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RESEARCH ARTICLE

InvS Coordinates Expression of PrgH and FimZ and Is Required for Invasion of Epithelial Cells by *Salmonella enterica* serovar Typhimurium

Lu Wang,[a] Xia Cai,[a]* Shuyan Wu,[a]* Rajdeep Bomjan,[a] Ernesto S. Nakayasu,[b]* Kristian Händler,[c]* Jay C. D. Hinton,[d] Daoguo Zhou[a]

Department of Biological Sciences[a] and Bindley Bioscience Center,[b] Purdue University, West Lafayette, Indiana, USA; Department of Microbiology, School of Genetics & Microbiology, Moyne Institute of Preventive Medicine, Trinity College Dublin, Dublin, Ireland[c]; Institute of Integrative Biology, University of Liverpool, Liverpool, United Kingdom[d]

ABSTRACT Deep sequencing has revolutionized our understanding of the bacterial RNA world and has facilitated the identification of 280 small RNAs (sRNAs) in *Salmonella*. Despite the suspicions that sRNAs may play important roles in *Salmonella* pathogenesis, the functions of most sRNAs remain unknown. To advance our understanding of RNA biology in *Salmonella* virulence, we searched for sRNAs required for bacterial invasion into nonphagocytic cells. After screening 75 sRNAs, we discovered that the ablation of InvS caused a significant decrease of *Salmonella* invasion into epithelial cells. A proteomic analysis showed that InvS modulated the levels of several type III secreted *Salmonella* proteins. The level of PrgH, a type III secretion apparatus protein, was significantly lower in the absence of InvS, consistent with the known roles of PrgH in effector secretion and bacterial invasion. We discovered that InvS modulates *fimZ* expression and hence flagellar gene expression and motility. We propose that InvS coordinates the increase of PrgH and decrease in FimZ that promote efficient *Salmonella* invasion into nonphagocytic cells.

IMPORTANCE Salmonellosis continues to be the most common foodborne infection reported by the CDC in the United States. Central to *Salmonella* pathogenesis is the ability to invade nonphagocytic cells and to replicate inside host cells. Invasion genes are known to be regulated by protein transcriptional networks, but little is known about the role played by small RNAs (sRNAs) in this process. We have identified a novel sRNA, InvS, that is involved in *Salmonella* invasion. Our result will likely provide an opportunity to better understand the fundamental question of how *Salmonella* regulates invasion gene expression and may inform strategies for therapeutic intervention.

KEYWORDS *Salmonella*, gene regulation, host cell invasion, noncoding RNA

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) remains a leading cause of foodborne illness and poses a major public health problem worldwide. *Salmonella* harbors several pathogenicity islands (SPIs) scattered throughout the chromosome, which comprise functionally distinct virulence genes. Virulent *Salmonella* strains possess pathogenicity island 1 (SPI-1) and pathogenicity island 2 (SPI-2), encoding two separate type III secretion systems (TTSSs). These TTSSs function to deliver bacterial effectors into the host cell to reprogram host cell functions to promote invasion and intracellular survival, respectively (1). *Salmonella* TTSSs are composed of more than 20 proteins, including a highly conserved group of integral membrane proteins, a family of cytoplasmic chaperones, and several accessory proteins. The core unit of *Salmonella*

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Address correspondence to Daoguo Zhou, zhoud@purdue.edu.

* Present address: Xia Cai, Shanghai Medical College, Fudan University, Shanghai, China; Shuyan Wu, Department of Microbiology, Medical College of Soochow University, Suzhou, Jiangsu, China; Ernesto S. Nakayasu, Biological Sciences Division, Pacific Northwest National Laboratory, Richland, Washington, USA; Kristian Händler, Life and Medical Sciences Institute, Rheinische-Friedrich-Wilhelms University Bonn, Bonn, Germany.

SPI-1 TTSS is the needle complex. The multiring base of the complex is anchored to the bacterial envelope, which is composed of proteins, including InvG, PrgH, and PrgK (2). The filamentous needle is composed of PrgI, which is linked to the base by another substructure, the inner rod. It is known that the deletion of PrgH or PrgK impairs the SPI-1 TTSS assembly and hence effector secretion (3, 4). SPI-1 effectors include Salmonella invasion protein A (SipA), SipB, SipC, Salmonella outer protein B (SopB), SopD, SopE, and SopE2. These SPI-1 effectors work in concert to rearrange the host actin cytoskeleton to facilitate *Salmonella* invasion (5). By contrast, SPI-2 effectors are responsible for *Salmonella* replication inside phagocytic cells to promote bacterial survival and systemic infection.

The process of *Salmonella* infection of mammals involves the transition of the bacteria through multiple environmental conditions, from the acidity of the stomach to the low-oxygen environment of the gastrointestinal tract. The pathogen relies on an intricate transcriptional network to stimulate invasion when the pathogen interacts with the nonphagocytic cells associated with the gut wall. The SPI-1 invasion genes are tightly regulated by several SPI-1-encoded classic transcription factors. HilC and HilD are two AraC-like transcriptional regulators which activate HilA expression. In turn, HilA, an OmpR/ToxR family member, directly activates the transcription of several SPI-1 operons involved in effector secretion and bacterial invasion. These operons encode the type III secretory apparatus, secreted effectors, and transcriptional regulators such as InvF, an AraC-like transcriptional regulator (6). InvF activates the expression of SPI-1 *Salmonella* effector genes from a second HilA-independent promoter (7).

Bacterial sRNAs are small (50 to 250 nucleotides) noncoding RNA molecules. sRNAs usually regulate gene expression through base pairing with a corresponding mRNA target(s) and thereby repress or activate the target genes at the posttranscriptional level. Many *trans*-acting sRNAs require the Hfq RNA chaperone to form stable base pairing with target mRNAs.

Although rapid progress has been made in the identification of novel *Salmonella* sRNA transcripts (8–11), the majority of the identified sRNAs are of unknown biological function, and very few sRNAs have been shown to play a role in the regulation of *Salmonella* virulence (12, 13). IsrM is a pathogenicity island-encoded sRNA that is important for bacterial invasion and intracellular replication inside macrophages (14). We hypothesized that a newly discovered sRNA might control the expression of genes required for *Salmonella* invasion.

Chao et al. have performed deep sequencing of Hfq-bound transcripts from *Salmonella* and identified 280 sRNAs (8). The majority of those sRNAs have never been functionally characterized. In this study, we surveyed the role of the previously identified sRNAs for their roles in *Salmonella* invasion. Using an exhaustive screening approach, we discovered that *Salmonella* sRNA InvS is essential for *Salmonella* invasion. Several of the type III effector proteins known to be involved in bacterial invasion were secreted at lower levels in the absence of InvS. InvS also modulates the protein levels of PrgH and FimZ, a type III secretion apparatus protein and a negative regulator that suppresses the expression of *Salmonella* invasion genes, respectively. We suggest that InvS regulates *Salmonella* invasion via PrgH and FimZ.

RESULTS

InvS is essential for *Salmonella* invasion. To identify *S.* Typhimurium sRNAs involved in bacterial invasion, we generated chromosomal deletions of 75 sRNA-encoding genes in strain SL1344. The resulting null mutant strains were tested for their ability to invade cultured epithelial cells using the classic gentamicin protection assay (see Table S1 in the supplemental material). The deletion of STnc470 had the biggest impact on the invasion of HeLa cells, with a reduction of approximately 70% compared with that of the wild-type strain (Fig. 1). Accordingly, we renamed STnc470 as InvS. The invasion defect of the Δ*invS* mutant was restored when InvS was expressed in *trans* from a plasmid (Fig. 1), proving that InvS is required for efficient *Salmonella* invasion.

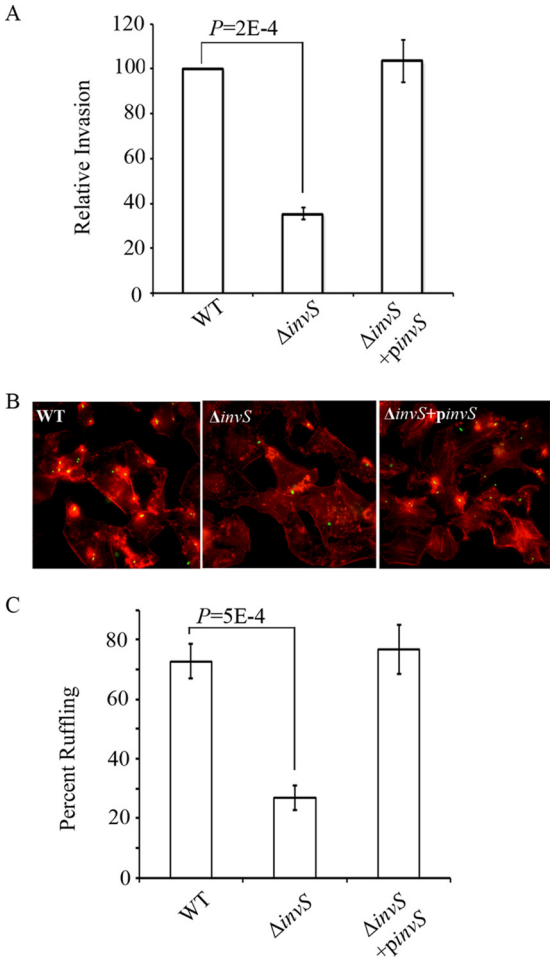

FIG 1 InvS is essential for *Salmonella* invasion. (A) HeLa cells were infected with *Salmonella* strains for 15 min at an MOI of 10. Relative bacterial invasion was determined by the gentamicin protection assay as described in Materials and Methods. The invasion rate of the wild-type strain was defined as 100%. The data are the averages from three independent experiments with error bars indicating the standard deviations. (B) HeLa cells were infected with *Salmonella* for 15 min at an MOI of 10. Actin staining was conducted as described in Materials and Methods to indicate *Salmonella*-induced ruffling formation. (C) Percentages of infected cells with ruffles were calculated. The data shown were obtained from three independent experiments. Error bars indicate standard deviations. *P* values were calculated using the Student *t* test.

InvS is an 89-nucleotide sRNA that was first identified as STnc470 (11). Further characterization has shown that InvS binds Hfq and is derived from the 3′ untranslated region (UTR) of *srfN* (STM0082) (8). A Northern blot analysis confirmed the size of InvS and that it is cotranscribed with *srfN* and present as a discrete transcript, consistent with processing of the transcript (Fig. 2).

Overexpression of HilD or InvF rescues the Δ*invS* invasion defect. SPI-1 genes encode proteins involved in the secretion and injection of bacterial effectors into the host cell that promote *Salmonella* invasion (15, 16). The expression of SPI-1 genes is tightly regulated by a number of transcriptional regulators, including HilD, HilA, and InvF. HilD activates HilA, which in turn upregulates the expression of genes encoding the TTSS, such as proteins encoded by the *prg-org* and the *inv-spa* operons (6, 17, 18). The first gene of the *inv-spa* gene cluster encodes the AraC-like regulator InvF, which activates the expression of genes encoding secreted effectors that are essential for *Salmonella* invasion, including the *sic-sip* operon, *sopE*, and *sopB* (19).

As a first step toward understanding how InvS facilitates *Salmonella* invasion, we looked for a role of InvS in the regulation of the transcription factors mentioned above.

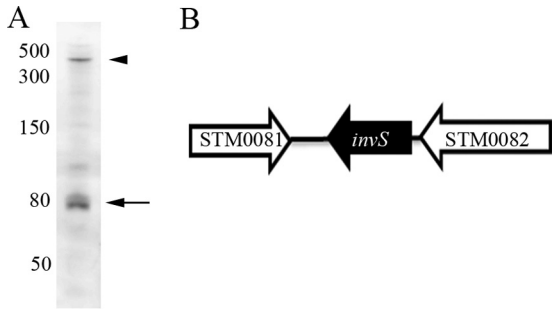

FIG 2 Validation of the InvS transcript. (A) Northern blot analysis identification of the InvS transcript. Comparison with comigrating markers suggests that InvS accumulates as an 89-nt transcript (arrow). Arrowhead, STM0082-STn470 mRNA (400 nt). RNA was isolated from *S.* Typhimurium 4/74 grown to early stationary phase in LB medium. (Republished from the *Proceedings of the National Academy of Sciences* [10].) (B) Schematic diagram showing that InvS sRNA is encoded in the 3′ UTR of STM0082.

For this, we tested whether the overexpression of these regulators would rescue the invasion defect of the Δ*invS* mutant. HeLa cells were infected with wild-type *Salmonella*, a Δ*invS* null mutant, or the Δ*invS* mutant strain expressing one of the following regulators from a plasmid: HilA, HilC, SirA, HilD, or InvF. Invasion rates were assessed using the classic gentamicin protection assay. We found that the overexpression of HilD or InvF restored the invasion defect of the Δ*invS* mutant (Fig. 3A) while HilA, HilC, or SirA did not. Our gentamicin protection assay showed plasmids expressing HilA (p*hilA*), HilC (p*hilC*), and SirA (p*sirA*) were able to restore the invasion deficiency of Δ*hilA*, Δ*hilC*, and Δ*sirA* mutant strains, which indicates that the plasmids are functional (Fig. 3B).

It is also reported that HilD is able to activate the transcription of *invF* from a promoter that is far upstream of its HilA-dependent promoter (6). The loss of HilD results in a more severe effect on invasion than the loss of HilA (6). Using a β-galactosidase fusion, we showed that overexpressing HilD activated *invF* expression more profoundly than overexpressing HilA. Thus, overexpressing only *hilA* may not be sufficient to restore the invasion phenotype of the Δ*invS* mutant.

We next explored whether InvS regulates the transcription of *hilD* or *invF*. A *lacZ* reporter gene was placed under transcriptional control of the *hilD* or *invF* promoter in either the wild type or the Δ*invS* mutant *Salmonella* strain background. The β-galactosidase activities were then monitored under SPI-1-inducing conditions. We found that the expression of *lacZ* transcribed from the *hilD* or the *invF* promoter remained at similar levels in both the wild-type strain and the Δ*invS* mutant background (Fig. 3C), suggesting that neither *hilD* nor *invF* is regulated by InvS at the transcriptional level. Furthermore, we generated plasmids expressing the HilD-green fluorescent protein (GFP) or the InvF-GFP fusion proteins and monitored their levels in the presence and absence of InvS in *E. coli*. The HilD-GFP and InvF-GFP levels were not InvS dependent (Fig. 3D). We conclude that *hilD* and *invF* are unlikely to be the direct targets of InvS.

Proteomic analysis of secreted proteins with and without InvS. Type III secreted effector proteins are known to be involved in promoting *Salmonella* invasion. An altered secretion of these effectors could potentially affect bacterial invasion. To explore whether InvS affected the levels of the secreted proteins and to identify the potential targets of InvS, we carried out a quantitative proteomic analysis using isobaric tags for relative and absolute quantification (iTRAQ) (20) in the wild-type (WT) and Δ*invS* null mutant strains. Proteins were assessed from the pellets to determine total expression levels and from the supernatants to identify secreted amounts. In the bacterial pellets, we calculated the relative protein abundance in Δ*invS* versus WT. Since iTRAQ has known issues of underestimating fold changes (21), a threshold *P* value of $\leq$0.1 in combination with a minimum of a 1.3-fold change in protein abundance was used (a Δ*invS*/WT ratio of $<$0.7 or $>$1.3 was considered significant). In the bacterial pellets, we detected more than 200 proteins whose abundances were changed in the

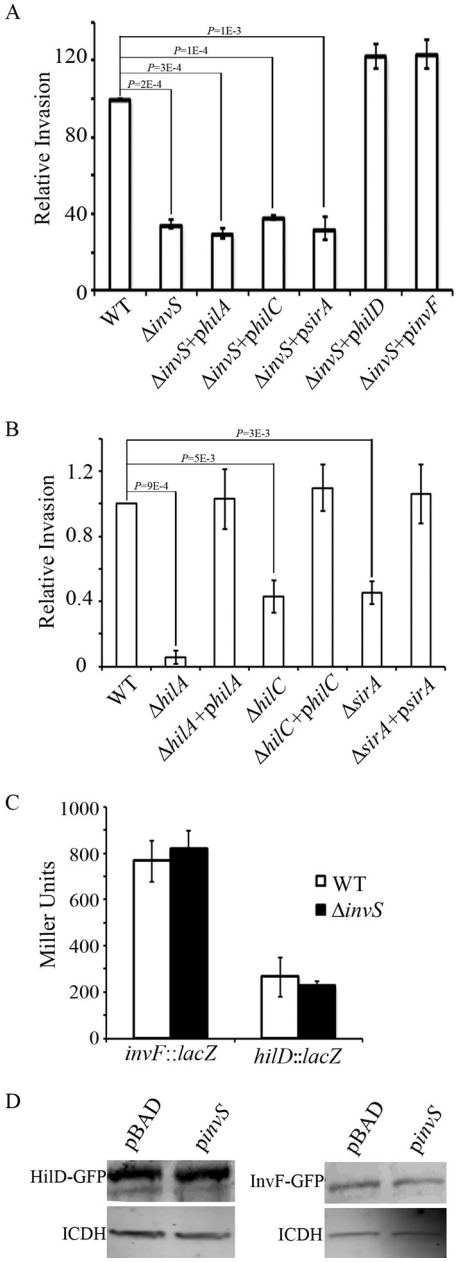

FIG 3 Overexpression of *hilD* or *invF* rescues the Δ*invS* mutant invasion defect. (A, B) HeLa cells were infected with the indicated *Salmonella* strains for 15 min at an MOI of 10. Relative bacterial invasion was determined by the gentamicin protection assay. The data shown were obtained from three independent experiments. Error bars indicate standard deviations. *P* values were calculated using the Student *t* test. (C) InvS does not change the expression level of *hilD* or *invF*. The *lacZ* reporter gene was placed under transcriptional control of the *hilD* or *invF* promoter in either the wild type or the Δ*invS* mutant *Salmonella* strain. β-Galactosidase activity assay was measured as described in Materials and Methods. The data shown were obtained from three independent experiments. Results are presented as the means in Miller units. Error bars indicate standard deviations. (D) InvS does not change the expression of *hilD-gfp* or *invF-gfp*. The 5′ UTR along with the full ORFs of HilD and InvF were translationally fused to GFP. Plasmids expressing HilD-GFP or InvF-GFP were cotransformed with plasmids expressing InvS or a vector control as indicated. HilD-GFP or InvF-GFP was detected by Western blotting with polyclonal anti-GFP antibodies. Bacterial isocitrate dehydrogenase (ICDH) was similarly detected using anti-ICDH polyclonal antibodies as the loading control.

Δ*invS* mutant (see Table S2). While the majority of these were uncharacterized hypothetical proteins or proteins not known to be related to invasion, we detected a significant decrease in flagellar proteins in the Δ*invS* mutant compared with that in the wild-type *Salmonella*. We also found the level of FlhD was markedly decreased in the

Δ*invS* mutant compared with that in the wild type. FlhD is a transcriptional regulator that is known to regulate flagellar expression to promote *Salmonella* invasion. Interestingly, FimZ, a regulator known to facilitate fimbrial protein expression and to repress the expression of flagellar genes by binding to the *flhD* promoter, was found to be 3.8-fold more abundant in the pellet fraction from the Δ*invS* mutant than from the wild type. This is consistent with the increased amount of fimbrial proteins and the decrease of flagellar proteins in pellet fractions from the Δ*invS* mutant (Table S2). Flagella have been indicated as essential for efficient bacterial adhesion. It is also reported that flagellum-driven motility forces the bacterium into a "near surface swimming" mode, which promotes *Salmonella* invasion through "scanning" of the host cell surface (22). In addition, FimZ is known to downregulate *Salmonella* invasion by activating *hilE*, which represses the expression of several of the *Salmonella* invasion genes. Thus, we reasoned that InvS may function to downregulate *fimZ* to promote *Salmonella* invasion.

We performed a similar analysis on supernatant fractions, with the exception that peptides were not labeled with iTRAQ due to the challenges of consistently derivatizing secreted proteins of low abundance. The analysis of secreted proteins revealed that the amounts of several *Salmonella* SPI-1 secreted effectors, including SipA, SopA, SipC, and SopB, in the supernatant fractions of the Δ*invS* mutant strain were lower than those of the wild-type bacteria. By contrast, the levels of many other *Salmonella* effector proteins remain unchanged in the pellet fractions in the Δ*invS* mutant strain compared with those of the wild-type bacteria (Table S2). These results indicate that InvS might regulate *Salmonella* effector secretion. In the bacterial pellets, we failed to detect most of the type III apparatus proteins, which might be due to the low abundances of these proteins in the pellet samples.

InvS regulates *Salmonella* effector secretion. Our proteomics data suggested that InvS is involved in *Salmonella* effector secretion. We sought to examine the expression and secretion of SipA, SipB, and SipC, the three main invasion-related effectors, by Western blotting. Consistent with the proteomics results, the InvS null mutant strain secreted dramatically reduced levels of SipA, SipB, and SipC (Fig. 4A and B). By contrast, the expressions of SipA, SipB, and SipC in the cell-associated fraction were unchanged in both the InvS null mutant strain and the wild type. Taken together, we conclude that InvS is important for the secretion of effector proteins.

To examine if InvS affects *Salmonella* effector translocation *per se*, we carried out a β-lactamase-based translocation assay using SipA-TEM1 fusion as a translocation reporter (23). The SipA-TEM1 fusion protein was expressed at similar levels in the wild-type *Salmonella* and the Δ*invS* mutant strain (Fig. 4D). Next, HeLa cells were infected with wild-type *Salmonella* and the Δ*invS* mutant strain expressing SipA-TEM1, and the translocation efficiency was evaluated as previously described (23). As shown in Fig. 4C and E, SipA was translocated at a much lower level from the Δ*invS* null mutant than from the wild-type *Salmonella*. These results support the proteomics data and indicate that InvS is involved in type III effector secretion and translocation during *Salmonella* infection.

InvS controls the level of PrgH. One of the possibilities that might lead to the decreased secretion of a group of type III effectors is the dysfunction of the TTSS apparatus. A GFP-based plasmid assay is available to study sRNA-mediated translational control and to verify potential sRNA targets (24). We used GFP translational fusions to determine whether InvS can modulate the levels of the TTSS apparatus proteins SpaO, InvA, PrgK, and PrgH at the posttranscriptional level. The *Salmonella invS* null mutant strain was transformed with a P_{BAD}-based sRNA expression vector (with or without InvS) and a constitutive GFP fusion expression vector (pXG30) that carried the 5′ UTR and the full open reading frames (ORFs) of SpaO, InvA, PrgK, and PrgH translationally fused to GFP. The expression of the GFP fusion proteins was examined in the presence or absence of InvS. While the levels of SpaO-GFP, InvA-GFP, and PrgK-GFP remain unchanged with and without InvS, the PrgH-GFP level was decreased in the absence of InvS (Fig. 5A and B). When the PrgH-GFP expression plasmid was introduced into

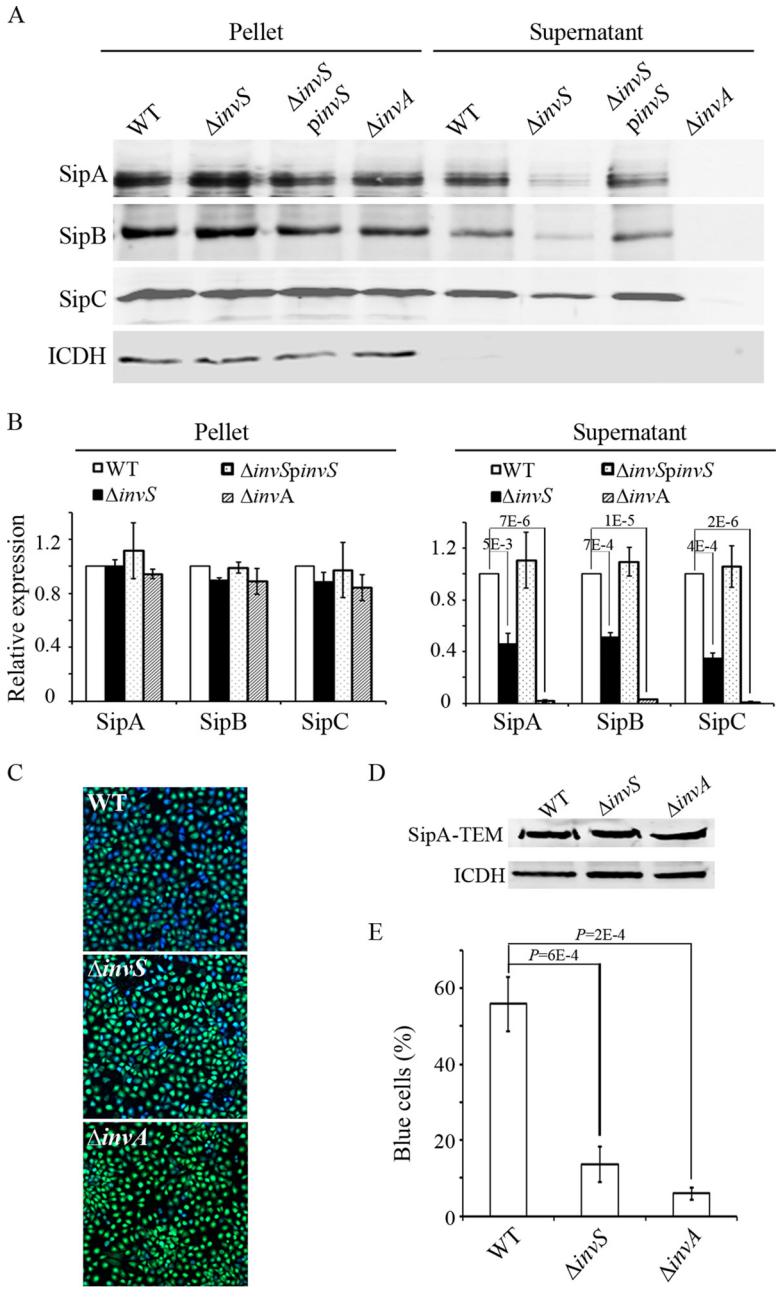

FIG 4 InvS regulates *Salmonella* effector secretion and translocation. (A) Expression and secretion of invasion-related effectors in *Salmonella* WT, Δ*invS*, Δ*invS*/p*invS*, and Δ*invA* strains. Bacterial strains were grown under SPI-1-inducing conditions and equal amounts of bacterial lysates or culture supernatants were analyzed by Western blotting. (B) Quantification of protein expression in panel A. Protein levels in the WT strain were defined as 1. Values represent relative protein levels after normalization with the expression in the WT. Data are representative of three experiments. (C) HeLa cells were infected with various *Salmonella* strains carrying a plasmid expressing the SipA-TEM fusion protein. Fifteen minutes after the infection, cells were loaded with CCF4-AM and incubated at room temperature for 2 h. The translocation efficiency was evaluated under a fluorescence microscope. (D) Western blot showing the expression of SipA-TEM in different strains. (E) Quantification of SipA-TEM translocation. Percentages of blue cells were used to measure the translocation efficiency. The data shown were obtained from three independent experiments. Standard deviations are shown. *P* values were calculated using the Student *t* test.

Escherichia coli, no PrgH-GFP was detected by Western blotting (data not shown). The lack of PrgH-GFP expression could be because additional *Salmonella* factors may be required to maintain a higher level of PrgH-GFP in *Salmonella*. We also transformed pXG30 expressing PrgH-GFP into the WT and Δ*invS* strains and examined the expres-

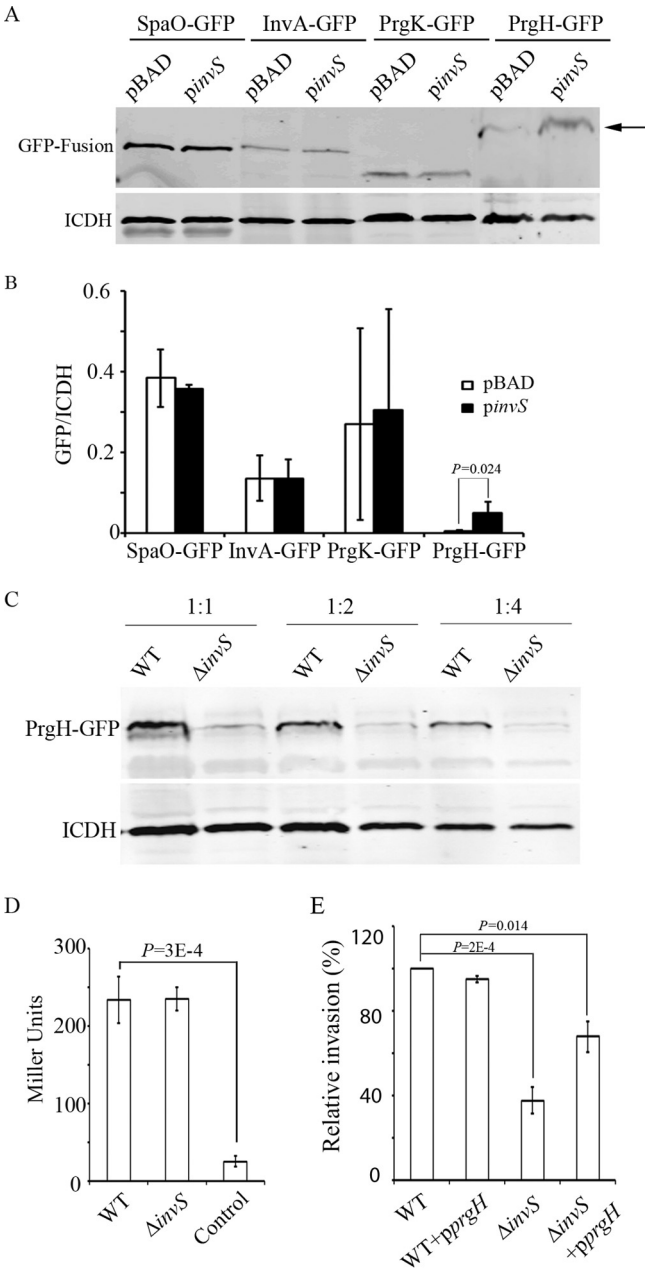

FIG 5 InvS regulates the level of PrgH. (A) InvS upregulates *prgH-gfp* expression in *Salmonella*. The 5′ UTR along with the full ORFs of SpaO, InvA, PrgK, and PrgH were translationally fused to GFP. Plasmids expressing the GFP fusion proteins were cotransformed with plasmids expressing InvS or the vector control into the Δ*invS* strain. The background strain eliminates the potential effect that can be caused by chromosomal *invS*. Bacterial isocitrate dehydrogenase (ICDH) was detected using anti-ICDH polyclonal antibodies as the loading control. The levels of GFP fusion proteins were determined by Western blotting with polyclonal anti-GFP antibodies. The arrow indicates the expression of PrgH-GFP. (B) Quantification of GFP fusion protein expression from three independent experiments. Values represent GFP fusion protein expression levels after normalization to the expression of ICDH. The *P* value was calculated using the Student *t* test. (C) The expression of *prgH-gfp* was decreased in the absence of InvS. pXG30-derived PrgH-GFP was transformed into the WT or Δ*invS* strain. Western blot of the 2-fold dilution series showing the expression of *prgH-gfp*. (D) InvS does not regulate the transcriptional level of *prgH*. A promoterless *lacZ* gene was placed under the *prgH* promoter in either the wild-type *Salmonella* or the Δ*invS* mutant strain. β-Galactosidase activity was measured as described in Materials and Methods. The *Salmonella* WT strain without *lacZ* was used as the negative control. The data shown were obtained from three independent experiments. Results are presented as the means in Miller units. Error bars indicate standard deviations. (E) Overexpression of *prgH* partially restores the Δ*invS* mutant invasion defect. HeLa cells were infected with *Salmonella* for 15 min at an MOI of 10. Relative bacterial invasion was determined by the gentamicin protection assay. The data shown were obtained from three independent experiments. The *P* values were calculated using the Student *t* test.

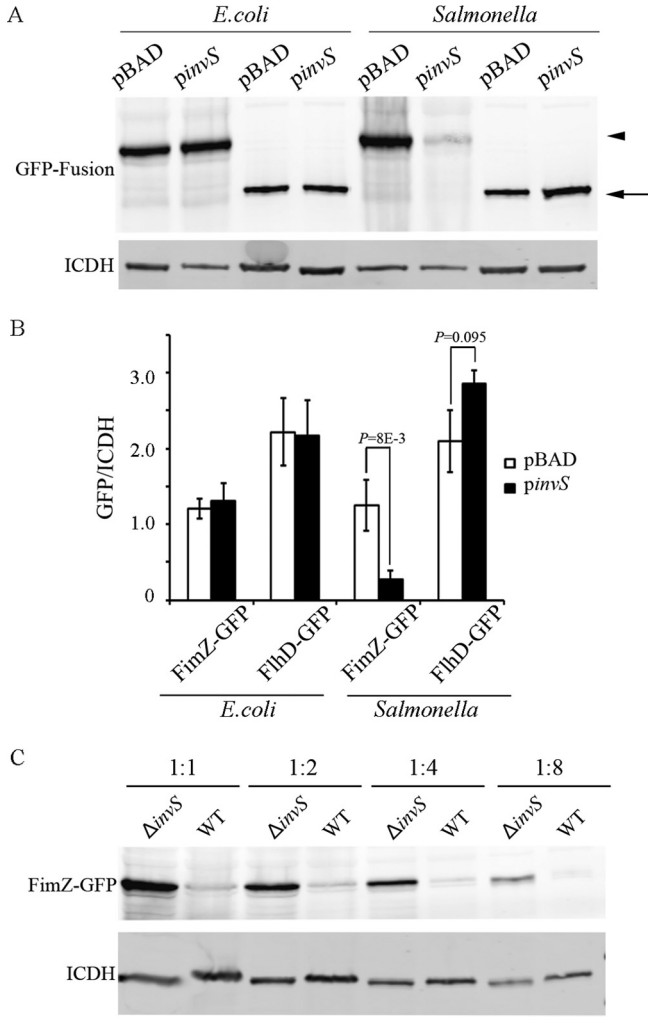

FIG 6 InvS regulates the level of FimZ. (A) InvS downregulates *fimZ-gfp* expression in *Salmonella*. The 5′ UTR along with the full ORFs of FimZ and FlhD were translationally fused to GFP. Plasmids derived from pXG30, expressing GFP fusion proteins, were cotransformed with pBAD-derived plasmids expressing InvS or the vector control. Bacterial ICDH was detected using polyclonal anti-ICDH antibodies as the loading control. The levels of GFP fusion proteins were determined by Western blotting with polyclonal anti-GFP antibodies. (B) Quantification of GFP fusion proteins from three independent experiments. Values represent GFP fusion protein levels after normalization with that of ICDH. Data are representative of three experiments. The *P* values were calculated using the Student *t* test. (C) The expression of FimZ-GFP was decreased in the presence of InvS. pXG30-derived FimZ-GFP was transformed into the WT or the Δ*invS* strain. Western blot of the 2-fold dilution series showing decreases of FimZ-GFP levels in the presence of InvS.

sions of the fusion proteins by Western blotting. We detected a smaller amount of PrgH-GFP in Δ*invS*, which further confirmed that InvS functions to upregulate *prgH-gfp* expression (Fig. 5C). On the other hand, similar levels of *prgH* promoter activity were detected in the WT and the Δ*invS* mutant strain (Fig. 5D), indicating that InvS may indirectly regulate *prgH* at the posttranscriptional level. To investigate whether the overexpression of PrgH is able to rescue the InvS-dependent invasion phenotype, we overexpressed PrgH in the Δ*invS* mutant strain, and its invasion efficiency was found to be partially restored compared with that of the wild-type *Salmonella* (Fig. 5E). Overexpressing PrgH in the wild-type strain did not significantly influence invasion levels (Fig. 5E). This result suggests that InvS is required for maintaining PrgH expression and *Salmonella* invasion. The partial rescue of invasion by the overexpression of *prgH* in the Δ*invS* mutant suggests that InvS may influence additional target genes involved in *Salmonella* invasion.

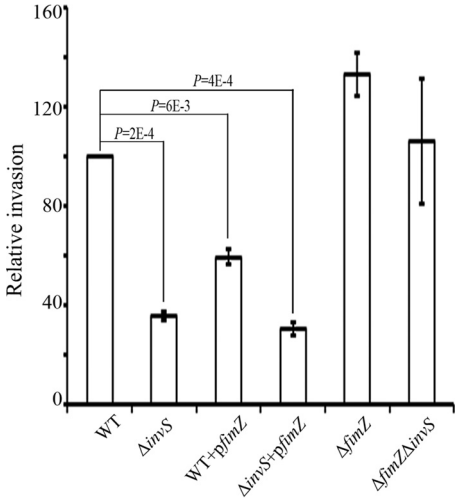

FIG 7 FimZ downregulates *Salmonella* invasion. HeLa cells were infected with *Salmonella* for 15 min at an MOI of 10. Relative bacterial invasion was determined by the gentamicin protection assay. The data shown were obtained from three independent experiments. The *P* values were calculated using the Student *t* test.

InvS regulates the level of FimZ. Our proteomic analysis showed a higher level of FimZ and lower level of FlhD in the absence of InvS. Next, we used the GFP-based plasmid assay to test if InvS affects the levels of FimZ and FlhD. The 5′ UTR along with the full ORFs of FimZ and FlhD were translationally fused to GFP. The assay was performed in both *E. coli* and *Salmonella* Δ*invS*. When tested in the Δ*invS* background strain, we detected a lesser amount of FimZ-GFP when InvS was coexpressed from a plasmid, p*invS*. Interestingly, the difference in the FimZ-GFP levels disappeared when the same plasmids were coexpressed in the *E. coli* background (Fig. 6A and B). This result suggests that InvS may indirectly repress *fimZ* expression and that additional cofactors (from *Salmonella*) may be required for InvS to regulate *fimZ* expression. The expression of the GFP fusion proteins was also examined in the WT and the Δ*invS* mutant strain. We found that the level of FimZ-GFP was higher in the Δ*invS* mutant than in the WT strain (Fig. 6C). FimZ is known to negatively regulate *flhD* expression. It is possible that the increase in FimZ in the Δ*invS* background strain led to the decrease of *flhD* expression. We then performed a gentamicin protection assay to examine if the alteration of the FimZ level is able to work against the effect of InvS and rescue the invasion phenotype of the Δ*invS* mutant. We found the double deletion Δ*fimZ* Δ*invS* strain showed an invasion level similar to that of the Δ*fimZ* strain. The overexpression of FimZ in the wild-type strain resulted in a decrease in the invasion rate (Fig. 7). Western blotting confirmed the decrease of flagella in the Δ*invS* strain compared with the wild-type strain (Fig. 8A and B), which is consistent with the result showing that the deletion of *invS* impaired *Salmonella* motility (Fig. 8C). The overexpression of FimZ produced a larger amount of FimZ than the *invS* deletion strain and drastically inhibited flagellar gene expression (Fig. 8). Although the detailed mechanism remains unclear, our results suggest *fimZ* is an important regulatory component linking InvS and its effects on flagellar expression and *Salmonella* invasion. InvS facilitates invasion in a *fimZ*- and flagellum-dependent manner.

DISCUSSION

Small RNAs represent a relatively new set of posttranscriptional regulatory molecules that are gaining interest in bacteria. A few bacterial sRNAs are reported to regulate the bacterial stress response and are involved in the regulation of virulence genes. Gong et al. reported that IsrM negatively regulates *Salmonella* HilE and is essential for *Salmonella* invasion (14). Ryan et al. have demonstrated that the small RNA DsrA influences the acid tolerance response and virulence of *Salmonella* (25, 26, 49).

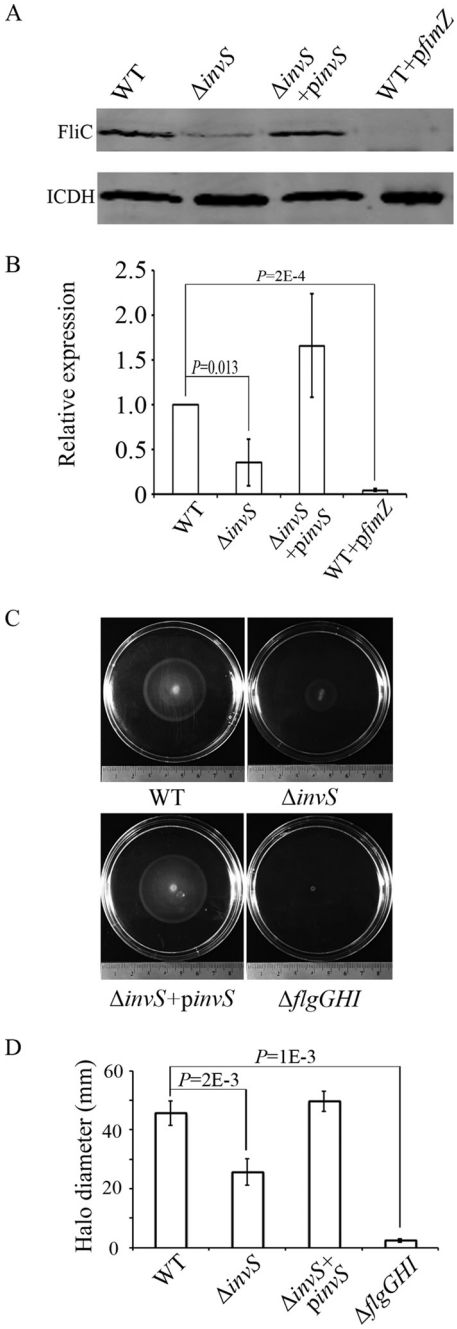

FIG 8 InvS regulates flagellar expression. (A) InvS upregulates the expression of *fliC*. The expression of FliC protein was detected by Western blotting with monoclonal anti-FliC antibodies. Bacterial ICDH was detected using anti-ICDH polyclonal antibodies as the loading control. (B) Quantification of FliC expression. FliC expression in the WT strain was defined as 1. Values represent relative protein expression levels after normalization with that in the WT. Data are representative of three experiments. The *P* values were calculated using the Student *t* test. (C) Two-microliter samples of *Salmonella* cultures (optical density at 600 nm, 1.0) were inoculated onto LB plates made up of 0.3% Bacto agar (Difco), and cultures were grown at 37°C. Photos were taken 6 h postinoculation. (D) Halos around the colonies were measured after 6 h of incubation at 37°C. Data are representative of three experiments. The *P* values were calculated using the Student *t* test.

Recently, hundreds of novel sRNAs have been identified in *Salmonella*, but few have been functionally characterized (8, 11, 14, 27). To identify the involvement of these small RNAs in *Salmonella* virulence, we screened recently identified *Salmonella* sRNAs for their roles in *Salmonella* invasion and found that InvS is essential for *Salmonella*

entry into nonphagocytic cells. InvS was originally identified by Hfq coimmunoprecipitation sequencing (Hfq-CoIP-seq) and showed a 2- to 47-fold enrichment under various stress conditions (8, 11). Colgan et al. performed transcriptome sequencing (RNA-seq) to study the differential expression of *Salmonella* sRNAs. InvS was shown to be positively regulated by two-component regulatory systems, including SsrA/B, PhoP/Q and OmpR/EnvZ (28). The details of the InvS regulatory pathways are not clear. It is known that PhoP/Q regulates both SPI-1 and SPI-2 expression, while SsrA/B and OmpR/EnvZ are able to activate SPI-2 expression (29). It is not known if InvS plays any roles in the cross talk between SPI-1 and SPI-2.

Many classic transcription factors are known to regulate *Salmonella* invasion by controlling the transcription of invasion-related genes. For example, the transcription of SPI-1 genes can be activated by HilA, HilC, HilD, InvF, and SirA (30). Our results showed that overexpression of HilD and InvF were able to restore the invasion defect of the Δ*invS* mutant. So far, there is no evidence to suggest that *hilD* or *invF* is a direct target of InvS. Interestingly, we found that the overexpression of HilA failed to rescue the invasion deficiency. This may indicate that InvS is able to regulate invasion in a HilD-dependent but HilA-independent pathway. Singer et al. have demonstrated that HilD directly activates the expression of flagellar genes, while HilA does not affect flagellar gene expression (31); this is consist with our data showing that the deletion of InvS results in a decrease of flagellar expression. Furthermore, it was reported HilD is able to activate the transcription of *invF* from a promoter that is far upstream of its HilA-dependent promoter. The loss of *hilD* resulted in a more severe effect on the expression of a subset of SPI-1 genes than the loss of *hilA* (6). Our data show that overexpressing HilD activates *invF* expression more profoundly than overexpressing HilA. In addition, it is also possible that additional factors are involved in the InvS-mediated regulation of invasion. InvS may have multiple targets, which might balance out the effect of HilA overexpression. This may explain why overexpressing only *hilA* is not sufficient to restore the invasion defect of the *invS* mutant.

We speculated that InvS exerts its function by regulating genes downstream of *hilD* and *invF*. These downstream genes may include the *Salmonella* SPI-1 type III secretion system and type III effectors that are known to play direct roles in *Salmonella* invasion. To identify the targets of InvS, we noticed that type III effector secretion and translocation are decreased in the absence of InvS. Further analysis revealed that InvS activates the expression of *prgH*, which is required for the assembly of the type III secretion needle complex. It is known that the deletion of *prgH* impairs SPI-1 TTSS assembly and effector secretion (3, 4). Consistent with its effect in type III secretion, overexpressing PrgH in the Δ*invS* mutant partially rescued the invasion deficiency. It is still unclear how *prgH* is regulated by InvS. Our results showed similar levels of *prgH* promoter activity in the WT and the Δ*invS* mutant background (Fig. 5D). Thus, it is possible that InvS regulates *prgH* indirectly or at the protein level. In addition, it is possible that InvS affects additional target genes to regulate *Salmonella* invasion.

Our proteomic analysis showed higher levels of FimZ in the absence of InvS. FimZ is known as a transcriptional activator responsible for promoting the expression of type I fimbriae and downregulating flagellar synthesis (32). While fimbriae are known to play a role in adhering to infected cells, flagella have been associated with *Salmonella* motility and invasion. It has been reported that flagellum-driven motility forces the bacterium into a near surface swimming mode, which promotes *Salmonella* invasion by scanning the host cell surface (22). Our proteomics analysis indicated an increase in *fimZ* and a decrease in *fliC* expression in the absence of InvS. The deletion of *invS* impairs *Salmonella* motility, suggesting that InvS might function to promote motility to facilitate bacterial invasion. Consistent with this notion, previous reports found that HilD activates the transcription of flagellar genes while HilA does not (31). Our data show that the overexpression of HilD rescued the Δ*invS* invasion defect while HilA did not alter the invasion levels. This is in agreement with our data showing that uncontrolled or overexpression of *fimZ* (in the absence of InvS) leads to a decrease in

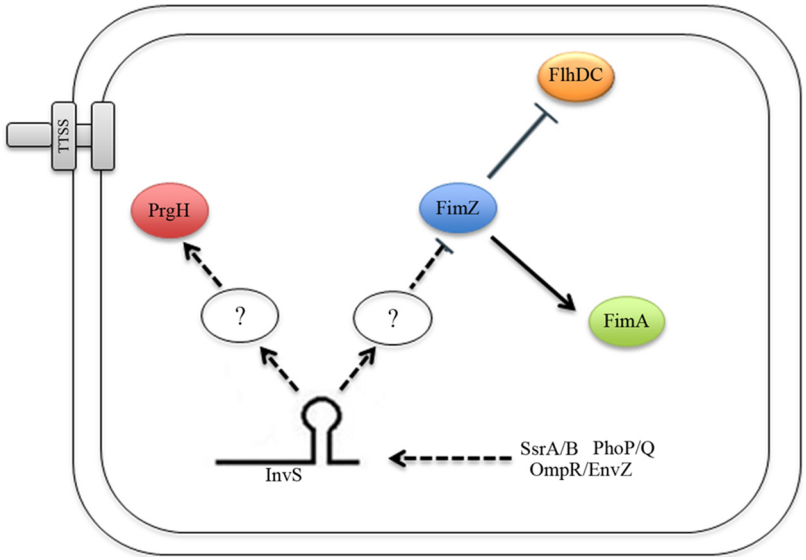

FIG 9 Model for InvS-mediated *Salmonella* invasion. InvS facilitates *Salmonella* effector secretion and translocation by positively regulating *prgH*, which encodes a type III secretion apparatus protein. Furthermore, InvS negatively regulates *fimZ*, a global regulator that is known to repress *Salmonella* SPI-1 gene expression by activating HilE. FimZ activates FimA, the major fimbrial unit. FimZ also negatively regulates flagellar synthesis by repressing expression of the master flagellar regulator FlhDC. The regulation of these target mRNAs or proteins by InvS, in turn, promotes *Salmonella* to invade the host cell. Arrows represent activation while the assertion signs represent inhibition. Dotted lines indicate indirect regulation. The symbol "?" indicates unknown factors or a signaling cascade that may be involved in the pathway.

invasiveness. Taken together, we conclude that InvS coordinates the increase in PrgH and decrease in FimZ leading to more efficient *Salmonella* invasion (Fig. 9).

The exact mechanism by which InvS activates the expression of PrgH and reduces the expression of FimZ is currently unclear. Corcoran et al. have established the GFP-based plasmid assay for the validation of sRNA-mediated target regulation (24). When tested in *E. coli*, we failed to detect any PrgH-GFP by Western blotting, suggesting that additional factors present in *Salmonella* might be involved in maintaining the stability of PrgH. Interestingly, our results indicate that InvS downregulates *fimZ* expression in *Salmonella* but not in *E. coli*. The regulation of *fimZ* and *prgH* expression is remarkably complex. It is known that *prgH* is under the regulation of many global regulators, such as HilA, InvF, PhoP, and SirA. Furthermore, Bailey et al. have shown that *prgH* and other SPI-1 genes are expressed at higher levels in a *ramA* mutant (33). Previous work showed FimY acts upstream of FimZ to activate the *fim* operon, while FliZ functions to repress FimZ posttranscriptionally (34). In addition, FimW and FimZ form a coupled feedback loop where they activate their own and each other's expression. Recently, it was reported that the two-component system PhoBR is also capable of inducing *fimZ* expression (35). Thus, it is possible that additional factors (from *Salmonella*) are required for InvS to regulate *fimZ* and *prgH* expression. Furthermore, Chao et al. showed InvS is associated with Hfq on the basis of results from their coimmunoprecipitation experiments (8). Ansong et al. detected a decrease in FimZ in Δ*hfq Salmonella* (36). In addition, previous studies suggested that cellular RNAs compete for Hfq, and one abundant sRNA can indirectly impact the targets of others by disrupting Hfq-mediated effects (37, 38). It is also possible that InvS indirectly regulates *fimZ* expression by disrupting the binding of Hfq to *fimZ* or other RNAs that target *fimZ* and *prgH*.

To date, only a fraction of published sRNAs has been functionally characterized, and the roles in bacterial virulence have only been elucidated for a few. We showed that InvS functions to positively regulate *prgH* expression and negatively regulate *fimZ* expression, which led to more efficient *Salmonella* invasion. InvS is highly conserved at

the DNA sequence level in all *Salmonella enterica* serovars, including Typhimurium, Newport, Typhi, Paratyphi, and Enteritidis (39). This pattern of conservation is consistent with the involvement of InvS in SPI-1-mediated invasion throughout the *Salmonella enterica* species. Our study expands the known sRNA-mediated regulatory network of *Salmonella*. Additional work on the remaining sRNAs and other regulatory factors will likely describe a coordinated regulatory network revealing the intricate regulation of virulence factors in *Salmonella*.

MATERIALS AND METHODS

Bacterial strains and mammalian cell lines. The *Salmonella* strains used in this study are isogenic derivatives of virulent wild-type (WT) strain SL1344 of *Salmonella* Typhimurium (40). In-frame chromosomal deletions of genes in *Salmonella* strains were generated using an allelic-exchange suicide vector pSB890 (41). Briefly, a DNA fragment with the in-frame deletion was cloned into the conjugative suicide vector pSB890. Plasmid constructs were introduced into *Salmonella* by conjugation and were subsequently integrated into the chromosome by homologous recombination. PCR-generated *invS* from the *Salmonella* chromosome was inserted into pBAD via EcoRI and XmaI sites to generate p*invS*. Translational *gfp* fusions were constructed by cloning a PCR insert amplified from the *Salmonella* chromosome and cloned into pXG30 via NsiI and NheI sites (24). DNA oligomer primers for these PCRs are listed in Table S3 in the supplemental material.

E. coli and *Salmonella* strains were routinely cultured in Luria-Bertani (LB) broth. *Salmonella* strains were cultured under SPI-1 TTSS-inducing conditions (LB broth with 0.3 M NaCl) for all of the invasion experiments. Antibiotics were used at the indicated concentrations: ampicillin, 120 $\mu g \cdot ml^{-1}$; streptomycin, 25 $\mu g \cdot ml^{-1}$; kanamycin, 40 $\mu g \cdot ml^{-1}$; and tetracycline, 12 $\mu g \cdot ml^{-1}$.

The mammalian cell line HeLa (CCL-2) was purchased from ATCC (Manassas, VA). HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (VWR) supplemented with 10% fetal bovine serum.

Fluorescent F-actin staining. An F-actin staining assay was conducted as described previously (42). HeLa cells were infected with *Salmonella* at a multiplicity of infection (MOI) of 10 unless indicated otherwise. Infected cells were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) for 15 min and permeabilized with 0.2% Triton X-100 in PBS. *Salmonella* was stained using rabbit anti-*Salmonella* O-antigen group B (Difco), and then visualized with Alexa Fluor 488 (Invitrogen). F-actin was visualized by staining with Texas Red-conjugated phalloidin (Molecular Probes, Eugene, OR).

Gentamicin protection assay. *Salmonella* infection of HeLa cells was conducted as previously described (43). Briefly, *Salmonella* cells were cultured to an optical density at 600 nm (OD_{600}) of 1.0 in LB broth with 0.3 M NaCl at 37°C. Bacteria were then added to HeLa cells at an MOI of 10 and incubated for 15 min at 37°C in 5% CO_2. After the infection, cells were washed twice with PBS to remove extracellular bacteria and were incubated further in DMEM containing 10% fetal bovine serum and 16 μg of gentamicin per ml. At different time points after gentamicin treatment, infected cells were washed three times in PBS and lysed with 1% Triton X-100 and 0.1% sodium dodecyl sulfate (SDS). Cell lysates were then serially diluted and plated on selective medium.

GFP-based two-plasmid assay. Bacteria were transformed with a P_{BAD}-based sRNA expression vector (with or without *invS*) and a expression vector (pXG30) that constitutively expresses corresponding GFP fusion proteins. Double transformants were grown overnight in LB medium containing appropriate antibiotics at 37°C, followed by subculture (1:200 dilution) until OD_{600} of 1.0 in LB broth containing 0.2% L-arabinose for the induction of *invS* expression. Western blotting was performed using polyclonal anti-GFP antibodies to monitor the expression of GPF fusion proteins (24).

Protein translocation assay. *Salmonella* strains expressing the β-lactamase fusions were used to infect monolayers of HeLa cells seeded in 96-well plates at an MOI of 20. Fifteen minutes after the infection, CCF4-AM (Invitrogen, Carlsbad, CA) was added to the wells. CCF4-AM enters cells and is cleaved by intracellular esterase, leading to the accumulation of CCF4. CCF4, emitting green fluorescence, is a β-lactamase substrate and emits blue fluorescence upon cleavage. After incubating with CCF4-AM for 2 h at room temperature, infected cells were examined under a fluorescence microscope to quantify the numbers of green and blue cells. Experiments were performed in triplicates. Approximately 300 cells were counted in each sample.

β-Galactosidase assay. *Salmonella* carrying LacZ fusions were grown at 37°C overnight, followed by subculture in an SPI-1-inducing condition until the OD_{600} reached 1.0. The β-galactosidase activity was measured according to standard protocols (44).

RNA isolation and Northern hybridization. RNA isolation and Northern hybridization experiments were performed as previously described (11, 45). Briefly, RNA was prepared by hot phenol extraction, followed by DNase I treatment. Five to 10 micrograms of total RNA was denatured for 5 min at 95°C in RNA loading buffer (95% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue, and 10 mM EDTA), separated on polyacrylamide gels, and transferred onto Hybond-XL membranes (GE Healthcare). The 5′-end γ-^{32}P-labeled oligonucleotides (Fermentas) were hybridized to membranes overnight at 42°C, and then washed with 5× saline-sodium citrate buffer (SSC) with 0.1% SDS, 1× SSC with 0.1%SDS, and 0.5× SSC with 0.1% SDS for 15 min each. Signals were visualized using a phosphorimager (Typhoon FLA 7000; GE Healthcare). The probes used are listed in Table S3.

Protein digestion, isobaric labeling, and peptide fractionation. WT and Δ*invS Salmonella* strains were cultured under SPI-1-inducing conditions to an OD_{600} of 1.0 and were centrifuged to separate the supernatant and pellet. *Salmonella* cells were lysed by vortexing with silica beads in 50 mM NH_4HCO_3 buffer, while supernatant proteins were obtained by precipitation with trichloroacetic acid. Cell lysates and supernatant proteins were then denatured in 8 M urea prepared in 50 mM NH_4HCO_3 containing 5 mM dithiothreitol for 30 min at 37°C. Samples were then alkylated by adding 400 mM iodoacetamide to a final concentration of 10 mM and incubating for 30 min at room temperature protected from light. The reaction was diluted 8-fold with 50 mM NH_4HCO_3 and incubated for 4 h at 37°C with trypsin at an enzyme/protein ratio of 1/50 (*m*/*m*). Samples were desalted with C_{18} SPE cartridges (Discovery C18, 1 ml, 50 mg; Sulpelco) as previously described (36). Peptides derived from cell lysates were labeled with 4-plex isobaric tags for relative and absolute quantification (iTRAQ) reagent (Applied Biosystems) according to the manufacturer's recommendations and were fractionated by high-pH reverse-phase liquid chromatography as previously described (46), while peptides derived from the supernatant fraction were left unlabeled and unfractionated. Briefly, peptides were loaded into a C_{18} column (Eclipse XDB C18, 5 μm, 4.6 by 150 mm; Agilent Technologies) connected to a high-performance liquid chromatograph (Waters 1525 binary HPLC pump) and eluted at 0.5 ml/min with the following gradient: 0 to 5% solvent B (solvent A, 10 mM ammonium formate [FA]; solvent B, 10 mM FA in 90% acetonitrile [ACN]) in 10 min, 5 to 35% solvent B in 60 min, 35 to 70% solvent B in 15 min, and holding at 70% for 10 min. Peptides were collected into 60 fractions, further concatenated into 15 fractions, and dried in a vacuum centrifuge. The supernatant was left unfractionated but was subjected to two steps of clean-up with C_{18} reverse-phase and strong cation exchange (SCX) cartridges to eliminate small molecule contamination (36).

Quantitative proteomic analysis. Peptides were dissolved in 0.1% formic acid and loaded into a C_{18} trap column (200 μm by 0.5 mm, ChromXP C18-CL, 3 μm, 120 Å; Eksigent) connected to a nanoHPLC system (Ekspert nanoLC 400; Eksigent). The separation was performed in a capillary C_{18} column (75 μm by 15 cm, ChromXP C18-CL, 3 μm, 120 Å) at 200 nl/min with the following gradient: 1 min in 5% solvent B (solvent A, 0.1% FA; solvent B, 80% ACN:0.1% FA), 5 to 35% solvent B in 60 min, 35 to 80% solvent B in 1 min, 6 min in 80% solvent B, 80 to 5% solvent B in 1 min, and hold in 5% for 11 min. Eluting peptides were directly analyzed in an electrospray ionization mass spectrometer (5600 TripleTOF; AB Sciex). Full mass spectrometry spectra were collected in the range of 400 to 2000 *m/z*, and the top 20 most intense parent ions were submitted to fragmentation for 100 ms each using rolling-collision energy.

The identification and quantification of peptides were performed with Paragon software as part of the ProteinPilot package (AB Sciex) by searching tandem mass spectra against *Salmonella enterica* serovar Typhimurium SL1344 sequences downloaded from Uniprot KnowledgeBase on 11 November 11 2014. Database searches were performed considering trypsin digestion, cysteine residue alkylation with iodoacetamide, and biological modifications asfactors. Peptides were filtered with a confidence score above 95, which resulted in a false-discovery rate of ~1.3% in protein level. The iTRAQ channel intensities were extracted using ProteinPilot and intensities from different peptide-spectrum matches and peptides from the same protein were summed together. Sample load was then normalized by total channel intensity and significance was tested by analysis of variance (ANOVA) using InfernoRDN (formerly, DAnTE) (47). For the label-free supernatant samples, peak areas were extracted with Skyline (48) before being normalized by linear regression and central tendency, and were tested by ANOVA using InfernoRDN.

Accession number(s). The raw proteomic data were deposited in the Proteomics Identifications (PRIDE) public repository under accession numbers PXD003589 and PXD003590.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00824-16.

SUPPLEMENTAL FILE 1, XLSX file, 0.1 MB.
SUPPLEMENTAL FILE 2, XLSX file, 0.3 MB.
SUPPLEMENTAL FILE 3, XLSX file, 0.1 MB.
SUPPLEMENTAL FILE 4, PDF file, 0.1 MB.

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