-galactosidase activity used

5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside ("X-gal", Sigma Chemical Co.) as indicator.

Construction of relevant strains

P_{LtetO}-*stdEF* and P_{LtetO}-*stdF* constructions were achieved by inserting P_{LtetO} promoter (Lutz and Bujard, 1997) upstream *stdE* and *stdF* respectively, in *Salmonella* chromosome. P_{LtetO} insertion removed the upstream genes in *std* operon and *stdA* native promoter. A fragment containing the *cat* gene and P_{LtetO} promoter was amplified by PCR using pXG1 as template (Urban and Vogel, 2007). The primers were labelled P_{LtetO}UP and P_{LtetO}DO (**Table S1**). The PCR product was treated with DpnI to remove template traces. The construction was inserted in the chromosome by λRed recombinase-mediated recombination (Datsenko and Wanner, 2000) and Cm^r colonies were selected. Insertion of the construction was verified by PCR, using a couple of primers specific for *cat* gene and the target gene (**Table S1**).

β-galactosidase assays

Levels of β-galactosidase activity were assayed using the CHCl3-sodium dodecyl sulfate permeabilization procedure (MILLER 1972). Unless otherwise indicated, β-galatosidase activity data are the average and standard deviation of 3 independent experiments.

Protein extracts and Western blot analysis

Total protein extracts were prepared from bacterial cultures grown at 37°C in LB until stationary phase (final O.D.600 ~2.5). Bacterial cells taken according to 1 O.D.600 were collected by centrifugation (16,000 g, 2 min, 4°C) 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, using 12% gels. Conditions for protein transfer have been described elsewere (JAKOMIN *et al.* 2008). Optimal dilutions of primary antibodies were as follows: anti-Flag M2 monoclonal antibody (1:5,000, Sigma Chemical Co, St. Louis, MO), and anti-GroEL polyclonal antibody (1:20,000, Sigma). Goat anti-mouse horseradish peroxidase-conjugated antibody (1:5,000, BioRad, Hercules, CA) or Goat anti-rabbit horseradish peroxidase conjugated antibody (1:20,000, Santa Cruz

Biotechnology, Heildelberg, Germany) were used as secondary antibodies. Proteins recognized by the antibodies were visualized by chemoluminescence using the luciferin-luminol reagents.

***std* genes cotranscription analysis**

RNA used for retrotranscription was extracted from *S. enterica dam* mutant cultures grown in LB up to stationary phase (O.D.600 ~2.5) using the SV total RNA isolation system (Promega Co., Madison, WI) as described at http://www.ifr.ac.uk/safety/microarrays/protocols.h 1 tml. The quantity and quality of the extracted RNA were determined using a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). To get rid of genomic DNA contamination, the preparation was treated twice with DNase I (Turbo DNA free, Applied Biosystems/Ambion, Austin, TX), following the manufacturer instructions. 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). 1 µl of retrotranscribed cDNA was used as template for PCR with couples of primers specific for contiguous *std* ORFs (**Table S1**, **Figure 3**). Non-retrotrancribed RNA and genomic DNA were used as positive and negative controls, respectively.

RNA extraction and Northern analysis

2 ml of *S. enterica* cells reaching stationary phase were taken by centrifugation, and the pellet resuspended in 100 ul of a lysozyme solution (3 mg/ml in water). Cells lysis was facilitated by three consecutive freeze-thaw cycles. After lysis, RNA was extracted using 1ml of Trizol reagent (Invitrogen), according to manufacter's instructions. Finally, total RNA was resuspended in 30 ul of RNase-free water for subsequent uses. Quality and quantity of the obtained RNA was determined using a Nanodrop instrument. For northern blot analysis, 10 µg of total RNA was loaded per lane and electrophoresed in denaturing 1% agarose formaldehyde gels. Transfer and fixation to Hybond-N^+ membranes (GE Healthcare) were performed by vacuum using 0.05M NaOH. Filters were then hybridized using an internally labelled ([α-^{32}P]UTP) riboprobe specific for the first 300 nts of the *hilD* 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 of the RNase P RNA gene (*rnpB*). Images of radioactive filters were obtained with a Fuji, and quantification was performed using the Multy Gauge software.

Motility assays

Motility assays were carried out in motility agar plates, containing 10 g/l triptone (Difco), 5 g/l NaCl, and 0.25 % Bacto-agar (Cano et al., 2002). A sterile stick was soaked in saturated bacterial cultures grown in LB, and used to inoculate motility agar plates. Bacterial motility halos were compared after growth at 37ºC.

RESULTS

Genetic screens for regulators of *hilD* expression using a plasmid library of *Salmonella* genome

We reported previously that SPI-1 regulation by Dam methylation is transmitted via HilD (Lopez-Garrido et al., 2010). However, several lines of evidence suggest that the regulation is indirect: (i) Dam methylation regulates *hilD* expression at postranscriptional level, while direct regulation is usually transcriptional; (ii) *hilD* promoter and regulatory regions lack GATC sites; and (iii) although *hilD* coding sequence contains 3 GATC sites, site directed mutagenesis has demonstrated that they are dispensable for regulation by Dam methylation (not shown). We made two alternative hypotheses to explain Dam-dependent expression of *hilD*: (i) Dam^+ hosts produce a factor necessary for sustaining a high level of *hilD* expression; (ii) Dam^- host produce a factor able to repress *hilD* expression at postranscriptional level. Those hypothesis entail that the expression of the factor itself must be Dam-dependent. In addition, its ectopic expression would lead to SPI-1 repression in Dam^+ background, or SPI-1 induction in Dam^- background, depending on the case. Thus, we decided to perform a genetic screen for SPI-1 regulators in Dam^+ and Dam^- backgrounds, using a pBR328-based multicopy plasmid library of *Salmonella* genome. As reporter, we used *hilD::lac930* fusion. That fusion has *lacZ* is inserted right after *hilD* stop codon, and its expression is Dam-dependent. (**Figure S1**). Dam^+ and Dam^- isogenic strains carrying the *hilD::lac930* fusion were transduced with 9 pools of plasmid library, each containing around 1000 independent plasmids. Chloramphenicol resistant transductants were selected on LB plates with X-gal. We looked for colonies with reduced β-galactosidase activity (white colonies) in Dam^+ background, and colonies with increased β-galactosidase activity (intense blue) in Dam^- background. The cloned fragments were sequenced using specific primers flanking the insertion point (**Table S1**). Then we checked if the expression of any of the genes contained in the cloned fragments was differentially regulated by Dam methylation, comparing the sequencing results with transcriptomic data from Dam^+ and Dam^- isogenic backgrounds (Balbontin et al. 2006). In Dam^- background, we selected 12 independent candidates with increased β-galactosidase activity. Sequencing of the candidates revealed that all of them carried the same fragment cloned in pBR328 (**Figure S2B**). That fragment contained the gene encoding RtsA protein, known to activate *hilD* transcription (Ellermeier et al., 2005),

amongst other genes (**Figure S2B**). Thus, we concluded that increased *hilD::lac930* expression was due to overproduction of RtsA. However, neither *rtsA* nor the rest of the genes contained in the plasmid were regulated by Dam methylation, according to transcriptomic data. In Dam$^+$ background, five different plasmids reduced *hilD::lac930* β-galactosidase activity (**Figure S2A**). One of them contained a fragment with the *std* fimbrial gene cluster, amongst other genes (**Figure S2A**). According to transcriptomic data, *std* mRNA is more that 100-folds increased in Dam$^-$ background (Balbontin et al. 2006). We speculated that overexpression of *std* gene cluster could be the cause of SPI-1 repression in *dam* mutants.

All the genes in *std* gene cluster are overexpressed in *dam* mutants

Transcriptomic analyses have shown that *stdA*, *stdB*, *stdC*, and the uncharacterized ORFs STM3026, and STM3025 are repressed by Dam methylation. Dam-dependent expression of *stdA*, *stdB*, and *stdC* has been confirmed by independent methods (Balbontin et al., 2006; Jakomin et al., 2008). However, regulation by Dam methylation of STM3026 and STM3025 has not been further analyzed. DNA sequence indicates the existence of an additional uncharacterized ORF, designed STM3025.1N, in the intergenic region between STM3026 and STM3025. We studied Dam-dependent expression of STM3026, STM3025.1N, and STM3025 by two independent methods: (i) analysis of β-galactosidase activity of STM3026::*lac*, STM3025.1N::*lac*, and STM3025::*lac* translational fusions in Dam$^+$ and Dam$^-$ backgrounds (**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**). Both, β-galactosidase assay and Western blot analysis show that the 3 genes are overexpressed in Dam$^-$ background, thus confirming the transcriptomic data. In addition, detection of the proteins by western blot demonstrates that the 3 ORFs are translated. We renamed STM3026, STM3025.1N and STM3025 as *stdD*, *stdE*, and *stdF* respectively in *Salmonella enterica* serovar Typhimurium.

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

stdA expression is driven by a promoter whose transcription is Dam-dependent (Jakomin et al., 2008; Jakomin et al., in preparation). The fact that expression all the genes in *std* cluster is induced in a *dam* mutant provides evidence that they constitute a polycistronic operon transcribed from *stdA* promoter. In order to confirm that, we experimentally analyzed cotranscription of contiguous ORFs by retroranscription and PCR. Total RNA was extracted from a *dam* mutant. Traces of DNA were removed by a treatment with Turbo DNase (Ambion). The RNA sample was split in two fractions: one fraction was retrotranscribed to cDNA using random primers; the other fraction suffered the same treatment, but water was added instead of retrotranscriptase. Then, we performed PCR with couples of primers specific for contiguous ORFs (**Figure 3A**, **Table S1**), in the presence of different templates: *Salmonella* genomic DNA as positive control, non-retrotranscribed RNA as negative control, and cDNA as query. The PCR product were resolved in a 2 % agarose with 0.5 µg/ml ethydium bromide, and visualized under UV light. As shown in **Figure 3B,** a PCR product of the expected size was obtained using either genomic DNA or cDNA. No band was observed when RNA was used as template. That indicates that *std* gene cluster constitute a polycistronic operon, coordinately transcribed from the promoter identified upstream *stdA* (Jakomin et al., 2008; **Figure 3A**). However, internal promoters may also exist.

stdE and *stdF* are the molecular link between Dam methylation and SPI-1

As discussed above, we considered the possibility that overexpression of *std* operon was the cause of SPI-1 repression in *dam* mutants. If such were the case, we reasoned, SPI-1 repression in Dam$^-$ background would be suppressed by deletion of *std* operon. We examined the expression of *invF::lac* and *sipB::lac* fusions in isogenic Dam$^+$ and Dam$^-$ strains with either an intact *std* operon or a deletion covering the whole operon. As shown in **Figure 4A**, β-galactosidase activities of *invF::lac* and *sipB::lac* fusions were reduced more than two folds in Dam$^-$ background in the strain with a functional *std* operon (Control). However, in a strain lacking the *std* operon, both fusions displayed similar β-galactosidase activities in Dam$^+$ and Dam$^-$ backgrounds. That result suggests that one or more proteins encoded in *std* operon are involved in the transmission of Dam-dependent regulation to SPI-1. In order to identify such protein(s), Dam-

dependent regulation of an *invF::lac* fusion was monitored in a set of mutants carrying in frame deletions in individual *std* genes (**Figure 4B**). *invF::lac* expression remains Dam-dependent in strains lacking *stdA*, *stdB*, *stdC*, and *stdD*, suggesting that those genes are not directly required for Dam-dependent control of SPI-1. However, repression of *invF::lac* expression in Dam$^-$ background is suppressed in strains lacking either *stdE* or *stdF*. That indicates that the product of both genes are necessary for SPI-1 repression in *dam* mutants.

StdE and StdF independently repress SPI-1 expression

A conceivable model SPI-1 for regulation by Dam methylation is that, in Dam$^-$ background, *std* operon is overexpressed and *stdE* and *stdF* gene products repress SPI-1. If that were the case, overexpression of *stdE* and *stdF* would repress SPI-1 expression in Dam$^+$ background as well. To test that hypothesis, we placed P_{LtetO} promoter (Lutz and Bujard, 1997) upstream *stdE* and *stdF*, to get a constitutive Dam-independent expression of those genes. To avoid possible interferences, *stdA* native promoter and all the upstream genes in the operon were removed. We made two basic constructions: P_{LtetO}-*stdEF* in which P_{LtetO} was placed upstream *stdE* in the chromosome and, in consequence, both *stdE* and *stdF* were constitutively expressed; and P_{LtetO}-*stdF* in which P_{LtetO} was inserted right upstream *stdF*, thus expressing constitutively only that gene. As controls, we used the same strains carrying in frame deletions in *stdE*, *stdF*, or both, depending on the case (**Figure 5A**). To check if the constructions were working, we analyzed the levels of StdE and StdF in protein extracts from P_{LtetO}-*stdEF* and P_{LtetO}-*stdF* strains by Western blot, using protein variants tagged with the 3xFLAG epitope (**Figure S3**). The level of StdE was around 160 and 40 folds higher in P_{LtetO}-*stdEF* extracts compared to wild type and *dam* mutants extracts respectively. The level of StdF in P_{LtetO}-*stdEF* and P_{LtetO}-*stdF* extracts was around 16 folds higher that in wild type extracts, but similar to that of *dam* mutants. Thus, P_{LtetO} constructs overexpress both, *stdE* and *stdF*, but the overexpression is higher for *stdE* than for *stdF* (**Figure S3**). We examined the expression the SPI-1 genes *hilA*, *invF*, and *sipB* in strains carrying P_{LtetO}-*stdE*, P_{LtetO}-*stdF*, and their respective controls. We analyzed the expression of the selected genes by two independent methods: (i) using *lac* fusions and measuring β-galactosidase activity; and (ii) determining protein levels by Western blot, using protein

variants tagged with the 3xFLAG epitope. Equivalent results were obtained by both methods for the 3 genes studied (**Figure 5B**). The results can be summarized as follows: expression *hilA*, *invF*, and *sipB* are strongly repressed when P_{LtetO} is inserted upstream *stdE*. The repression is partially relieved when *stdE* is deleted. However, deletion of *stdF* alone does not relieve the repression. Deletion of both genes completely restores SPI-1 expression to wild type levels, suggesting that SPI-1 repression is due to *stdE* and *stdA* expression and not to polar effects. Insertion of P_{LtetO} upstream *stdF* considerably represses the expression of the selected genes, but less than insertion upstream *stdE*. Deletion of *stdF* completely suppresses such repression. Those results provide evidence that both, StdE and StdF, can repress SPI-1 expression. In addition, both proteins can repress SPI-1 independently, since each of them can do it in the absence of the other. However, the impact of StdE seems to be quantitatively bigger than StdF, but it may be due to its higher overexpression (**Figure S**3)

StdE and StdF regulate *hilD* expression at postranscriptional level

We previously reported that Dam methylation was necessary to sustain high levels of the SPI-1 transcriptional activator HilD (Lopez-Garrido and Casadesus, 2010). In addition, regulation of *hilD* by Dam methylation was not transcriptional, but postranscriptional. Taking that into account, we would expect that StdE and StdF repressed *hilD* expression at postranscriptional level. We examined the expression of *hilD* using *hilD::lac1* transcriptional, in which *lacZ* was inserted right in *hilD* transcription start site. We have determined that *hilD*::lac1 fusion reflects *hilD* regulation at transcriptional level, since it is activated in the presence of a multicopy plasmid encoding RtsA (**Figure S1**), a known transcriptional activator of *hilD* (Ellermeier et al., **Figure S4**). Expression of *hilD::Lac1* fusion was determined in wild type (Control), P_{LtetO}-*stdEF*, P_{LtetO}-*stdEF* Δ*stdEF*, P_{LtetO}-*stdF*, and P_{LtetO}-*stdF* Δ*stdF* backgrounds (**Figure 5A, Figure6A**). β-galactosidase activities were similar in all the strains, suggesting that StdE and StdF do not regulate *hilD* transcription initiation. In order to study if StdE and StdF repressed *hilD* expression at postranscriptional level, we analyzed *hilD* mRNA levels by Northern blot in the following backgrounds: wild type (Control), P_{LtetO}-*stdEF,* P_{LtetO}-*stdEF* Δ*stdE*, P_{LtetO}-*stdEF* Δ*stdF*, P_{LtetO}-*stdEF* Δ*stdEF*, P_{LtetO}-*stdF*, and P_{LtetO}-*stdF* Δ*stdF*. As shown in **Figure 6B**, the level of *hilD* mRNA is

reduced around 4 folds in $P_{Ltet O}$-*stdEF* strain compared to wild type. Deletion of *stdE* partially recovers *hilD* mRNA level, and simultaneous deletion of *stdE* and *stdF* completely restores *hilD* mRNA to wild type level. The amount of *hilD* mRNA is reduced around two folds in $P_{Ltet O}$-*stdF* background, and such reduction is completely abolished by *stdF* deletion. Taking together, those results support the idea that StdE and StdF repress *hilD* expression at postranscriptional level.

Inhibition of motility by StdE and StdF

Inhibition of motility by proteins encoded in fimbrial operons has been reported in different bacterial species. (Lin et al., 2001; Simms and Mobley, 2008). We wandered if StdE and StdF were also able to inhibit motility. We examined motility of strain carrying the constructions $P_{Ltet O}$-*stdEF*, $P_{Ltet O}$-*stdF* and their respective deletion controls in motility agar plates (**Figure 7A**). Bacteria simultaneously expressing *stdE* and *stdF* are non-motile. A partial recovery of motility is observed when either *stdE* or *stdF* is deleted, and deletion of both genes completely restores motility to wild type levels. Motility is also reduced when $P_{Ltet O}$ is placed right upstream *stdF*, and deletion of *stdF* completely suppresses such reduction. We considered the possibility that StdE and StdF were somehow repressing flagellar gene expression. We analyzed the expression of an *flgK::lac* fusion in strains carrying either $P_{Ltet O}$-*stdEF* or $P_{Ltet O}$-*stdF*, and their respective deletion controls (**Figure 7B**). The pattern of *flgK* expression correlates with the defects observed in motility, what suggests that StdE and StdF inhibit motility by repressing flagellar gene expression.

DISCUSSION

We have characterized three new ORFs in the *std* gene cluster: STM3026, STM3025.1N, and STM3025, renamed *stdD*, *stdE*, and *stdF* respectively. Western blot analyses have demonstrated that those genes are expressed in *dam* mutants (**Figure 2B**). *stdD* encodes a predicted outer membrane protein, while StdE and StdF are predicted cytoplasmic proteins. Our results indicate that StdE and StdF repress SPI-1 and flagellar gene expression, suggesting the existence of a regulatory cross-talk that might coordinate Std fimbriae production, invasion and motility.

We have provided evidence that *stdA*, *stdB*, *stdC*, *stdD*, *stdE*, and *stdF* constitute a polycistronic operon: (i) expression of all those genes is activated in Dam⁻ background (**Figure 2**); and (ii) retrotranscription and PCR show that they are cotranscribed (**Figure 3**). *std* transcription is driven by a promoter located upstream *stdA* (Jakomin et al., 2008). Transcription from P_{stdA} is activated by direct binding of HdfR protein to a regulatory region upstream the promoter. However, methylation of two GATC sites in the regulatory region prevents binding of HdfR, thus repressing *std* expression (Jakomin et al., 2008; Jakomin et al., in preparation). It is likely that all *std* genes are coordinately regulated by Dam methylation due to a common transcription from P_{stdA}. However, internal promoters may also exist.

Salmonella enterica dam mutants 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 (Marinus and Casadesus, 2009). We previously reported that SPI-1 expression was repressed in Dam⁻ background (Balbontin et al., 2006; Lopez-Garrido et al., 2010). Dam methylation activates SPI-1 by controlling *hilD* expression at postranscriptional level (Lopez-Garrido and Casadesus, 2010), what together with the absence of GATC sites in *hilD* regulatory regions, suggests that the regulation is indirect. Our genetic screens and subsequent experiments have identified *std* fimbrial operon as the link between Dam methylation and SPI-1: (i) a multicopy plasmid containing the whole *std* operon represses *hilD* expression; (ii) *std* genes are upregulated in Dam⁻ background (**Figure 2**; Balbontin et al., 2006; Jakomin et al., 2008); and (iii) SPI-1 regulation by Dam methylation is completely suppressed in a strain lacking the whole *std* operon (**Figure 4A**). Altogether, those results suggest that overexpression of *std* in *dam* mutants leads to SPI-1 repression. It has been previously

shown that the extreme attenuation of *Salmonella enterica* serovar Typhimurium *dam* mutants upon oral infection (Garcia-del Portillo et al., 1999; Heithoff et al., 1999) is partially suppressed by deletion of *std*, suggesting that overexpression of Std fimbriae is detrimental for *Salmonella* virulence (Jakomin et al., 2008). The regulatory link between *std* and SPI-1 provide evidence that the detrimental effect of *std* overexpression for *Salmonella* virulence may be due to SPI-1 repression.

Epistasis analysis indicates that Dam-dependent control of SPI-1 requires the last two genes of *std* operon, StdE and StdF. That is further supported by the following observations: (i) constitutive expression of *stdE* and *stdF* in Dam^+ background represses SPI-1 expression (**Figure 5**); (ii) StdE and StdF are overproduced in Dam^- background (**Figure 2**); (iii) Dam methylation, StdE, and StdF regulate SPI-1 expression through HilD; and (iv) as happens in the case of Dam methylation, StdE and StdF does not regulate *hilD* transcription, but controls the level of *hilD* mRNA (**Figure 6**). A conceivable model to explain regulation by Dam methylation of SPI-1 is depicted in **Figure 8**: in Dam^+ background, GATC sites of P_{stdA} regulatory region are methylated, preventing binding of HdfR and activation of *std* transcription. In the absence of Dam methylation, HdfR activates transcription from P_{stdA} and all the proteins encoded in the operon are overproduced. Then, StdE and StdF repress *hilD* expression at posttranscriptional level, and as a consequence, the whole SPI-1 is downregulated.

When constitutively expressed, StdE and StdF can regulate *hilD* expression independently, since each one can do that in the absence of the other (**Figure 5**). However, deletion of any of them suppresses SPI-1 repression in *dam* mutants, suggesting that both are necessary for repression. It may be possible that both trigger the same regulatory pathway, but they can do that independently when overproduced. StdE shares around 40 % and 50 % identity with the transcriptional activators GrlA and CaiF from *E. coli* and *Enterobacter cloacae* respectively. Interestingly, StdF is similar to an uncharacterized protein encoded just downstream CaiF in *Enterobacter cloacae* chromosome, that is part of a hypothetical fimbrial gene cluster which genetic organization resembles that of *std* operon, suggesting that they may have a common origin. StdF is also 27 % similar to the SPI-1 encoded protein SprB from *Salmonella*. SprB is a transcriptional regulator able to bind to *hilD* and *siiA* promoters and repress and activate their expression respectively (Saini and Rao, 2010). Even though StdE and StdF are similar to known transcriptional regulators, they do not regulate *hilD* at

transcriptional level, but at postranscriptional level. Thus, either they have acquired the ability to control gene expression at postranscriptional level in *Salmonella enterica*, or they regulate transcription of a postranscriptional regulator of *hilD*.

In addition to repress SPI-1 expression, StdE and StdF can also inhibit motility. Expression of an *flgK::lac* fusion is repressed by constitutive expression of *stdE* and *stdF*, suggesting that motility inhibition is the consequence of flagellar gene repression. Overexpression of either *stdE* or *stdF* produces a mild motility inhibition (**Figure 7**). However, simultaneous expression of both genes has a synergistic effect and completely inhibiting motility and *flhK* expression (**Figure 7**). Thus, it may be possible that StdE and StdF regulate flagellar gene expression through the same pathway.

Several studies have reported coordinated expression of fimbrial, flagellar and invasion genes: in *Proteus mirabilis*, the protein encoded by the last gene in the *mrp* fimbrial operon, MrpJ, inhibits motility when the fimbrial operon is expressed (Lin et al., 2001). Similarly, PapX, the product of the last gene in the *pap* fimbrial operon of uropathogenic *Escherichia coli*, represses the expression of the flagellar mater regulator FlhDC by direct binding to its promoter region (Simms and Mobley, 2008). One case particularly interesting is the coordinated expression of type I fimbriae, flagellum and invasion genes mediated by FimY and FimZ. Those two proteins are encoded in independent transcriptional units next to the type I fimbrial operon *fim*. FimY and FimZ are essential for *fim* operon transcription (Yeh at al., 1995; Tynker and Clegg, 2000). In addition, FimZ represses SPI-1expression by activating transcription of the gene encoding the SPI-1 negative regulator HilE (Baxter and jones, 2005; Saini et al., 2009), and inhibits motility by repressing *flhDC* expression (Clegg and Hughes, 2002). That situation resembles that of StdE and StdF, suggesting that coordinated expression of fimbrial, flagellar and invasion genes is important for *Salmonella* virulence and persistence in the intestine (Saini et al., 2010)

A tempting speculation derived from the above results is that invasion and motility would be inhibited when *std* operon was expressed. *std* is not expressed under laboratory growth conditions in wild type *Salmonella* (Humphries et al., 2003; Humphries et al., 2005; Jakomin et al., 2008). However, several lines of evidence suggest that Std fimbriae is produced in the animal intestine: (i) mice infected with serovar Typhimutium seroconvert to StdA, the major fimbrial component of Std

fimbriae (Humphries et al., 2005); and (ii) *std* deletion reduces the ability of *Salmonella* to colonize and persist in the cecum of infected mice, while producing no defect in colonizing the small intestine. (Weening et al., 2005). According to that, it has been reported that Std fimbriae bind α(1,2)fucose residues, which are abundant in the cecal mucosa (Chessa et al., 2008). *Salmonella* invasion takes place preferentially in the ileum, while in the cecum invasion is inhibited. *std* expression in the cecum might contribute to inhibition of invasion. In addition, fimbriated bacteria would inhibit motility and live attached to cecal mucosa, what could help to the persistence of *Salmonella* in the host intestine.

The genome of *Salmonella* has evolved by the acquisition of genetic modules that provided new abilities to interact with eukaryotic cells and exploit different niches (Ochman and Groisman, 1997; Prowllik and McLelland, 2003). A critical point of that modular evolution is to get a coordinate expression of the different genetic modules. In some cases, the modules themselves carry regulatory genes of its own expression, which serve as connection with the core genome (Ochman and Groisman, 1997). In addition, there are some examples of cross-talk between genetic modules independently acquired: the SPI-1 encoded regulator HilD can activate SPI-2 expression during late stationary growth phase (Bustamante et al., 2008); expression of SPI-4 genes is activated by the SPI-1-encoded SprB transcriptional regulator (Saini and Rao, 2010); HilE, a SPI-1 negative regulator, is encoded in a region of *Salmonella* chromosome that has been proposed to be a pathogenicity island (Baxter et al., 2003); SPI-1 and SPI-2-encoded transcriptional regulators control the expression of effector proteins located outside those islands (Darwin and Miller, 2001; Knodler et al., 2002), and some are located in horizontally-acquired DNA fragments (Hardt et al., 1998; Wood et al., 1998). *std* genes are well conserved amongst *Salmonella* serovars, but are absent in closely related species (Prowllik and McCLelland, 2003), suggesting that the cluster has been acquired by horizontal gene transfer. Thus, the connection between *std* and SPI-1 provides an additional example of cross talk between horyzontally-aquired genes.

LEGENDS TO FIGURES

FIGURE 1. Diagram showing SPI-1 regulation. Arrows represent genes, and circles represent proteins. Grey arrows and circles mean SPI-1-encoded regulators. Regulators encoded outside SPI-1 are in white.

FIGURE 2. Expression of STM3026 (*stdD*), STM3025.1N (*stdE*), and SSTM3025 (*stdF*) in Dam^{+} and Dam^{-} backgrounds. A. β-galactosidase activity of STM3026::*lac*, STM3025.1N::*lac*, and STM3025::*lac* translational fusions. Black histograms represent the activity in Dam^{+} background, and white histograms represent the activity in Dam^{-} background. β-galactosidase activity has been relativized to 100 in Dam^{-} background. B. Levels of STM3026, STM3025.1N, and STM3025 proteins in extracts from Dam^{+} and Dam^{-} hosts. 3xFLAG-tagged proteins were detected 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 Dam^{-} background

FIGURE 3. A. Diagram of *std* operon. Opposite arrows below the diagram represent couple of primers used to examine cotranscription of contiguous coding sequences. B. Cotranscription of contiguous coding sequences in *std* operon. PCR fragments generated with couples of primers specific for contiguous coding sequences were resolved in a 2 % agarose gel and stained with ethidium bromide.

FIGURE 4. β-galactosidase activities of *invF::lac* and *sipB::lac* fusions in a strain containing an intact *std* operon (Control) and in a strain lacking the whole *std* operon (Δ*std*). Black and white histograms represent β-galactosidase activities in Dam^{+} and Dam^{-} backgrounds respectively. B. Regulation by Dam methylation of an *invF::lac* fusion in strains carrying in frame deletions in individual *std* genes. Black histograms represent β-galactosidase activities in Dam^{+} background, and white histograms represent β-galactosidase activities in Dam^{-} background.

FIGURE 5. A. Diagram representing P_{LtetO}-*stdEF* and P_{LtetO}-*stdF* constructions, and their respective deletion controls lacking *stdE*, *stdF*, or both. B. Expression of *hilA*, *invF*, and *sipB* in strains carrying a native *std* operon (Control), P_{LtetO}-*stdEF*, P_{LtetO}-*stdF*, and their respective control constructions. Histograms represent β-galactosidase activities of *hilA::lac*, *invF::lac*, and *sipB::lac* fusions. HilA-3XFLAG, InvF-3xFLAG

and SipB.3xFLAG levels in protein extracts from appropriate strains were determined by western blotting using anti-FLAG antibodies. GroEL level was used as loading control. For quantification, the ratio tagged protein / GroEL was relativized to 100 in Control strains. The symbols "+" and "-"indicate presence or absence respectively of StdE or StdF.

FIGURE 6. A. β-galactosidase activity of *hilD::lac* fusion in a strain with a native *std* operon (Control), and in strains carrying P_{LtetO}-*stdEF*, P_{LtetO}-*stdEF* Δ*stdEF*, P_{LtetO}-*stdF* or P_{LtetO}-*stdF* Δ*stdF* constructions. The symbols "+" and "-"indicate presence or absence respectively of StdE or StdF. B. Level of *hilD* mRNA in RNA extracts from wild type, P_{LtetO}-*stdEF*, P_{LtetO}-*stdEF*, and their respective control strains. *hilD* mRNA was detected by Northern blotting, using a riboprobe specific for the first 300 nucleotides of *hilD* coding sequence. The symbols "+" and "-"indicate presence or absence respectively of StdE or StdF.

FIGURE 7. A. Growth of wild type, P_{LtetO}-*stdEF*, P_{LtetO}-*stdEF* Δ*stdE*, P_{LtetO}-*stdEF* Δ*stdF*, P_{LtetO}-*stdEF* Δ*stdEF*, P_{LtetO}-*stdF* and P_{LtetO}-*stdF* Δ*stdF* strains on motility agar plates. B. Expression of an *flgK::lac* fusion in wild type background (Control), and in strains carrying P_{LtetO}-*stdEF*, P_{LtetO}-*stdF*, and their respective deletion controls. The symbols "+" and "-"indicate presence or absence respectively of StdE or StdF.

FIGURE 8. Model of SPI-1 regulation by Dam methylation.

FIGURE S1. B-galactosidase activity of *hilD::lac930* fusion in Dam^{+} background (black histogram), and Dam^{-} background (white histogram).

FIGURE S2. β-galactosidase activity of *hilD::lac930* fusion in control strains (carrying pBR328 empty plasmid), or candidates with reduced β-galactosidase activity in Dam^{+} background (A) and increased β-galactosidase activity in Dam^{-} background (B). Diagrams representing the fragments cloned in the different candidates are also shown.

FIGURE S3. A. Diagram showing tagging of StdE and StdF with 3xFLAG epitope in strains carrying P_{LtetO}-*stdEF* and P_{LtetO}-stdF constructions. B. Level of StdE-3xFLAG and StdF3xFLAG in protein extracts from wild type, Dam^{-}, P_{LtetO}-*stdEF* and P_{LtetO}-*stdF* backgrounds. 3xFLAG tagged proteins were detected 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 Dam^{-} background.

FIGURE S4. β-galactosidase activity of *hilD::lac1* fusion in a strain carrying pBR328 empty plasmid (black histogram), and pBR328 with a fragment containing *rtsA* gene (white histogram).

Strain designation	**Genotype or description**	**Reference or source**
14028	Wild type	ATCC
SV5264	Δ*dam-231*	This study
SV6530	Φ(*stdD'-lacZ*$^+$)(Hyb)	This study
SV6531	Δ*dam-231* Φ(*stdD'-lacZ*$^+$)(Hyb)	This study
SV6532	Φ(*stdE'-lacZ*$^+$)(Hyb)	This study
SV6533	Δ*dam-231* Φ(*stdE'-lacZ*$^+$)(Hyb)	This study
SV6534	Φ(*stdF'-lacZ*$^+$)(Hyb)	This study
SV6535	Δ*dam-231* Φ(*stdF'-lacZ*$^+$)(Hyb)	This study
SV6662	*stdD*::3xFLAG	This study
SV6663	Δ*dam-231 stdD*::3xFLAG	This study
SV6748	*stdE*::3xFLAG	This study
SV6749	Δ*dam-231 stdE*::3xFLAG	This study
SV6501	*stdF*::3xFLAG	This study
SV6502	Δ*dam-231 stdF*::3xFLAG	This study
SV5297	Φ(*invF'-lacZ*$^+$)(Hyb)	This study
SV5298	Δ*dam-231* Φ(*invF'-lacZ*$^+$)(Hyb)	This study
SV5382	Φ(*sipB'-lacZ*$^+$)(Hyb)	This study
SV5383	Δ*dam-231* Φ(*sipB'-lacZ*$^+$)(Hyb)	This study
SV6473	Δ*std* Φ(*invF'-lacZ*$^+$)(Hyb)	This study
SV6474	Δ*std* Δ*dam-231* Φ(*invF'-lacZ*$^+$)(Hyb)	This study
SV6475	Δ*std* Φ(*sipB'-lacZ*$^+$)(Hyb)	This study
SV6476	Δ*std* Δ*dam-231* Φ(*sipB'-lacZ*$^+$)(Hyb)	This study
SV6477	Δ*stdA* Φ(*invF'-lacZ*$^+$)(Hyb)	This study
SV6478	Δ*stdA* Δ*dam-231* Φ(*invF'-lacZ*$^+$)(Hyb)	This study
SV6479	Δ*stdB* Φ(*invF'-lacZ*$^+$)(Hyb)	This study
SV6480	Δ*stdB* Δ*dam-231* Φ(*invF'-lacZ*$^+$)(Hyb)	This study
SV6481	Δ*stdC* Φ(*invF'-lacZ*$^+$)(Hyb)	This study
SV6482	Δ*stdC* Δ*dam-231* Φ(*invF'-lacZ*$^+$)(Hyb)	This study
SV6483	Δ*stdD* Φ(*invF'-lacZ*$^+$)(Hyb)	This study
SV6484	Δ*stdD* Δ*dam-231* Φ(*invF'-lacZ*$^+$)(Hyb)	This study
SV6485	Δ*stdE* Φ(*invF'-lacZ*$^+$)(Hyb)	This study
SV6486	Δ*stdE* Δ*dam-231* Φ(*invF'-lacZ*$^+$)(Hyb)	This study
SV6487	Δ*stdF* Φ(*invF'-lacZ*$^+$)(Hyb)	This study
SV6488	Δ*stdF* Δ*dam-231* Φ(*invF'-lacZ*$^+$)(Hyb)	This study
SV6503	P_{LtetO}-*stdEF*	This study
SV6506	P_{LtetO}-*stdEF* Δ*stdE*	This study
SV6508	P_{LtetO}-*stdEF* Δ*stdF*	This study
SV6634	P_{LtetO}-*stdEF* Δ*stdEF*	This study
SV6504	P_{LtetO}-*stdF*	This study
SV6635	P_{LtetO}-*stdF* Δ*stdF*	This study
SV5284	Φ(*hilA'-lacZ*$^+$)(Hyb)	This study
SV6513	P_{LtetO}-*stdEF* Φ(*hilA'-lacZ*$^+$)(Hyb)	This study
SV6750	P_{LtetO}-*stdEF* Δ*stdE* Φ(*hilA'-lacZ*$^+$)(Hyb)	This study
SV6751	P_{LtetO}-*stdEF* Δ*stdF* Φ(*hilA'-lacZ*$^+$)(Hyb)	This study

SV6752	P_{LtetO}-*stdEF* Δ*stdEF* Φ(*hilA'-lacZ*$^{+}$)(Hyb)	This study
SV6519	P_{LtetO}-*stdF* Φ(*hilA'-lacZ*$^{+}$)(Hyb)	This study
SV6753	P_{LtetO}-*stdF* Δ*stdF* Φ(*hilA'-lacZ*$^{+}$)(Hyb)	This study
SV6512	P_{LtetO}-*stdEF* Φ(*sipB'-lacZ*$^{+}$)(Hyb)	This study
SV6754	P_{LtetO}-*stdEF* Δ*stdE* Φ(*sipB'-lacZ*$^{+}$)(Hyb)	This study
SV6755	P_{LtetO}-*stdEF* Δ*stdF* Φ(*sipB'-lacZ*$^{+}$)(Hyb)	This study
SV6756	P_{LtetO}-*stdEF* Δ*stdEF* Φ(*sipB'-lacZ*$^{+}$)(Hyb)	This study
SV6518	P_{LtetO}-*stdF* Φ(*sipB'-lacZ*$^{+}$)(Hyb)	This study
SV6757	P_{LtetO}-*stdF* Δ*stdF* Φ(*sipB'-lacZ*$^{+}$)(Hyb)	This study
SV6511	P_{LtetO}-*stdEF* Φ(*invF'-lacZ*$^{+}$)(Hyb)	This study
SV6758	P_{LtetO}-*stdEF* Δ*stdE* Φ(*invF'-lacZ*$^{+}$)(Hyb)	This study
SV6759	P_{LtetO}-*stdEF* Δ*stdF* Φ(*invF'-lacZ*$^{+}$)(Hyb)	This study
SV6760	P_{LtetO}-*stdEF* Δ*stdEF* Φ(*invF'-lacZ*$^{+}$)(Hyb)	This study
SV6517	P_{LtetO}-*stdF* Φ(*invF'-lacZ*$^{+}$)(Hyb)	This study
SV6761	P_{LtetO}-*stdF* Δ*stdF* Φ(*invF'-lacZ*$^{+}$)(Hyb)	This study
SV6762	P_{LtetO}-*stdEF* *hilA*::3xFLAG	This study
SV6763	P_{LtetO}-*stdEF* Δ*stdE* *hilA*::3xFLAG	This study
SV6764	P_{LtetO}-*stdEF* Δ*stdF* *hilA*::3xFLAG	This study
SV6765	P_{LtetO}-*stdEF* Δ*stdEF* *hilA*::3xFLAG	This study
SV6766	P_{LtetO}-*stdF* *hilA*::3xFLAG	This study
SV6767	P_{LtetO}-*stdF* Δ*stdF* *hilA*::3xFLAG	This study
SV6768	P_{LtetO}-*stdEF* *sipB*::3xFLAG	This study
SV6769	P_{LtetO}-*stdEF* Δ*stdE* *sipB*::3xFLAG	This study
SV6770	P_{LtetO}-*stdEF* Δ*stdF* *sipB*::3xFLAG	This study
SV6771	P_{LtetO}-*stdEF* Δ*stdEF* *sipB*::3xFLAG	This study
SV6772	P_{LtetO}-*stdF* *sipB*::3xFLAG	This study
SV6773	P_{LtetO}-*stdF* Δ*stdF* *sipB*::3xFLAG	This study
SV6774	P_{LtetO}-*stdEF* *invF*::3xFLAG	This study
SV6775	P_{LtetO}-*stdEF* Δ*stdE* *invF*::3xFLAG	This study
SV6776	P_{LtetO}-*stdEF* Δ*stdF* *invF*::3xFLAG	This study
SV6777	P_{LtetO}-*stdEF* Δ*stdEF* *invF*::3xFLAG	This study
SV6778	P_{LtetO}-*stdF* *invF*::3xFLAG	This study
SV6779	P_{LtetO}-*stdF* Δ*stdF* *invF*::3xFLAG	This study
SV5457	*invF*::3xFLAG	This study
SV5456	*hilA*::3xFLAG	This study
SV5459	*sipB*::3xFLAG	This study
SV6410	Φ(*hilD-lacZ1*)	This study
SV6515	P_{LtetO}-*stdEF* Φ(*hilD-lacZ1*)	This study
SV6780	P_{LtetO}-*stdEF* Δ*stdEF* Φ(*hilD-lacZ1*)	This study
SV6521	P_{LtetO}-*stdF* Φ(*hilD-lacZ1*)	This study
SV6781	P_{LtetO}-*stdF* Δ*stdF* Φ(*hilD-lacZ1*)	This study
SV4917	Φ(*flgK-lacZ*)	This study
SV6782	P_{LtetO}-*stdEF* Φ(*flgK-lacZ*)	This study
SV6783	P_{LtetO}-*stdEF* Δ*stdE* Φ(*flgK-lacZ*)	This study
SV6784	P_{LtetO}-*stdEF* Δ*stdF* Φ(*flgK-lacZ*)	This study
SV6785	P_{LtetO}-*stdEF* Δ*stdEF* Φ(*flgK-lacZ*)	This study

SV6786	P_{LtetO}-*stdF* Φ(*flgK-lacZ*)	This study
SV6787	P_{LtetO}-*stdF* Δ*stdF* Φ(*flgK-lacZ*)	This study
SV6413	Φ(*hilD-lacZ930*)	This study
SV6788	Δ*dam-231* Φ(*hilD-lacZ930*)	This study
SV6509	P_{LtetO}-*stdE*::3xFLAG	This study
SV6510	P_{LtetO}-*stdEF*::3xFLAG	This study
SV6664	P_{LtetO}-*stdF*::3xFLAG	This study
SV6524	Φ(*hilD-lacZ1*)/pBR328	This study
SV6525	Φ(*hilD-lacZ1*)/pBR328-*rtsA*	This study

Figure 1

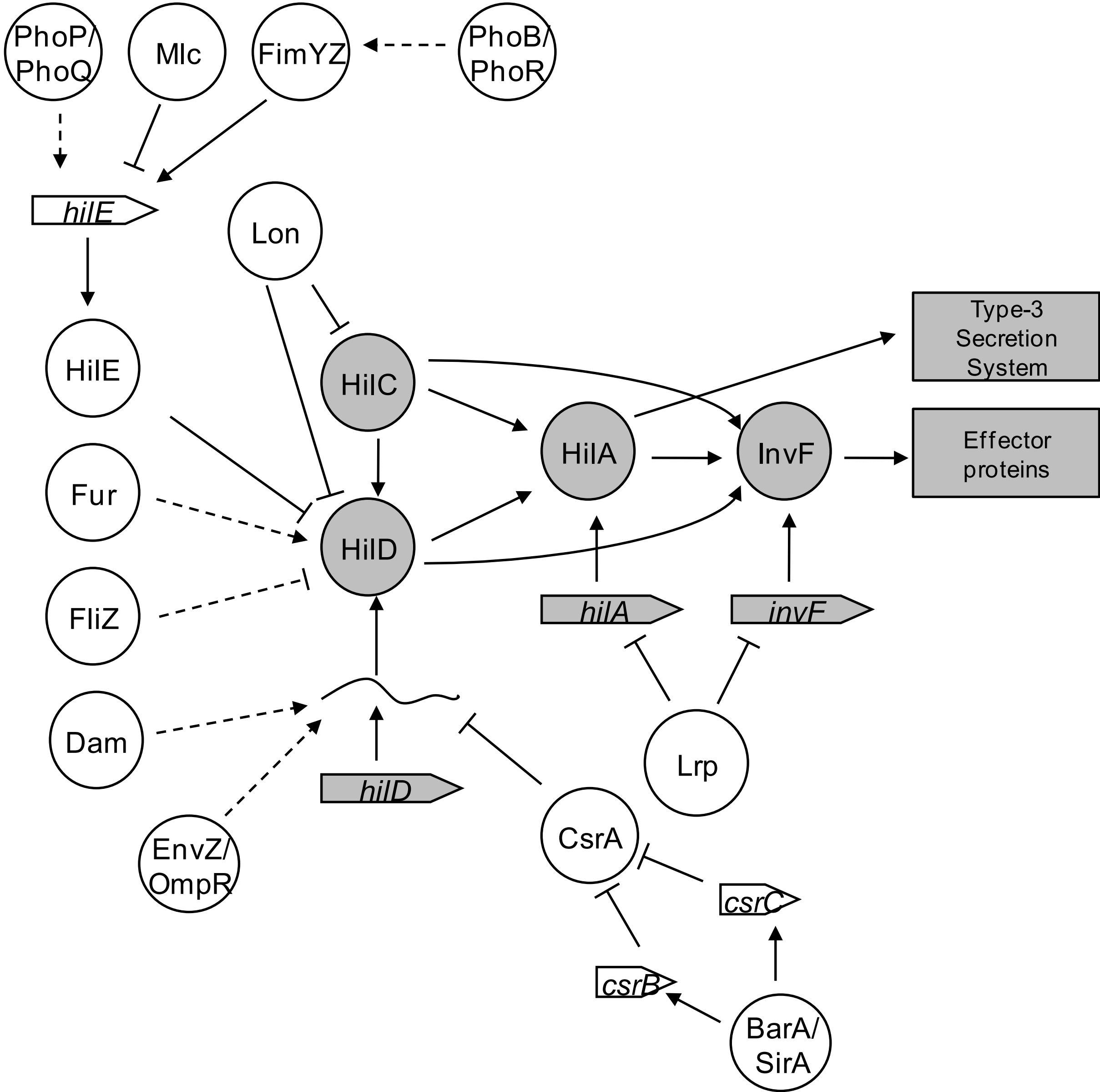
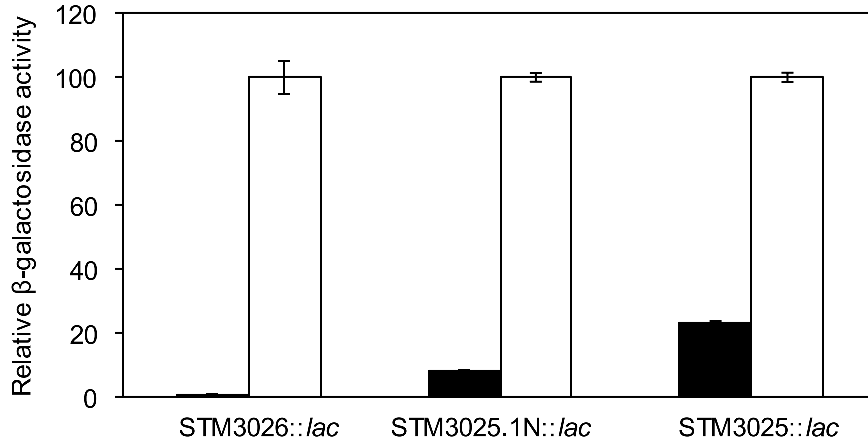


Figure 2

A



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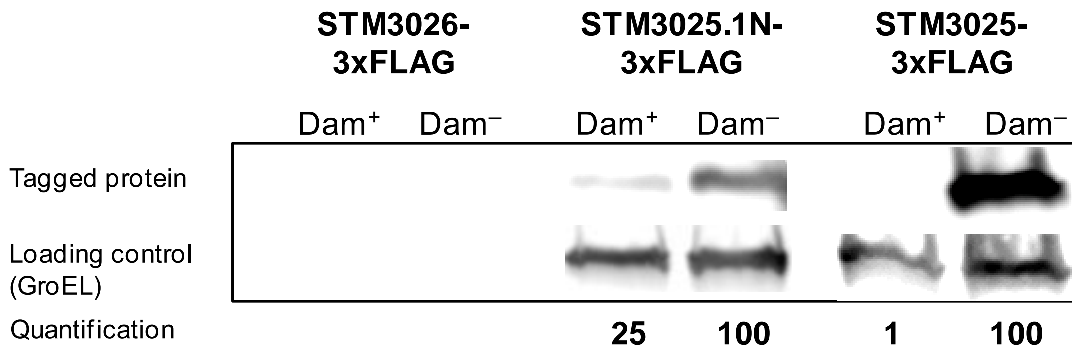
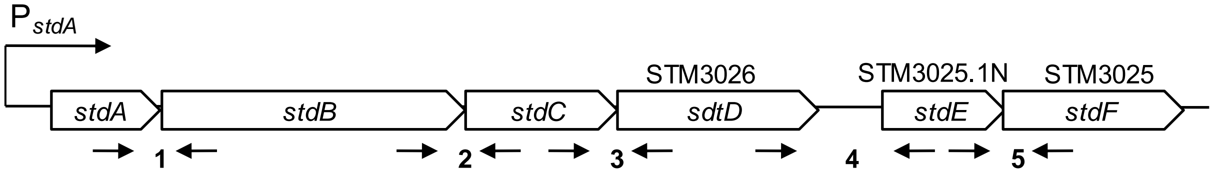


Figure 3

A



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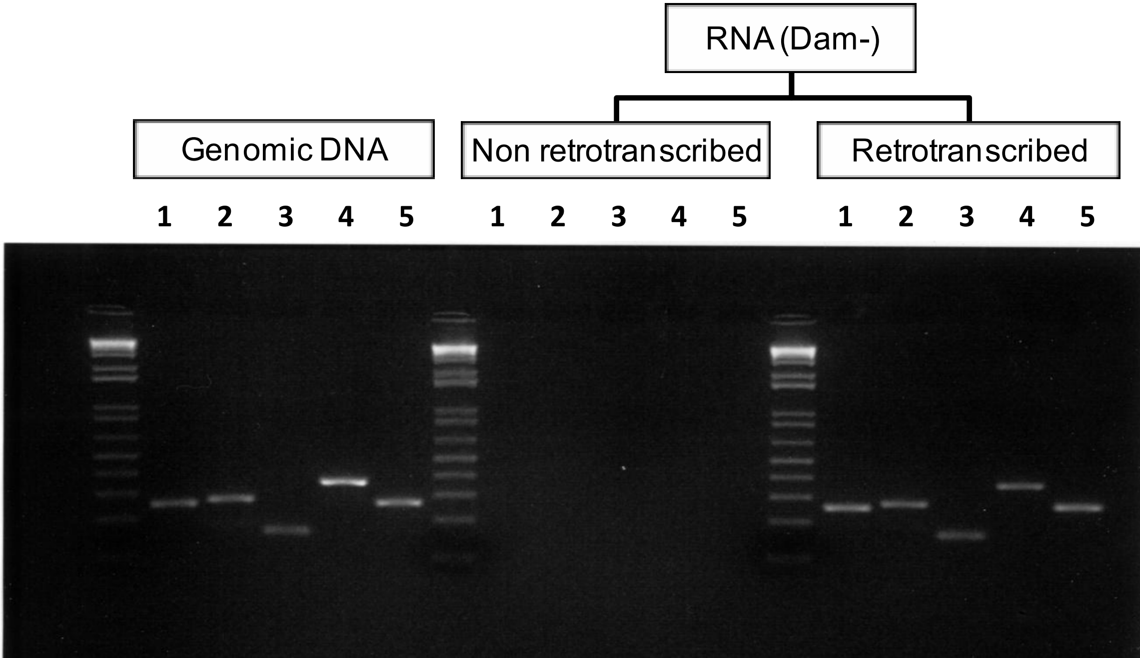


Figure 4

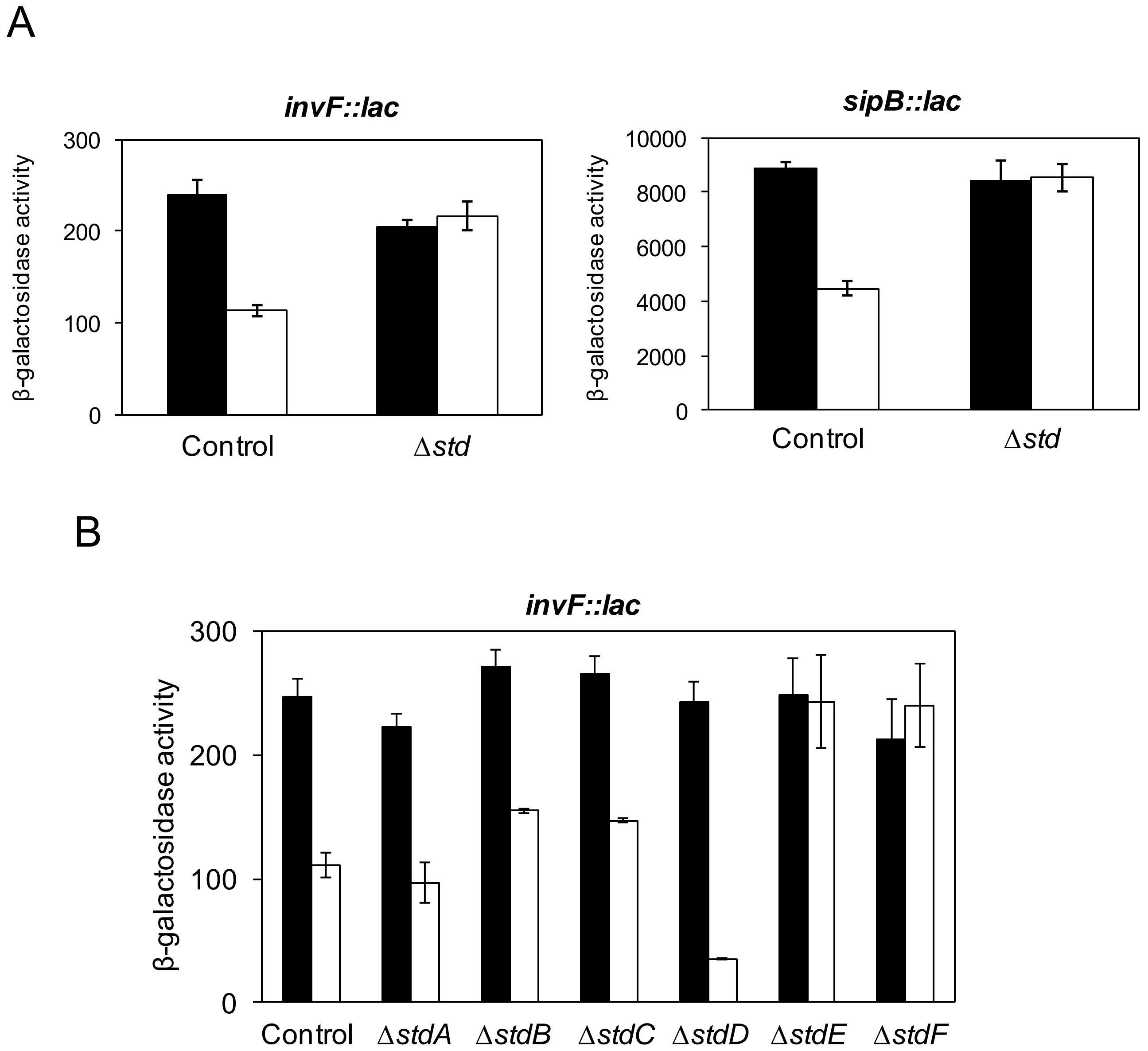
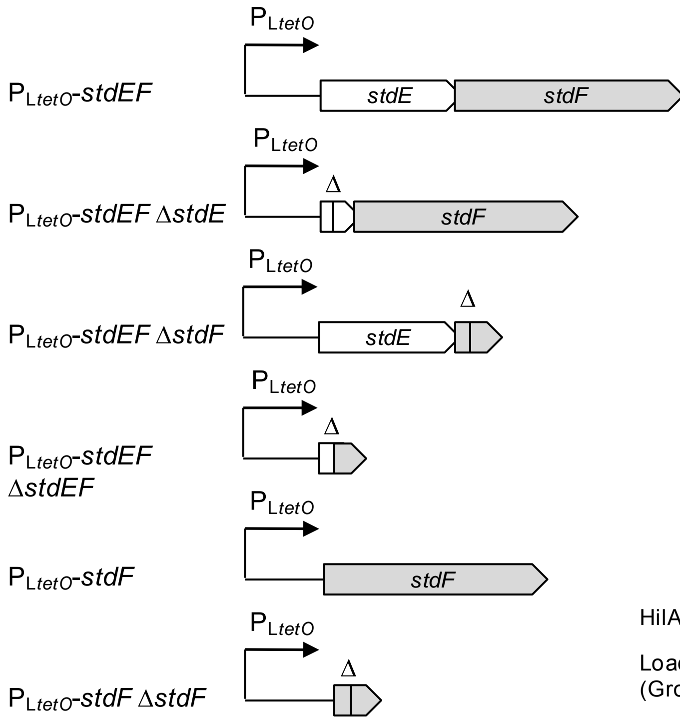
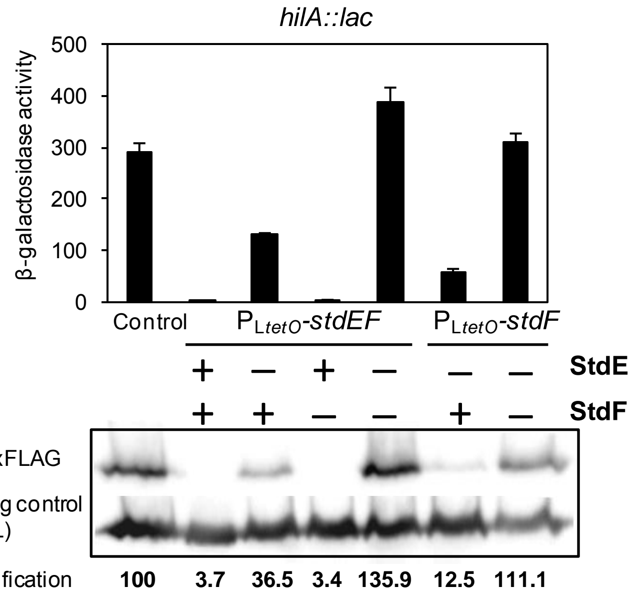
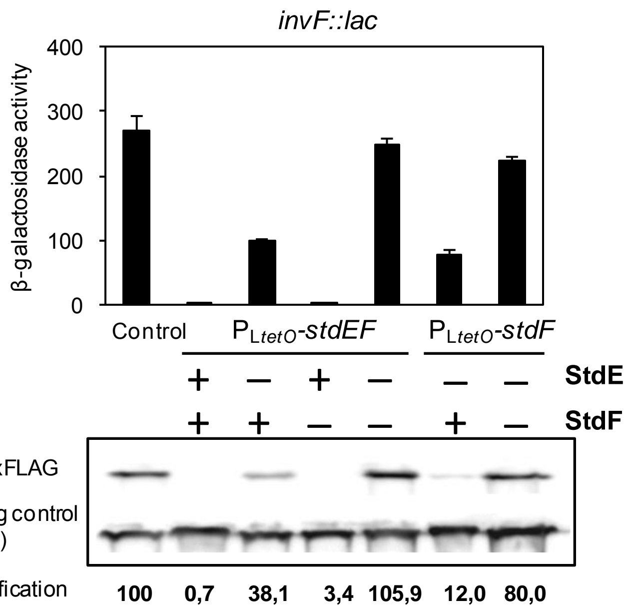


Figure 5







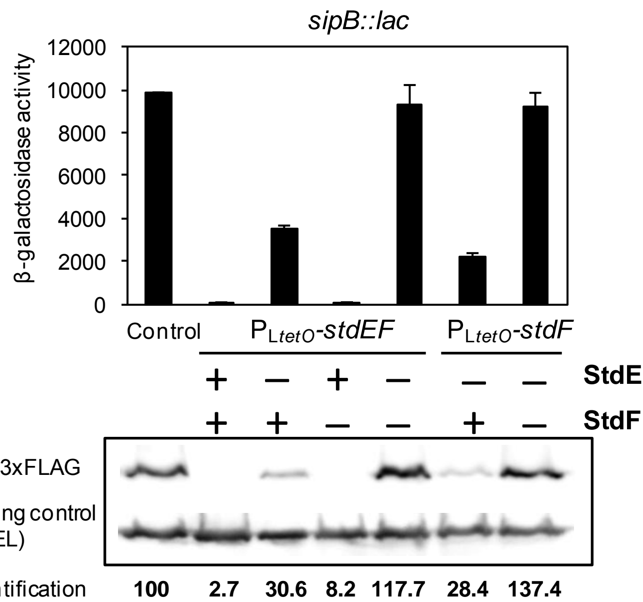
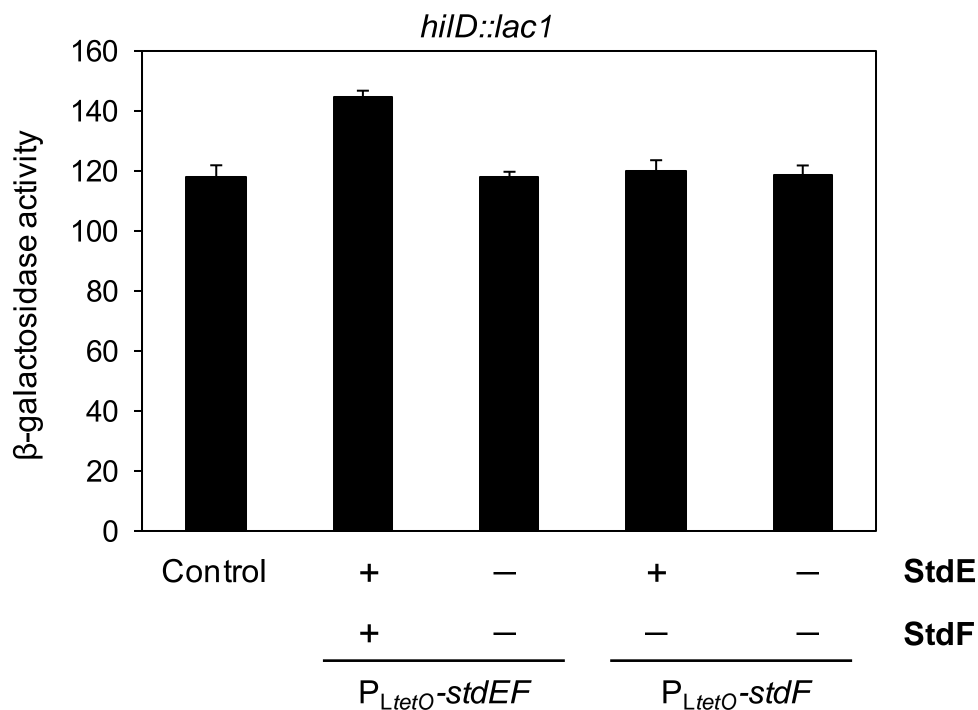


Figure 6

A



B

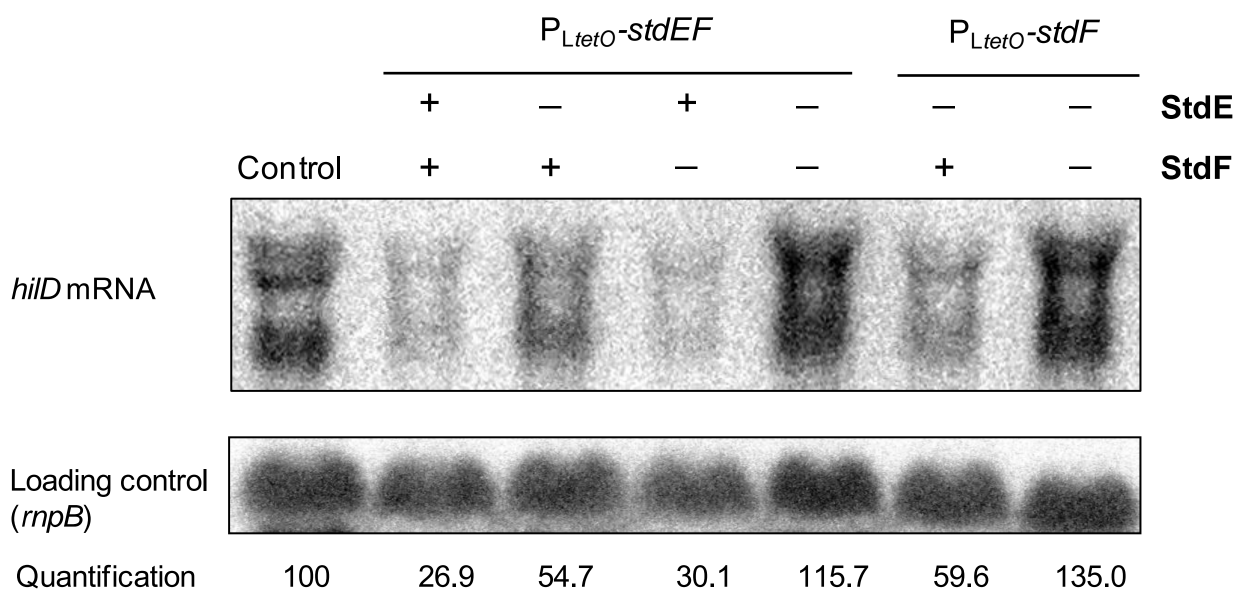
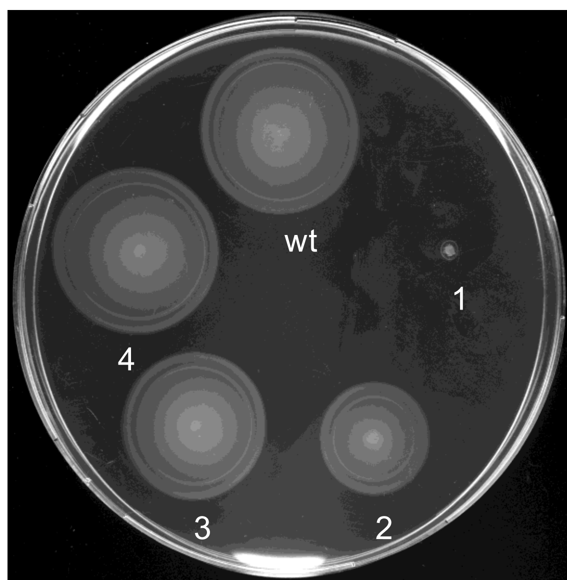
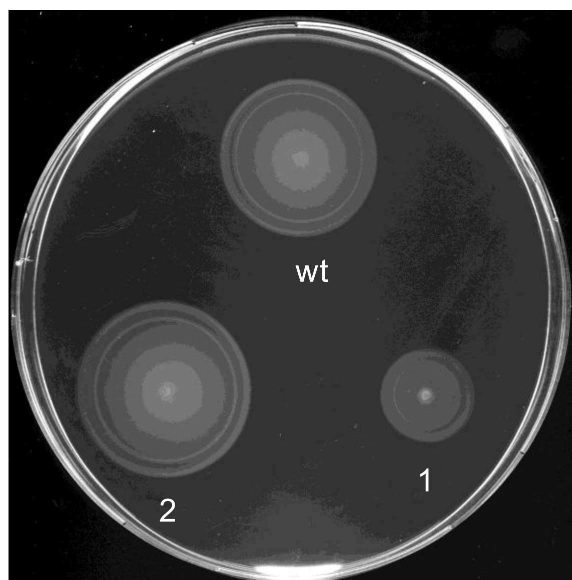


Figure 7

A





1. P_{LtetO}-*stdEF*
2. P_{LtetO}-*stdEF* Δ*stdE*
3. P_{LtetO}-*stdEF* Δ*stdF*
4. P_{LtetO}-*stdEF* Δ*stdEF*

1. P_{LtetO}-*stdF*
2. P_{LtetO}-*stdF* Δ*stdF*

B

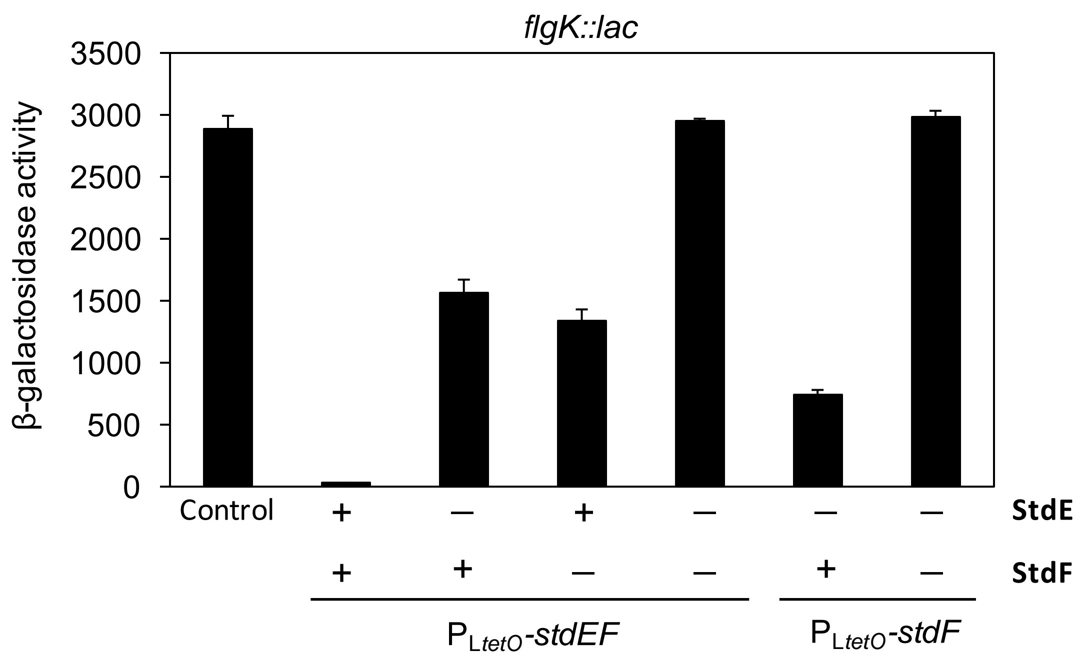
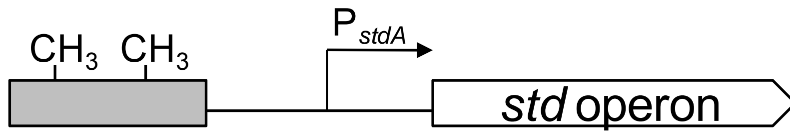


Figure 8

Dam+ background



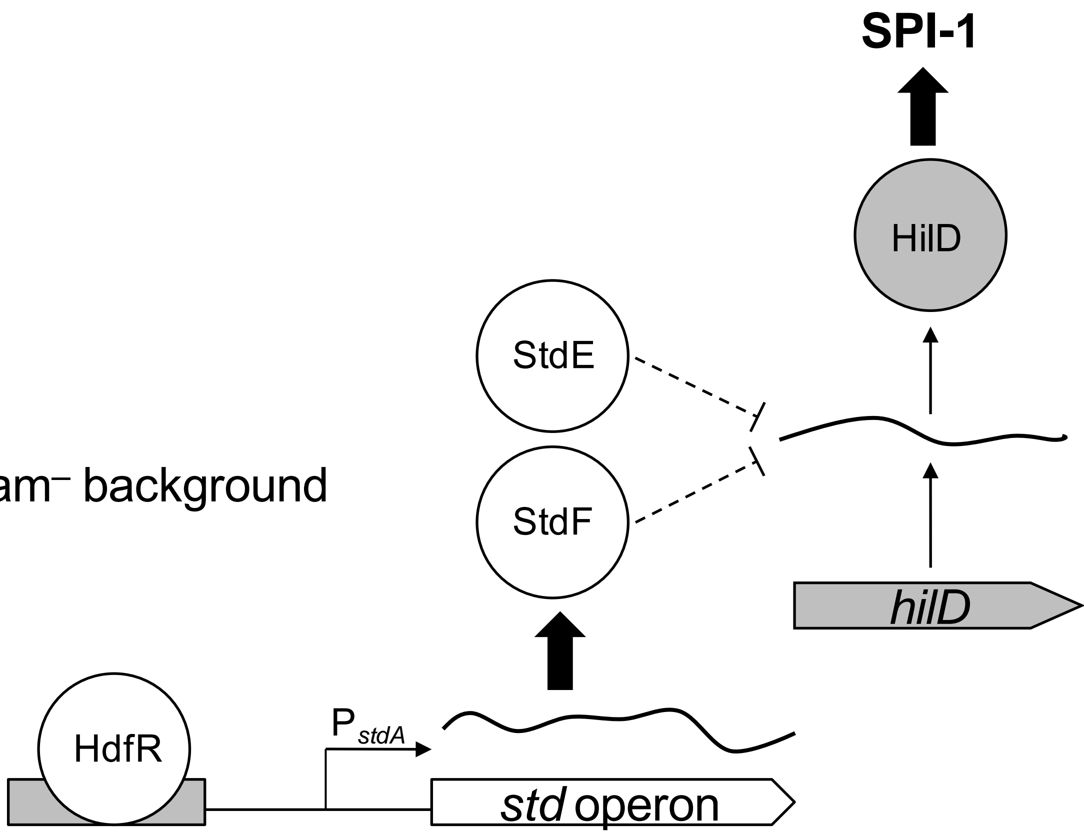
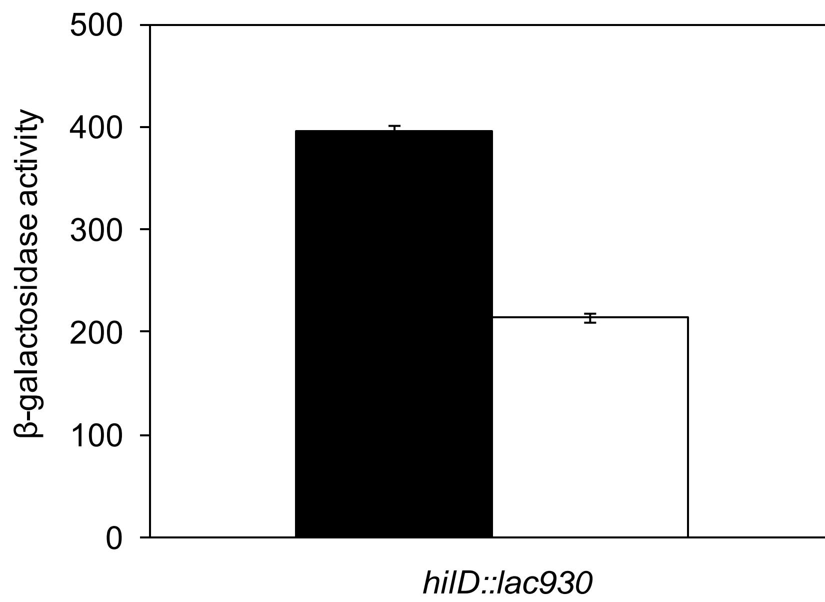


Table S1. Oligonucleotides used in this study (5'→3')

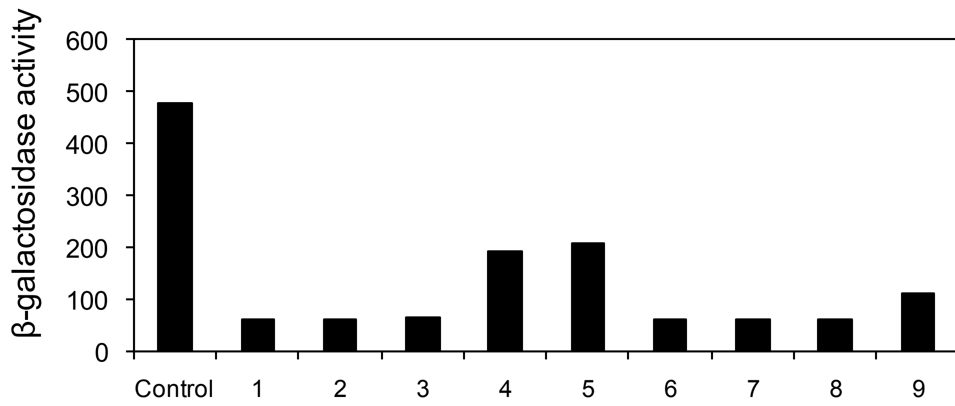
Oligonucleotide	**Sequence**
stdDUP	acgcaggggcgacatcatgacagaatggatttttaatctgattccggggatccgtcgacc
stdDDO	gattagttataggtaacagtaacgggtattgcagcagaaagtgtaggctggagctgcttc
stdD-E1	tgcagatgaatcgctacacc
stdD-E1	ttccccgataactcagtcag
stdEUP	ccagttatggagaggttttatgtgccctgataatacacacattccggggatccgtcgacc
stdEDO	ttaccgacccggcgttttgataccagcggcggtccggcttgtgtaggctggagctgcttc
stdE-E1	tgctgcaatacccgttactg
stdE-E2	caggctgcctgtatgcg
stdFUP	ggtccggagatttatgccgggctgcaactgtgaaaccgcaattccggggatccgtcgacc
stdFDO	tgtcagtgtttctggatagggtcgccggaggcgggttattgtgtaggctggagctgcttc
stdF-E1	ggtcggtaatggtgacagg
stdF-E2	gaaaggccatacattcagcg
stdF-3xFLAGUP	ttgtaaatcactgcagcgaaccccgtttatcgctccgcaagactacaaagaccatgacgg
stdF-3xFLAGDO	cggcgtgtcagtgtttctggatagggtcgccggaccgccccatatgaatatcctccttag
stdE-3xFLAGUP	gaagatcctgaaaaagaaggaggatgaggatgacggaacagactacaaagaccatgacgg
PLtetO-std UP	tacattaaaaagtatttctttgatgattattcttaaattaaggcttacccgtcttactgtc
PLtetO-stdEF-DO	ttcagggcacataaaacctctccataactgggtaaatgatgtgctcagtatctctatcactgatag
PLtetO-stdF-DO	cccgcatttctgttactgcacagccggttccacagttcatgtgctcagtatctctatcactgatag
stdA-FOR	atagccctgacagatgccg
stdB-REV	ggcctgcgacttcaggac
stdB-FOR	ctacctgacaggtctcagc
stdC-REV	gggtccggtcaacattgac
stdC-FOR	tgcagatgaatcgctacacc
stdD-REV	cctagctcaaccgcatacac
stdD-FOR	ctattacaggacgtgtcacc
stdE-REV	catcatggttggtctgtccg
stdE-FOR	ggtcggtaatggtgacagg
stdF-REV	gtttccgacgtaattgctgc

stdAUP	taaaattcttttcactggtaccatcaccaactcaccctgtattccggggatccgtcgacc
stdADO	ctgtcgttatttaccgcgtgaaatcacaggtatttcaggggtgtaggctggagctgcttc
stdAUP2	taaaattcttttcactggtaccatcaccaactcaccctgtcatatgaatatcctccttag
stdA-E1	ggaaagttcaggtgcttcg
stdA-E2	gctttcgtgttgtcgtcc
stdBUP	gcgccatgccatgaaaattacccggcttgccattctgattattccggggatccgtcgacc
stdBDO	tttagacctgctctgtgacagggatatttttatctgcagggtgtaggctggagctgcttc
stdB-E1	acgacagggagaagccg
stdB-E2	cgcatccatgataatacgg
stdCUP	cacagagcaggtctaaaggagaaaacaggtgaaaaacagcattccggggatccgtcgacc
stdCDO	aatccattctgtcatgatgtcgcccctgcgtccttaaacggtgtaggctggagctgcttc
stdC-E1	cggaaacggtgacactcag
stdC-E2	tgaccggaagtgactgcac
hilDriboprobeUP	atggaaaatgtaacctttgtaag
hilDriboprobeDO	gttttttaatacgactcactatagggaggtatatcgaaatccatgtggc
rnpBriboprobeUP	
rnpBriboprobeDO	
pBR328-Fw	actgtccgaccgctttgg
pBR328-Rv	gccagcaaccgcacctg
lacZ	
hilDUP930	aactacgccatcgacattcataaaaatggcgaaccattaacatatgaatatcctccttag
hilDUP1	agagcatttacaactcagatttttcagtaggataccagtcatatgaatatcctccttag
hilDDO2	gcaaatagttctcagagggaacggatgatgtataaatatggtgtaggctggagctgcttc
hilD-E1	agaccattgccaacacacgc
hilD-E2’	atcatcctcaggctggctcc

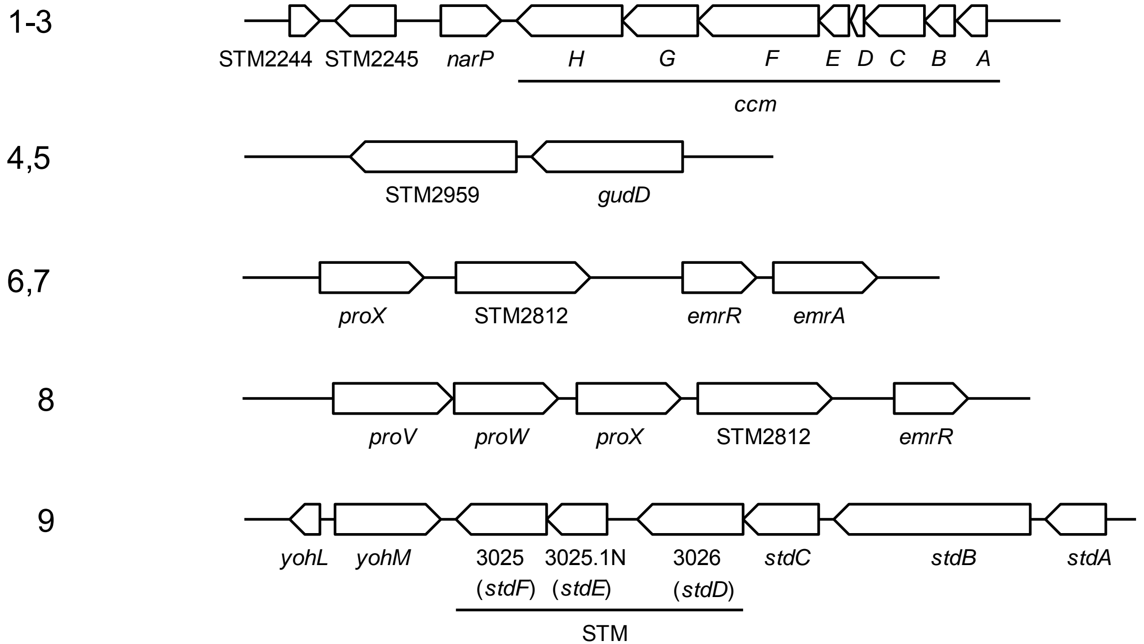
Figure S1



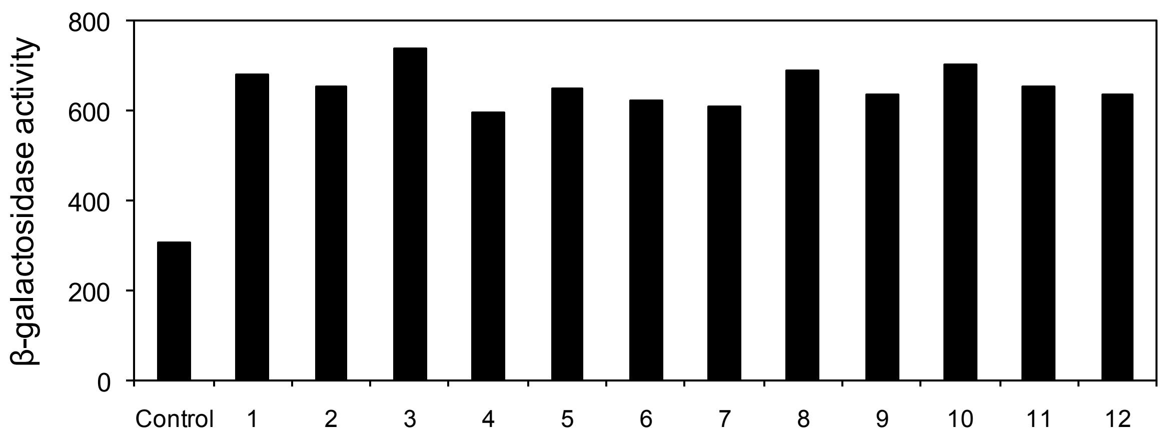
A



Candidates **Complete ORFs in the cloned fragment**



B



Candidates **Complete ORFs in the cloned fragment**

1-12

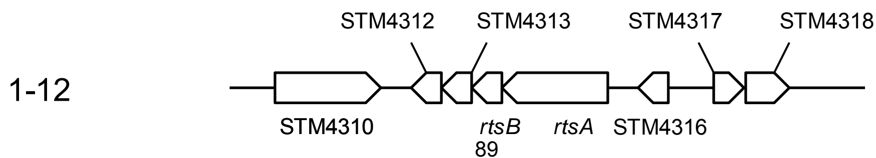
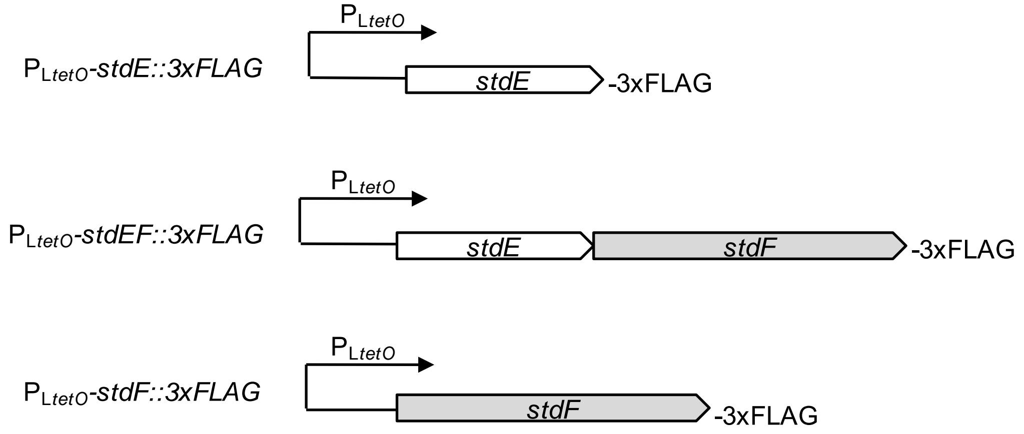
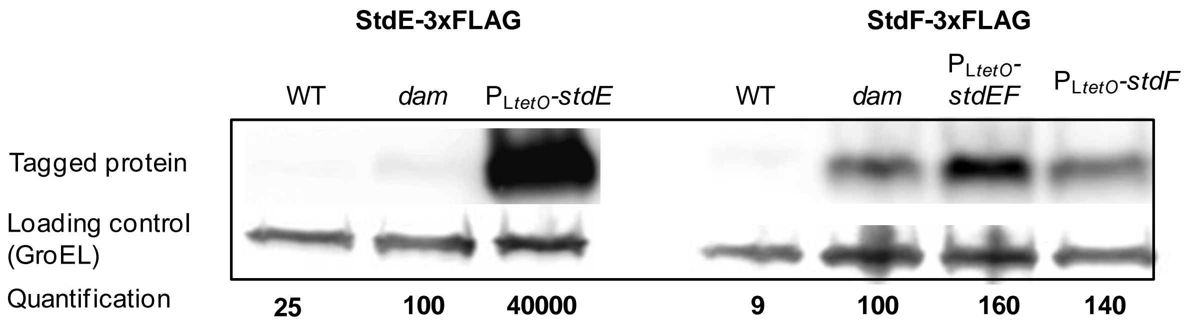


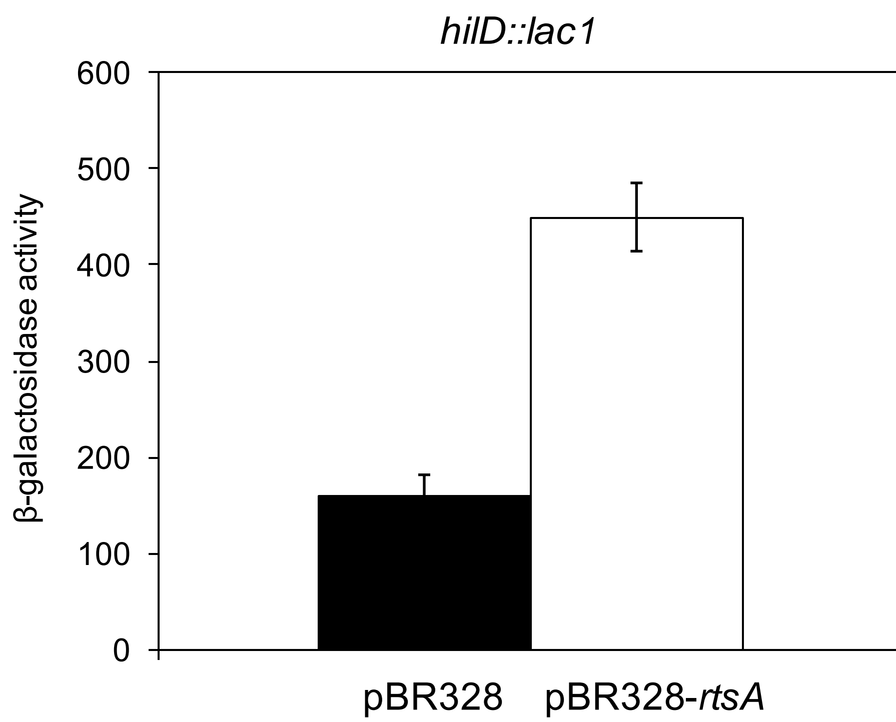
Figure S

A



B




hilD::lac1
600
500
400
300
200
100
0
β-galactosidase activity
pBR328
pBR328-rtsA

hilD mRNA possesses a long regulatory 3' unstranslated region involved in the postranscriptional integration of signals for SPI-1 regulation

Javier López-Garrido, Elena Puerta-Fernández, Jörg Vogel and Josep Casadesús

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). SPI-1 expression is tightly controlled by several molecular and environmental factors through a regulatory network made of the SPI-1-encoded transcriptional activators HilA, HilC, HilD and InvF. In most cases, global regulators target *hilD* expression at postranscriptional level, but the mechanisms of regulation are poorly understood. We have found that *hilD* mRNA possesses a long 3'UTR of 310 nt. Deletion of *hilD* 3'UTR increases *hilD* mRNA levels what correlates with SPI-1 overexpression, suggesting that targeting *hilD* 3'UTR might be an efficient way to control SPI-1 expression. In such context, we provide evidence that *hilD* 3'UTR may be a target for *hilD* mRNA degradation, and is necessary for *hilD* and SPI-1 regulation by the RNA binding protein Hfq. Thus, *hilD* 3'UTR may be involved in the postranscriptional integration of signals for SPI-1 regulation.

INTRODUCTION

Salmonella enterica is a gram negative bacterium able to produce gastric and systemic infections in a variety of animal hosts, including humans. An important trait of *Salmonella* virulence is its ability to penetrate inside non-phagocytic epithelial cells in the animal small intestine. Such process, known as invasion, requires the translocation of bacterial proteins directly from bacterial cytoplasm to epithelial cell cytoplasm through a type-3 secretion system (TTSS) (Darwin and Miller, 1999; Lostroh and Lee, 2001). Those proteins, called effectors, interact with specific targets inside intestinal epithelial cells, triggering a cascade of molecular events that culminates with *Salmonella* invasion (Darwin and Miller, 1999).

Some effector proteins and all the components of TTSS are encoded in a 40-kb region of *Salmonella* chromosome, known as *Salmonella* pathogenicity island 1 (SPI-1) (Altier, 2005; Jones, 2005; Lostroh and lee, 2001). Expression of SPI-1 genes is coordinately regulated by several environmental and molecular factors through a regulatory network made of SPI-1-encoded transcriptional activators: HilA, HilC, HilD, and InvF (Altier, 2005; Ellermeier and Slauch, 2007; Jones, 2005). InvF activates transcription of genes that encode effector proteins (Darwin and Miller, 1999; Eichelberg and Galan, 1999). HilA directly activates transcription of genes encoding TTSS components, and indirectly transcription of genes encoding effector proteins by activating transcription of the gene for InvF (Bajaj et al., 1996). HilC and HilD redundantly activate transcription of *hilA* and *invF* (Akbar et al., 2003; Olekhnovich and Kadner, 2006; Rakeman et al., 1999; Schechter and Lee, 2001). In addition, HilD can activate *hilC* transcription and its own transcription, by direct binding to both promoters (Ellermeier et al., 2005; Olekhnovich and Kadner, 2002). That regulatory network serves as link between SPI-1 expression and general regulatory systems encoded elsewhere in the chromosome (Altier, 2005; Ellermeier and Slauch, 2007; Jones, 2005).

Most of the regulatory systems known to control SPI-1 primarily target HilD expression, and then regulation is transmitted to the rest of SPI-1 genes (Ellermeier and Slauch, 2007). Surprisingly, those regulatory systems seem to control *hilD* expression at postranscriptional or postranslational level, rather than at the level of transcription initiation (Ellermeier and Slauch, 2007). In such context, it has been shown that HilD

protein is the target for several regulatory systems: HilD is degraded by the ATP-dependent Lon protease (Takaya et al., 2005); HilE, a negative SPI-1 regulator (Fahlen et al., 2000), physically interacts with HilD (Baxter and Jones, 2003), likely interfering with its function; it has been recently proposed that FliZ activates SPI-1 expression by somehow controlling HilD activity (Chubitz et al., 2010). Regulation of *hilD* expression at mRNA level has also been proposed: overproduction of the RNA binding protein CsrA represses SPI-1 expression (Altier et al., 2000; Martinez et al., 2011), and it has been shown that CsrA binds to a region in *hilD* mRNA that overlaps with the SD sequence, likely preventing translation and accelerating mRNA decay (Martinez et al., 2011); DNA adenine (Dam) methylation contribute to sustain high levels of SPI-1 expression by decreasing *hilD* mRNA turnover (López-Garrido and Casadesus, 2010). Hence, postranscriptional control of *hilD* expression seems to be a key event for SPI-1 regulation.

Here we report that *hilD* mRNA possesses a long 3'unstranslated region (3'UTR) of 310 nucleotides. Long 3'UTR are common in eukaryotes, where they can play regulatory roles, but are not well characterized in prokaryotes. Our results suggest that *hilD* 3'UTR is a target for *hilD* mRNA degradation, and is necessary for *hilD* and SPI-1 regulation by the RNA binding protein Hfq. Thus, *hilD* 3'UTR may be involved in the postranscriptional integration of signals for SPI-1 regulation.

MATHERIALS AND METHODS

Bacterial strains, bacteriophages, and standard strain construction:

All the *Salmonella enterica* strains listed in **Table 1** 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 plasmid pKD4 or 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 **Table S1**, together with the oligonucleotides (labeled "E") used for allele verification by the polymerase chain reaction. For the construction of *lac* fusions in the *Salmonella* chromosome, FRT sites generated by excision of Kmr cassettes (DATSENKO and WANNER 2000) were used to integrate either plasmid pCE37 or pCE40 (ELLERMEIER *et al.* 2002). Unless otherwise specified, all *lac* fusions used in this study are translational. Addition of 3xFLAG epitope tag to protein-coding DNA sequences was carried out using plasmids pSUB11 (Kmr, 3xFLAG) as template (UZZAU *et al.* 2001). Transductional crosses using phage P22 HT 105/1 *int201* [(SCHMIEGER 1972) and G. Roberts, unpublished] 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.

Growth conditions

Luria-Bertani (LB) broth was used as standard liquid medium. Solid media were prepared by the addition of 1.5 % agar. For determination of expression of SPI-1 genes by β-galactosidase assay, Western blot, or Northern blot, saturated cultures were diluted 1:50 in LB and incubated at 37 ºC with shaking (200 rpm). Samples were taken when the cultures had reached stationary phase (O.D. 2-2.5). When required, Km (50 µg/ml) or Cm (20 µg/ml) were added to the culture medium. Green plates were prepared according to Chan and co-workers (CHAN *et al.* 1972), except that methyl blue (Sigma Chemical Co, St. Louis, MO) substituted for aniline blue.

Construction of relevant strains

For construction of *hilD* Δ3'UTR allele, Km^r gene was amplified from pKD13 with the primers JVO5462 and JVO5463 and inserted in *Salmonella* chromosome by λRed recombinase-dependent recombination, deleting a 231 nt fragment of *hilD* 3'UTR starting from the first nucleotide after *hilD* stop codon. Km^r gene was healed by recombination of flanking FRT sequences mediated by pCP20-encoded FLP recombinase, leaving a scar of 82 nt. As result, a shorter 3' UTR of 162 nt was produced, keeping the native *hilD* Rho-independent transcriptional terminator.

Expression of *hilD* from a heterologous promoter was achieved replacing its native promoter by P_{LtetO} promoter (Lutz and Bujard, 1997). A fragment containing the *cat* gene and P_{LtetO} promoter was amplified by PCR using pXG1 as template (Urban and Vogel, 2007). The primers were labelled P_{LtetO}-*hilD* UP and P_{LtetO}-*hilD* DO (**Table S1**). The PCR product was treated with DpnI to remove template traces. The construction was inserted in the chromosome by λRed recombinase-mediated recombination (Datsenko and Wanner, 2000) and Cm^r colonies were selected. Insertion of the construction was verified by PCR, using a couple of primers specific for *cat* gene and the target gene (**Table S1**).

RNA extraction procedures

2 ml of *S. enterica* cells reaching stationary phase were taken by centrifugation, and the pellet resuspended in 100 ul of a lysozyme solution (3 mg/ml in water). Cells lysis was facilitated by three consecutive freeze-thaw cycles. After lysis, RNA was extracted using 1ml of Trizol reagent (Invitrogen), according to manufacter's instructions. Finally, total RNA was resuspended in 30 ul of RNase-free water for subsequent uses. Quality and quantity of the obtained RNA was determined using a Nanodrop instrument.

3'RACE

3'RACE was performed as described by Argaman and collaborators (2001). The *hilD*-specific primer used for PCR was JVO5536. Specific RCR products were cloned in pTOPO vector, and 4 independent clones were sequenced using external primers.

Northern blots in polyacrylamide gels

10 µg of total RNA resuspended in 2X RPA loading buffer (98 % Formamid, 1 mM EDTA, 0.1 % Xylene cyanole, 0.1 % Bromphenol blue) were loaded in a 4.5 % polyacrylamide 7 M urea gel, prepared in TBE, and solidified with 0.08 % (w/V) APS and 0.106 % (V/V) TEMED. Electrophoresis was carried out at room temperature in TBE buffer, at 300 V for 4 hours. RNA was transferred onto Hybond-XL membranes (Amersham) by wet electrophoresis in TBE buffer (50 V, 1 h, 4 ºC), and crosslinked with UV light. *hilD* mRNA was detected using a ^{32}P-labelled riboprobe specific for the first 300 nt of *hilD* coding sequence.

Northern blot in agarose gels

For northern blot analysis, 10 µg of total RNA was loaded per lane and electrophoresed in denaturing 1% agarose formaldehyde gels. Transfer and fixation to Hybond-N^{+} membranes (GE Healthcare) were performed by vacuum using 0.05M NaOH. Filters were then hybridized using an internally labelled ([α-^{32}P]UTP) riboprobe specific for the first 300 nts of the *hilD* 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 of the RNase P RNA gene (*rnpB*). Images of radioactive filters were obtained with a Fuji, and quantification was performed using the Multy Gauge software.

β-galactosidase assays

Levels of β-galactosidase activity were assayed using the CHCl3-sodium dodecyl sulfate permeabilization procedure (MILLER 1972). Unless otherwise indicated, the results shown are the average and standard deviation of 3 independent experiments.

Protein extracts and Western blot analysis

Total protein extracts were prepared from bacterial cultures grown at 37°C in LB until stationary phase (final O.D.600 ~2.5). Bacterial cells taken according to 1 O.D.600 were collected by centrifugation (16,000 g, 2 min, 4°C) 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, using 12% gels. Conditions for protein transfer have been described elsewhere (JAKOMIN *et al.* 2008). Optimal dilutions of primary antibodies were as follows: anti-Flag M2 monoclonal antibody (1:5,000, Sigma Chemical Co, St. Louis, MO), and anti-GroEL polyclonal antibody (1:20,000, Sigma). Goat anti-mouse horseradish peroxidase-conjugated antibody (1:5,000, BioRad, Hercules, CA) or Goat anti-rabbit horseradish peroxidase conjugated antibody (1:20,000, Santa Cruz Biotechnology, Heildelberg, Germany) were used as secondary antibodies. Proteins recognized by the antibodies were visualized by chemoluminescence using the luciferin-luminol reagents.

RESULTS

hilD mRNA has a long 3'UTR

hilD transcription start point has been identified previously (Olekhnovich and Kadner, 2002), and leaves a 5'UTR of 35 nt in *hilD* mRNA. As postranscriptional control of *hilD* expression seems to be a key event in SPI-1 regulation, we decided to determine *hilD* transcription termination point. Sequence analysis with RNAfold software predicts the existence of a secondary structure similar to a Rho-independent transcriptional terminator around 300 nt downstream *hilD* stop codon, consisting on a stem of 12 nt with a 1-nt bulge in its 3' side, and a hairpin loop of 4 nt, followed by a U-rich region (**Figure 1A**). That fits with the proposed structure for a Rho independent transcriptional terminator (Carafa et al., 1990; Lesnik et al., 2001). We experimentally determined *hilD* transcription stop point by 3'RACE. As shown in **Figure 1B**, *hilD* transcription stops right at the end of the U-rich region downstream the predicted steam-loop, suggesting that such structure constitute a functional Rho-independent transcriptional terminator. According to that, of *hilD* mRNA should be 1275 nt length. In order to estimate the size of *hilD* mRNA, we detected *hilD* mRNA by Northern blot in a 4.5 % polyacrylamide gel, using a P^{32}-labelled riboprobe specific for *hilD*. Comparison of *hilD* size mRNA with pUC8 DNA marker (Fermentas) shows that full-length *hilD* RNA runs slower than the higher band in the marker (1118 nt), supporting the expected size of *hilD* mRNA (**Figure 1C**). Hence, *hilD* mRNA molecule includes a 5'UTR of 35 nt, a coding sequence of 930 nt, and an unusually long 3'UTR of 310 nt (**Figure 1D**).

Deletion of *hilD* 3'UTR results in increased levels of *hilD* mRNA

Although it is known that 3'UTRs are important for postranscriptional regulation in eukaryotes (Grzybowska et al., 2001), long 3'UTRs are poorly studied in prokaryotes. We considered the possibility that *hilD* 3'UTR was playing a role in postranscriptional control of *hilD* expression. Thus, we constructed a strain carrying a modified *hilD* 3'UTR (*hilD* Δ3'UTR): a 231-nt fragment of *hilD* 3'UTR starting from the first nucleotide after *hilD* stop codon was deleted, leaving the Rho-independent transcriptional terminator intact. The deleted fragment was exchanged by the 82-nt pKD4 scar, resulting in a shorter 3'UTR with a different sequence, keeping the native

hilD Rho-independent transcriptional terminator (**Figure 2A**). We detected *hilD* mRNA in RNA extracts from wild type and *hilD*Δ3'UTR strains by Northern blotting in agarose gels, using a P^{32}-labelled riboprobe specific for the first 300 nt of *hilD* coding sequence. As shown in **Figure 2B**, *hilD* Δ3'UTR mRNA is shorter than *hilD* native mRNA, thereby confirming the existence of a long 3'UTR in *hilD* mRNA. In addition, the level of *hilD* mRNA is around 11 folds higher in the absence of its 3'UTR than in wild type strain, suggesting that the presence of the 3'UTR somehow represses *hilD* expression. Since *hilD* 3'UTR is part of *hilD* mRNA, it is conceivable to speculate that it may control *hilD* expression at postranscriptional level. In order to confirm that, we examined the level of *hilD* mRNA in isogenic *hilD* $3'UTR^{+}$ and *hilD* $3'UTR^{-}$ strains in which *hilD* was transcribed from P_{LtetO} promoter (**Figure 2C**). The absence of a native 3'UTR increases *hilD* mRNA level 3.5 folds even if *hilD* is transcribed from a heterologous promoter, indicating that 3'UTR influences *hilD* expression at postranscriptional level. Note that *hilD* overexpression upon 3'UTR deletion is higher when *hilD* is transcribed from its own promoter than when transcription is driven by P_{LtetO}, suggesting that autoactivation of *hilD* transcription may amplify *hilD* overexpression in the absence of its 3'UTR.

SPI-1 is overexpressed in *hilD* Δ3'UTR hosts

The above results show that *hilD* mRNA is overproduced in the absence of its native 3'UTR. We wandered if such overproduction was functional, and triggered overexpression of the whole SPI-1. We examined the expression of 4 SPI-1 genes (*invF*, *invH*, *sipB*, and *sipC*) in isogenic *hilD* $3'UTR^{+}$ and *hilD* $3'UTR^{-}$ strains by two independent methods: (i) measurement of β-galactosidase activity of *invF::lac*, *invH::lac*, *sipB::lac*, and *sipB::lac* fusions (**Figure 2D**); and (ii) determination of InvF, InvH, SipB, and SipC levels by Western blotting, using protein variants tagged with the 3xFLAG epitope (**Figure 2E**). β-galactosidase activities are around 3-4 folds higher in *hilD* $3'UTR^{-}$ than *hilD* $3'UTR^{+}$ hosts, for the 4 genes analyzed. Similarly, InvF, InvH, SipB and SipC levels are also from 2.6 to 9.6 folds higher in *hilD* $3'UTR^{-}$ strains, compared to *hilD* $3'UTR^{+}$ background. Hence, *hilD* mRNA overproduction observed in the absence of its native 3'UTR correlates with SPI-1 overexpression, suggesting that *hilD* 3'UTR may be a target to control SPI-1 expression.

Inactivation of RNA-degradosome components ribonuclease E and polynucleotide phosphorylase suppresses *hilD* overexpression in the absence of its 3'UTR

In eukaryotes, mRNA degradation usually depends on their long 3'UTRs (Barreau et al., 2005; Beelman and Parker, 1995). We hypothesized that the long 3'UTR of *hilD* mRNA might be a target for mRNA degradation. If such were the case, we reasoned, inactivation of factors involved in RNA turnover might suppress the differences observed in the level of *hilD* mRNA with and without its native 3'UTR. We constructed deletion mutants in genes encoding the endonucleases ribonuclease E (RNase E) and ribonuclease G (RNase G), the exonuclease polynucleotide phosphorylase (Pnp), and the poly(A) polymerase I (PapI). Since RNase E is essential in *Salmonella*, we only removed the C-terminal region of the protein (Viegas et al., 2007), involved in the assembly of RNA degradosome (Carpousis, 2002). *hilD* and *hilD* Δ3'UTR mRNA levels were determined in strains lacking RNase E, RNase G, Pnp, or PapI (**Figure 3**). *hilD* Δ3'UTR mRNA levels are higher than *hilD* mRNA levels in control, RNase G^{-} and PapI^{-} backgrounds, suggesting that *hilD* 3'UTR is not involved in *hilD* mRNA degradation by RNase G or polyadenilation-dependent pathways. However, similar levels of *hilD* and *hilD* Δ3'UTR mRNAs are detected in strains lacking either RNase E or Pnp. Those two proteins are component of the RNA degradosome (Carposius, 2002), suggesting that *hilD* 3'UTR may be a target for mRNA degradation by RNA degradosome.

Hfq regulates *hilD* expression at posttranscriptional level

We considered the possibility that *hilD* 3'UTR was involved on SPI-1 regulation by some of the signals that control SPI-1 expression. In that sense, we looked at the RNA binding protein Hfq. It has been reported that SPI-1 expression is repressed in mutants lacking Hfq (Sittka et al., 2007). In addition, Hfq CoIP experiments have determined that Hfq binds to *hilD* mRNA (Sittka et al., 2008). In order to determine if *hilD* expression was regulated by Hfq, we examined the level of *hilD* mRNA in Hfq^{+} and Hfq^{-} backgrounds by Northern blotting (**Figure 4A**). Lack of Hfq reduces the level of *hilD* mRNA almost 5 folds. However, the expression of a *hilD::lac* transcriptional

fusion is similar in Hfq$^+$ and Hfq$^-$ backgrounds (**Figure 4B**), suggesting that the regulation is postranscriptional. That is further supported by the observation that the level of *hilD* mRNA is still Hfq-dependent when *hilD* is transcribed from a heterologous promoter (**Figure 4C**). Those results support the idea that Hfq may control *hilD* expression by direct binding to *hilD* mRNA molecule.

Deletion of *hilD* 3'UTR suppresses regulation of *hilD* expression by Hfq

Interestingly, RNA fragments belonging to *hilD* 3'UTR were enriched after Hfq CoIP, suggesting that Hfq may directly bind that region (Sittka et al., 2008). That opens the interesting possibility that *hilD* 3'UTR is required for the regulation of *hilD* expression by Hfq. To test that hypothesis, we analyzed the levels of *hilD* mRNA and *hilD* Δ3'UTR mRNA in Hfq$^+$ and Hfq$^-$ backgrounds. As shown in **Figure 4D**, the level of *hilD* mRNA is 5-fold reduced in Hfq$^-$ compared to Hfq$^+$ background. However, similar levels of *hilD* and *hilD* Δ3'UTR mRNAs are detected in Hfq$^+$ and Hfq$^-$ backgrounds, indicating that the 3'UTR of *hilD* mRNA is important for regulation of *hilD* expression by Hfq.

SPI-1 regulation by Hfq is transmitted via HilD

It has been previously reported that SPI-1 expression is repressed in *hfq* mutants (Sittka et al., 2007), and that correlates with reduced levels of *hilD* mRNA in Hfq$^-$ background (**Figure 4A, C**). Since HilD is one of the main transcriptional activators of SPI-1 expression, it is conceivable that Hfq regulates SPI-1 expression through HilD. To study that possibility, we examined regulation by Hfq of selected SPI-1 genes in a set of mutants lacking the main SPI-1-encoded transcriptional activators (HilA, HilC, or HilD). SPI-1 expression was monitored using *lac* fusions in representative genes. β-galactosidase activity of each fusion was determined in Hfq$^+$ and Hfq$^-$ strains, containing all SPI-1-encoded transcriptional activators (Control), or lacking individual activators. As shown in **Figure 5A**, β-galactosidase activities of *hilA::lac*, *invF::lac*, *sipB::lac* and *invH::lac* fusions are reduced in strains lacking HilC, but their expression is still Hfq-dependent. Similarly, deletion of *hilA* reduces expression of *invF::lac* and *sipB::lac* fusions, but Hfq-dependent regulation is still observed. Those results indicate

that HilC and HilA are dispensable for SPI-1 regulation by Hfq. In HilD⁻ background, expression of *hilA::lac*, *invF::lac*, and *sipB::lac* fusions is completely abolished, making it impossible to get any information about regulation by Hfq. However, expression of *invH::lac* and *hilC::lac* fusions are not completely abolished in *hilD* mutants, but regulation by Hfq is suppressed, suggesting that SPI-1 regulation by Hfq is transmitted through HilD.

SPI-1 repression by Hfq is dependent on *hilD* 3'UTR

Since *hilD* 3'UTR is necessary for regulation of *hilD* expression by Hfq, we studied if it was also required for Hfq-dependent regulation of SPI-1. We examined the expression of *invF*, *sipB*, and *sipC* in the following backgrounds: Hfq^+ *hilD* $3'UTR^+$, Hfq^- *hilD* $3'UTR^+$, Hfq^+ *hilD* $3'UTR^-$, and Hfq^- *hilD* $3'UTR^-$. We measured β-galactosidase activity of *invF::lac*, *sipB::lac*, and *sipC::lac* fusions. Likewise, InvF, SipB, and SipC protein levels were determined by Western blotting, using 3xFLAG-tagged protein versions. The results were consistent for the 3 genes analyzed by both methods (**Figure 5B and C**): *invF*, *sipB*, and *sipC* expression is reduced in Hfq^- background in strains containing a native *hilD* 3'UTR. As expected, *hilD* $3'UTR^-$ strains display higher levels of *invF*, *sipB*, and *sipC* expression. However, repression in *hfq* mutants is almost completely abolished. We considered the possibility that suppression of regulation by Hfq was the result of saturation due to *hilD* overexpression in the absence of its 3'UTR. To check that possibility, we examined regulation by Dam methylation of an *invF::lac* fusion in *hilD* $3'UTR^+$ and *hilD* $3'UTR^-$ backgrounds (**Figure S1**). We previously reported that Dam methylation regulates SPI-1 expression through HilD (Lopez-Garrido and Casadesus, 2010). *invF::lac* expression is still Dam-dependent in the absence of *hilD* 3'UTR, suggesting that lost of Hfq regulation is a specific effect rather that an artifact due to HilD overproduction. Altogether, those results indicate that *hilD* 3'UTR is necessary for SPI-1 regulation by Hfq

DISCUSSION

Postranscriptional control of *hilD* expression is essential for SPI-1 regulation by different regulatory systems (Ellermeier and Slauch, 2007). However, despite its importance in SPI-1 regulation, the mechanisms of postranscriptional control of *hilD* are poorly understood. Our results indicate that *hilD* 3'UTR may mediate *hilD* regulation at postranscriptional level: deletion of *hilD* 3'UTR increases *hilD* mRNA levels what correlates with SPI-1 overexpression, suggesting that targeting *hilD* 3'UTR might be an efficient way to control SPI-1 expression. In such backgrounds, we provide evidence that *hilD* 3'UTR may be a target for *hilD* mRNA degradation and regulation by the RNA chaperone Hfq.

Higher levels of *hilD* mRNA are detected upon deletion of its 3'UTR even when transcription is driven from a heterologous promoter, suggesting that *hilD* 3'UTR does not affect *hilD* mRNA synthesis. Furthermore, inactivation of RNA degradosome components RNase E and Pnp suppresses the differences in *hilD* mRNA levels with and without 3'UTR. Altogether, that suggests that *hilD* 3'UTR may be a target for *hilD* mRNA degradation by the RNA degradosome. According to that, it has been reported that *Salmonella* mutants lacking a functional RNase E undergo increased SPI-1 expression (Fahlen et al., 2000). It may be possible that it was due to 3'UTR-directed *hilD* mRNA degradation.

SPI-1 expression is repressed in *Salmonella* mutants lacking the RNA binding protein Hfq (Sittka et al., 2007). Epistasis analysis have shown that Hfq-dependent regulation of SPI-1 is transmitted through HilD and we have evidences that Hfq regulates *hilD* expression at postranscriptional level: (i) Lowered levels of *hilD* mRNA are detected in *hfq* mutants; (ii) however expression of a *hilD::lac* transcriptional fusion is not reduced in Hfq$^-$ background; and (iii) *hilD* mRNA levels are reduced in *hfq* mutants even when *hilD* is transcribed from a heterologous promoter. Deletion of *hilD* 3'UTR suppresses regulation of *hilD* and SPI-1 by Hfq. According to that, RNA fragments corresponding to *hilD* 3'UTR have been recovered upon Hfq CoIP, suggesting that Hfq directly binds to that region (Sittka et al., 2008). Hence, it is tempting to speculate that Hfq needs to interact with *hilD* 3'UTR in order to regulate *hilD* expression. Those results open the possibility that *hilD* 3'UTR serves to integrate regulatory signals at postranscriptional

level. Future studies might reveal new regulators that target *hilD* 3'UTR to control SPI-1 expression.

It is well known that eukaryotic mRNAs sometimes have long 3'UTRs with regulatory properties (Grzybowska et al., 2001): mRNA stability can be modulated by controlling polyadenylation status of 3' end (Beelman and Parker, 1995). In addition, binding of certain proteins to specific sequences located in 3'UTRs of mRNAs can modulate mRNA stability, translation and localization (Barreau et al., 2005; Sonenberg and Hinnebusch, 2009; St Johnston, 1995; Wilkie et al., 2003). In prokaryotes it has been traditionally thought that 3'UTR harbor mainly a transcriptional terminator that contributes to RNA stabilization, preventing degradation by exonucleases. However, recent advances in transcriptomic analysis have possibilities the identification of long 3'UTRs in some bacterial transcripts (Toledo-Arana et al., 2009; Rasmussen et al., 2009; Broeke-Smits et al., 2010), raising the possibility that they have regulatory roles (Gripenland et al., 2010). For example, in *Bacillus subtilits* there are 9 different mRNAs that harbors a conserved 3'UTR of around 220 nt (Rasmussen et al., 2009), and it has been speculated that it might have a functional relevance (Rasmussen et al., 2009). Furthermore, 3'UTR-derived sRNAs have been observed in *Escherichia coli* (Kawano et al., 2005), suggesting that they might regulate gene expression in trans. The results reported in this study provide an example of an eukaryotic-like 3'UTR in a bacterial mRNA. Apart from being a target for mRNA degradation, *hilD* 3'UTR may possibilite regulation of *hilD* expression by direct binding of Hfq. Thus, the presence of regulatory 3'UTRs in bacterial RNAs may be more frequent than previously thought. Future studies might uncover new regulatory functions associated with prokaryotic 3'UTRs.

LEGENDS TO FIGURES

FIGURE 1. A. Diagram of a Rho-independent transcriptional terminator-like structure predicted by RNAfold software around 300 nt downstream *hilD* stop codon. B. Sequence of *hilD* 3'UTR. The 3 first nucleotides (in bold capital letters) correspond to *hilD* stop codon. Nucleotides in bold at the end of the sequence constitute the Rho-independent transcriptional terminator. *hilD* transcription terminator point determined by 3'RACE is pointed out with an arrow. C. Northern blot of *hilD* mRNA in 4.5 % acrylamide gel. The first line corresponds to pUC8 DNA marker labelled with ^{32}P. The second line correspond to *hilD* mRNA, detected with a specific ^{32}P-labelled riboprobe. D. Diagram of the primary structure of *hilD* mRNA. 5'UTR, coding sequence, 3'UTR and Rho-independent transcriptional terminator are represented.

FIGURE 2. A. Diagram of *hilD* mRNA and *hilD* Δ3'UTR mRNA. The thick line in the 3'region of *hilD* Δ3'UTR mRNA represents the 82-nt pKD4 scar. The Rho-independent transcriptional terminator is left intact. B, C. Northern blot of *hilD* and *hilD* Δ3'UTR mRNAs MOPS-formaldehyde agarose gel, when *hilD* transcribed from its own promoter (B), and when transcription is driven from P_{LtetO} promoter (C). *rnpB* mRNA has been used as loading control. For quantification the ratio *hilD / rnpB* was relativized to 1 in *hilD* mRNA carrying a native 3'UTR. D. β-galactosidase activities of *invF::lac*, *invH::lac*, *sipB::lac*, and *sipC::lac* fusions in strains with a native *hilD* 3'UTR (black histograms), or carrying *hilD* Δ3'UTR allele (white histograms). Due to disparate activities of the different fusions, β-galactosidase activities have been relativized to 100 in 3'UTR$^+$ background. E. Western blot of InvF-3xFLAG, InvH-3xFLAG, SipB-3xFLAG, and SipC-3xFLAG in protein extracts from *hilD* 3'UTR$^+$ and *hilD* 3'UTR$^-$ hosts. GroEL has been used as loading control. For quantification the ratio 3xFLAG-tagged protein / GroEL has been relativized to 1 in *hilD* 3'UTR$^+$ background.

FIGURE 3. Levels of *hilD* and *hilD* Δ3'UTR mRNAs in strains lacking RNase G, PapI, RNase E, or Pnp. For simplification, *hilD* and *hilD* Δ3'UTR mRNAs has been labelled 3'UTR$^+$ and 3'UTR$^-$ respectively. Both mRNAs were detected by Northern blotting in MOPS-formaldehyde agarose gels, using a ^{32}P-labelled riboprobe specific for *hilD*. For quantification, *hilD / rnpB* ration was relativized to 1 in 3'UTR$^+$ background.

FIGURE 4. A. *hilD* mRNA levels in RNA extracts from Hfq^+ and Hfq^- isogenic strains. *hilD* mRNA was detected by Northern blot in MOPS-formaldehyde agarose gel, using a ^{32}P-labelled *hilD* riboprobe. *rnpB* mRNA was used as loading control. B. β-galactosidase activity of a *hilD::lac* transcriptional fusion in Hfq^+ (black histogram) and Hfq^- (white histogram) background. C. *hilD* mRNA levels in Hfq^+ and Hfq^- strains that express *hilD* from P_{LtetO} promoter, detected by Northern blot in MOPS-formaldehyde agarose gel. As loading control, *rnpB* mRNA was detected. D. *hilD* mRNA levels in Hfq^+ *hilD* 3'UTR$^+$, Hfq^+ *hilD* 3'UTR$^-$, Hfq^- *hilD* 3'UTR$^+$, and Hfq^- *hilD* 3'UTR$^-$ isogenic strains, detected by Northern blot in MOPS-formaldehyde agarose gel. The ratio *hilD* mRNA / *rnpB* mRNA was relativized to 1 in Hfq^+ *hilD* 3'UTR$^+$ background for quantification.

FIGURE 5. A. Epistasis analysis of SPI-1 regulation by Hfq. Black histograms represent β-galactosidase activities in Hfq^+ background, and white histograms represent β-galactosidase activities in Hfq^- background. B. Regulation by Hfq of *invF::lac*, *sipB::lac*, and *sipC::lac* fusions in strains with a native *hilD* 3'UTR or carrying the *hilD* Δ3'UTR allele. Black and white histograms represent β-galactosidase activities in Hfq^+ and Hfq^- backgrounds, respectively. C. Levels of InvF, SipB, and SipC protein versions tagged with the 3xFLAG epitope, in protein extracts from Hfq^+ *hilD* 3'UTR$^+$, Hfq^- *hilD* 3'UTR$^+$, Hfq^+ *hilD* 3'UTR$^-$, and Hfq^- *hilD* 3'UTR$^-$ backgrounds. Tagged proteins were specifically detected by Western blotting, using commercial anti-FLAG antibodies. For quantification, the ratio tagged-protein / GroEL was relativized to 1 in Hfq^+ *hilD* 3'UTR$^+$.

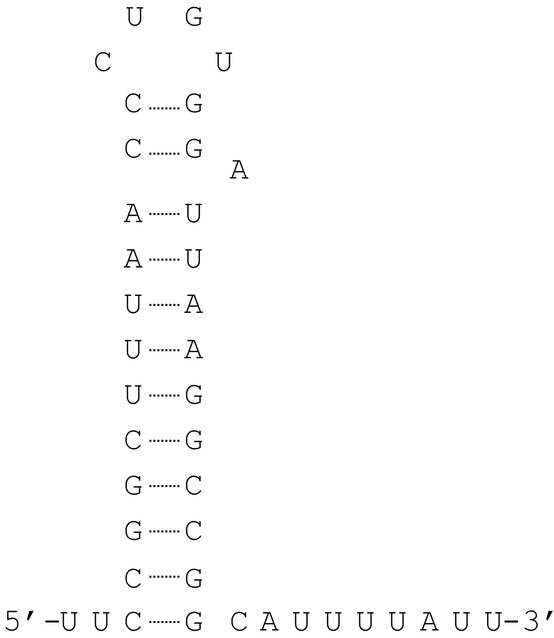
FIGURE S1. β-galactosidase activity of an *invF::lac* fusion in Dam^+ (black histograms) and Dam^- (white histograms) backgrounds. The assays were performed in strains with a native *hilD* 3'UTR (3'UTR$^+$) or carrying the *hilD* Δ3'UTR allele (3'UTR$^-$).

Strain designation	**Genotype or description**	**Reference or source**
14028	Wild type	ATCC
SV6190	*hilD* Δ3'UTR	This study
SV6636	P_{LtetO}-*hilD*	This study
SV6637	P_{LtetO}-*hilD* Δ3'UTR	This study
SV5297	Φ(*invF'-lacZ*$^+$)	This study
SV6227	*hilD* Δ3'UTR Φ(*invF'-lacZ*$^+$)	This study
SV5301	Φ(*invH'-lacZ*$^+$)	This study
SV6656	*hilD* Δ3'UTR Φ(*invH'-lacZ*$^+$)	This study
SV5382	Φ(*sipB'-lacZ*$^+$)	This study
SV6646	*hilD* Δ3'UTR Φ(*sipB'-lacZ*$^+$)	This study
SV5293	Φ(*sipC'-lacZ*$^+$)	This study
SV6651	*hilD* Δ3'UTR Φ(*sipC'-lacZ*$^+$)	This study
SV5457	*invF*::3xFLAG	This study
SV6642	*hilD* Δ3'UTR *invF*::3xFLAG	This study
SV5458	*invH*::3xFLAG	This study
SV6645	*hilD* Δ3'UTR *invH*::3xFLAG	This study
SV5459	*sipB*::3xFLAG	This study
SV6648	*hilD* Δ3'UTR *sipB*::3xFLAG	This study
SV5460	*sipC*::3xFLAG	This study
SV6653	*hilD* Δ3'UTR *sipC*::3xFLAG	This study
SV6789	Δ*rng*::*cat*	This study
SV6790	*hilD* Δ3'UTR Δ*rng*::*cat*	This study
SV6791	Δ*pcnB*::*cat*	This study
SV6792	*hilD* Δ3'UTR Δ*pcnB*::*cat*	This study
SV5961	Δ*rne*::*cat*	This study
SV6640	*hilD* Δ3'UTR Δ*rne*::*cat*	This study
SV5963	Δ*pnp*::*cat*	This study
SV6639	*hilD* Δ3'UTR Δ*pnp*::*cat*	This study
SV6193	Δ*hfq*::*kan*	This study
SV6638	P_{LtetO}-*hilD* Δ*hfq*::*kan*	This study
SV6410	Φ(*hilD-lacZ1*)	This study
SV6422	Δ*hfq*::*cat* Φ(*hilD-lacZ1*)	This study
SV6192	*hilD* Δ3'UTR Δ*hfq*::*kan*	This study
SV5284	Φ(*hilA'-lacZ*$^+$)(Hyb)	This study
SV5848	Δ*hfq*::*cat* Φ(*hilA'-lacZ*$^+$)(Hyb)	This study
SV5401	Δ*hilC* Φ(*hilA'-lacZ*$^+$)(Hyb)	This study
SV6793	Δ*hfq*::*cat* Δ*hilC* Φ(*hilA'-lacZ*$^+$)(Hyb)	This study
SV5399	Δ*hilD* Φ(*hilA'-lacZ*$^+$)(Hyb)	This study
SV6794	Δ*hfq*::*cat* Δ*hilD* Φ(*hilA'-lacZ*$^+$)(Hyb)	This study
SV5850	Δ*hfq*::*cat* Φ(*invF'-lacZ*$^+$)(Hyb)	This study
SV5403	Δ*hilA* Φ(*invF'-lacZ*$^+$)(Hyb)	This study
SV6795	Δ*hfq*::*cat* Δ*hilA* Φ(*invF'-lacZ*$^+$)(Hyb)	This study
SV5405	Δ*hilC* Φ(*invF'-lacZ*$^+$)(Hyb)	This study
SV6796	Δ*hfq*::*cat* Δ*hilC* Φ(*invF'-lacZ*$^+$)(Hyb)	This study
SV5407	Δ*hilD* Φ(*invF'-lacZ*$^+$)(Hyb)	This study

SV6797	Δ*hfq*::*cat* Δ*hilD* Φ(*invF'-lacZ*$^+$)(Hyb)	This study
SV5852	Δ*hfq*::*cat* Φ(*sipB'-lacZ*$^+$)(Hyb)	This study
SV5316	Δ*hilA* Φ(*sipB'-lacZ*$^+$)(Hyb)	This study
SV6798	Δ*hfq*::*cat* Δ*hilA* Φ(*sipB'-lacZ*$^+$)(Hyb)	This study
SV5318	Δ*hilC* Φ(*sipB'-lacZ*$^+$)(Hyb)	This study
SV6799	Δ*hfq*::*cat* Δ*hilC* Φ(*sipB'-lacZ*$^+$)(Hyb)	This study
SV5312	Δ*hilD* Φ(*sipB'-lacZ*$^+$)(Hyb)	This study
SV6800	Δ*hfq*::*cat* Δ*hilD* Φ(*sipB'-lacZ*$^+$)(Hyb)	This study
SV6801	Δ*hfq*::*cat* Φ(*invH'-lacZ*$^+$)(Hyb)	This study
SV5419	Δ*hilA* Φ(*invH'-lacZ*$^+$)(Hyb)	This study
SV6802	Δ*hfq*::*cat* Δ*hilA* Φ(*invH'-lacZ*$^+$)(Hyb)	This study
SV5417	Δ*hilC* Φ(*invH'-lacZ*$^+$)(Hyb)	This study
SV6803	Δ*hfq*::*cat* Δ*hilC* Φ(*invH'-lacZ*$^+$)(Hyb)	This study
SV5415	Δ*hilD* Φ(*invH'-lacZ*$^+$)(Hyb)	This study
SV6804	Δ*hfq*::*cat* Δ*hilD* Φ(*invH'-lacZ*$^+$)(Hyb)	This study
SV5384	Φ(*hilC'-lacZ*$^+$)(Hyb)	This study
SV6805	Δ*hfq*::*cat* Φ(*hilC'-lacZ*$^+$)(Hyb)	This study
SV5386	Δ*hilD* Φ(*hilC'-lacZ*$^+$)(Hyb)	This study
SV6806	Δ*hfq*::*cat* Δ*hilD* Φ(*hilC'-lacZ*$^+$)(Hyb)	This study
SV5854	Δ*hfq*::*cat* Φ(*sipC'-lacZ*$^+$)(Hyb)	This study
SV6641	Δ*hfq*::*cat* *hilD* Δ3'UTR Φ(*invF'-lacZ*$^+$)	This study
SV6647	Δ*hfq*::*cat* *hilD* Δ3'UTR Φ(*sipB'-lacZ*$^+$)	This study
SV6652	Δ*hfq*::*cat* *hilD* Δ3'UTR Φ(*sipC'-lacZ*$^+$)	This study
SV6643	Δ*hfq*::*cat* *invF*::3xFLAG	This study
SV6644	Δ*hfq*::*cat* *hilD* Δ3'UTR *invF*::3xFLAG	This study
SV6649	Δ*hfq*::*cat* *sipB*::3xFLAG	This study
SV6650	Δ*hfq*::*cat* *hilD* Δ3'UTR *sipB*::3xFLAG	This study
SV6654	Δ*hfq*::*cat* *sipC*::3xFLAG	This study
SV6655	Δ*hfq*::*cat* *hilD* Δ3'UTR *sipC*::3xFLAG	This study
SV5298	Δ*dam-231* Φ(*invF'-lacZ*$^+$)(Hyb)	This study
SV6228	*hilD* Δ3'UTR Δ*dam-231* Φ(*invF'-lacZ*$^+$)(Hyb)	This study

Figure 1

A



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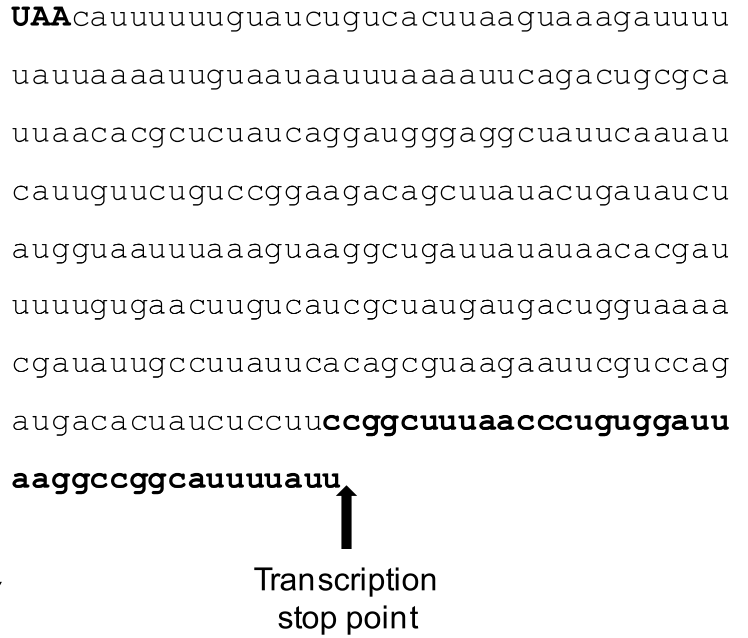

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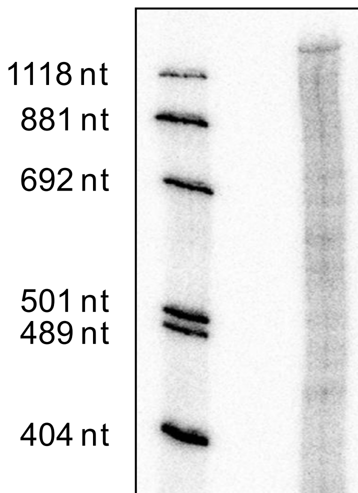

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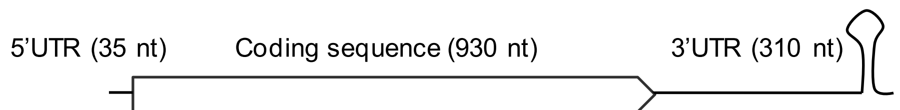

Figure 2

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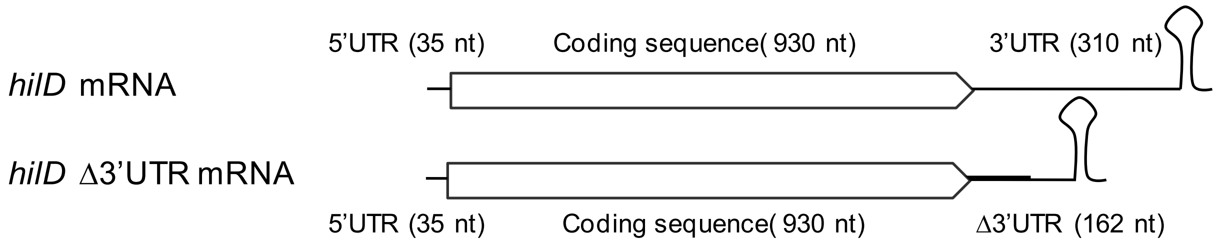

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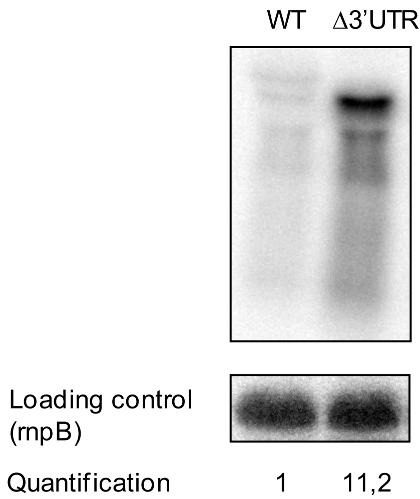

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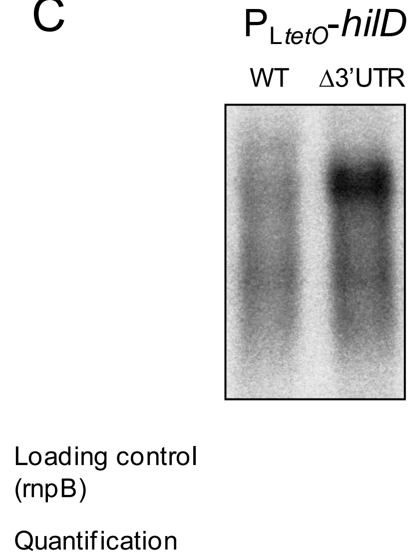

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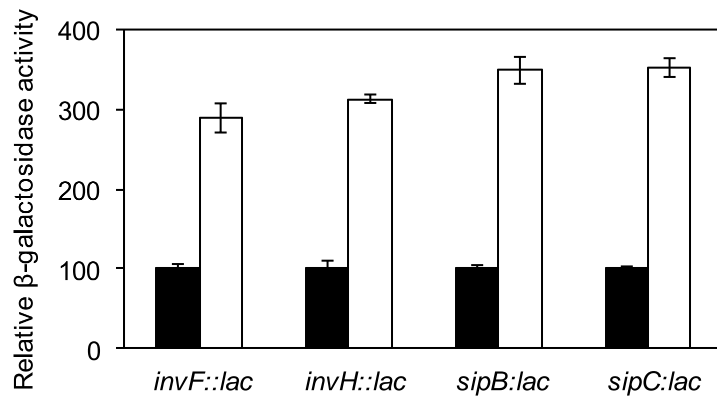

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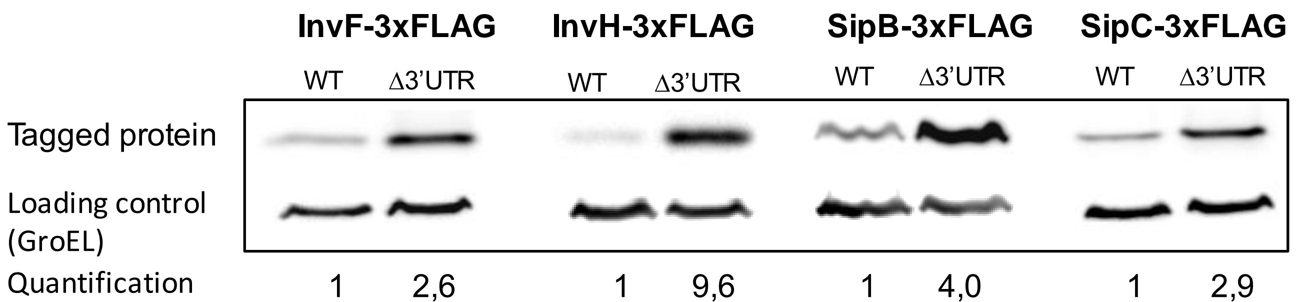

Figure 3

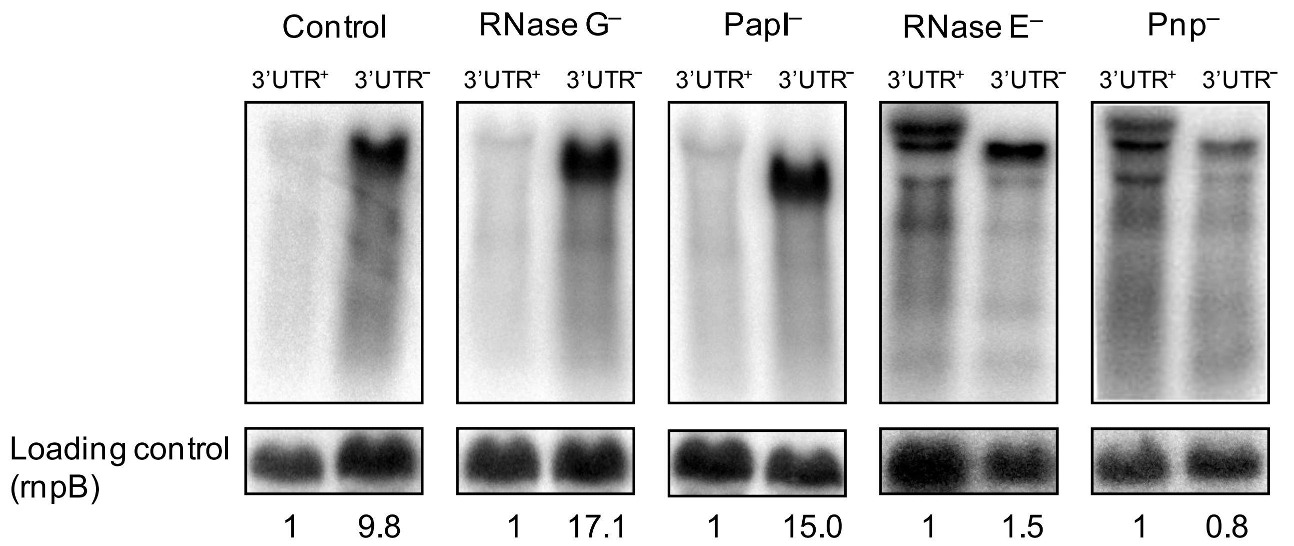

Figure 4

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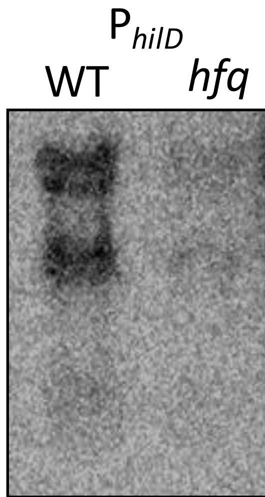

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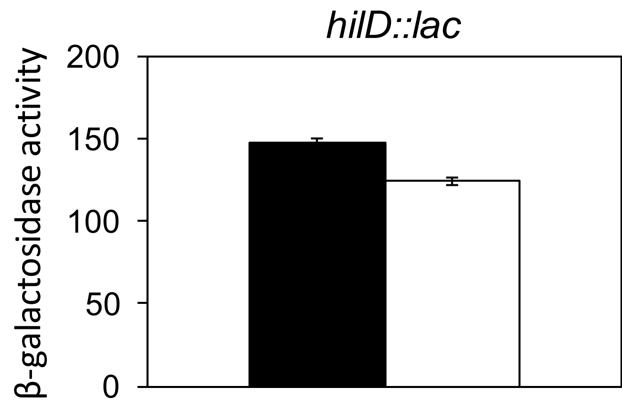

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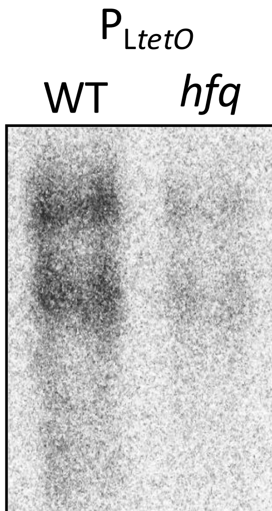

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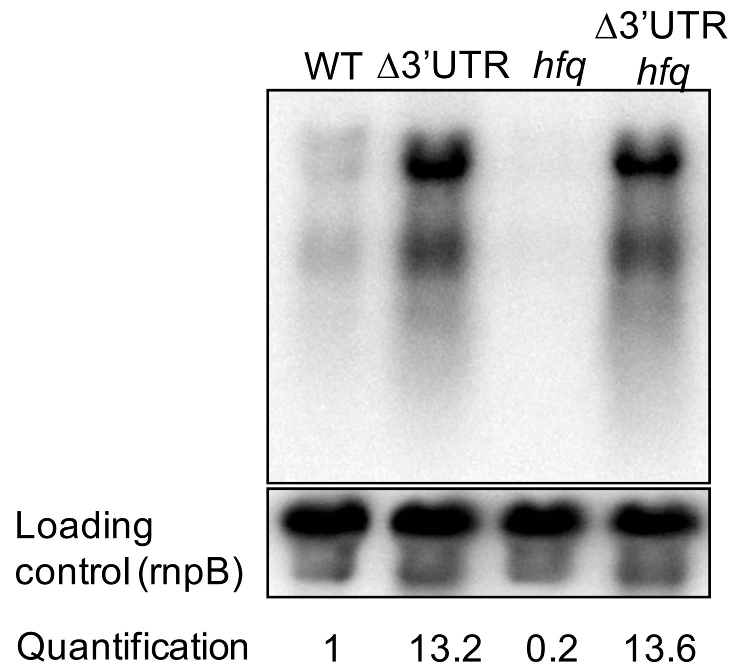

Figure 5

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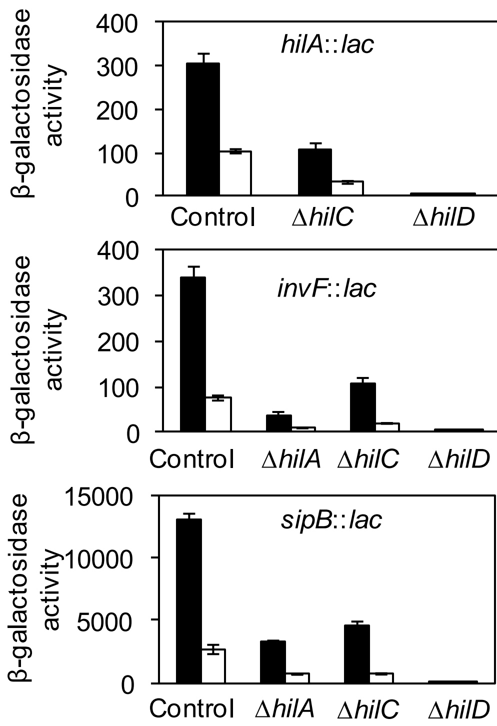

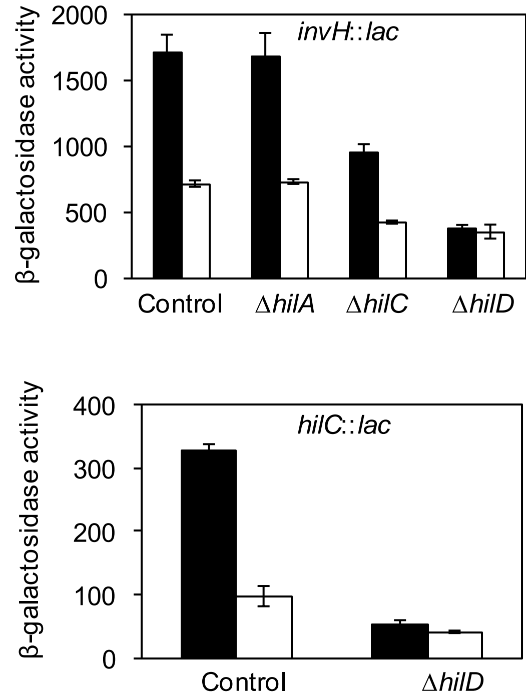

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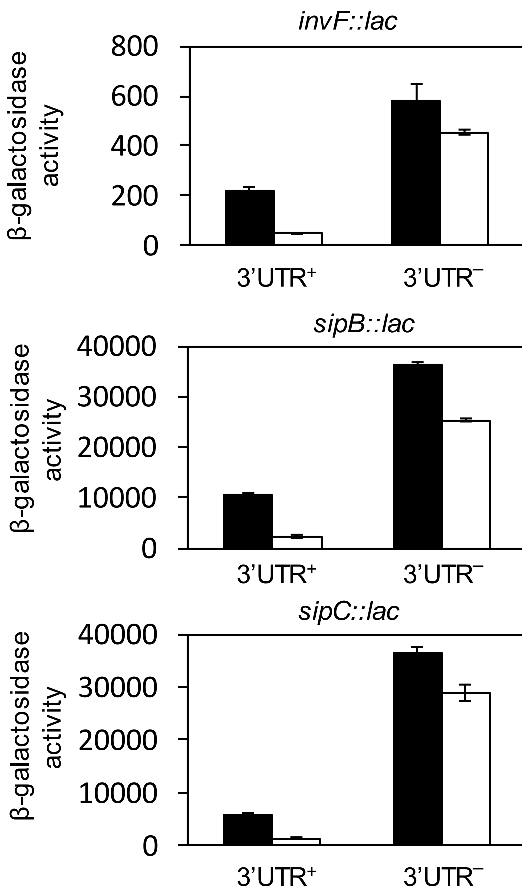

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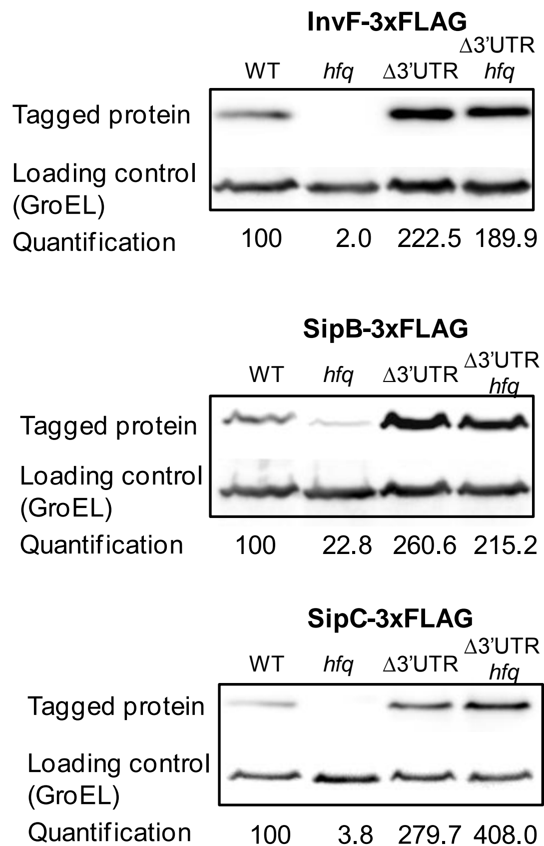
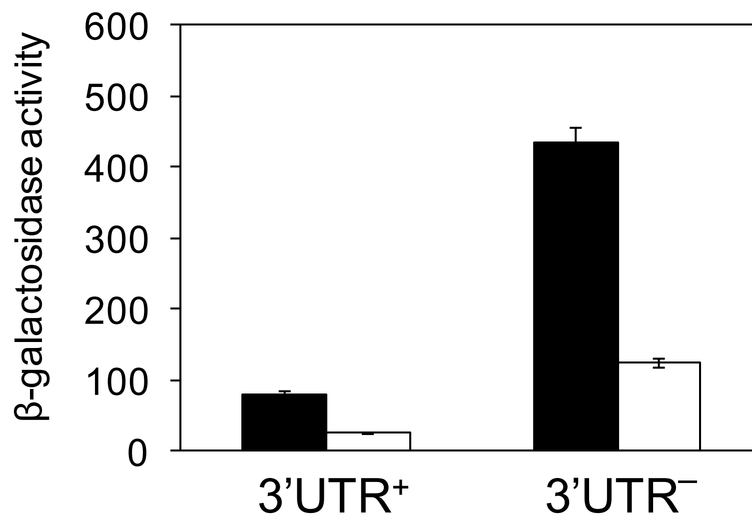


Table S1. Oligonucleotides used in this study (5'→3')

Oligonucleotide	**Sequence**
hilDriboprobeUP	atggaaaatgtaacctttgtaag
hilDriboprobeDO	gtttttttaatacgactcactatagggaggtatatcgaaatccatgtggc
rnpBriboprobeUP	gaagaagtgaaactgaccgataagc
rnpBriboprobeDO	taatacgactcactataggccgaagctgaccagacagtcg
hilD-E2’	atcatcctcaggctggctcc
RT-hilD3’-UP	agcttacggatgttgccgatc
JVO5462	aactacgccatcgacattcataaaaatggcgaaccattaaattccggggatccgtcgacc
JVO5463	ggagatagtgtcatctggacgaattcttacgctgtgaatagtgtaggctggagctgcttc
rngUP	accgaattcgcgtcgactcacgtctgacctatgagtcgctcatatgaatatcctccttag
rngDO	cggctgatggccactgtaatgctccagcttactggtcatttgtaggctggagctgcttcg
rng-E1	ggtgcatacggtattcaggg
rng-E2	tcgtcgagattacggtgtcc
pcnBUP	gcccgtctgccgtaatgcgaaagacatagagtaagttgatcatatgaatatcctccttag
pcnBDO	tgggatgttccatcagtttccaggcgcgtttgccctgacgtgtaggctggagctgcttcg
pcnB-E1	cgctgagctatgattagccg
pcnB-E2	tccggtctaatgacgcaagc


invF::lac
β-galactosidase activity
600
500
400
300
200
100
0
3'UTR+
3'UTR–

Inhibition of *Salmonella enterica* invasion by L-arabinose

Javier Lopez-Garrido, Elena Puerta-Fernández, and Josep Casadesus

ABSTRACT

Invasion of intestinal epithelial cells is a critical step in *Salmonella* infection. Invasion requires the expression of genes located the *Salmonella* Pathogenicity Island 1 (SPI-1). SPI-1 expression is controlled by environmental factors allowing high expression of SPI-1 in the ileum, the portion of the small intestine where invasion takes place. Several evidences suggest that the pentose L-arabinose is an specific signal for SPI-1 repression: (i) the expression of SPI-1 genes is repressed in the presence of L-arabinose, even at low concentration; (ii) that effect is independent of L-arabinose metabolism, since it is observed in a mutant lacking L-arabinose isomerase, the first enzyme involved in L-arabinose degradation; and (iii) addition of other pentoses such as D-arabinose and D-xylose does not repress SPI-1 expression. Deletion of the gene encoding the L-arabinose permease, *araE,* suppresses SPI-1 repression by L-arabinose, suggesting that L-arabinose needs to be inside the cell to repress SPI-1. Once inside the cell, L-arabinose represses SPI-1 independently of the L-arabinose-responsive regulator AraC. Epistasis analysis shows that SPI-1 repression by L-arabinose depends on the SPI-1-encoded transcriptional activator HilD, and our results suggest that L-arabinose controls HilD activity. L-arabinose is produced by plants and is poorly absorbed in the digestive tract of mammals and chicks. The presence of sugars that can be metabolized by *Salmonella enterica* in the small intestine might be a signal for inhibition of invasion. L-arabinose might also play a signaling role during the recently discovered colonization of plants by *Salmonella*.

INTRODUCTION

Salmonella enterica is a Gram-negative bacterium that causes intestinal and systemic diseases in humans and other animal hosts. Infection by *Salmonella* usually starts with the ingestion of contaminated food. After passage through the stomach, duodenum and jejunum, *Salmonella* reaches the ileum, where it has the ability to penetrate inside intestinal epithelial cells, in a process known as invasion. Depending on the *Salmonella* strain and the animal host, after invasion the infection can remain localized in the intestine, or *Salmonella* can cross the intestinal epithelium and disseminate inside the hosts. Thus, invasion of intestinal epithelial cells is a critical step for both, intestinal and systemic infections by *Salmonella.*

Invasion requires the expression of genes located in a 40-kb region of *Salmonella* chromosome, known as *Salmonella* pathogenicity island 1 (SPI-1) (Jones, 2005; Altier, 2005; Lostroh and Lee, 2001). SPI-1 is a set of at least 38 genes organized in 8 or more transcriptional units, located at centisome 63 on the *Salmonella enterica* chromosome (McClelland et al., 2001). It encodes a whole type 3 secretion system (TTSS) and several effector proteins that are translocated directly into intestinal epithelial cell through the TTSS (Lostroh and Lee, 2001). The effector proteins interact with specific targets inside epithelial cells and triggers cytoskeleton rearrangements necessary for *Salmonella* invasion (Darwin and Miller, 1999). SPI-1 also encodes four transcriptional activators responsible for its own expression: HilA, HilC, HilD, and InvF (**Figure S1**) (Lostroh and lee, 2001; Ellermeier and Slauch, 2007). HilA is a member of the OmpR/ToxR family (Bajaj et al., 1995; Lee et al., 1992) and directly activates transcription of genes encoding components of the TTSS and the transcriptional activator InvF (Bajaj et al., 1996). In association with the SicA chaperone, InvF boost transcription of the *sicA/sip* operon, mainly encoding effector proteins (Darwin and Miller, 1999; Eichelberg and Galan, 1999). *hilA* transcription is directly activated by HilC and HilD, both members of the AraC/XylS family of transcriptional regulators (Schechter and Lee, 2001). HilC and HilD relieve repression of *hilA* promoter by the nucleoid proteins H-NS and Hha (Olekhnovich and Kadner, 2006). They are also able to activate the expression of *invF* and *sicA/sip* transcriptional units independently of HilA (Akbar et al., 2003; Rakeman et al., 1999). Furthermore, HilD can activate *hilC* and its own transcription (Ellermeier et al., 2005) by direct binding to both promoters

(Olekhnovich and Kadner, 2002). Together with the outside transcriptional activator RtsA (Ellermeier and Slauch, 2003), SPI-1-encoded transcriptional factors form a regulatory network that governs SPI-1 expression in response to environmental stimuli and regulatory factors (**Figure S1**) (Ellermeier et al., 2005; Jones, 2005).

During its passage through the digestive tract, *Salmonella* finds several environmental conditions that can affect SPI-1 expression. In the stomach, *Salmonella* has to survive in an extremely acid pH. SPI-1 expression is repressed at acid pH (Bajaj et al., 1996; Behlau and Miller, 1993), thus preventing invasion in the stomach. The proximal part of the small intestine is under the influence of digestive fluids coming from the stomach, and the pH remains slightly acid. In addition, Salmonella has to deal with the antimicrobial activities of bile, secreted in the duodenum. Bile concentration decreases as it is being reabsorbed along the small intestine during digestion. It has been reported that bile represses SPI-1 expression, and it can be a signal for inhibition of invasion in the proximal small intestine (Prouty and Gunn, 2000). Another environmental factor that represses SPI-1 expression is the short-chain fatty acids propionate and butyrate (Lawhon et al., 2002). Mammalian digestive tract is rich in short chain fatty acids, due to digestion of nutrients and the metabolism of intestinal microbiota. The concentration of short-chain fatty acids is higher in the large intestine. In the colon propionate and butyrate concentration reaches 70 and 20 mM respectively (Lawhon et al., 2002). Such concentration may be sufficient for inhibition of invasion. Therefore, along the digestive tract there are several gradients of environmental factors that repress SPI-1 expression. Those gradients leave a region, the ileum, in which repressor concentrations are relatively low. It is supposed that the environmental control of SPI-1 expression makes it to be highly expressed in the ileum, the portion of the small intestine where *Salmonella* preferentially invades.

We have found that the pentose L-arabinose represses SPI-1 expression. L-arabinose is the second most abundant pentose in nature and is found in hemicellulose and pectin in plant cell walls. *Salmonella* can use L-arabinose as sole carbon source (Gutnick et al., 1969). L-arabinose catabolism by *Salmonella* starts with its transport inside the cell through a permease encoded by the *araE* gene (Lee et al., 1981; Lee et al., 1982). Once inside the cell, L-arabinose is sequentially transformed into L-ribulose, L-ribulose-5P, and D-xylulose-5P, by the action of L-arabinose isomerase, ribulokinase, and L-

ribulose-5P-4-epimerase respectively (Englesberg, 1961; Englesberg et al., 1962). L-ribulose-5P and D-xylulose-5P are substrates for the pentoses phosphate pathway, which produces glycolytic intermediates (**Figure S2**). L-arabinose isomerase, ribulokinase, and L-ribulose-5P-4-epimerase are encoded in by the *araA, araB,* and *araD* genes respectively (Englesberg, 1961; Englesberg et al., 1962), which are part of the same transcriptional unit, known as *araBAD* operon or arabinose operon (Gross and Englesberg, 1959; Englesberg, 1961; Englesberg et al., 1962; Lee et al., 1984). The expression of *araBAD* operon and *araE* are induced in the presence of L-arabinose (Lee et al., 1980; Lee et al., 1982). That regulation requires the transcriptional regulator AraC (Engelsberg et al., 1965; Lee et al., 1981), encoded immediately upstream *araBAD* operon but in divergent orientation (Lee et al., 1984). The mechanism of regulation has been extensively studied (see Schleif, 2010 for a recent review): shortly, AraC bound to L-arabinose directly activates transcription from *araBAD* and *araE* promoters. However, in the absence of L-arabinose, AraC acts as a repressor of *araBAD* and its own promoter.

Here, we investigate the role of L-arabinose in SPI-1 expression and *Salmonella* invasion. Genetic analysis reported below shows that SPI-1 expression is repressed in the presence of L-arabinose, and such repression is independent of L-arabinose catabolism and the regulatory protein AraC. Furthermore, SPI-1 repression by L-arabinose has a single target, the *hilD* gene. We present evidence that L-arabinose regulates *hilD* expression at posttranscriptional level, suggesting a new mechanism of gene expression control by L-arabinose.

MATERIALS AND METHODS

Bacterial strains, plasmids, bacteriophages, and standard strain construction

All the *Salmonella enterica* strains listed in **Table 1** 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 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 **Table S1**, together with the oligonucleotides (labeled "E") used for allele verification by the polymerase chain reaction. For the construction of most transcriptional and translational *lac* fusions in the *Salmonella* chromosome, FRT sites generated by excision of Kmr cassettes (DATSENKO and WANNER 2000) were used to integrate either plasmid pCE37 or pCE40 (ELLERMEIER *et al.* 2002). *hilD::lac477* translational fusion was constructed using the method described by Gerlach and Hensel (2005). Unless specified otherwise, all *lac* fusions used in this study are translational. Addition of 3xFLAG and HA epitope tags to protein-coding DNA sequences was carried out using plasmids pSUB11 (Kmr, 3xFLAG) and pSU314 (Cmr, HA) as templates (UZZAU *et al.* 2001). The plasmid pXG10-*hilD* was constructed by cloning a DNA fragment encompassing from *hilD* transcription start point to *hilD* transcription terminator in BrfBI-NheI restriction sites in pXG10 (Urban and Vogel, 2007) Transductional crosses using phage P22 HT 105/1 *int201* [(SCHMIEGER 1972) and G. Roberts, unpublished] 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.

Growth conditions

Luria-Bertani (LB) broth was used as standard liquid medium. Solid media were prepared by the addition of 1.5 % agar. L-arabinose, D-arabinose, D-xylose, or sucrose were added from 20 % stocks prepared in distilled water. For determination of expression of SPI-1 genes by β-galactosidase assay, western blot, or northern blot, saturated cultures were diluted 1:50 in LB or LB supplemented with the appropriate

sugar and incubated at 37 ºC with shaking (200 rpm). Samples were taken when the cultures had reached stationary phase (O.D. 2-2.5). Carbon-free medium (NCE) (Maloy and Roth, 1983), supplemented with the appropriate carbon source was used as minimal medium. Green plates were prepared according to Chan and co-workers (CHAN *et al.* 1972), except that methyl blue (Sigma Chemical Co, St. Louis, MO) substituted for aniline blue. Plate tests for monitoring β-galactosidase activity used 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside ("X-gal", Sigma Chemical Co.) as indicator.

Construction of relevant strains

Expression of *araE* and *hilD* from a heterologous promoter was achieved replacing their native promoters by P_{LtetO} promoter (Lutz and Bujard, 1997). A fragment containing the *cat* gene and P_{LtetO} promoter was amplified by PCR using pXG1 as template (Urban and Vogel, 2007). The primers were labelled P_{LtetO}UP and P_{LtetO}DO (**Table S1**). The PCR product was treated with DpnI to remove template traces. The construction was inserted in the chromosome by λRed recombinase-mediated recombination (Datsenko and Wanner, 2000) and Cm^r colonies were selected. Insertion of the construction was verified by PCR, using a couple of primers specific for *cat* gene and the target gene (**Table S1**).

pH curves

An overnight culture of *Salmonella* was 1:50 diluted in LB and LB plus 0.01; 0.02; 0.05; 0.1; 0.2; 0.5; and 1 % L-arabinose. The cultures were incubated at 37 ºC with 200 rpm shaking until they reached O.D. 600 2.5. Then, the cultures were centrifuged 20 min at 4000 rpm, and pH of the supernatant was determined using a pH-meter.

Protein extracts and Western blot analysis

Total protein extracts were prepared from bacterial cultures grown at 37°C in LB or LB plus the appropriate amount of L-arabinose until stationary phase (final O.D.600 ~2.5). Bacterial cells taken according to 1 O.D.600 were collected by centrifugation (16,000 g, 2 min, 4°C) 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, using 12% gels. Conditions for protein

transfer have been described elsewhere (JAKOMIN *et al.* 2008). Optimal dilutions of primary antibodies were as follows: anti-Flag M2 monoclonal antibody (1:5,000, Sigma Chemical Co, St. Louis, MO), anti-HA HA.11 monoclonal antibody (1:1,000, Covance, Princeton, NJ) and anti-GroEL polyclonal antibody (1:20,000, Sigma). Goat anti-mouse horseradish peroxidase-conjugated antibody (1:5,000, BioRad, Hercules, CA) or Goat anti-rabbit horseradish peroxidase conjugated antibody (1:20,000, Santa Cruz Biotechnology, Heildelberg, Germany) were used as secondary antibodies. Proteins recognized by the antibodies were visualized by chemoluminescence using the luciferin-luminol reagents.

RNA extraction and Northern analysis

2 ml of *S. enterica* cells reaching stationary phase were taken by centrifugation, and the pellet resuspended in 100 ul of a lysozyme solution (3 mg/ml in water). Cells lysis was facilitated by three consecutive freeze-thaw cycles. After lysis, RNA was extracted using 1ml of Trizol reagent (Invitrogen), according to manufacter's instructions. Finally, total RNA was resuspended in 30 ul of RNase-free water for subsequent uses. Quality and quantity of the obtained RNA was determined using a Nanodrop instrument. For northern blot analysis, 10 µg of total RNA was loaded per lane and electrophoresed in denaturing 1% agarose formaldehyde gels. Transfer and fixation to Hybond-N^+ membranes (GE Healthcare) were performed by vacuum using 0.05M NaOH. Filters were then hybridized using an internally labelled ([α-^{32}P]UTP) riboprobe specific for the first 300 nts of the *hilD* 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 of the RNase P RNA gene (*rnpB*). Images of radioactive filters were obtained with a Fuji, and quantification was performed using the Multy Gauge software.

β-galactosidase assays

Levels of β-galactosidase activity were assayed using the CHCl3-sodium dodecyl sulfate permeabilization procedure (Miller, 1972).

RESULTS

SPI-1 expression is downregulated in the presence of L-arabinose

pBAD vectors are a set of plasmids which contain the P_{BAD} promoter of the L-arabinose operon and its regulatory gene, *araC* (Guzman et al., 1995). In the presence of L-arabinose, transcription from P_{BAD} promoter is turned on, and in its absence transcription levels are very low. That provides a system that allows conditional expression of genes cloned under the control of P_{BAD} promoter, dependent on L-arabinose (Guzman et al., 1995). We considered the possibility of using the pBAD vectors for studying how overproduction of different proteins affected SPI-1 expression. Since expression from P_{BAD} promoter depends on L-arabinose, we decided to examine whether SPI-1 expression was affected by the presence of L-arabinose in a strain without pBAD as a control. For this purpose, we studied the expression level of an *invF::lac* fusion in LB and in LB plus 0.2 % of L-arabinose. As shown in **Figure 1 A** (left panel), *invF::lac* expression is reduced around 4 folds in LB plus L-arabinose compared to LB. That observation suggests that the presence of L-arabinose in the culture medium somehow represses the expression of SPI-1 genes.

SPI-1 repression in the presence of L-arabinose is independent of L-arabinose catabolism

Luria-Bertani (LB) broth contains a low concentration of sugars fermentable by *Salmonella.* The carbon sources for Salmonella in LB are catabolizable amino acids, not sugars (Sezonov et al., 2007). In such conditions, bacterial metabolism undergoes gluconeogenesis, and culture-medium pH is slightly alkalinized during growth. However, if we add a catabolizable sugar to LB exogenously, *Salmonella* will use it as a carbon source, and bacterial metabolism will undergo glycolysis. As a consequence, pyruvic acid will be produced and culture medium will be acidified (**Figure S2**). *Salmonella enterica* is able to regulate SPI-1 expression in response to pH. It has been reported that SPI-1 expression is almost 10 folds higher at pH 8 than at pH 6 (Bajaj et al., 1996). Thus, we considered the possibility that repression of SPI-1 expression in the presence of L-arabinose could be an indirect effect due to the acidification of the culture medium. We measured pH of LB and LB plus 0.2 % of L-arabinose before inoculation

of bacteria (before growth) and when bacterial population had reached an OD_{600} of 2.5 (after growth) (**Figure 1 B**). Before growth, both LB-pH and the pH of LB plus L-arabinose were near neutrality (7.04 and 7.08 respectively). However, after bacterial growth pH of LB was slightly alkaline (7.56) while pH of LB plus L-arabinose was 4.97. That suggests that catabolism of L-arabinose by *Salmonella* acidifies the culture medium. Hence, SPI-1 repression in the presence of L-arabinose could be a consequence of culture medium acidification. To address that hypothesis, we used a mutant with a deletion in the *araA* gene which encodes the L-arabinose isomerase, the first enzyme involved in L-arabinose catabolism (Englesberg, 1961). That mutant is unable to metabolize L-arabinose and cannot grow in minimal medium with L-arabinose as sole carbon source (**Figure S3**). In addition, culture-medium pH is not acidified after growth in the presence of 0.2 % of L-arabinose (pH of 7.56 compared to 7.49 in LB without L-arabinose) (**Figure 1 B, Figure S3**). Thus, if SPI-1 repression in the presence of L-arabinose was due to culture medium acidification, it would not be observed in the *araA* mutant. However, to our surprise, an *invF::lac* fusion is repressed more than 10 folds in the presence of 0.2 % of L-arabinose (**Figure 1 A**, right panel). That suggests that is L-arabinose itself rather than culture medium acidification what is responsible for SPI-1 repression. Indeed, repression is bigger in the *araA* mutant than in wild type background (10 folds compared to 4 folds) what could reflect a reduction in L-arabinose concentration due to its utilization as carbon source by $AraA^+$ bacteria. Taken together, those results open the possibility that L-arabinose is a signal for SPI-1 repression.

In order to avoid indirect effects due to culture medium acidification, the following experiments were done in $AraA^-$, unless otherwise indicated.

SPI-1 in general is repressed by L-arabinose

The above results show that expression of an *invF::lac* fusion is downregulated in the presence of L-arabinose. We wanted to know if such downregulation affected just to a subset of SPI-1 genes, or if the whole island was repressed by L-arabinose. To address that question, we analyzed the expression of different SPI-1 genes (**Figure 2**). We selected genes encoded in separated transcriptional units, and representing different functional categories. The selected genes were *hilA,* encoding a key transcriptional

activator of SPI-1 expression; *invF*, encoding a transcriptional activator of a subset of SPI-1 genes; *sipB*, encoding an effector protein; and *prgH*, encoding a component of the type-three secretion system. We analyzed the expression of those genes in LB and in LB plus 0.2% of L-arabinose by two independent methods: (i) by measuring β-galactosidase activity of *lac* fusions in selected genes (**Figure 2 A**); and (ii) by determining protein levels by western blot using protein versions tagged with 3xFLAG epitope (**Figure 2 B**). In order to avoid acidification of the culture medium due to L-arabinose catabolism, the experiments were carried out in AraA$^-$ background. **Figure 2** shows that both, β-galactosidase activity and protein level are reduced in the presence of L-arabinose in the four genes studied (from 3 to almost 20 folds, depending on the gene and the method used for determination of expression). That suggests that L-arabinose represses SPI-1 in general, affecting to different transcriptional units and functional categories.

It has been previously shown that HilA plays a central role in the co-ordinate regulation of invasion genes by environmental and regulatory conditions (Bajaj et al 1996). *hilA* is strongly repressed in the presence of L-arabinose (more than 10 folds in β-galactosidase assays and almost 20 folds in western blot assays). For that reason, in the following experiments we will use *hilA* as a reporter of SPI-1 expression.

Repression of SPI-1 expression by L-arabinose is dose-dependent

So far we have been using a concentration of L-arabinose of 0.2 % (equivalent to 13.3 mM). That concentration has been traditionally used for induction of P_{BAD} expression (Guzman et al, 1995), but lower concentrations also work. If L-arabinose is a signal for SPI-1 repression it could have a repressive effect at concentrations lower than 0.2 %.

A *hilA::lac* fusion and a HilA-3xFLAG protein version were used to determine SPI-1 repression with increasing concentrations of L-arabinose by β-galactosidase assay and western blot respectively (**Figure 2 C; Figure 2 D**). As above, the experiments were carried out in AraA$^-$ background to avoid culture-medium acidification. A gradual decrease of *hilA* expression as L-arabinose concentration increases is observed both by β-galactosidase assay (**Figure 2 C**) and by western blot analysis (**Figure 2 D**). Note that at the lower concentration of L-arabinose assayed (0.01 %, equivalent to 66.7 μM) *hilA*

expression is still significantly reduced (more that 3 folds in β-galactosidase assay and more than 2 folds in western blot), further supporting the idea that L-arabinose is a signal for SPI-1 repression.

Effect of other pentoses on SPI-1 expression

L-arabinose is a pentose metabolizable by *Salmonella* (Gutnick et al., 1969). We wandered if other pentoses were able to repress SPI-1 expression or if the effect was specific of L-arabinose. Thus, we studied the effect of two additional pentoses: D-xylose and D-arabinose.

(i) D-xylose is the most abundant pentose in nature, and can be used by *Salmonella* as sole carbon source (Gutnick et al., 1969). The first enzyme involved in D-xylose catabolism is D-xylose isomerase, encoded by the *xylA* gene (Shamanna and Sanderson, 1979). To examine the effect of D-xylose on SPI-1 expression, we constructed a strain with a deletion in the *xylA* gene. The *xylA* mutant is unable to use D-xylose as sole carbon source, avoiding the change of culture-medium pH due to D-xylose catabolism (**Figure S4**). We monitored the expression of a *hilA::lac* fusion on a $XylA^-$ background, grown on LB, or LB plus 0.2 % or 1 % of D-xylose. As shown in **Figure 3 B**, the presence of D-xylose in the culture medium does not have a significant effect on *hilA::lac* expression. However, the same fusion is strongly repressed in the presence of either 0.2 % or 1 % of L-arabinose (**Figure 3 A**).

(ii) D-arabinose is the D isomer of arabinose and it cannot be used by *Salmonella* as sole carbon source (Gutnick et al., 1969). However, some *Salmonella* mutants display a D-Ara^+ phenotype (Old and Morlock, 1977). In such mutants, D-arabinose induces the synthesis of two enzymes usually involved in L-fucose metabolism: L-fucose isomerase and L-fuculokinase. Those enzymes have a bifunctional activity, and can convert D-arabinose into D-ribulose I phosphate. Those observations imply that, unless it cannot be metabolized by wild type *Salmonella*, D-arabinose may enter wild type *Salmonella* cells. **Figure 3 C** shows that hilA*::lac* expression is similar in LB and LB plus 0.2 % or 1 % of D-arabinose, suggesting that it is not involved in SPI-1 regulation.

Thus, neither D-xylose nor D-arabinose is able to repress SPI-1 expression, as happens with L-arabinose. That suggests that the effect of L-arabinose is specific.

Intracellular L-arabinose is responsible for SPI-1 repression

In an attempt to understand the mechanism of SPI-1 repression by L-arabinose, we started by analyzing if L-arabinose could exert is repressive effect from outside the cell, or if it needed the cell. In *Salmonella*, L-arabinose enters the cell through a specific permease encoded by the *araE* gene (Lee et al., 1981; Lee et al., 1982). We constructed an *araE* mutant. That mutant cannot grow in minimal medium plus 0.1 % of L-arabinose as sole carbon source, grows slowly in the presence of 0.2 %, and grows as wild type strain in the presence of 1 % of L-arabinose (**Figure S3**). That suggests that AraE permease is essential for transport of L-arabinose when the extracellular concentration is low. However, when we raise the extracellular concentration, L-arabinose may enter by other routes. With that background, we made the following reasoning: if L-arabinose needed to be inside the cell to repress SPI-1, the repression would be suppressed in an *araE* mutant at low L-arabinose concentrations. However, as we increased the extracellular concentration of L-arabinose, it would enter the cell through AraE-independent pathways, and SPI-1 would be repressed. If, on the contrary, extracellular L-arabinose could repress SPI-1 expression, the repression would be similar in $AraE^{+}$ and $AraE^{-}$ backgrounds at low and high L-arabinose concentrations. We examined the expression of a *hilA::lac* fusion in isogenic *araA* and *araA araE* backgrounds. The strains were grown in LB and LB plus 0.2; 1; 2; or 3 % of L-arabinose (**Figure 4 A,** two first columns). *araE* mutation almost completely suppresses *hilA* repression by L-arabinose at 0.2 %. However, in the presence of higher concentrations, *hilA* is significantly repressed. Nevertheless, repression is higher in $AraE^{+}$ than in $AraE^{-}$ background in all L-arabinose concentrations assayed. To be sure that the repression observed at high L-arabinose concentrations was not due to the increase in osmolarity, we examined *hilA::lac* expression in the presence of up to 3 % of sucrose. *hilA::lac* expression was not repressed under any of the sucrose concentrations assayed (**Figure S5**) Those results indicate that L-arabinose needs to be inside the cell in order to repress SPI-1 expression.

SPI-1 repression by L-arabinose is AraC-independent

L-arabinose binds to AraC in bacterial cytoplasm. That binding modifies AraC structure, which can then activate the expression of genes involved in L-arabinose

catabolism, including *araE* (Scheilf 2010). The evidence that L-arabinose needs to be inside the cell to repress SPI-1 expression opens an interesting possibility: it might be possible that activation of AraC by L-arabinose in bacterial cytoplasm was directly or indirectly responsible for SPI-1 repression. To study that hypothesis, we analyzed repression by L-arabinose of a *hilA::lac* fusion in isogenic *araA* and *araA araC* backgrounds, in the presence of increasing concentrations of L-arabinose. Mutation of *araC* suppresses *hilA* repression by 0.2 % L-arabinose (**Figure 4 A,** compare first and third column). However, *hilA* expression is increasingly repressed as L-arabinose concentration increases. The repression pattern is very similar to the pattern observed in the *araE* mutant (**Figure 4 A**, second column). As AraC activates *araE* transcription, the suppression observed in $AraC^{-}$ background could be due to a decrease of intracellular L-arabinose due to lack of AraE permease. To circumvent that problem we did two different approximations:

(i) If suppression of SPI-1 regulation by L-arabinose in $AraC^{-}$ background was due to lack of AraE permease, mutation of *araE* would be epistatic over *araC* mutation. Thus, the double mutant *araE araC* would display the same phenotype than the *araE* single mutant. However, if the effect of *araC* mutation was independent of AraE permease, *araE* and *araC* mutations would have additive effects. As shown in **Figure 4 A** *araE araC* double mutant display the same phenotype than *araE* single mutant.

(ii) We constructed a strain that express *araE* constitutively, independently of AraC. To achieve that we placed P_{LtetO} promoter (Lutz and Bujard, 1997) upstream *araE* gene, replacing its native promoter. That strain grows similarly to wild type in minimal medium plus 0.1 % of L-arabinose, suggesting that AraE permease is being produced (**Figure S6**). When *araE* is expressed constitutively, mutation of *araC* no longer suppresses *hilA* repression by L-arabinose (**figure 4 B**).

The above results suggests that, once inside the cell, L-arabinose represses SPI-1 expression through an AraC-independent pathway

Evidence for SPI-1 repression at low intracellular concentration of L-arabinose

We have previously shown that a concentration of L-arabinose of 0.01 % in the culture medium was sufficient to significantly repress *hilA* expression. However, the fact that the production of AraE permease depends on the presence of L-arabinose produces a curious effect: cells that randomly transport L-arabinose will produce more AraE permease that will transport of more L-arabinose inside the cell. However, if L-arabinose does not enter the cell, production of AraE permease will not be induced and L-arabinose will not be transported. Thus, in the presence of L-arabinose there are two subpopulations in a *Salmonella* culture: one producing AraE permease and with a high level of intracellular L-arabinose, and another with low levels of AraE permease and intracellular L-arabinose (**Figure 5 A**). What changes when bacteria are cultured with different concentrations of L-arabinose is not the intracellular concentration of L-arabinose of single cells, but the proportion of cells of each subpopulation (Siegele and Hu, 1997; **Figure 5 A**). Hence, it is conceivable that SPI-1 repression by L-arabinose actually requires high concentrations intracellular L-arabinose. To circumvent that potential problem, we used the strain that expresses constitutively *araE*, independently of the presence of L-arabinose (described above). It has been reported that constitutive expression of *araE* avoid the formation of subpopulations in the presence of L-arabinose, and intracellular L-arabinose concentration increases gradually with extracellular concentration (Khlebinkov et al., 2001; **Figure 5 A**). We examined the expression of a *hilA::lac* fusion in isogenic *araA* and *araA* P_{LtetO}-*araE* backgrounds, cultured in the presence of increasing concentrations of L-arabinose (**Figure 5 B**). Both strains shows a repression of more than two folds at the minimal L-arabinose concentration assayed (0.005 %, equivalent to 33 μM), and the repression is bigger as the concentration of L-arabinose increases. That suggests that low intracellular concentrations of L-arabinose are sufficient to significantly repress SPI-1 expression.

L-arabinose-dependent expression of SPI-1 is transmitted via HilD

SPI-1 expression is controlled by a regulatory network of SPI-1-encoded transcriptional activators. On top of the network are the transcriptional activators HilA, HilC and HilD (**Figure S1**). We have shown that *hilA* expression is regulated by L-arabinose. However, that regulation might not by direct, and could be transmitted via HilC and/or

HilD. Mutation of *hilC* reduces *hilA* expression 2-3 folds (Lopez-Garrido and Casadesus, 2010), while addition of 0.2 % of L-arabinose to the culture medium represses *hilA* more than 10 folds. That makes unlikely that HilC is responsible for transmission of L-arabinose regulation. However, mutation of *hilD* reduces *hilA* expression more than 100 folds (Lopez-Garrido and Casadesus, 2010). Thus, we examined the possibility that L-arabinose regulated SPI-1 expression via HilD. To do that, we analyzed the regulation by L-arabinose of three HilD-activated genes (*hilC*, *rtsA*, and *invH*) using *lac* fusions, in $AraA^{-}$ $HilD^{+}$ and $AraA^{-}$ $HilD^{-}$ backgrounds (**Figure 6**) Because *lac* fusions in those genes have disparate β-galactosidase activities, the activity of each fusion was normalized to 100 in the $HilD^{+}$ background growing LB. Expression of all of them is reduced in the presence of L-arabinose in $HilD^{+}$. In the absence of HilD, expression of those genes is reduced but not completely abolished, and similar levels of β-galactosidase activity are detected in LB and LB plus L-arabinose (**Figure 6**). The lost of L-arabinose repression in a *hilD* mutant provides evidence that L-arabinose-dependent regulation of SPI-1 requires a functional *hilD* gene.

L-arabinose regulates *hilD* expression

The evidence that L-arabinose regulates SPI-1 expression through HilD suggests that *hilD* expression itself may be controlled by L-arabinose. We monitored the expression of two different *hilD::lac* fusion in LB and LB plus 0.2 % L-arabinose: (i) *hilD::lac1*, a transcriptional fusion in which *lacZ* has been inserted right in the transcription start point; and (ii) *hilD::lac477*, a translational fusion inserted at position 477 of *hilD* coding sequence (**Figure 7 A**). To our surprise, similar β-galactosidase levels were observed in the absence and in the presence of L-arabinose in both fusions. That suggests that L-arabinose does not regulate neither *hilD* transcription nor translation initiation. We then analyzed the level of *hilD* mRNA by northern blot (**Figure 7 B**). The amount *hilD* mRNA is reduced in the presence of L-arabinose. That indicates that L-arabinose regulates *hilD* expression. In addition, the regulation might be at posttranscriptional level.

L-arabinose regulates HilD at protein level

The discrepancy between *hilD::lac* fusions and *hilD* mRNA level in L-arabinose-dependent regulation made us consider the following possibility: the *hilD::lac* fusions used above generated *hilD* null mutations, while the northern blot was carried out in $HilD^+$ background. It has been described that *hilD* is under the control of an autogenous transcriptional activation (Ellermeier et al., 2005). If L-arabinose were somehow impairing the ability of HilD to activate gene transcription, the reduction in *hilD* mRNA level observed in the presence of L-arabinose might indeed have a transcriptional origin, due to lack of HilD autoactivation. If such were the case, *hilD* transcription would be L-arabinose-dependent in a strain carrying a functional HilD protein. We constructed a *hilD::lac* transcriptional fusion right after *hilD* stop codon (*hilD::lac930*). That fusion leaves an intact *hilD* coding sequence, thus allowing autoactivation of *hilD* transcription. As shown in **Figure 8A**, β-galactosidase activity of the *hilD::lac930* fusion is reduced almost 5 folds in the presence of 0.2 % L-arabinose. To determine if such regulation was in fact due to HilD autoregulation, we expressed *hilD* from a heterologous promoter. We inserted P_{LtetO} promoter upstream *hilD*, deleting its native promoter. We designed the construction to keep the same transcription start site than the native promoter. β-galactosidase activity of *hilD::lac930* fusion transcribed from P_{LtetO} is similar in LB and LB plus 0.2 % L-arabinose (**Figure 8A**). Furthermore, the level of *hilD* mRNA is not reduced in the presence of L-arabinose when *hilD* is transcribed from P_{Lteto} (**Figure 8B**). Taken together, those results suggest that the primary target of L-arabinose is HilD protein. We hypothesized that L-arabinose might interfere with HilD function, what could indirectly affects transcription of *hilD* itself and the rest of SPI-1 genes. To further analyze that possibility, we followed two different strategies: (i) we examined the regulation by L-arabinose of *hilA::lac* and *invF::lac* fusions when *hilD* was expressed from P_{LtetO}. Expression of both fusions is reduced around four folds in the presence of 0.2% L-arabinose (**Figure 8C**). Note that the regulation is smaller than when *hilD* is expressed from its native promoter, suggesting that autoactivation of *hilD* may serve to amplify regulatory inputs; (ii) we analyzed regulation by L-arabinose of *rtsA* gene. It is encoded outside SPI-1 but is directly activated by HilD. That provides the possibility to study *rtsA* regulation in the absence of SPI-1, expressing *hilD* ectopically. For that purpose, we cloned *hilD* in a plasmid called pXG-10, under the control of P_{LtetO} promoter. We examined regulation by L-arabinose of an *rtsA::lac*

fusion in three different backgrounds: *araA* with pXG-10 empty vector, *araA* ΔSPI-1 with pXG-10 empty vector, and *araA* ΔSPI-1 *with* pXG10-*hilD* vector. As shown in **Figure 8D**, *rtsA::lac* is regulated by L-arabinose is a strain with SPI-1. Deletion of SPI-1 decreases β-galactosidase activity, and suppresses regulation by L-arabinose. However, ectopic expression of *hilD* from a plasmid restores *rtsA::lac* expression and regulation by L-arabinose in the absence of SPI-1. That supports that L-arabinose affects HilD protein, and confirms that HilD alone is sufficient for transmission of L-arabinose-dependent regulation.

Translocation of SPI-1 effectors is reduced in the presence of L-arabinose

To determine if SPI-1 repression by L-arabinose was relevant for *Salmonella* interactions with host cells, we analyzed translocation of SPI-1 effectors into eukaryotic HeLa cells (**Figure 9**). For that purpose, we used a fusion of the SPI-1 effector encoding gene *sipA* with the *cya* gene of *Bordetella pertusis*. *Bordetella* adenylate cyclase requires Calmodulin in order to synthesize cAMP. Thus, cAMP will be produced only if the SipA-Cya fusion protein is translocated into eukaryotic cells. The *sipA::cya* fusion was introduced in isogenic *araA*, *araA araE*, and *araA prgH* backgrounds, and the strains were grown in LB and LB plus 0.2% L-arabinose before mixing with HeLa cells. Translocation was estimated by measuring the amount of cAMP. Translocation is strongly reduced in the presence of L-arabinose in *araA* background (**Figure 9**). However, in *araA araE* background, addition of L-arabinose does not reduce translocation, reflecting that L-arabinose needs to be inside the cell to repress SPI-1 expression (**Figure 9**). *araA prgH* background was used as negative control, since *prgH* encodes an essential component of SPI-1 type-three secretion system. Those results confirm that SPI-1 repression caused by L-arabinose impairs *Salmonella* interaction with host cells.

DISCUSSION

SPI-1 is regulated by different environmental factors. Here, we have reported that L-arabinose, even at low concentrations, represses SPI-1 expression, and that effect is independent of L-arabinose catabolism. Furthermore, other pentoses such as D-xylose and D-arabinose fail to repress SPI-1. That opens the interesting possibility that L-arabinose is a specific signal for SPI-1 repression.

Regulation of virulence genes by sugars has been reported in different bacteria. In the Gram-positive bacterium *Listeria monocytogenes,* the expression of virulence genes regulated by the master regulator PrfA is repressed in the presence of sugars transported through the phosphoenolpyruvate-sugar phosphotransferase system (PTS) (Park and Kroll, 1993; Milenbachs et al., 1997; De las Heras et al. 2011). However, such repression is not observed in the presence of non-PTS sugars (Ripio et al, 1997; Stoll et al, 2008; Joseph et al, 2008; de las Heras et al., 2009). In *Streptococcus pyogenes*, production of surface M protein, a major virulence determinant, is affected by the sugar source (Pine and Reeves, 1978). Transcription the gene encoding the surface M protein is indirectly activated is by carbon catabolic repression (CCR) through the virulence gene regulator Mga. CCR also controls virulence gene expression in *Clostridium prefringes* (Varga et al, 2004) and *Staphylococcus aureus* (Morse at al., 1969). In *Salmonella enterica* there are evidences that PTS-dependent sugars repress invasion gene expression: *crp cya* mutants of *Salmonella enterica* serovar Choleraesuis are attenuated in pigs (*Kennedy* et al., 1999), and that correlates with the inability of *crp* mutants to secrete SPI-1 TTSS effectors (Zeng-Weng et al., 2010). In addition, it has been reported that Mlc, a global regulator of carbon metabolism, activates SPI-1 expression by directly repressing the transcription of SPI-1 negative regulator HilE (Lim et al., 2007). Mlc regulon can be induced by the PTS-sugars glucose and mannose (Plumbridge, 2002). According to that, it has been shown that *hilD* expression is slightly reduced in the presence of glucose and mannose (Lim et al., 2007). However, L-arabinose is a non-PTS sugar and its transport inside the cell does not induce Mlc regulon or reduces the level of cAMP. Furthermore, regulation of HilD by L-arabinose is independent of HilE (not shown). Hence, L-arabinose must regulate SPI-1 by a different mechanism.

The observation that L-arabinose can regulate gene expression is not new. It is well known that genes necessary for L-arabinose catabolism are activated the presence of L-arabinose, and such activation depends on the transcriptional regulator AraC (reviewed in Schleif, 2010). However, the traditional model of L-arabinose-dependent gene expression does not fit in the case of SPI-1 repression. We have shown that L-arabinose needs the AraE permease in order to efficiently repress SPI-1. As expression of *araE* depends on AraC, AraC is indirectly required for the transport of L-arabinose. However, once L-arabinose is inside the cell, AraC is no longer necessary for SPI-1 repression. That provides evidence of the existence of a new way to control gene expression by L-arabinose in *Salmonella*.

The requirement of the AraE permease for SPI-1 repression by L-arabinose admits two interpretations: (i) L-arabinose has to be inside the cell to repress SPI-1; or (ii) the transport of L-arabinose through AraE necessary for SPI-1 repression. However, the last possibility seems unlikely, since it would involve the existence of a signal transduction system associated to AraE that, to our knowledge has not been described. The observation that *Salmonella araE* mutants can grow with 1 % of L-arabinose as sole carbon source provide evidence that L-arabinose enters through alternative pathways at high concentrations. L-arabinose can repress SPI-1 expression in *Salmonella araE* mutants when provided at concentrations of 1 % or higher, thereby confirming the hypothesis that intracellular L-arabinose, rather that the transport through AraE permease, is responsible for SPI-1 repression.

We have determined that L-arabinose regulates SPI-1 expression through HilD. According to that, we have evidences that L-arabinose regulates HilD at protein level, either controlling its stability or activity. HilD is an AraC-like transcriptional activator. It is tempting to propose that L-arabinose might regulate HilD activity by direct binding to the protein, but further experiments are required to study that hypothesis.

L-arabinose is a plant-derived sugar, and the presence of a specific system in *Salmonella* for its catabolism indicates that *Salmonella* finds L-arabinose during its life cycle and uses it as carbon source. Our results suggest that, apart from as a carbon source, *Salmonella* might use L-arabinose as a signal for SPI-1 repression under certain circumstances. We propose two different scenario in which the sensing of L-arabinose could repress SPI-1 expression: (i) the animal intestine; and (ii) outside the animal host.

(i) The observation that *Slamonella* grown in the presence of L-arabinose fail to translocate the SPI-1 effector *sipA* into fibroblats provide evidence that L-arabinose might inhibit invasion *in vivo*. L-arabinose is poorly absorbed during digestion in mammals (Cori, 1925) and chicken (Wagh andWaibel, 1967), and there are evidences that free L-arabinose is present in the intestine: P_{BAD} promoter expression is induced in the intestine of mice that receive food with plant components (Loessner et al., 2009). Furthermore, L-arabinose catabolism is requires for an efficient colonization of the large intestine be commensal and pathogenic strains of *E. coli* (Fabich et al., 2008). L-arabinose supports efficient growth of *Salmonella in vitro* and might be a preferred carbon source in the intestine. The presence of L-arabinose in the intestine could be detected by *Salmonella* as a signal for repression of invasion. If that were true, L-arabinose-rich compounds in the diet could prevent infections by *Salmonella*. Consistent with that idea, it has been observed that dietary addition of arabinoxylooligosaccharides, made of few molecules of L-arabinose and D-xylose, provides protection against oral infections by *Salmonella enterica* serovar Enteritidis in poultry (Eeckhaut et al., 2008). However, we have observed that repression of *hilA* by L-arabinose is smaller in when *Salmonella* grows on SPI-1inducing conditions than of SPI-1 standard conditions (notshown). SPI-1 inducing conditions are thought to mimic the conditions in the ileum. Therefore, it could be possible that L-arabinose contributed to keep low levels of SPI-1 in the large intestine and the first portion of the small intestine, allowing *Salmonella* to invade in the ileum.

(ii) SPI-1 repression by L-arabinose could also play a role outside the animal host. As a plant-derived sugar, L-arabinose accumulates in the soil. It has been shown that *Salmonella* is able to persist in the soil for long periods (Islam et al., 2004). It may be possible that SPI-1 repression by L-arabinose in the soil may improve the fitness of *Salmonella* in that niche. One interesting feature of *Salmonella* is its ability to colonize plant surfaces (epiphytic colonization) (Barak et al., 2002; Brandl and Mandrell, 2002) and the spaces between cells inside the plants (endophytic colonization) (Franz et al., 2007). Plant colonization may be part of *Salmonella* life-cycle, and it could be used as a way for recolonizing animal hosts (Tyler and Triplett, 2008). *Salmonella* mutants lacking components of the SPI-1 TTSS perform a better plant colonization than wild type strains (Iniquez et al., 2005). It seems that the presence of a functional TTSS in the surface of *Salmonella* triggers a defense response by the plant (Iniquez et al., 2005). In

such context, the detection of L-arabinose by *Salmonella* in plants might contribute to turn down SPI-1 expression for efficient plant colonization.

Hence, our report of SPI-1 repression by L-arabinose suggests new roles of the sugar on *Salmonella* physiology. A deeper study in the molecular mechanism could reveal new mechanism of regulation of gene expression by L-arabinose.

FIGURE 1. A. β-galactosidase activities of an *invF::lac* fusion in LB (black histograms) and in LB plus 0.2 % L-arabinose (white histograms). The graphic on the left represents the activities measured in $AraA^+$ background. The graphic on the right represents the activities measured in $AraA^-$ background. Histograms represent the average and standard deviations from 3 experiments. B. Culture-medium pH measured before inoculation of bacteria (before growth), and after bacterial culture had reached an O.D. 600 of 2.5 (after growth).

FIGURE 2. A. β-galactosidase activities of *hilA::lac*, *invF::lac*, *sipB::lac*, and *prgH::lac* fusions in the absence (black histograms) and in the presence (white histograms) of 0.2 % L-arabinose, measured in $AraA^-$ background. Data represent the average and standard deviations of 3 experiments. B. Levels of HilA, InvF, SipB, and PrgH in protein extracts from $AraA^-$ strains grown in LB with and without 0.2 % L-arabinose. 3xFLAG-tagged proteins were detected by Western blotting using anti-FLAG commercial antibodies. The loading control was GroEL in all cases. C. β-galactosidase activity of a *hilA::lac* fusion in an $AraA^-$ strain grown in the presence of increasing concentrations of L-arabinose. Histograms represent the average and standard deviations of 3 experiments. D. Level of HilA-3xFLAG in protein extracts from an $AraA^-$ strain grown in increasing concentrations of L-arabinose. HilA level was normalized to GroEL for quantification.

FIGURE 3. Chemical structure and effect on SPI-1 expression of 3 different pentoses: A. L-arabinose; B. D-xylose; and C. D-arabinose. β-galactosidase activity of a *hilA::lac* fusion was monitored in LB, and LB plus 0.2 % and 1 % of the appropriate pentose. In the cases of L-arabinose and D-xylose, the experiments were performed in $AraA^-$ background and $XylA^-$ background respectively. Histograms represent the average and standard deviation of 3 experiments.

FIGURE 4. Role of AraE and AraC on SPI-1 regulation by L-arabinose. A. β-galactosidase activity of a *hilA::lac* fusion in $AraA^-$ (control), $AraA^-$ $AraE^-$, $AraA^-$ $AraC^-$, and $AraA^-$ $AraE^-$ $AraC^-$ isogenic backgrounds. β-galactosidase activity was measured in LB, and LB plus 0.2 %, 1 %, 2 %, and 3 % L-arabinose. Results are the average and standard deviations of 3 experiments. B. β-galactosidase activity of an *hilA::lac* fusion in $AraA^-$ (control), $AraA^-$ $AraE^-$, $AraA^-$ $AraC^-$, $AraA^-$ $P_{L\mathit{tetO}}$-*araE*, and

AraA$^-$ AraC$^-$ $P_{L\textit{tetO}}$-*araE* isogenic backgrounds, grown in LB and LB plus 0.2 % L-arabinose. Data represent the average and standard deviations of three experiments.

FIGURE 6. β-galactosidase activities of *hilC::lac*, *rtsA::lac*, and *invH::lac* fusions in isogenic AraA$^-$ HilD$^+$ and AraA$^-$ HilD$^-$ backgrounds. Black histograms represent β-galactosidase activities in LB. White histograms represent β-galactosidase activities in LB plus 0.2 % L-arabinose. Results are the average and standard deviations of 3 experiments.

FIGURE 7. A. β-galactosidase activities of *hilD::lac1* transcriptional fusion and *hilD::lac 477* translational fusion in LB (black histograms) and LB plus 0.2 % L-arabinose (white histograms). Measurements were performed in AraA$^-$ background. Histograms represent the average and standard deviations of 3 experiments. B. Level of *hilD* mRNA in RNA extracts from an AraA$^-$ strain grown in LB and LB plus 0.2 % L-arabinose. *hilD* mRNA was detected by Northern blotting using a P^{32}-lebelled riboprobe complementary to the 5' region of *hilD* mRNA. *rnpB* mRNA was used as loading control.

FIGURE 8. A. β-galactosidase activity of *hilD::lac 930* fusion in LB (black histograms) and LB plus 0.2 % L-arabinose (white histograms). β-galactosidase activity was measured in AraA$^-$ (control) and AraA$^-$ $P_{L\textit{tetO}}$-*hilD* strains. Data represent the average and standard deviations of 3 experiments. B. Level of *hilD* mRNA in RNA extracts from AraA$^-$ (control) and AraA$^-$ $P_{L\textit{tetO}}$-*hilD* strains, grown in LB and LB plus 0.2 % L-arabinose. *rnpB* mRNA was used as loading control. C. β-galactosidase activities of *hilA::lac* and *invF::lac* fusions in LB (black histograms) and LB plus 0.2 % L-arabinose (white histograms). Measurements were done in AraA$^-$ (control) and AraA$^-$ $P_{L\textit{tetO}}$-*hilD* strains. Results represent the average and standard deviations of three experiments. C. β-galactosidase activity of an *rtsA::lac* fusion in LB (black histograms) and LB plus 0.2 % L-arabinose (white histograms), in the following backgrounds: AraA$^-$ with pXG10 empty plasmid, AraA$^-$ ΔSPI-1 with pXG10 empty plasmid, and AraA$^-$ ΔSPI-1 with pXG10-*hilD* plasmid. Data represent the average and standard deviations of three experiments.

Strain designation	**Genotype or description**	**Reference or source**
14028	Wild type	ATCC
SV5999	Δ*araA*	This study
SV5297	Φ(*invF'-lacZ*$^+$)(Hyb)	This study
SV6000	Δ*araA* Φ(*invF'-lacZ*$^+$)(Hyb)	This study
SV6205	Δ*araA* Φ(*hilA'-lacZ*$^+$)(Hyb)	This study
SV6206	Δ*araA* Φ(*sipB'-lacZ*$^+$)(Hyb)	This study
SV6207	Δ*araA* Φ(*prgH'-lacZ*$^+$)(Hyb)	This study
SV6209	Δ*araA* *invF*::3xFLAG	This study
SV6208	Δ*araA* *hilA*::3xFLAG	This study
SV6210	Δ*araA* *sipB*::3xFLAG	This study
SV6211	Δ*araA* *prgH*::3xFLAG	This study
SV5284	Φ(*hilA'-lacZ*$^+$)(Hyb)	This study
SV6218	Δ*xylA* Φ(*hilA'-lacZ*$^+$)(Hyb)	This study
SV6213	Δ*araA* Δ*araE* Φ(*hilA'-lacZ*$^+$)(Hyb)	This study
SV6212	Δ*araBAD* Δ*araC* Φ(*hilA'-lacZ*$^+$)(Hyb)	This study
SV6807	Δ*araBAD* Δ*araE* Δ*araC* Φ(*hilA'-lacZ*$^+$)(Hyb)	This study
SV6244	Δ*araA* P_{LtetO}-*araE* Φ(*hilA'-lacZ*$^+$)(Hyb)	This study
SV6245	Δ*araBAD* Δ*araC* P_{LtetO}-*araE* Φ(*hilA'-lacZ*$^+$)(Hyb)	This study
SV6219	Δ*araA* Φ(*hilC'-lacZ*$^+$)(Hyb)	This study
SV6222	Δ*araA* Δ*hilD* Φ(*hilC'-lacZ*$^+$)(Hyb)	This study
SV6220	Δ*araA* Φ(*rtsA'-lacZ*$^+$)(Hyb)	This study
SV6223	Δ*araA* Δ*hilD* Φ(*rtsA'-lacZ*$^+$)(Hyb)	This study
SV6221	Δ*araA* Φ(*invH'-lacZ*$^+$)(Hyb)	This study
SV6224	Δ*araA* Δ*hilD* Φ(*invH'-lacZ*$^+$)(Hyb)	This study
SV6417	Δ*araA* Φ(*hilD-lacZ1*)	This study
SV6419	Δ*araA* Φ(*hilD'-lacZ*$^+$*477*)(Hyb)	This study
SV6421	Δ*araA* Φ(*hilD-lacZ930*)	This study
SV5808	Δ*araA* P_{LtetO}-*hilD* Φ(*hilD-lacZ930*)	This study
SV5809	Δ*araA* P_{LtetO}-*hilD*	This study
SV6657	Δ*araA* P_{LtetO}-*hilD* Φ(*hilA'-lacZ*$^+$)(Hyb)	This study
SV6658	Δ*araA* P_{LtetO}-*hilD* Φ(*invF'-lacZ*$^+$)(Hyb)	This study
SV6659	Δ*araA* Φ(*rtsA'-lacZ*$^+$)(Hyb)/pXG10	This study
SV6660	Δ*araA* Δ*spi-1* Φ(*rtsA'-lacZ*$^+$)(Hyb)/pXG10	This study
SV6661	Δ*araA* Δ*spi-1* Φ(*rtsA'-lacZ*$^+$)(Hyb)/pXG10-*hilD*	This study
SV6199	Δ*araE*	This study
SV6197	Δ*araC*	This study
SV6243	P_{LtetO}-*araE*	This study
SV6201	Δ*xylA*	This study
SV6423	Δ*araA* Φ(*sipA'-cya*$^+$)(Hyb)	This study
SV6424	Δ*araA* Δ*araE* Φ(*sipA'-cya*$^+$)(Hyb)	This study
SV6425	Δ*araA* Δ*prgH* Φ(*sipA'-cya*$^+$)(Hyb)	This study

Figure 1.

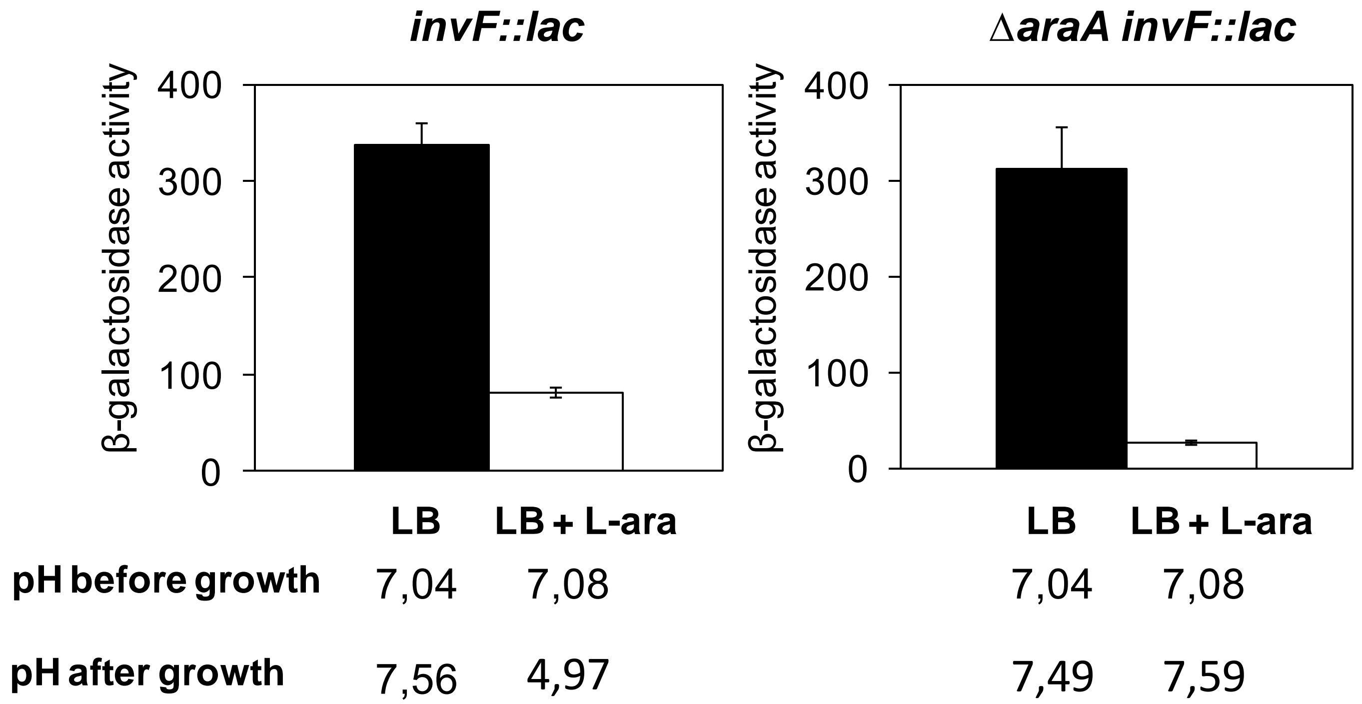


Fig 2.

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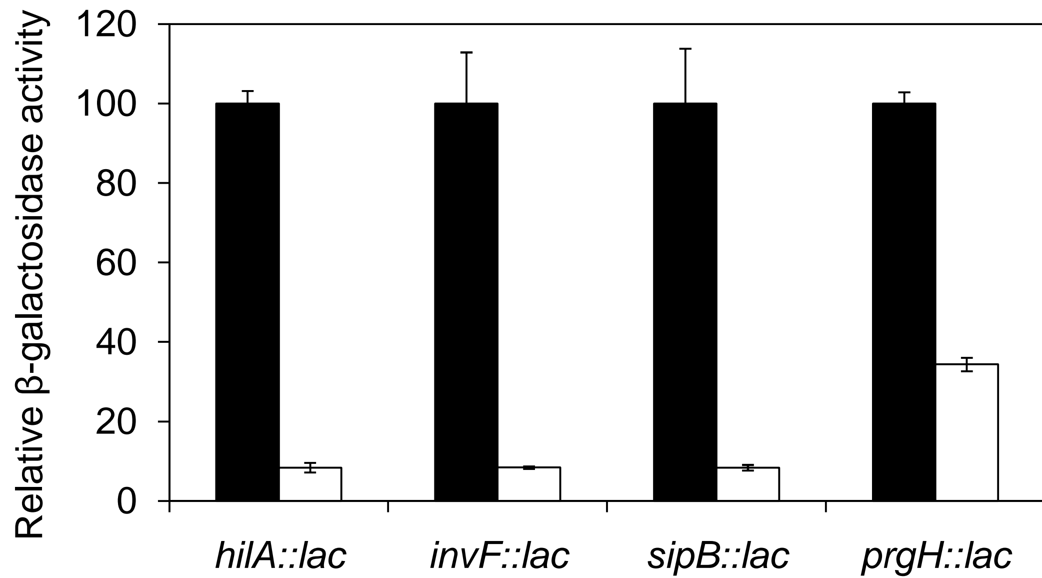

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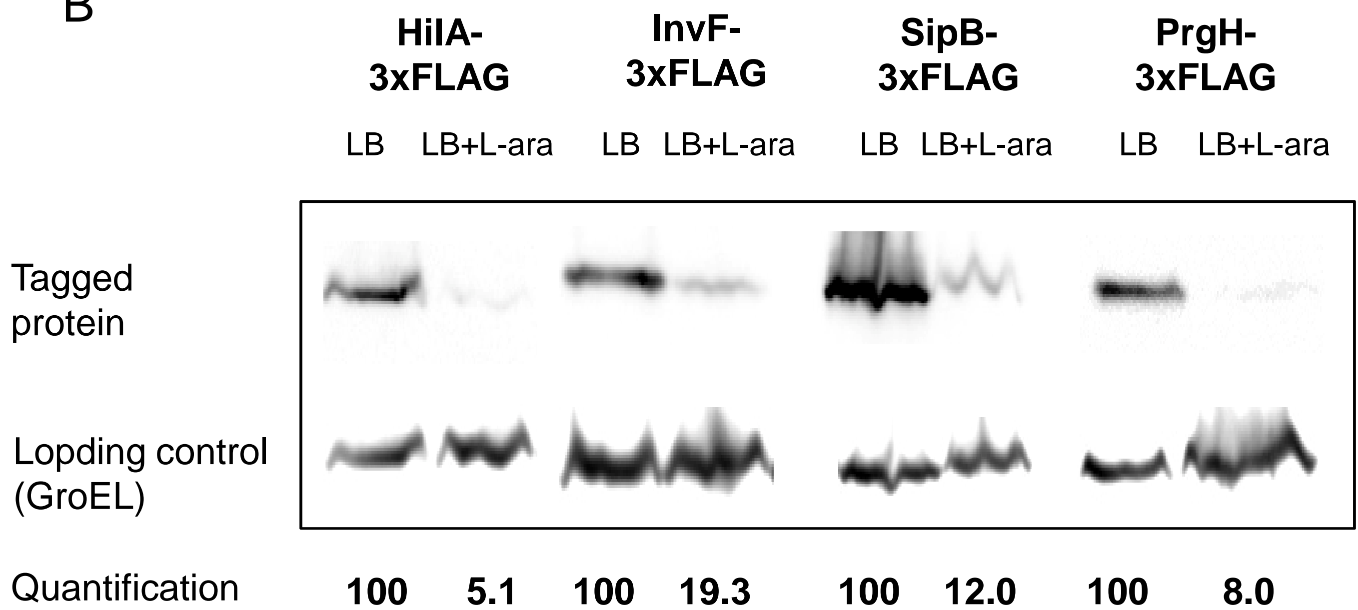

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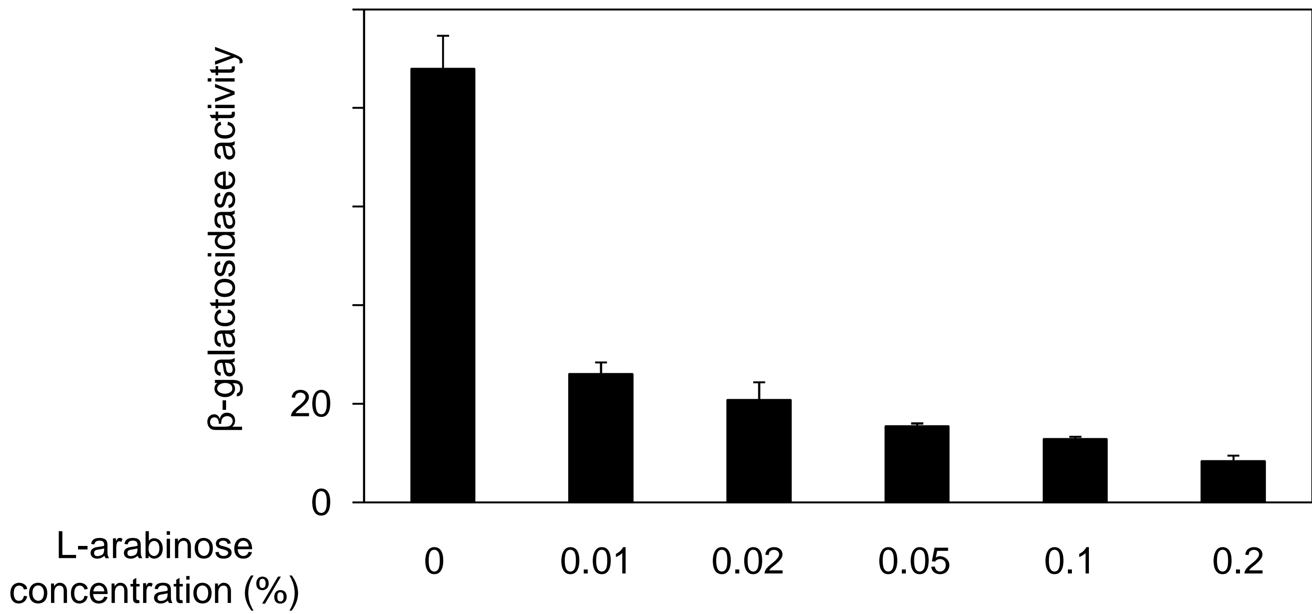

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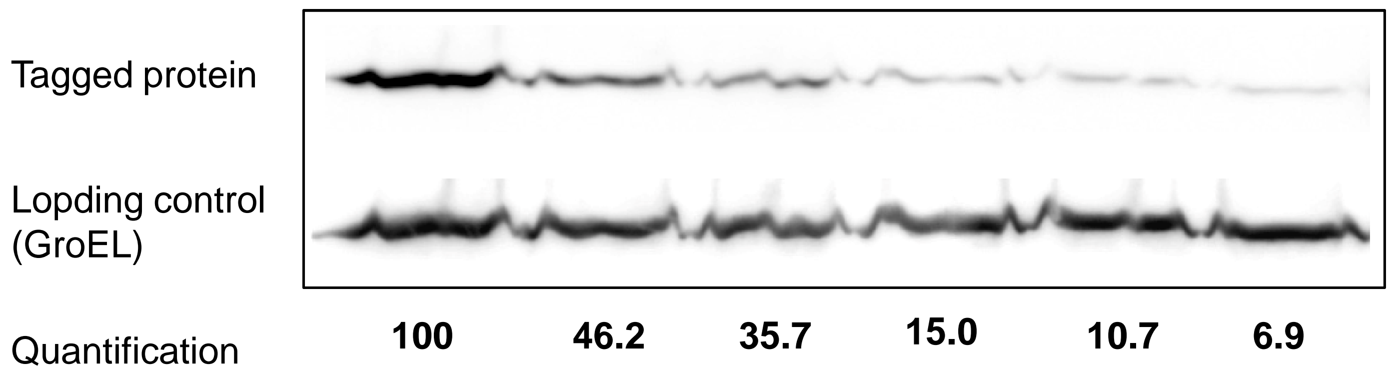

Fig. 3

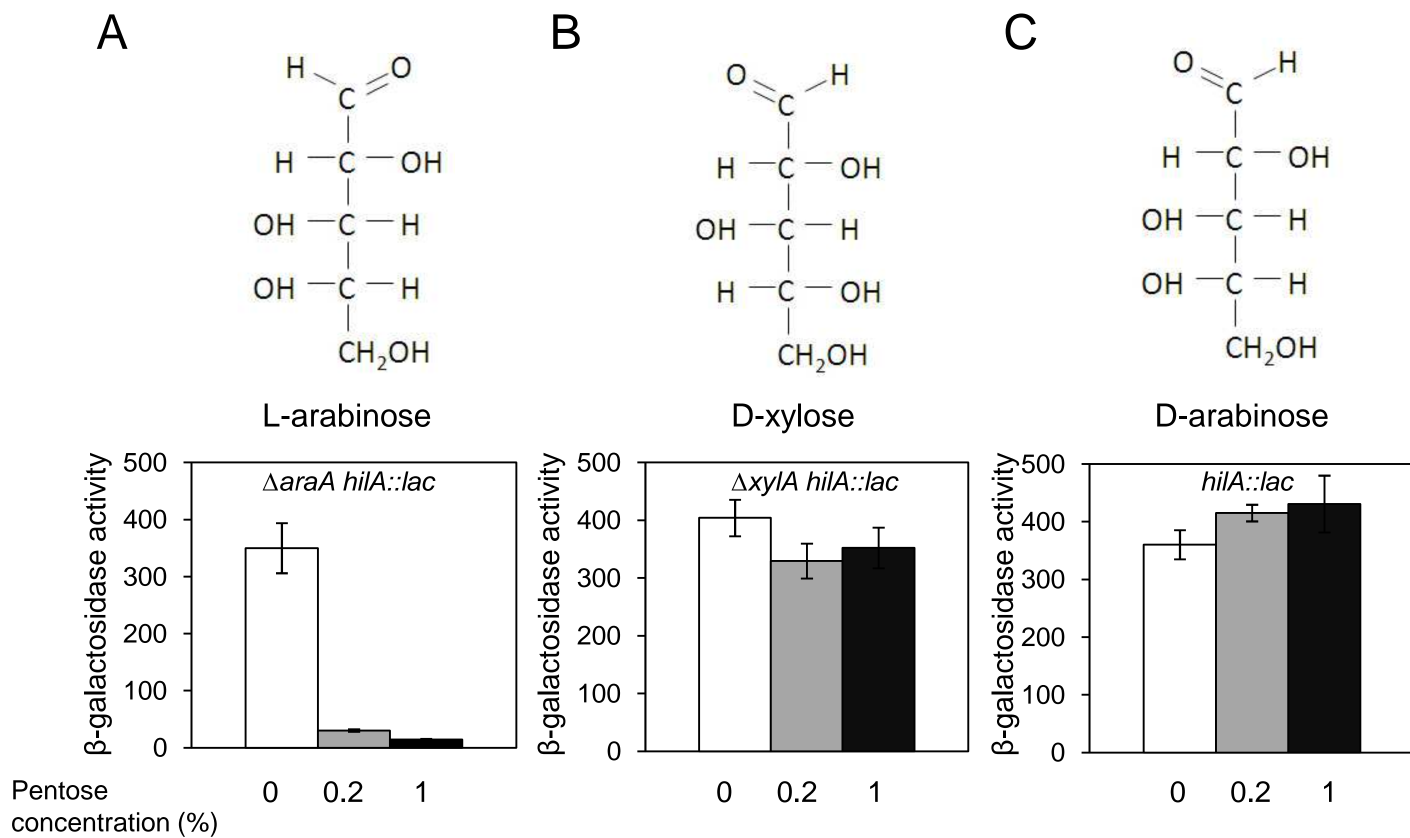

Fig 4.

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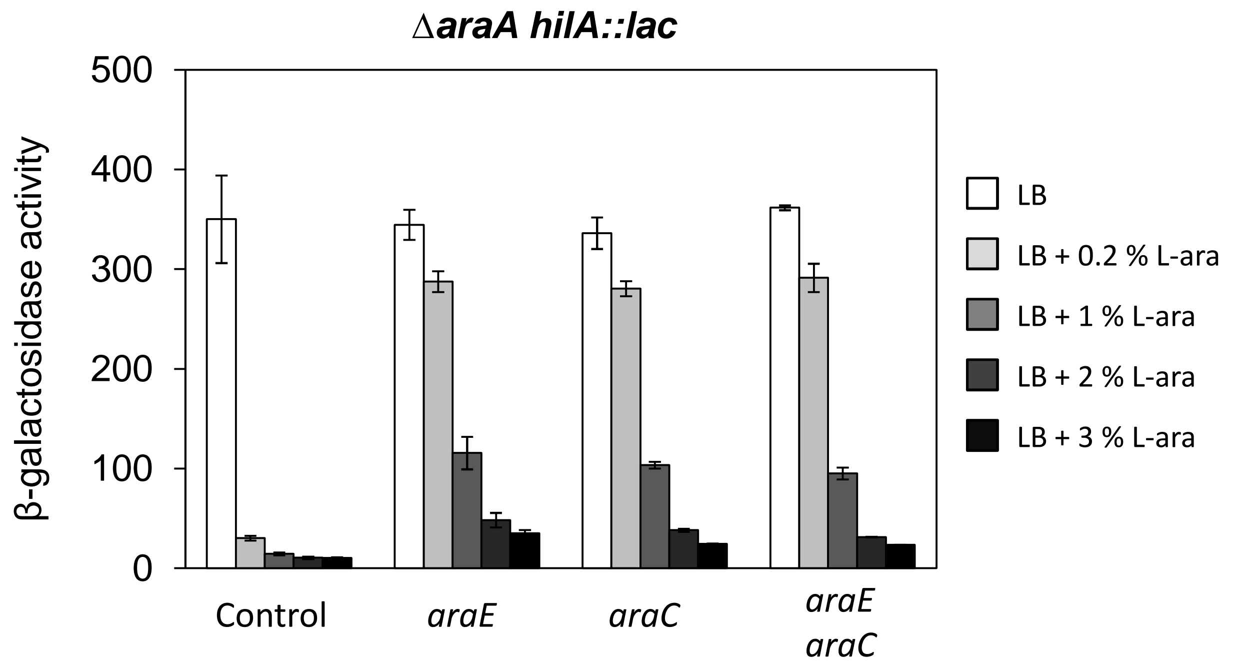

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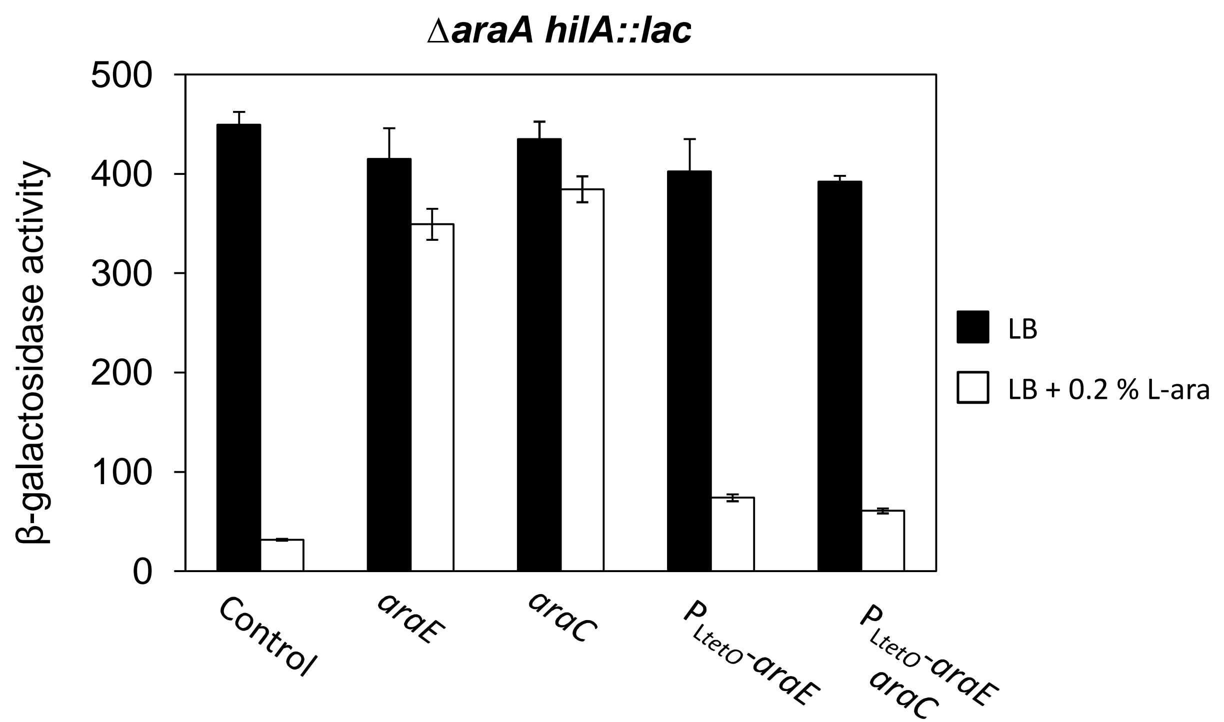

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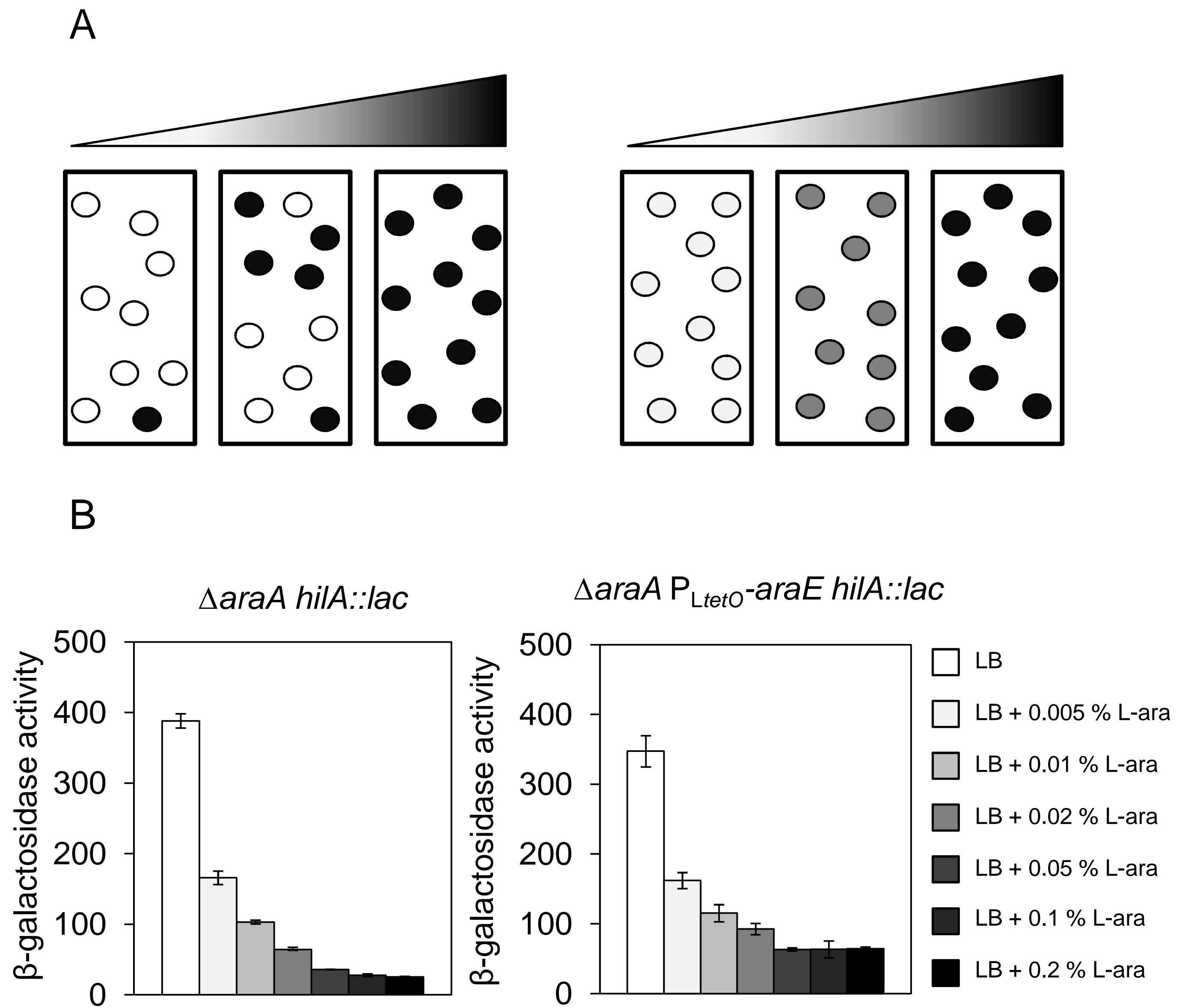


Fig. 6

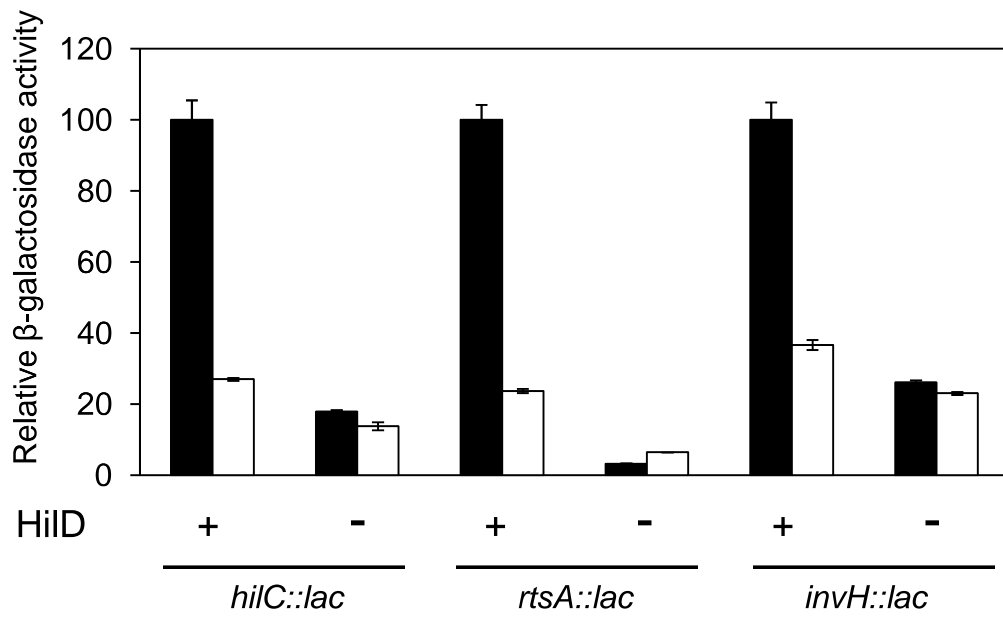
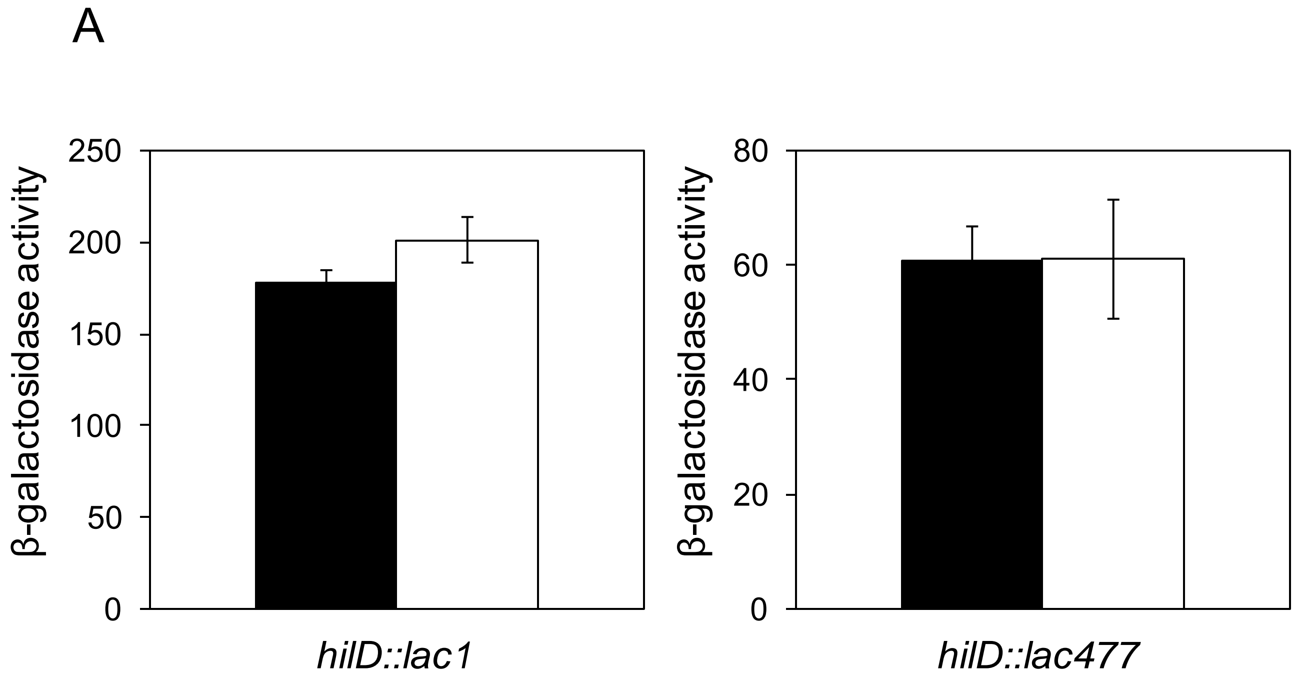


Fig. 7

A



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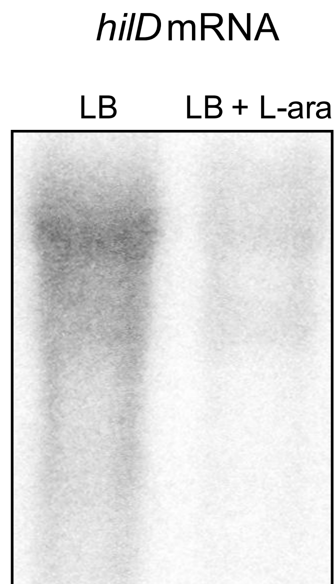
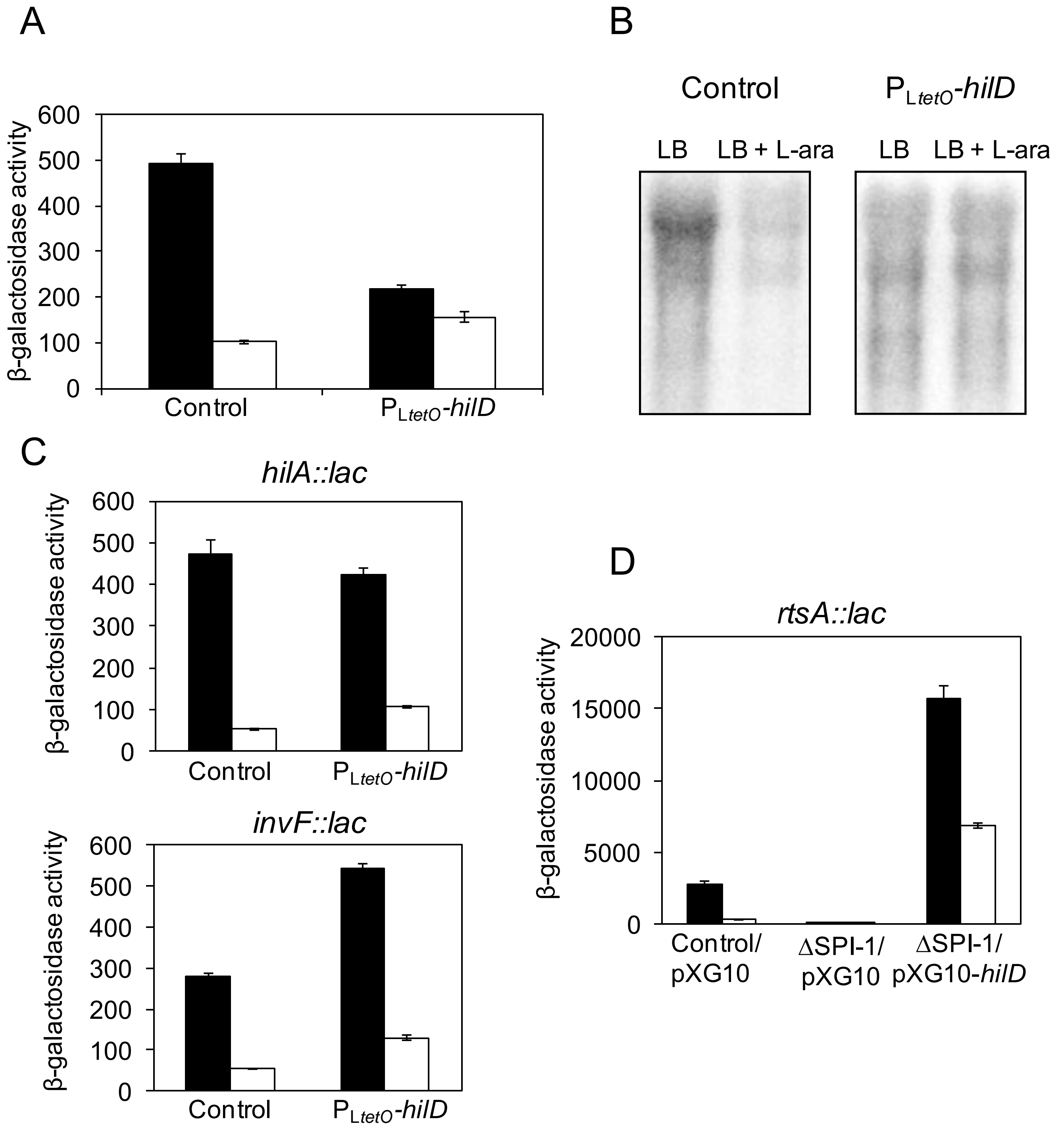


Fig. 8



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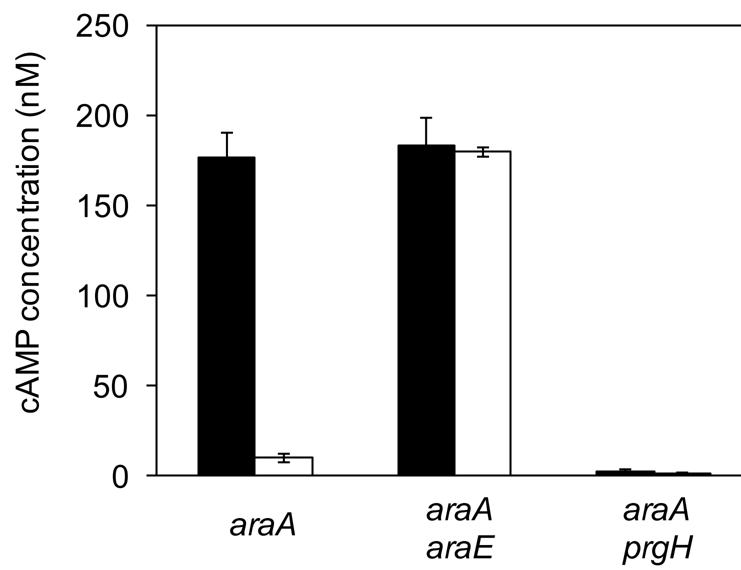


Table S1. Oligonucleotides used in this study (5'→3')

Oligonucleotide	Sequence
araAUP	ctgactcattaaggacacgacaatgacgatttttgataatattccggggatccgtcgacc
araADO	gcaatccgtttcaccaattaacgtttgaacccgtaatacagtgtaggctggagctgcttc
araA-E1	tgcgacgtactgaatcgtcc
araA-E2	accaccaccatatcgtcagc
xylAUP	cgtttacttgccgtcttatctgattatggagctcactatgattccggggatccgtcgacc
xylADO	ggccgggctaacgcggagtcgcccggtagatagggttattgtgtaggctggagctgcttc
xylA-E1	aattcatcacagcaaacgg
xylA-E2	caggatagcttttacacccg
PLtetO-araE-UP	tgggtttaacttaatccatatattgttaaataatagctataggcttacccgtcttactgtc
PLtetO-araE-DO	ttaatagagaccatattttcctgccacaacagagtaagacgtgctcagtatctctatcactgatag
araEUP	tttcaggctatgtcttactctgttgtggcaggaaaatatgattccggggatccgtcgacc
araEDO	cggataacaggcgtcatccggcatgggagggggattacagtgtaggctggagctgcttc
araE-E1	tataccatagcggtagatggc
araE-E2	agtcgattcccagctcatc
araCUP	tttgtttcttctctgaacatcggggggtagagaaatcatgattccggggatccgtcgacc
araCDO	gcttatgacatctttgtggacacatcattcactttttattgtgtaggctggagctgcttc
araC-E1	tcaatgtggacattccagc
araC-E2	gataaagtgttccagcagtgc
hilDUP1	agagcatttacaactcagatttttcagtaggataccagtcatatgaatatcctccttag
hilDUP930	aactacgccatcgacattcataaaaatggcgaaccattaacatatgaatatcctccttag
hilD-lacZUP	tgaacatctgaaaacggcgttctcctgtacgaaggatacacccgtcgttttacaacgtcg
hilD-lacZDO	gcaaatagttctcagagggaacggatgatgtataaatatgcgtgtaggctggagctgcttc
hilDDO2	gcaaatagttctcagagggaacggatgatgtataaatatggtgtaggctggagctgcttc
hilD-E1	agaccattgccaacacacgc
hilD-E2’	atcatcctcaggctggctcc
PLtetO-hilD-UP	ttgggttcttttggtgtaacaatcagaccattgccaacacaggcttacccgtcttactgtc
PLtetO-hilD-DO	tccatattatccctttgttgatgttattttaatgttccttgtgctcagtatctctatcactgatag

pXG10-hilD- UP	gtttttatgcataaggaacattaaaataacatcaac
pXG10-hilD- DO	gtttttgctagcggcaaatagttctcagaggg
pXG10-FOR	ttggaacctcttacgtgcc
pXG10-REV	gcatcaccttcaccctctc
hilDriboprobeUP	atggaaaatgtaacctttgtaag
hilDriboprobeDO	gtttttttaatacgactcactatagggaggtatatcgaaatccatgtggc
rnpBriboprobeUP	
rnpBriboprobeDO	

Fig S1

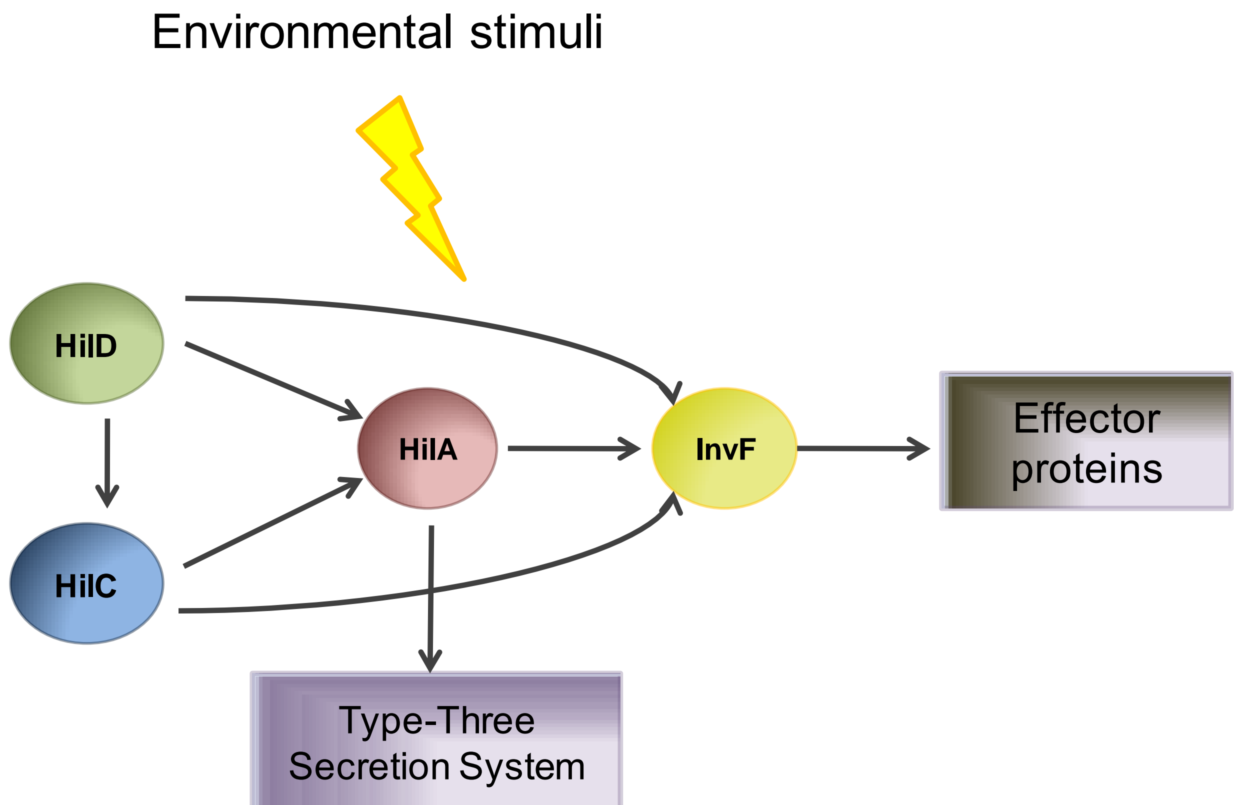


Fig S2

Glycolysis

Sugar (glucose) - - - - -→ Pyruvic acid → Acidification

L-arabinose catabolism

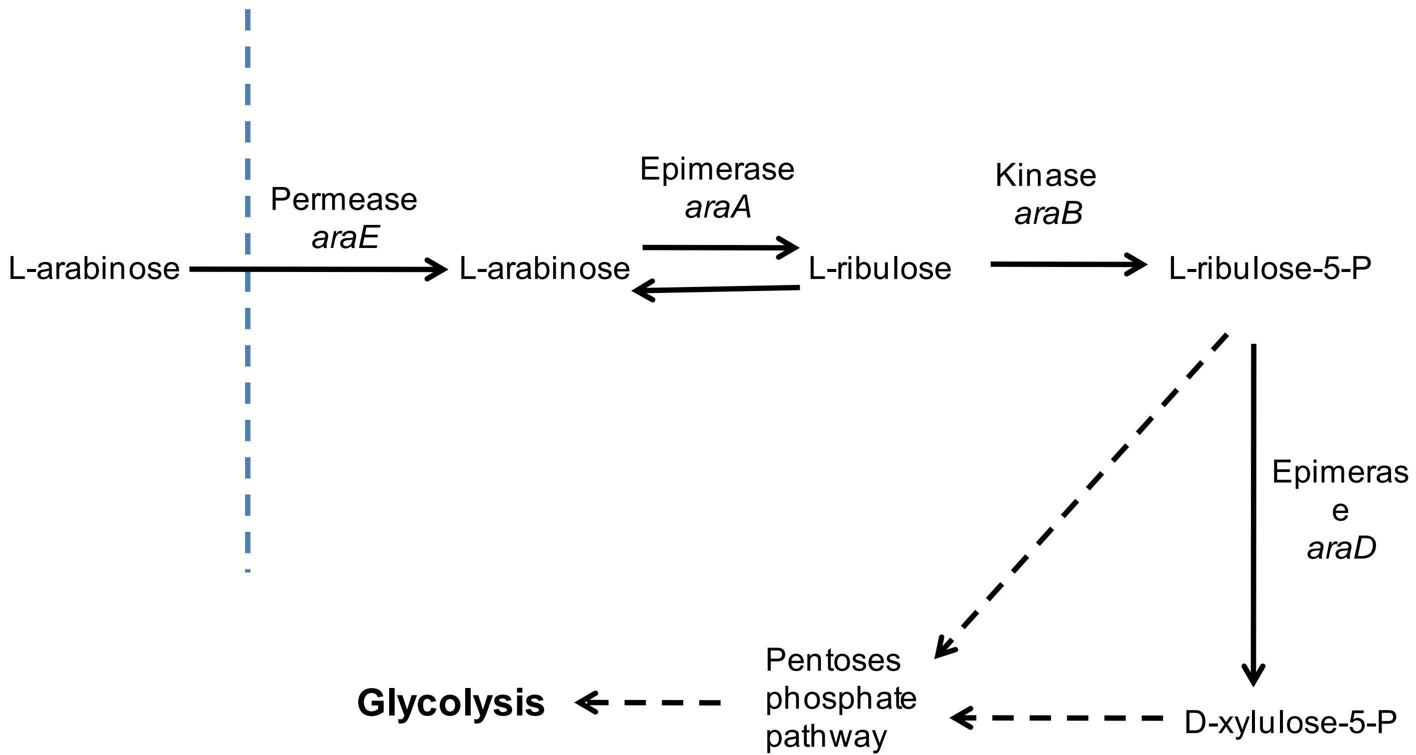
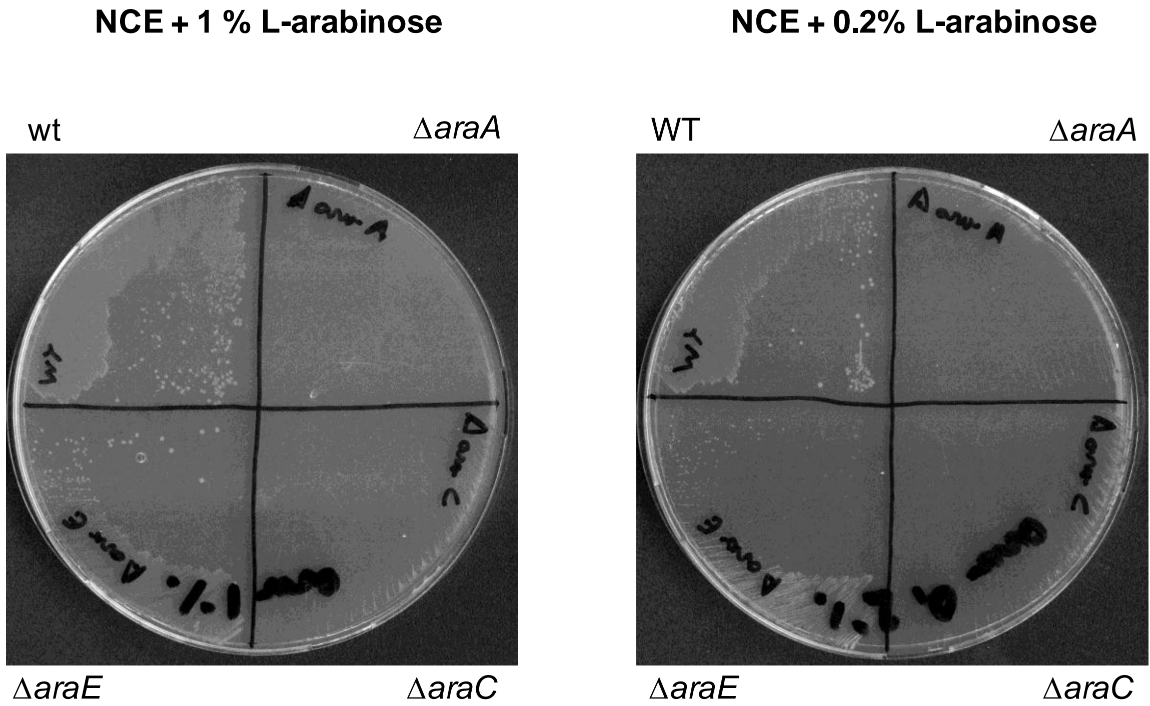


Figure S3

Phenotypes associated with *araA, araC* and *araE* mutations

A



B

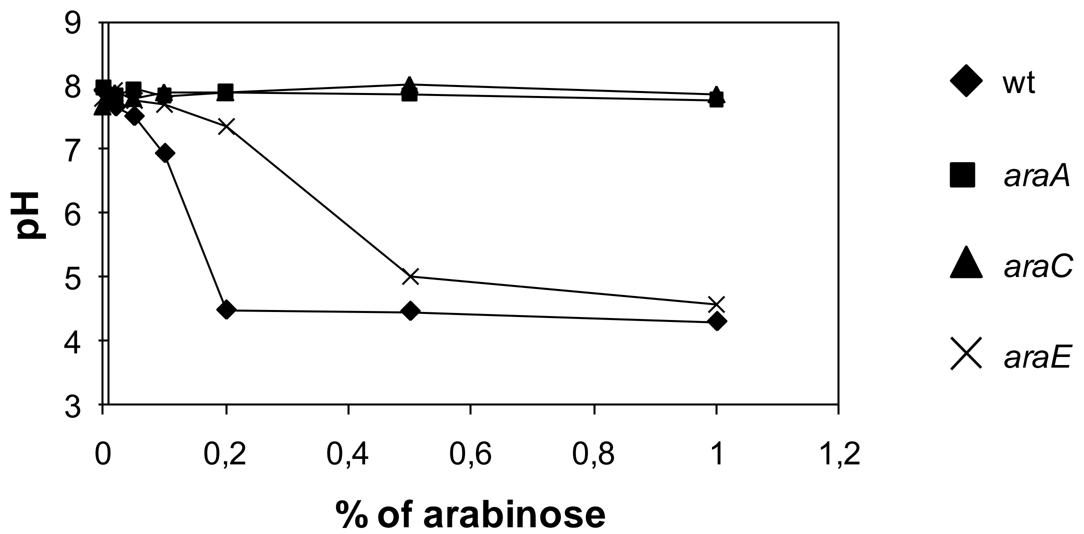


Fig S4

NCE + 1 % D-xylose

WT ΔxylA

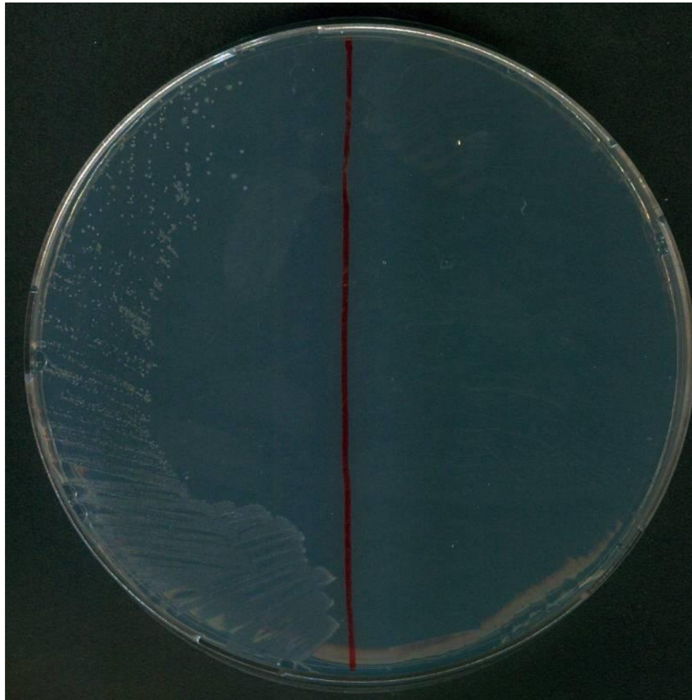


Figure S5

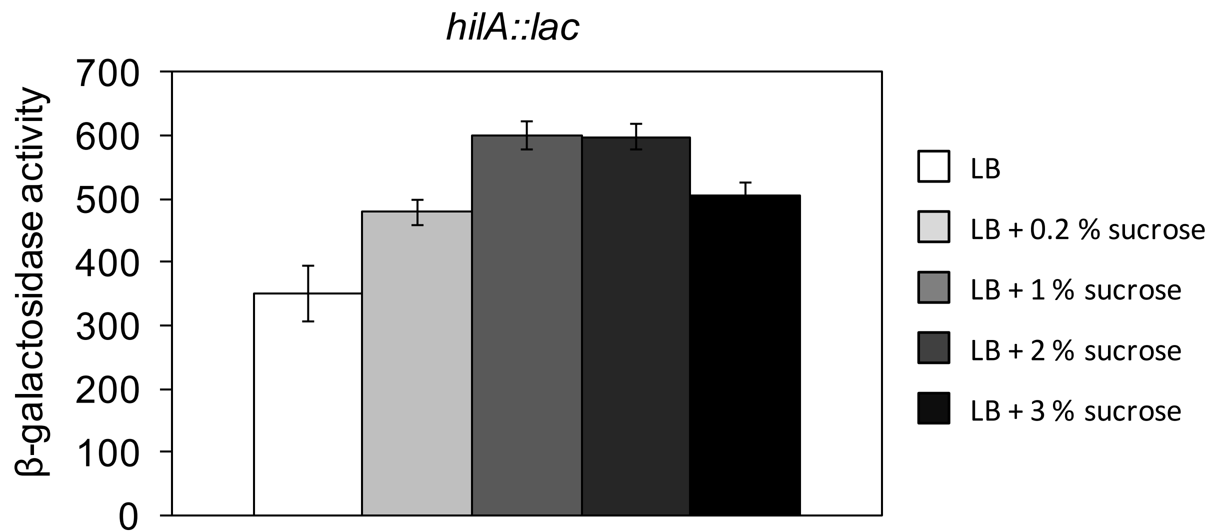
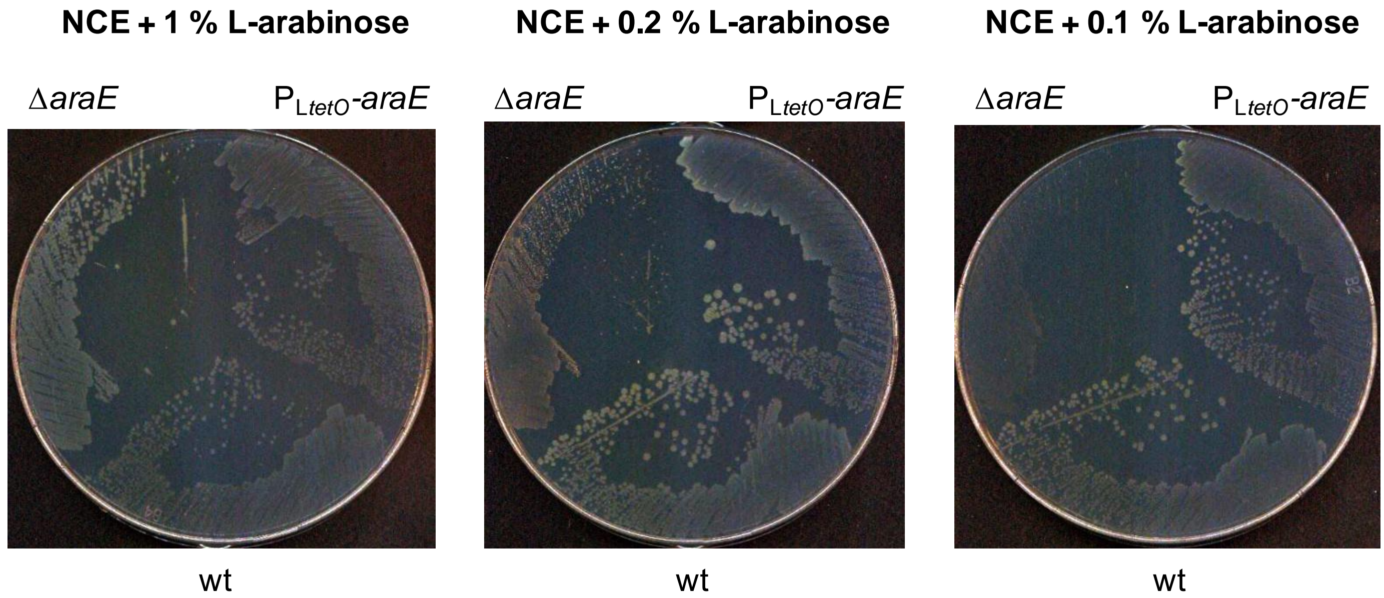


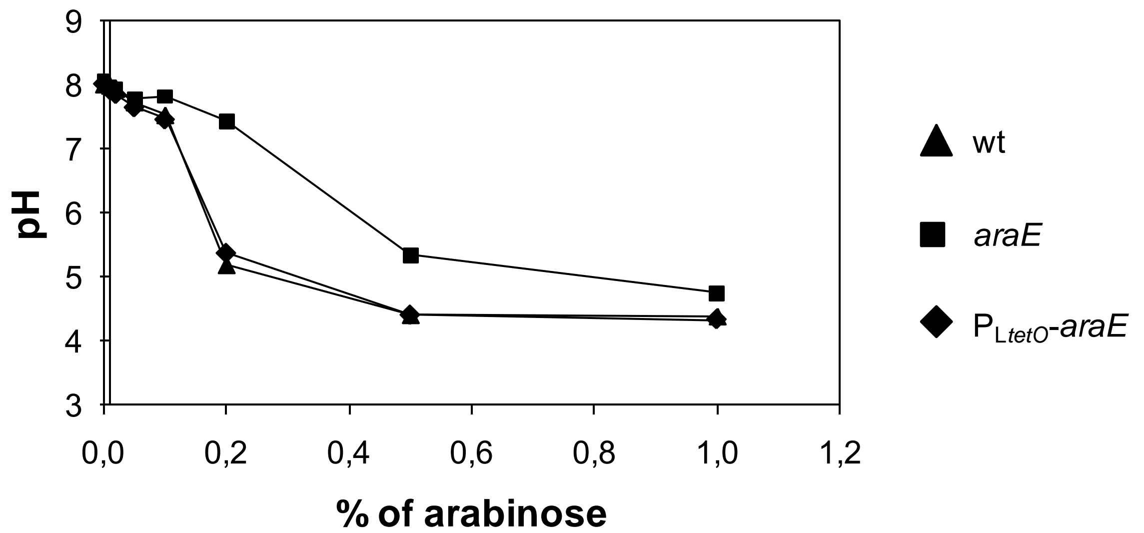
Figure S6

Phenotypes associated with P_{LtetO}-*araE* mutation

A



B



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 (BALBONTIN *et al.* 2006; BLYN *et al.* 1989; CAMACHO and CASADESUS 2002; HAAGMANS and VAN DER WOUDE 2000; JAKOMIN *et al.* 2008; KÜCHERER *et al.* 1986; ROBERTS *et al.* 1985; TORREBLANCA and CASADESUS 1996; WALDRON *et al.* 2002). 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 an 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 *hilD* mRNA turnover.

The hypothesis, at first sight odd, that Dam methylation is a postranscriptional 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 postranscriptional 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 (ELLERMEIER and SLAUCH 2008; KAGE *et al.* 2008; LUCAS and LEE 2001). However, with the potential exception of FliZ (KAGE *et al.* 2008) and CsrA (ALTIER *et al.* 2000; ELLERMEIER and SLAUCH 2007), postranscriptional regulators of *hilD* seem to affect either the HilD protein level (MATSUI *et al.* 2008; TAKAYA *et al.* 2005) 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 postranscriptional 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 *Yersinia 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 postranscriptional (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.

We have characterized three new ORFs in the *std* gene cluster: STM3026, STM3025.1N, and STM3025, renamed *stdD*, *stdE*, and *stdF* respectively. Western blot analyses have demonstrated that those genes are expressed in *dam* mutants (**Figure 2B**). *stdD* encodes a predicted outer membrane protein, while StdE and StdF are predicted cytoplasmic proteins. Our results indicate that StdE and StdF repress SPI-1 and flagellar gene expression, suggesting the existence of a regulatory cross-talk that might coordinate Std fimbriae production, invasion and motility.

We have provided evidence that *stdA*, *stdB*, *stdC*, *stdD*, *stdE*, and *stdF* constitute a polycistronic operon: (i) expression of all those genes is activated in Dam⁻ background (**Figure 2**); and (ii) retrotranscription and PCR show that they are cotranscribed (**Figure 3**). *std* transcription is driven by a promoter located upstream *stdA* (Jakomin et al., 2008). Transcription from P_{stdA} is activated by direct binding of HdfR protein to a regulatory region upstream the promoter. However, methylation of two GATC sites in the regulatory region prevents binding of HdfR, thus repressing *std* expression (Jakomin et al., 2008; Jakomin et al., in preparation). It is likely that all *std* genes are coordinately regulated by Dam methylation due to a common transcription from P_{stdA}. However, internal promoters may also exist.

Salmonella enterica dam mutants 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 (Marinus and Casadesus, 2009). We previously reported that SPI-1 expression was repressed in Dam⁻ background (Balbontin et al., 2006; Lopez-Garrido et al., 2010). Dam methylation activates SPI-1 by controlling *hilD* expression at postranscriptional level (Lopez-Garrido and Casadesus, 2010), what together with the absence of GATC sites in *hilD* regulatory regions, suggests that the regulation is indirect. Our genetic screens and subsequent experiments have identified *std* fimbrial operon as the link between Dam methylation and SPI-1: (i) a multicopy plasmid containing the whole *std* operon represses *hilD* expression; (ii) *std* genes are upregulated in Dam⁻ background (**Figure 2**; Balbontin et al., 2006; Jakomin et al.,

2008); and (iii) SPI-1 regulation by Dam methylation is completely suppressed in a strain lacking the whole *std* operon (**Figure 4A**). Altogether, those results suggest that overexpression of *std* in *dam* mutants leads to SPI-1 repression. It has been previously shown that the extreme attenuation of *Salmonella enterica* serovar Typhimurium *dam* mutants upon oral infection (Garcia-del Portillo et al., 1999; Heithoff et al., 1999) is partially suppressed by deletion of *std*, suggesting that overexpression of Std fimbriae is detrimental for *Salmonella* virulence (Jakomin et al., 2008). The regulatory link between *std* and SPI-1 provide evidence that the detrimental effect of *std* overexpression for *Salmonella* virulence may be due to SPI-1 repression.

Epistasis analysis indicates that Dam-dependent control of SPI-1 requires the last two genes of *std* operon, StdE and StdF. That is further supported by the following observations: (i) constitutive expression of *stdE* and *stdF* in Dam^+ background represses SPI-1 expression (**Figure 5**); (ii) StdE and StdF are overproduced in Dam^- background (**Figure 2**); (iii) Dam methylation, StdE, and StdF regulate SPI-1 expression through HilD; and (iv) as happens in the case of Dam methylation, StdE and StdF does not regulate *hilD* transcription, but controls the level of *hilD* mRNA (**Figure 6**). A conceivable model to explain regulation by Dam methylation of SPI-1 is depicted in **Figure 8**: in Dam^+ background, GATC sites of P_{stdA} regulatory region are methylated, preventing binding of HdfR and activation of *std* transcription. In the absence of Dam methylation, HdfR activates transcription from P_{stdA} and all the proteins encoded in the operon are overproduced. Then, StdE and StdF repress *hilD* expression at posttranscriptional level, and as a consequence, the whole SPI-1 is downregulated.

When constitutively expressed, StdE and StdF can regulate *hilD* expression independently, since each one can do that in the absence of the other (**Figure 5**). However, deletion of any of them suppresses SPI-1 repression in *dam* mutants, suggesting that both are necessary for repression. It may be possible that both trigger the same regulatory pathway, but they can do that independently when overproduced. StdE shares around 40 % and 50 % identity with the transcriptional activators GrlA and CaiF from *E. coli* and *Enterobacter cloacae* respectively. Interestingly, StdF is similar to an uncharacterized protein encoded just downstream CaiF in *Enterobacter cloacae* chromosome, that is part of a hypothetical fimbrial gene cluster which genetic organization resembles that of *std* operon, suggesting that they may have a common origin. StdF is also 27 % similar to the SPI-1 encoded protein SprB from *Salmonella*. SprB is a transcriptional regulator able to bind to *hilD* and *siiA* promoters and repress

and activate their expression respectively (Saini and Rao, 2010). Even though StdE and StdF are similar to known transcriptional regulators, they do not regulate *hilD* at transcriptional level, but at postranscriptional level. Thus, either they have acquired the ability to control gene expression at postranscriptional level in *Salmonella enterica*, or they regulate transcription of a postranscriptional regulator of *hilD*.

In addition to repress SPI-1 expression, StdE and StdF can also inhibit motility. Expression of an *flgK::lac* fusion is repressed by constitutive expression of *stdE* and *stdF*, suggesting that motility inhibition is the consequence of flagellar gene repression. Overexpression of either *stdE* or *stdF* produces a mild motility inhibition (**Figure 7**). However, simultaneous expression of both genes has a synergistic effect and completely inhibiting motility and *flhK* expression (**Figure 7**). Thus, it may be possible that StdE and StdF regulate flagellar gene expression through the same pathway.

Several studies have reported coordinated expression of fimbrial, flagellar and invasion genes: in *Proteus mirabilis*, the protein encoded by the last gene in the *mrp* fimbrial operon, MrpJ, inhibits motility when the fimbrial operon is expressed (Lin et al., 2001). Similarly, PapX, the product of the last gene in the *pap* fimbrial operon of uropathogenic *Escherichia coli*, represses the expression of the flagellar mater regulator FlhDC by direct binding to its promoter region (Simms and Mobley, 2008). One case particularly interesting is the coordinated expression of type I fimbriae, flagellum and invasion genes mediated by FimY and FimZ. Those two proteins are encoded in independent transcriptional units next to the type I fimbrial operon *fim*. FimY and FimZ are essential for *fim* operon transcription (Yeh at al., 1995; Tynker and Clegg, 2000). In addition, FimZ represses SPI-1expression by activating transcription of the gene encoding the SPI-1 negative regulator HilE (Baxter and jones, 2005; Saini et al., 2009), and inhibits motility by repressing *flhDC* expression (Clegg and Hughes, 2002). That situation resembles that of StdE and StdF, suggesting that coordinated expression of fimbrial, flagellar and invasion genes is important for *Salmonella* virulence and persistence in the intestine (Saini et al., 2010)

A tempting speculation derived from the above results is that invasion and motility would be inhibited when *std* operon was expressed. *std* is not expressed under laboratory growth conditions in wild type *Salmonella* (Humphries et al., 2003; Humphries et al., 2005; Jakomin et al., 2008). However, several lines of evidence suggest that Std fimbriae is produced in the animal intestine: (i) mice infected with serovar Typhimutium seroconvert to StdA, the major fimbrial component of Std

fimbriae (Humphries et al., 2005); and (ii) *std* deletion reduces the ability of *Salmonella* to colonize and persist in the cecum of infected mice, while producing no defect in colonizing the small intestine. (Weening et al., 2005). According to that, it has been reported that Std fimbriae bind α(1,2)fucose residues, which are abundant in the cecal mucosa (Chessa et al., 2008). *Salmonella* invasion takes place preferentially in the ileum, while in the cecum invasion is inhibited. *std* expression in the cecum might contribute to inhibition of invasion. In addition, fimbriated bacteria would inhibit motility and live attached to cecal mucosa, what could help to the persistence of *Salmonella* in the host intestine.

The genome of *Salmonella* has evolved by the acquisition of genetic modules that provided new abilities to interact with eukaryotic cells and exploit different niches (Ochman and Groisman, 1997; Prowllik and McLelland, 2003). A critical point of that modular evolution is to get a coordinate expression of the different genetic modules. In some cases, the modules themselves carry regulatory genes of its own expression, which serve as connection with the core genome (Ochman and Groisman, 1997). In addition, there are some examples of cross-talk between genetic modules independently acquired: the SPI-1 encoded regulator HilD can activate SPI-2 expression during late stationary growth phase (Bustamante et al., 2008); expression of SPI-4 genes is activated by the SPI-1-encoded SprB transcriptional regulator (Saini and Rao, 2010); HilE, a SPI-1 negative regulator, is encoded in a region of *Salmonella* chromosome that has been proposed to be a pathogenicity island (Baxter et al., 2003); SPI-1 and SPI-2-encoded transcriptional regulators control the expression of effector proteins located outside those islands (Darwin and Miller, 2001; Knodler et al., 2002), and some are located in horizontally-acquired DNA fragments (Hardt et al., 1998; Wood et al., 1998). *std* genes are well conserved amongst *Salmonella* serovars, but are absent in closely related species (Prowllik and McCLelland, 2003), suggesting that the cluster has been acquired by horizontal gene transfer. Thus, the connection between *std* and SPI-1 provides an additional example of cross talk between horyzontally-aquired genes.

Postranscriptional control of *hilD* expression is essential for SPI-1 regulation by different regulatory systems (Ellermeier and Slauch, 2007). However, despite its importance in SPI-1 regulation, the mechanisms of postranscriptional control of *hilD* are poorly understood. Our results indicate that *hilD* 3'UTR may mediate *hilD* regulation at postranscriptional level: deletion of *hilD* 3'UTR increases *hilD* mRNA

levels what correlates with SPI-1 overexpression, suggesting that targeting *hilD* 3'UTR might be an efficient way to control SPI-1 expression. In such backgrounds, we provide evidence that *hilD* 3'UTR may be a target for *hilD* mRNA degradation and regulation by the RNA chaperone Hfq.

Higher levels of *hilD* mRNA are detected upon deletion of its 3'UTR even when transcription is driven from a heterologous promoter, suggesting that *hilD* 3'UTR does not affect *hilD* mRNA synthesis. Furthermore, inactivation of RNA degradosome components RNase E and Pnp suppresses the differences in *hilD* mRNA levels with and without 3'UTR. Altogether, that suggests that *hilD* 3'UTR may be a target for *hilD* mRNA degradation by the RNA degradosome. According to that, it has been reported that *Salmonella* mutants lacking a functional RNase E undergo increased SPI-1 expression (Fahlen et al., 2000). It may be possible that it was due to 3'UTR-directed *hilD* mRNA degradation.

SPI-1 expression is repressed in *Salmonella* mutants lacking the RNA binding protein Hfq (Sittka et al., 2007). Epistasis analysis have shown that Hfq-dependent regulation of SPI-1 is transmitted through HilD and we have evidences that Hfq regulates *hilD* expression at postranscriptional level: (i) Lowered levels of *hilD* mRNA are detected in *hfq* mutants; (ii) however expression of a *hilD::lac* transcriptional fusion is not reduced in Hfq$^-$ background; and (iii) *hilD* mRNA levels are reduced in *hfq* mutants even when *hilD* is transcribed from a heterologous promoter. Deletion of *hilD* 3'UTR suppresses regulation of *hilD* and SPI-1 by Hfq. According to that, RNA fragments corresponding to *hilD* 3'UTR have been recovered upon Hfq CoIP, suggesting that Hfq directly binds to that region (Sittka et al., 2008). Hence, it is tempting to speculate that Hfq needs to interact with *hilD* 3'UTR in order to regulate *hilD* expression. Those results open the possibility that *hilD* 3'UTR serves to integrate regulatory signals at postranscriptional level. Future studies might reveal new regulators that target *hilD* 3'UTR to control SPI-1 expression.

It is well known that eukaryotic mRNAs sometimes have long 3'UTRs with regulatory properties (Grzybowska et al., 2001): mRNA stability can be modulated by controlling polyadenylation status of 3' end (Beelman and Parker, 1995). In addition, binding of certain proteins to specific sequences located in 3'UTRs of mRNAs can modulate mRNA stability, translation and localization (Barreau et al., 2005; Sonenberg and Hinnebusch, 2009; St Johnston, 1995; Wilkie et al., 2003). In prokaryotes it has been traditionally thought that 3'UTR harbor mainly a transcriptional terminator that

contributes to RNA stabilization, preventing degradation by exonucleases (Ref). However, recent advances in transcriptomic analysis have possibilities the identification of long 3'UTRs in some bacterial transcripts (Toledo-Arana et al., 2009; Rasmussen et al., 2009; Broeke-Smits et al., 2010), raising the possibility that they have regulatory roles (Gripenland et al., 2010). For example, in *Bacillus subtilits* there are 9 different mRNAs that harbors a conserved 3'UTR of around 220 nt (Rasmussen et al., 2009), and it has been speculated that it might have a functional relevance (Rasmussen et al., 2009). Furthermore, 3'UTR-derived sRNAs have been observed in *Escherichia coli* (Kawano et al., 2005), suggesting that they might regulate gene expression in trans. The results reported in this study provide an example of an eukaryotic-like 3'UTR in a bacterial mRNA. Apart from being a target for mRNA degradation, *hilD* 3'UTR may possibilite regulation of *hilD* expression by direct binding of Hfq. Thus, the presence of regulatory 3'UTRs in bacterial RNAs may be more frequent than previously thought. Future studies might uncover new regulatory functions associated with prokaryotic 3'UTRs.

SPI-1 is regulated by different environmental factors. Here, we have reported that L-arabinose, even at low concentrations, represses SPI-1 expression, and that effect is independent of L-arabinose catabolism. Furthermore, other pentoses such as D-xylose and D-arabinose fail to repress SPI-1. That opens the interesting possibility that L-arabinose is a specific signal for SPI-1 repression.

Regulation of virulence genes by sugars has been reported in different bacteria. In the Gram-positive bacterium *Listeria monocytogenes,* the expression of virulence genes regulated by the master regulator PrfA is repressed in the presence of sugars transported through the phosphoenolpyruvate-sugar phosphotransferase system (PTS) (Park and Kroll, 1993; Milenbachs et al., 1997; De las Heras et al. 2011). However, such repression is not observed in the presence of non-PTS sugars (Ripio et al, 1997; Stoll et al, 2008; Joseph et al, 2008; de las Heras et al., 2009). In *Streptococcus pyogenes*, production of surface M protein, a major virulence determinant, is affected by the sugar source (Pine and Reeves, 1978). Transcription the gene encoding the surface M protein is indirectly activated is by carbon catabolic repression (CCR) through the virulence gene regulator Mga. CCR also controls virulence gene expression in *Clostridium prefringes* (Varga et al, 2004) and *Staphylococcus aureus* (Morse at al., 1969). In *Salmonella enterica* there are evidences that PTS-dependent sugars repress invasion

gene expression: *crp cya* mutants of *Salmonella enterica* serovar Choleraesuis are attenuated in pigs (*Kennedy* et al., 1999), and that correlates with the inability of *crp* mutants to secrete SPI-1 TTSS effectors (Zeng-Weng et al., 2010). In addition, it has been reported that Mlc, a global regulator of carbon metabolism, activates SPI-1 expression by directly repressing the transcription of SPI-1 negative regulator HilE (Lim et al., 2007). Mlc regulon can be induced by the PTS-sugars glucose and mannose (Plumbridge, 2002). According to that, it has been shown that *hilD* expression is slightly reduced in the presence of glucose and mannose (Lim et al., 2007). However, L-arabinose is a non-PTS sugar and its transport inside the cell does not induce Mlc regulon or reduces the level of cAMP. Furthermore, regulation of HilD by L-arabinose is independent of HilE (not shown). Hence, L-arabinose must regulate SPI-1 by a different mechanism.

The observation that L-arabinose can regulate gene expression is not new. It is well known that genes necessary for L-arabinose catabolism are activated the presence of L-arabinose, and such activation depends on the transcriptional regulator AraC (reviewed in Schleif, 2010). However, the traditional model of L-arabinose-dependent gene expression does not fit in the case of SPI-1 repression. We have shown that L-arabinose needs the AraE permease in order to efficiently repress SPI-1. As expression of *araE* depends on AraC, AraC is indirectly required for the transport of L-arabinose. However, once L-arabinose is inside the cell, AraC is no longer necessary for SPI-1 repression. That provides evidence of the existence of a new way to control gene expression by L-arabinose in *Salmonella*.

The requirement of the AraE permease for SPI-1 repression by L-arabinose admits two interpretations: (i) L-arabinose has to be inside the cell to repress SPI-1; or (ii) the transport of L-arabinose through AraE necessary for SPI-1 repression. However, the last possibility seems unlikely, since it would involve the existence of a signal transduction system associated to AraE that, to our knowledge has not been described. The observation that *Salmonella araE* mutants can grow with 1 % of L-arabinose as sole carbon source provide evidence that L-arabinose enters through alternative pathways at high concentrations. L-arabinose can repress SPI-1 expression in *Salmonella araE* mutants when provided at concentrations of 1 % or higher, thereby confirming the hypothesis that intracellular L-arabinose, rather that the transport through AraE permease, is responsible for SPI-1 repression.

We have determined that L-arabinose regulates SPI-1 expression through HilD. According to that, we have evidences that L-arabinose regulates HilD at protein level, either controlling its stability or activity. HilD is an AraC-like transcriptional activator. It is tempting to propose that L-arabinose might regulate HilD activity by direct binding to the protein, but further experiments are required to study that hypothesis.

L-arabinose is a plant-derived sugar, and the presence of a specific system in *Salmonella* for its catabolism indicates that *Salmonella* finds L-arabinose during its life cycle and uses it as carbon source. Our results suggest that, apart from as a carbon source, *Salmonella* might use L-arabinose as a signal for SPI-1 repression under certain circumstances. We propose two different scenario in which the sensing of L-arabinose could repress SPI-1 expression: (i) the animal intestine; and (ii) outside the animal host.

(i) The observation that *Slamonella* grown in the presence of L-arabinose fail to translocate the SPI-1 effector *sipA* into fibroblats provide evidence that L-arabinose might inhibit invasion *in vivo*. L-arabinose is poorly absorbed during digestion in mammals (Cori, 1925) and chicken (Wagh andWaibel, 1967), and there are evidences that free L-arabinose is present in the intestine: P_{BAD} promoter expression is induced in the intestine of mice that receive food with plant components (Loessner et al., 2009). Furthermore, L-arabinose catabolism is requires for an efficient colonization of the large intestine be commensal and pathogenic strains of *E. coli* (Fabich et al., 2008). L-arabinose supports efficient growth of *Salmonella in vitro* and might be a preferred carbon source in the intestine. The presence of L-arabinose in the intestine could be detected by *Salmonella* as a signal for repression of invasion. If that were true, L-arabinose-rich compounds in the diet could prevent infections by *Salmonella*. Consistent with that idea, it has been observed that dietary addition of arabinoxylooligosaccharides, made of few molecules of L-arabinose and D-xylose, provides protection against oral infections by *Salmonella enterica* serovar Enteritidis in poultry (Eeckhaut et al., 2008). However, we have observed that repression of *hilA* by L-arabinose is smaller in when *Salmonella* grows on SPI-1inducing conditions than of SPI-1 standard conditions (notshown). SPI-1 inducing conditions are thought to mimic the conditions in the ileum. Therefore, it could be possible that L-arabinose contributed to keep low levels of SPI-1 in the large intestine and the first portion of the small intestine, allowing *Salmonella* to invade in the ileum.

(ii) SPI-1 repression by L-arabinose could also play a role outside the animal host. As a plant-derived sugar, L-arabinose accumulates in the soil. It has been shown that

Salmonella is able to persist in the soil for long periods (Islam et al., 2004). It may be possible that SPI-1 repression by L-arabinose in the soil may improve the fitness of *Salmonella* in that niche. One interesting feature of *Salmonella* is its ability to colonize plant surfaces (epiphytic colonization) (Barak et al., 2002; Brandl and Mandrell, 2002) and the spaces between cells inside the plants (endophytic colonization) (Franz et al., 2007). Plant colonization may be part of *Salmonella* life-cycle, and it could be used as a way for recolonizing animal hosts (Tyler and Triplett, 2008). *Salmonella* mutants lacking components of the SPI-1 TTSS perform a better plant colonization than wild type strains (Iniquez et al., 2005). It seems that the presence of a functional TTSS in the surface of *Salmonella* triggers a defense response by the plant (Iniquez et al., 2005). In such context, the detection of L-arabinose by *Salmonella* in plants might contribute to turn down SPI-1 expression for efficient plant colonization.

Hence, our report of SPI-1 repression by L-arabinose suggests new roles of the sugar on *Salmonella* physiology. A deeper study in the molecular mechanism could reveal new mechanism of regulation of gene expression by L-arabinose.

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