InvS coordinates expression of PrgH and FimZ, and is required for invasion of epithelial cells by *Salmonella enterica* serovar Typhimurium

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ABSTRACT

Deep sequencing has revolutionized our understanding of the bacterial RNA world, and has facilitated the identification of 280 sRNAs in *Salmonella*. Despite 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 non-phagocytic cells. After screening 75 sRNAs, we discovered that ablation of InvS caused a significant decrease of *Salmonella* invasion into epithelial cells. 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 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 promotes efficient *Salmonella* invasion into non-phagocytic cells.

IMPORTANCE

Salmonellosis continues to be the most common foodborne infection reported by the CDC in the USA. Central to *Salmonella* pathogenesis is the ability to invade non-phagocytic 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 sRNAs in this process. We have identified a novel sRNAs, InvS that is involved in *Salmonella* invasion. Our result will likely provide an opportunity to better understand the fundamental question of how *Salmonella* regulate invasion gene expression and may inform strategies for therapeutic intervention.

INTRODUCTION

Salmonella enterica serovar Typhimurium (*S. typhimurium*) remains a leading cause of foodborne illness and poses a major public health problem worldwide. *Salmonella* harbor several pathogenicity islands (SPIs) scattered on the chromosome, which comprise functionally distinct virulence genes. Virulent *Salmonella* strains possess pathogenicity island 1 (SPI-1) and the 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* T3SSs 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* SPI-1 T3SS is the needle complex. The multi-ring 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 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, SopE2. These SPI-1 effectors work in concert to rearrange the host actin cytoskeleton to facilitate *Salmonella* invasion (5). In 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 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 upon an intricate transcriptional network to stimulate invasion when the pathogen interacts with the non-phagocytic cells associated with the gut wall. The SPI-1 invasion genes are tightly regulated by several SPI-1 encoded classical 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 (50-250 nucleotides) non-coding RNA molecules. sRNAs usually regulate gene expression through base pairing with corresponding mRNA

target(s), and thereby repress or activate the target genes at the post-transcriptional 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 (9). The majority of those sRNAs have never been functional characterized. In this study, we survey the role of those 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.

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 (15). In-frame chromosomal deletions of genes in *Salmonella* strains were generated using an allelic-exchange suicide vector pSB890 (16). 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. Polymerase chain reaction (PCR)-generated *invS* from *Salmonella* chromosome was inserted into pBAD via EcoRI/XmaI sites to generate p*invS*. Translational *gfp* fusions were constructed by cloning of an PCR insert amplified from *Salmonella* chromosome and cloned into pXG30 via NsiI/NheI sites (17). DNA oligomer primers for these PCR reactions are listed in **Table S3**.

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 at 120 μg ml^{-1}, streptomycin at 25 μg ml^{-1}, kanamycin at 40 μg ml^{-1}, and tetracycline at 12 μg ml^{-1}.

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

Fluorescent F-actin staining

F-actin staining assay was conducted as described previously (18). HeLa cells were infected with *Salmonella* at MOI of 10 unless indicated otherwise. Infected cells were fixed with 3.7% formaldehyde in 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 (19). Briefly, *Salmonella* were cultured to an optical density of 1.0 at 600 nm 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 infection, cells were washed twice with phosphate-buffered saline (PBS) to remove extracellular bacteria and incubated further in DMEM containing 10% fetal bovine serum and 16 μg of gentamicin ml^{-1}. At different time point 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 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) till OD600 of 1.0 in LB containing 0.2% L-arabinose for induction of *invS* expression. Western blot was performed using polyclonal anti-GFP antibodies to monitor the expression of GPF fusion proteins (17).

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. 15 min after infection, CCF4-AM (Invitrogen, Carlsbad, CA) was added into 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 hours at room temperature, infected cells were examined under a fluorescent microscope to quantify the number of green and blue cells. Experiments were performed in triplicate. 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 SPI-1 inducing condition until OD600 reaches 1.0. The β-galactosidase activity was measured according to standard protocols (20).

RNA isolation and northern hybridization

RNA isolation and northern hybridization experiments were performed as previously described (11, 21). Briefly, RNA was prepared by hot phenol extraction, followed by DNAaseI treatment. 5–10 μg total RNA were denatured for 5 min at 95°C in RNA loading buffer (95% formamide, 0.1% xylene cyanole, 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 labelled oligos (Fermentas) were hybridized to membranes overnight at 42°C, and then washed with 5 × Saline-Sodium Citrate (SSC) buffer/0.1% SDS, 1 × SSC/0.1%SDS and 0.5 × SSC/0.1% SDS for 15 min each. Signals were visualized using a phosphor-imager (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 1.0 of OD_{600}, followed by centrifugation 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 ºC. 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 1/50 (m/m) enzyme/protein ratio. Samples were desalted with C18 SPE cartridges (Discovery C18, 1 ml, 50 mg, Sulpelco) as previously described (22). Peptides derived from cell lysates were labelled with 4-plex isobaric tags for relative and absolute quantification (iTRAQ) reagent (Applied Biosystems) following the manufacturer's recommendations and were fractionated by high pH reverse phase liquid chromatography as previously described (23), while peptides derived from the supernatant fraction were left unlabeled and unfractionated. Briefly, peptides were loaded into a C18 column (Eclipse XDB C18, 5 µm, 4.6x150 mm, Agilent Technologies) connected to an high performance liquid chromatograph (Waters 1525 binary HPLC pump) and eluted at 0.5 mL/min with the following gradient: 0-5% solvent B (solvent A: 10 mM ammonium formate, solvent B: 10 mM ammonium formante in 90% acetonitrile) in 10 min, 5-35% solvent B in 60 min, 35-70% 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 submitted to two steps of clean up with

C18 reverse phase and strong cation exchange (SCX) cartridges to eliminate small molecule contamination (22).

Quantitative proteomic analysis

Peptides were dissolved in 0.1% formic acid and loaded into a C18 trap column (200 µm x 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 C18 column (75 µm x 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 and solvent B: 80% ACN/ 0.1% FA), 5-35% solvent B in 60 min, 35-80% solvent B in 1 min, 6 min in 80% solvent B, 80-5% 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-MS 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 milliseconds each using rolling-collision energy.

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 November 11, 2014. Database searches were performed considering trypsin digestion, cysteine residue alkylation with iodoacetamide and biological modifications were considered as factors. 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 the ProteinPilot and were 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 ANOVA using InfernoRDN (former DAnTE) (24). For the label-free supernatant samples, peak areas were extracted with Skyline (25) before being normalized by linear regression and central tendency, and tested by ANOVA using InfernoRDN.

RESULTS

InvS (STnc470) 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 classical gentamicin protection assay (**Table. S1**). The deletion of STnc470 had the biggest impact upon invasion of HeLa cells, with a reduction of approximately 70% compared to 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.

InvS is an 89 nucleotide sRNA, first identified as STnc470 (11). Further

characterization has shown that InvS binds Hfq, and is derived from the 3'UTR of *srfN* (STM0082) (8). Northern blot analysis confirmed the size of InvS, and that it is co-transcribed with *srfN* and present as a discrete transcript, consistent with processing of the transcript (**Fig. 2AB**).

Overexpression of HilD or InvF rescues the ΔinvS invasion defect

SPI-1 genes encode proteins involved in the secretion and injection of bacterial effectors into the host cell that promotes *Salmonella* invasion (26, 27). 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, 28, 29). 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* (30).

As a first step towards understanding how InvS facilitates *Salmonella* invasion, we looked for a role of InvS in the regulation of the transcription factors mentioned above. For this, we tested whether overexpression of these regulators would rescue the invasion defect of Δ*invS*. HeLa cells were infected with wild-type *Salmonella*, 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

classical gentamycin protection assay. We found that overexpression of HilD or InvF restored the invasion defect of Δ*invS* (**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*) are able to restore the invasion deficiency of Δ*hilA*, Δ*hilC,* Δ*sirA* mutant strains, which indicates the plasmids are functional (**Fig. 3B**).

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

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* promoters 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* promoters remain at similar levels in both the wild-type strain and the Δ*invS* mutant background (**Fig. 3C**), suggesting that *hilD* or *invF* are not regulated by InvS at the transcription level. Furthermore, we generated plasmids expressing the HilD-GFP or the InvF-GFP fusion proteins and monitored their levels in the presence and absence of

279 InvS in *E. coli*. The HilD-GFP and InvF-GFP levels were not InvS-dependent (**Fig.**
280 **3D**). We conclude that *hilD* and *invF* are unlikely to be the direct targets of InvS.

281 **Proteomic analysis of secreted proteins with and without InvS**

282 Type III secreted effector proteins are known to be involved in promoting *Salmonella*
283 invasion. Altered secretion of these effectors could potentially affect the bacterial
284 invasion. To explore whether InvS affected the level of the secreted proteins and to
285 identify the potential targets of InvS, we carried out a quantitative proteomic analysis
286 using isobaric tags for relative and absolute quantification (iTRAQ) (31) in the WT and
287 Δ*invS* null mutant strains. Proteins from the pellets were assessed for total
288 expression levels and from the supernatants to identify secreted amounts. In the
289 bacterial pellets, we calculated the relative protein abundance in Δ*invS* versus WT
290 (Δ*invS*/WT). Since iTRAQ has known issues of underestimating fold changes (32), a
291 threshold of *p*-value $\leqslant$ 0.1 in combination with a minimum of 1.3-fold change in
292 protein abundance was used (Δ*invS*/WT<0.7 or >1.3 was considered to be significant).
293 In the bacterial pellets, we detected more than 200 proteins whose abundance were
294 changed in Δ*invS* (**Table S2**). While the majority of them were uncharacterized,
295 hypothetical proteins or proteins not known to be related to invasion, we detected a
296 significant decrease of flagellar proteins in Δ*invS* compared to the wild-type
297 *Salmonella*. We also found the level of FlhD was markedly decreased in the Δ*invS*
298 mutant as compared to that of the wild type. FlhD is a transcriptional regulator that is

299 known to regulate flagella expression to promote *Salmonella* invasion. Interestingly,
300 FimZ, a regulator known to facilitate fimbrial protein expression and repress the
301 expression of flagellar genes by binding to the *flhD* promoter, was found to be 3.8 fold
302 more abundant in the Δ*invS* mutant pellet fraction compared to that of the wild type.
303 This is consistent with the increased amount of fimbrial proteins and the decrease of
304 flagellar proteins in Δ*invS* pellet fractions (**Table S2**). Flagella have been indicated
305 as essential for efficient bacterial adhesion. It is also reported that flagella-driven
306 motility forces the bacterium into a "near surface swimming" mode, which promote
307 *Salmonella* invasion through "scanning" of the host cell surface (33). In addition,
308 FimZ is known to down regulate *Salmonella* invasion by activating *hilE*, which
309 represses the expression of several of the *Salmonella* invasion genes. Thus, we
310 reasoned that InvS may function to down regulate *fimZ* to promote *Salmonella*
311 invasion.

312 We performed similar analysis on supernatant fractions, with the exception that
313 peptides were not labeled with iTRAQ due to the challenges of consistently
314 derivatizing the low abundance of secreted proteins. The analysis of secreted
315 proteins revealed that the supernatant fractions have less amount of several
316 *Salmonella* SPI-1 secreted effectors, including SipA, SopA, SipC and SopB in the
317 Δ*invS* mutant strain compared to that of the wild-type bacteria. In contrast, levels of
318 many other *Salmonella* effector proteins remain unchanged in the pellet fractions in
319 the Δ*invS* mutant strain comparing to that of the wild-type bacteria (**Table S2).**

These results indicate 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 abundance of these proteins that are present 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, three main invasion-related effectors, by Western blot. Consistent with the proteomics results, the InvS null mutant strain secreted dramatically reduced levels of SipA, SipB, and SipC (**Fig. 4A and 4B**). In contrast, the expression of SipA, SipB, and SipC in the cell-associated fraction was 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 the β-lactamase-based translocation assay using SipA-TEM1 fusion as a translocation reporter (34). The SipA-TEM1 fusion protein is 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 (34). As shown in **Fig. 4C and 4E**, SipA was translocated at a much lower level from

340 the Δ*invS* null mutant as compared to the wild-type *Salmonella*. These results
341 support the proteomics data and indicate that InvS is involved in type III effector
342 secretion and translocation during *Salmonella* infection.

343 **InvS controls the level of PrgH**

344 One of the possibilities that might lead to the decreased secretion of a group of type III
345 effectors is the dysfunction of the T3SS apparatus. A GFP-based plasmid assay is
346 available to study sRNA-mediated translational control, and for the verification of
347 potential sRNA targets (17). We used GFP translational fusions to determine
348 whether InvS can modulate levels of T3SS apparatus proteins: SpaO, InvA, PrgK, and
349 PrgH at the post-transcriptional level. The *Salmonella invS* null mutant strain was
350 transformed with a P_{BAD}-based sRNA expression vector (with or without InvS) and a
351 constitutive GFP fusion expression vector (pXG30) that carried the 5'UTR and the full
352 ORFs of SpaO, InvA, PrgK, and PrgH, translationally fused to the GFP. Expression
353 of the GFP fusion proteins were examined in the presence or absence of InvS.
354 While the levels of SpaO-GFP, InvA-GFP, and PrgK-GFP remain unchanged with and
355 without InvS, the PrgH-GFP level was decreased in the absence of InvS **(Fig. 5AB)**.
356 When the PrgH-GFP expressing plasmid was introduced into *E. coli*, no PrgH-GFP
357 was detected by Western blot (Data not shown). The lack of PrgH-GFP expression
358 could be because additional *Salmonella* factors may be required in maintaining higher
359 level of PrgH-GFP in *Salmonella*. We also transformed pXG30 expressing

PrgH-GFP into the WT and Δ*invS* strains and examine the expressions of the fusion proteins by Western blot. We detected a lower amount of PrgH-GFP in Δ*invS,* which further confirmed that InvS function to up-regulate *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 post-transcriptional level. To investigate whether 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 to 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 overexpression of *prgH* in Δ*invS* suggests that InvS may influence additional target genes involved in *Salmonella* invasion.

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 less

amount of FimZ-GFP when InvS is co-expressed from a plasmid, p*invS*. Interestingly, the difference in FimZ-GFP level disappeared when the same plasmids were co-expressed in the *E. coli* background **(Fig. 6AB)**. 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. Expression of the GFP fusion proteins was also examined in the WT and Δ*invS* mutant strain. We found that the level of FimZ-GFP is higher in the Δ*invS* compared to 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 preformed gentamicin protection assay to examine if alteration of FimZ level is able to work against the effect of InvS and rescue the invasion phenotype of Δ*invS* . We found the double deletion Δ*fimZ*Δ*invS* strain showed a similar invasion level as compared to the Δ*fimZ* strain. Overexpression of FimZ in the wild-type strain resulted in a decrease in invasion rate (**Fig. 7**). Western blot confirmed the decrease of flagella in the Δ*invS* strain compared to that in the wild-type strain (**Fig. 8AB**), which is consistent with the result showing that deletion of *invS* impairs *Salmonella* motility (**Fig. 8C**). Overexpression of FimZ produces higher amount of FimZ than that in the *invS* deletion strain and drastically inhibited the flagellar gene expression (**Fig. 8**). Although the detailed mechanism remains unclear, our results suggest *fimZ* is an important regulatory component linking InvS

and its effect on flagellar expression and *Salmonella* invasion. InvS facilitates invasion in a *fimZ* and flagellar dependent manner.

DISCUSSION

Small RNAs represent a relatively new set of post-transcriptional regulatory molecules that are gaining increasing interest in bacteria. A few bacterial sRNAs are reported to regulate 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. Ryan *et al*. have demonstrated DsrA influences the acid tolerance response and virulence of *Salmonella* (14, 35, 36). Recently, hundreds of novel sRNAs have been identified in *Salmonella*, but few have been functionally characterized (8, 11, 14, 37). In an effort 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 non-phagocytic cells. InvS was originally identified by the Hfq-CoIP-Seq and showed a 2 to 47-fold enrichment under various stress conditions (8, 11). Colgan *et al*. performed 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 (38). The details of InvS regulatory pathway are not clear. It is known that PhoP/Q regulates both SPI-1 and SPI-2 expression while SsrA/B and OmpR/EnvZ is able to activate SPI-2

420 expression (39). It is not known if InvS play any roles in the cross-talk between
421 SPI-1 and SPI-2.

422 Many classical transcriptional factors are known to regulate *Salmonella* invasion by
423 controlling the transcription of invasion-related genes. For example, the transcription
424 of SPI-1 genes can be activated by HilA, HilC, HilD, InvF, and SirA (40). Our result
425 showed that overexpression of HilD and InvF was able to restore the invasion defect
426 of Δ*invS*. So far, there is no evidence to suggest that *hilD* or *invF* are the direct
427 target of InvS. Interestingly, we found overexpression of HilA failed to rescue the
428 invasion deficiency. This may indicate InvS is able to regulate invasion in a
429 HilD-dependent but HilA-independent pathway. Singer *et al.* have demonstrated that
430 HilD directly activates the expression of flagellar genes while HilA does not affect the
431 flagellar gene expression (41), this is consist with our data showing that deletion of
432 InvS results a decrease of flagellar expression. Furthermore, it was reported HilD is
433 able to activate the transcription of *invF* from a promoter that is far upstream of its
434 HilA-dependent promoter. Loss of *hilD* resulted a more severe effect on the
435 expression of a subset of SPI1 genes than the loss of *hilA* (6). Our data showed that
436 over-expressing HilD activate *invF* expression more profoundly than that from
437 overexpressing HilA. In addition, It is also possible that additional factors might be
438 involved in InvS-mediated regulation of invasion. InvS may have multiple targets,
439 which might balance out the effect of HilA over-expression. This may eaplain why
440 over expressing only *hilA* is not sufficient to restore the invasion defect of *invS* mutant.

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

455 Our proteomic analysis showed higher levels of FimZ in the absence of InvS. FimZ
456 is known as a transcriptional activator responsible for promoting the expression of
457 type I fimbriae and down regulating flagella synthesis (42). While fimbriae are known
458 to play a role in adhering to infected cells, flagella have been associated with
459 *Salmonella* motility and invasion. It has been reported that flagella-driven motility
460 forces the bacterium into a “near surface swimming” mode, which promotes
461 *Salmonella* invasion by “scanning” the host cell surface (33). Our proteomics

462 analysis indicated an increase in *fimZ* and a decrease in *fliC* expression in the
463 absence of InvS. Deletion of *invS* impairs *Salmonella* motility, suggesting that InvS
464 might function to promote motility to facilitate bacterial invasion. Consistent with this
465 notion, previous reports found that HilD activates the transcription of flagella genes
466 while HilA does not (41). Our data showed that overexpression of HilD rescued the
467 Δ*invS* invasion defect while HilA did not alter the invasion levels. This is in
468 agreement with our data showing that uncontrolled or overexpression of *fimZ* (in the
469 absence of InvS) leads to a decrease in invasiveness. Taken together, we conclude
470 that InvS coordinates the increase in PrgH and decrease in FimZ leading to more
471 efficient *Salmonella* invasion (**Fig. 9**).

472 The exact mechanism of how InvS activates the expression of PrgH and reduces the
473 expression of FimZ is currently unclear. Corcoran *et al*. have established the
474 GFP-based plasmid assay for validation of sRNA-mediated target regulation (17).
475 When tested in *E. coli*, we failed to detect any PrgH-GFP by Western blot, suggesting
476 that additional factors present in *Salmonella* might be involved in maintaining the
477 stability of PrgH. Interestingly, our results indicate that InvS down regulate *fimZ*
478 expression in *Salmonella* but not in *E.coli*. The regulation of *fimZ* and *prgH*
479 expression is remarkably complex. It is known that *prgH* is under the regulation of
480 many global regulators such as HilA, InvF, PhoP and SirA. Furthermore, Bailey *et al.*
481 have shown that *prgH* and other SPI-1 genes are expressed at higher levels in a
482 *ramA* mutant (43). Previous work showed FimY acts upstream of FimZ to activate

483 the *fim* operon, while FliZ functions to repress FimZ posttranscriptionally (44). In
484 addition, FimW and FimZ form a coupled feedback loop where they activate their own
485 and each other's expression. Recently, it has been reported that the two-component
486 system PhoBR is also capable of inducing *fimZ* expression (45). Thus, it is possible
487 that additional factors (from *Salmonella*) may be required for InvS to regulate *fimZ* and
488 *prgH* expression. Furthermore, Chao *et al*. showed InvS is associated with Hfq
489 based on their co-immunoprecipitation experiments (8). Ansong *et al*. detected a
490 decrease of FimZ in the Δ*hfq Salmonella* (22). In addition, previous studies
491 suggested that cellular RNAs compete for Hfq, and one abundant sRNA can indirectly
492 impact the targets of others by disrupting Hfq-mediated effects (46, 47). It is also
493 possible that InvS indirectly regulate *fimZ* expression through disrupting the binding of
494 Hfq to *fimZ* or other RNAs that targets *fimZ* and *prgH*.

495 To date, only a fraction of published sRNAs has been functionally characterized and
496 their roles in bacterial virulence have only been elucidated for a few. We showed
497 that InvS functions to positively regulate *prgH* expression, and negatively regulate
498 *fimZ* expression, which led to more efficient *Salmonella* invasion. The InvS is highly
499 conserved at the DNA sequence level in all *Salmonella enterica* serovars, including
500 Typhimurium, Newport, Typhi, Paratyphi and Enteritidis (48). This pattern of
501 conservation is consistent with the involvement of InvS in SPI1-mediated invasion
502 throughout the *Salmonella enterica* species. Our study expands the known
503 sRNA-mediated regulatory network of *Salmonella*. Additional work on the remaining

sRNAs and other regulatory factors will likely form a coordinated regulatory network revealing the intricate regulation of virulence factors in *Salmonella*.

Data availability

The raw proteomic data were deposited in the public repository PRIDE under accession numbers PXD003589 and PXD003590.

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672 **Figure legends**

673 **Fig. 1.** InvS is essential for *Salmonella* invasion. (**A**) HeLa cells were infected with
674 *Salmonella* strains for 15min at an MOI of 10. Relative bacterial invasion was
675 determined by the gentamicin protection assay as described in the experimental
676 procedures. Invasion rate of the wild-type strain was defined as 100%. The data
677 are the averages of three independent experiments with error bars indicate as
678 standard deviations. (**B**) HeLa cells were infected with *Salmonella* for 15min at an
679 MOI of 10. Actin staining was conducted as described in experimental procedures to
680 indicate *Salmonella* induced ruffling formation. (**C**) Percentage of infected cells with
681 ruffles was calculated. The data shown were obtained from three independent
682 experiments. Error bars indicate standard deviations. *P*-value was calculated
683 using the Student's *t*-test.

684 **Fig. 2.** Validation of the InvS transcript. (**A**) Northern Blot analysis identified the
685 transcript to be 89nt in length. Arrow: InvS, 89nt. Arrowhead: STM0082-STn470
686 mRNA, 400nt. (**B**) Schematic diagram showing that InvS sRNA is encoded in the
687 3'UTR of STM0082.

688 **Fig. 3.** Overexpression of *hilD* or *invF* rescues the Δ*invS* invasion defect. (**A**) HeLa
689 cells were infected with indicated *Salmonella* strains for 15 min at an MOI of 10.
690 Relative bacterial invasion was determined by the gentamicin protection assay. The
691 data shown were obtained from three independent experiments. Error bars indicate

692 standard deviations. *P*-value was calculated using the Student's *t*-test. (**B**) HeLa
693 cells were infected with indicated *Salmonella* strains for 15 min at an MOI of 10.
694 Relative bacterial invasion was determined by the gentamicin protection assay.
695 Error bars indicate standard deviations. *P*-value was calculated using the Student's
696 *t*-test. (**C**) InvS does not change the expression levels of *hilD* or *invF*. The *lacZ*
697 reporter gene was placed under transcriptional control of *hilD* or *invF* promoters into
698 either the wild-type or the Δ*invS* mutant *Salmonella* strains. β-galactosidase activity
699 assay was measured following the description in experimental procedures. The data
700 shown were obtained from three independent experiments. Results were presented
701 as the mean in Miller units. Error bars indicate standard deviations. (**D**) InvS does
702 not change the expression of *hilD-gfp* or *invF-gfp*. The 5'UTR along with the full
703 ORFs of HilD and InvF were translationally fused to GFP. Plasmids expressing
704 HilD-GFP or InvF-GFP were co-transformed with plasmids expressing InvS or vector
705 control as indicated. HilD-GFP or InvF-GFP was detected by Western blot with
706 polyclonal anti-GFP antibodies. Bacterial ICDH was similarly detected using
707 anti-ICDH polyclonal antibodies as the loading control.

708 **Fig. 4.** InvS regulates *Salmonella* effector secretion and translocation. (**A**) The
709 expression and secretion of invasion-related effectors in *Salmonella* strains including
710 the WT, Δ*invS,* Δ*invS*p*invS and* Δ*invA*. Bacterial strains were grown under SPI-1
711 inducing conditions and equal amounts of bacterial lysates or culture supernatants
712 were analyzed by Western blot. (**B**) Quantification of protein expression in panel A.

713 Protein levels in the WT strain was defined as 1. Values represent relative protein
714 levels after normalization with the expression in WT. Data are representative of
715 three experiments. (**C**) HeLa cells were infected with various *Salmonella* strains
716 carrying plasmid expressing SipA-TEM fusion protein. 15min post infection, cells
717 were loaded with CCF4-AM, incubated at room temperature for 2h. The translocation
718 efficiency was evaluated under a fluorescence microscopy. (**D**) Western blot
719 showing the expression of SipA-TEM in different strains. (**E**) Quantification of
720 SipA-TEM translocation. Percentage of blue cells was used to measure the
721 translocation efficiency. The data shown were obtained from three independent
722 experiments. Standard deviations are shown. *P*-value was calculated using the
723 Student's *t*-test.

724 **Fig. 5.** InvS regulates the level of PrgH. (**A**) InvS up regulates *prgH-gfp* expression
725 in *Salmonella*. The 5'UTR along with the full ORFs of SpaO, InvA, PrgK, and PrgH
726 were translationally fused to GFP. Plasmids expressing the GFP fusion proteins
727 were co-transformed with plasmids expressing InvS or vector control into Δ*invS.*
728 The background strain eliminates the potential effect that can be caused by
729 chromosomal *invS*. Bacterial ICDH (Isocitrate dehydrogenase) was detected using
730 anti-ICDH polyclonal antibodies as the loading control. The levels of GFP fusion
731 proteins were determined by Western blot with polyclonal anti-GFP antibodies.
732 Arrow represents the expression of PrgH-GFP. (**B**) Quantification of GFP fusion
733 proteins expression from three independent experiments. Values represent GFP

fusion protein expression level after normalization to the expression of ICDH. *P*-value was calculated using the Student's *t*-test. (**C**) The expression of *prgH-gfp* was decreased in the absence of InvS. pXG30-derived PrgH-GFP was transformed in to WT or Δ*invS* strain. Western blot of the two fold dilution series showing the expression of *prgH-gfp.* (**D**) InvS does not regulate the transcriptional level of *prgH*. Promotorless *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 the experimental procedures. *Salmonella* WT strain without *lacZ* was used as the negative control. The data shown were obtained from three independent experiments. Results were presented as the mean in Miller units. Error bars indicate standard deviations. (**E**) Overexpression of *prgH* partially restores the Δ*invS* invasion defect. HeLa cells were infected with *Salmonella* for 15min at an MOI of 10. Relative bacterial invasion was determined by the gentamicin protection assay. The data shown were obtained from three independent experiments. *P*-value was calculated using the Student's *t*-test.

Fig. 6. InvS regulates the level of FimZ. (**A**) InvS down regulates *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 co-transformed 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

755 determined by Western blot with polyclonal anti-GFP antibodies. (**B**) Quantification of
756 GFP fusion proteins from three independent experiments. Values represent GFP
757 fusion protein levels after normalization with that of ICDH. Data are representative of
758 three experiments. *P*-value was calculated using the Student's *t*-test. (**C**) The
759 expression of FimZ-GFP was decreased in the presence of InvS. pXG30-derived
760 FimZ-GFP was transformed into the WT or Δ*invS* strain. Western blot of the two fold
761 dilution series showing a decrease of FimZ-GFP level in the presence of InvS.

762 **Fig. 7.** FimZ down regulate *Salmonella* invasion. HeLa cells were infected with
763 *Salmonella* for 15min at an MOI of 10. Relative bacterial invasion was determined
764 by the gentamicin protection assay. The data shown were obtained from three
765 independent experiments. *P*-value was calculated using the Student's *t*-test.

766 **Fig. 8.** InvS regulates flagella expression. (**A**) InvS up regulates the expression of
767 *fliC*. The expressions of FliC protein was detected by Western blot with monoclonal
768 anti-FliC antibodies. Bacterial ICDH was detected using anti-ICDH polyclonal
769 antibodies as the loading control. (**B**) Quantification of FliC expression. FliC
770 expression in WT strain was defined as 1. Values represent relative protein
771 expression level after normalization with that in WT. Data are representative of three
772 experiments *P*-value was calculated using the Student's *t*-test. (**C**) **2**μl of
773 *Salmonella* culture (optical density at 600 nm, 1.0) was inoculated onto the LB plates
774 made up of 0.3% Bacto agar (Difco) and grown at 37°C. Photos were taken 6h post

775 inoculation. (**D**) Halos around the colonies were measured after 6 h of incubation at
776 37°C. Data are representative of three experiments. P-value was calculated using
777 the Student's t-test.

778 **Fig. 9.** Model for the InvS-mediated *Salmonella* invasion. InvS facilitates *Salmonella*
779 effector secretion and translocation by positively regulating *prgH*, which encode a
780 type III secretion apparatus protein. Furthermore, InvS negatively regulates *fimZ*, a
781 global regulator that is known to repress *Salmonella* SPI-1 gene expression by
782 activating HilE. FimZ activate FimA, which encodes the major fimbrial unit. FimZ
783 also negatively regulates flagella synthesis through repressing flagellar master
784 regulator FlhDC expression. The regulation of these target mRNAs or proteins by
785 InvS, in turn, promotes *Salmonella* to invade host cell. Arrows represent activation
786 while the flat arrows represent inhibition. Dotted lines indicate indirect regulation.
787 The symbol “?” indicates unknown factors or signaling cascade that may be involved
788 in the pathway.

Fig.1

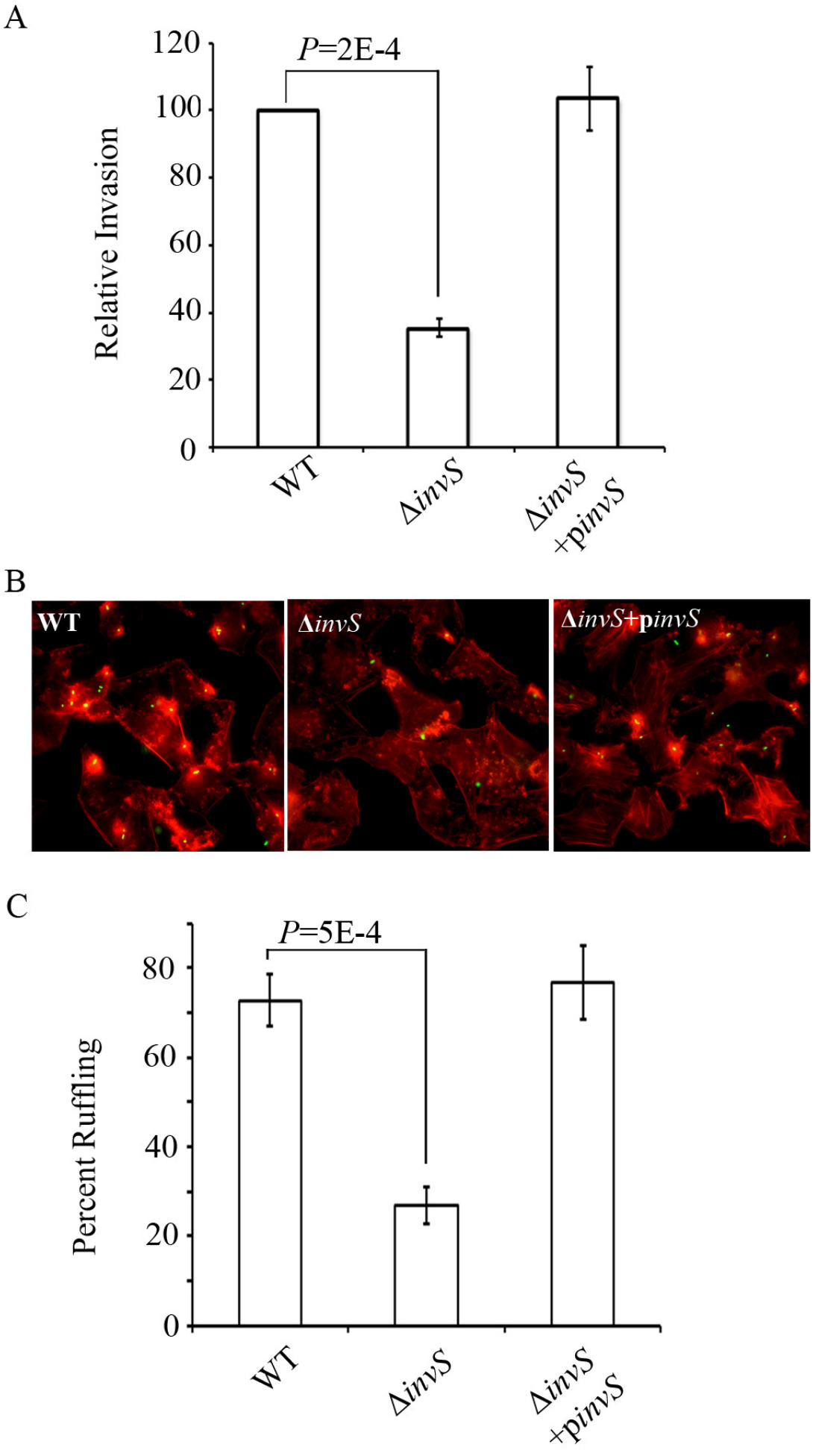

A
120
100
80
60
40
20
0
Relative Invasion
P=2E-4
WT
ΔinvS
ΔinvS +pinvS
B
WT
ΔinvS
ΔinvS+pinvS
C
80
60
40
20
0
Percent Ruffling
P=5E-4
WT
ΔinvS
ΔinvS +pinvS

Fig.2

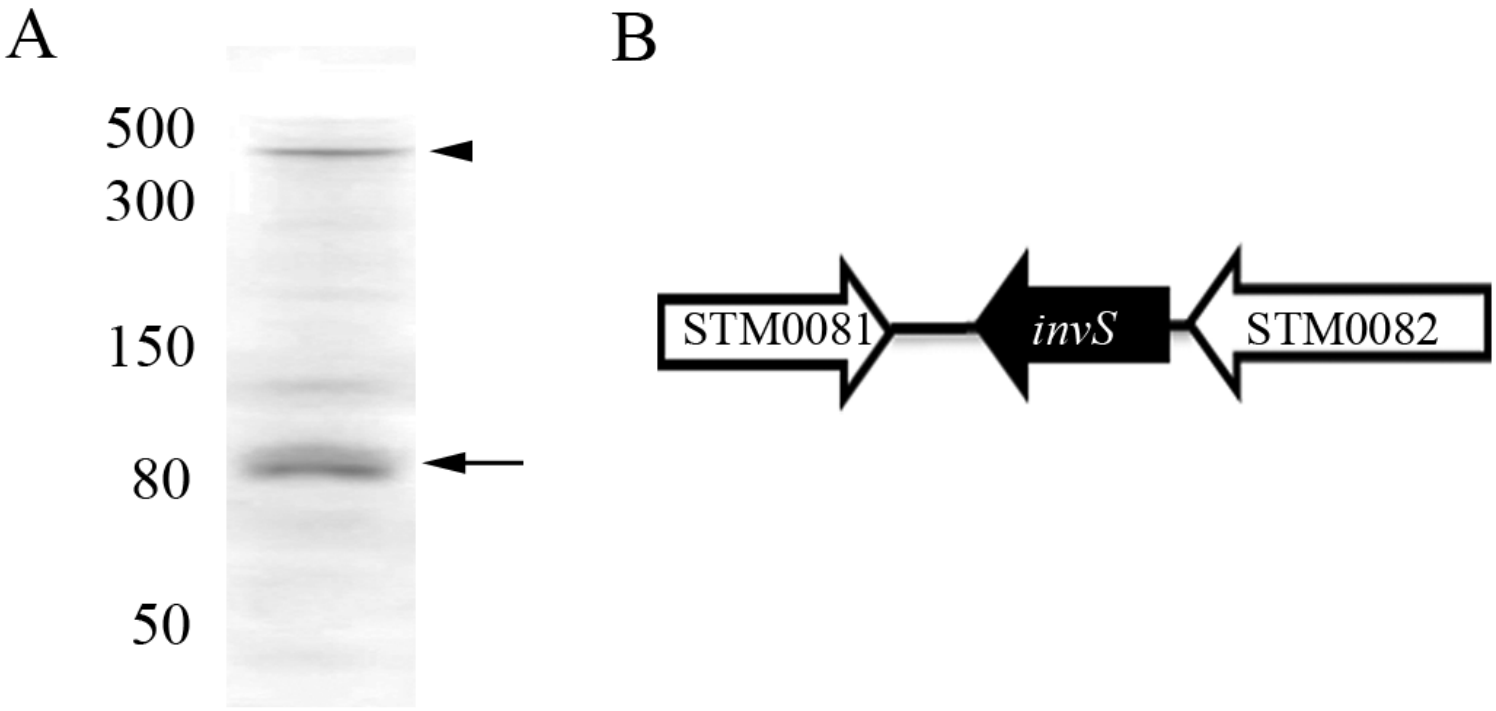


Fig.3

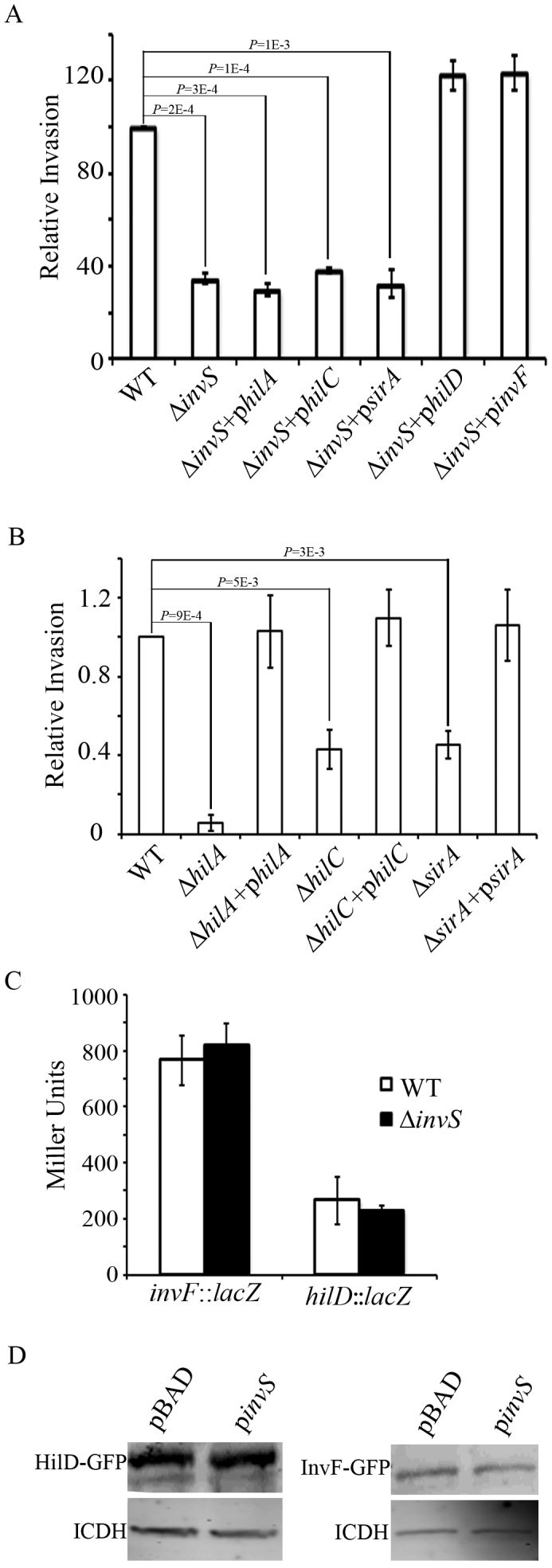

A
P=1E-3
P=1E-4
P=3E-4
P=2E-4
Relative Invasion
120
80
40
0
WT
ΔinvS
ΔinvS+philA
ΔinvS+philC
ΔinvS+psirA
ΔinvS+philD
ΔinvS+pinvF
B
P=3E-3
P=5E-3
P=9E-4
Relative Invasion
1.2
0.8
0.4
0
WT
ΔhilA
ΔhilA+philA
ΔhilC
ΔhilC+philC
ΔsirA
ΔsirA+psirA
C
Miller Units
1000
800
600
400
200
0
WT
ΔinvS
invF::lacZ
hilD::lacZ
D
pBAD
pinvS
HilD-GFP
ICDH
pBAD
pinvS
InvF-GFP
ICDH

Fig.4

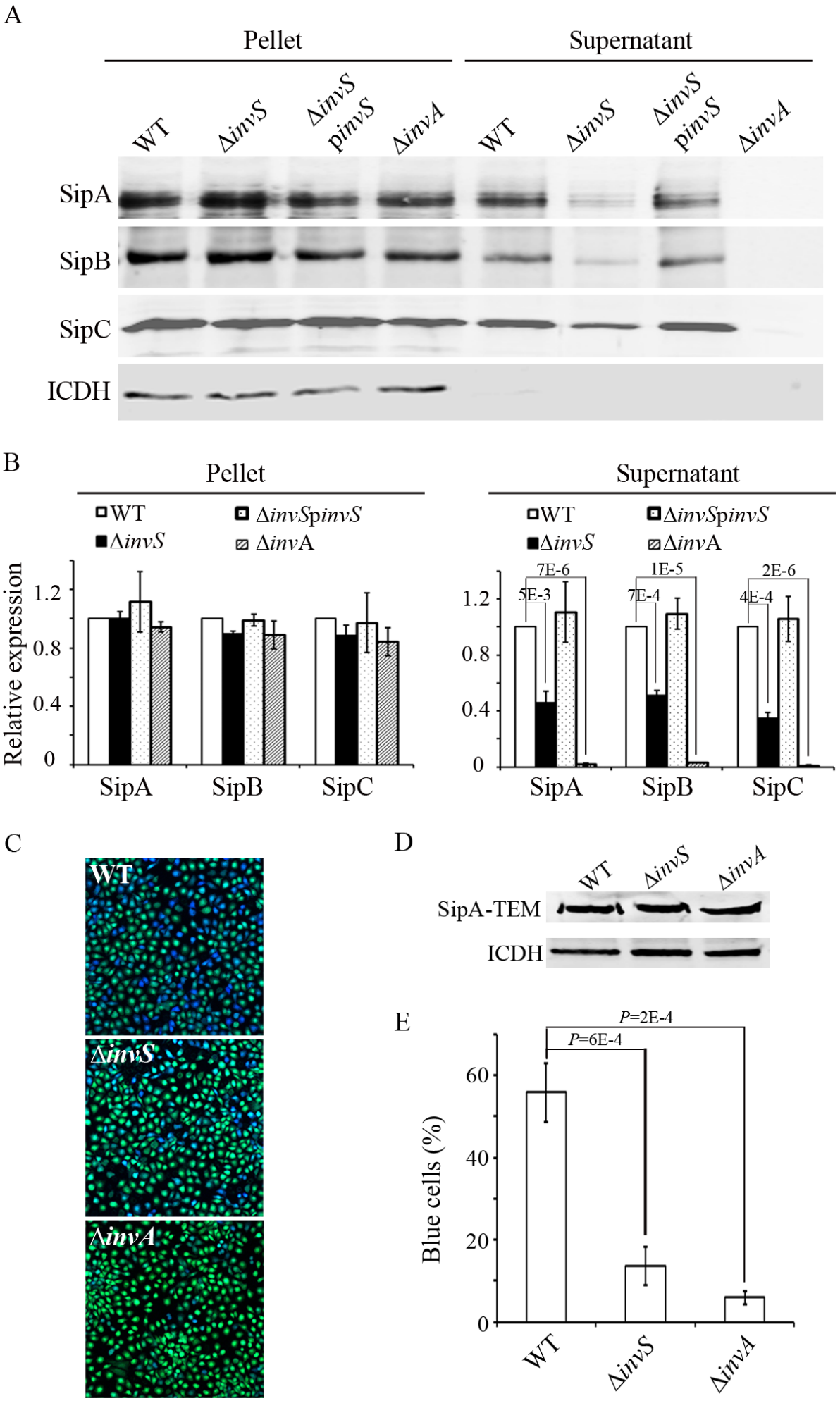

A
Pellet
Supernatant
WT
ΔinvS
ΔinvS pinvS
ΔinvA
SipA
SipB
SipC
ICDH
B
Pellet
Supernatant
WT
ΔinvSpinvS
ΔinvS
ΔinvA
Relative expression
1.2
0.8
0.4
0
7E-6
5E-3
1E-5
7E-4
2E-6
4E-4
SipA
SipB
SipC
C
WT
ΔinvS
ΔinvA
D
WT
ΔinvS
ΔinvA
SipA-TEM
ICDH
E
P=2E-4
P=6E-4
Blue cells (%)
60
40
20
0
WT
ΔinvS
ΔinvA

Fig.5

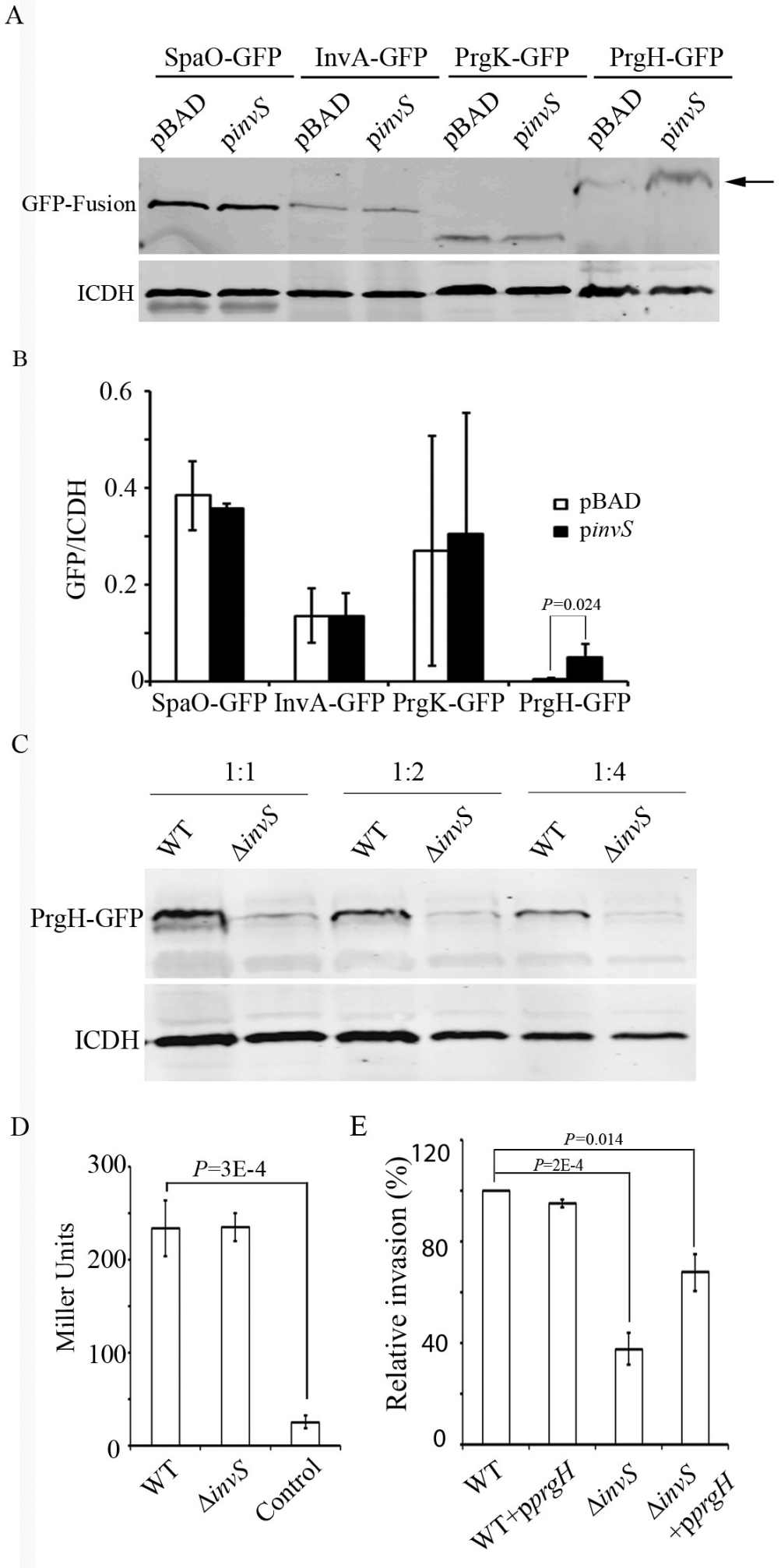

A
SpaO-GFP
InvA-GFP
PrgK-GFP
PrgH-GFP
pBAD
pinvS
GFP-Fusion
ICDH
B
GFP/ICDH
0.6
0.4
0.2
0
pBAD
pinvS
P=0.024
SpaO-GFP InvA-GFP PrgK-GFP PrgH-GFP
C
1:1
1:2
1:4
WT
ΔinvS
PrgH-GFP
ICDH
D
Miller Units
300
200
100
0
P=3E-4
WT
ΔinvS
Control
E
Relative invasion (%)
120
80
40
0
P=0.014
P=2E-4
WT
WT+pprgH
ΔinvS
ΔinvS +pprgH

Fig.6

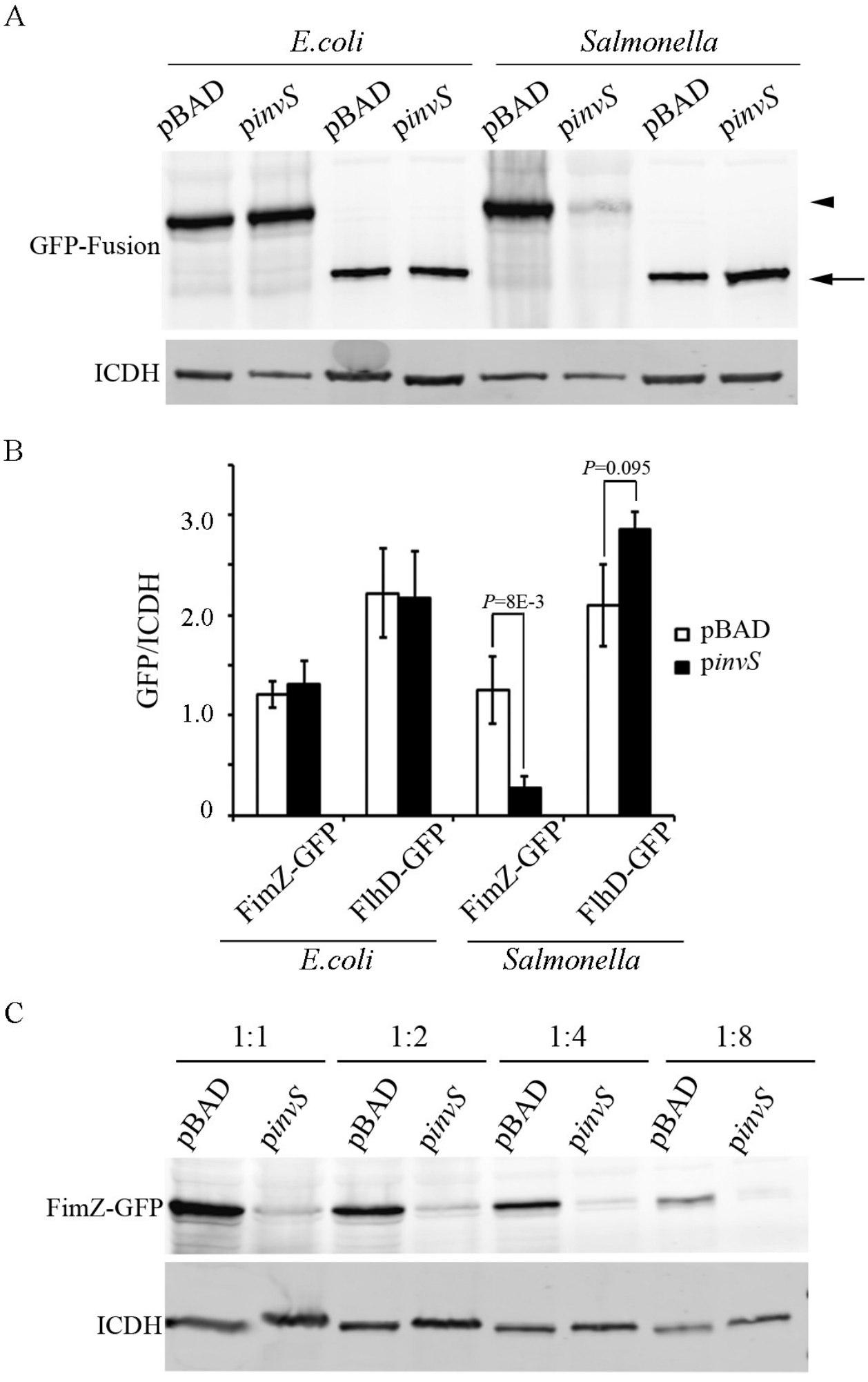

A
E.coli
Salmonella
pBAD
pinvS
pBAD
pinvS
pBAD
pinvS
pBAD
pinvS
GFP-Fusion
ICDH
B
P=0.095
P=8E-3
3.0
2.0
1.0
0
GFP/ICDH
pBAD
pinvS
FimZ-GFP
FlhD-GFP
FimZ-GFP
FlhD-GFP
E.coli
Salmonella
C
1:1
1:2
1:4
1:8
pBAD
pinvS
pBAD
pinvS
pBAD
pinvS
pBAD
pinvS
FimZ-GFP
ICDH

Fig.7

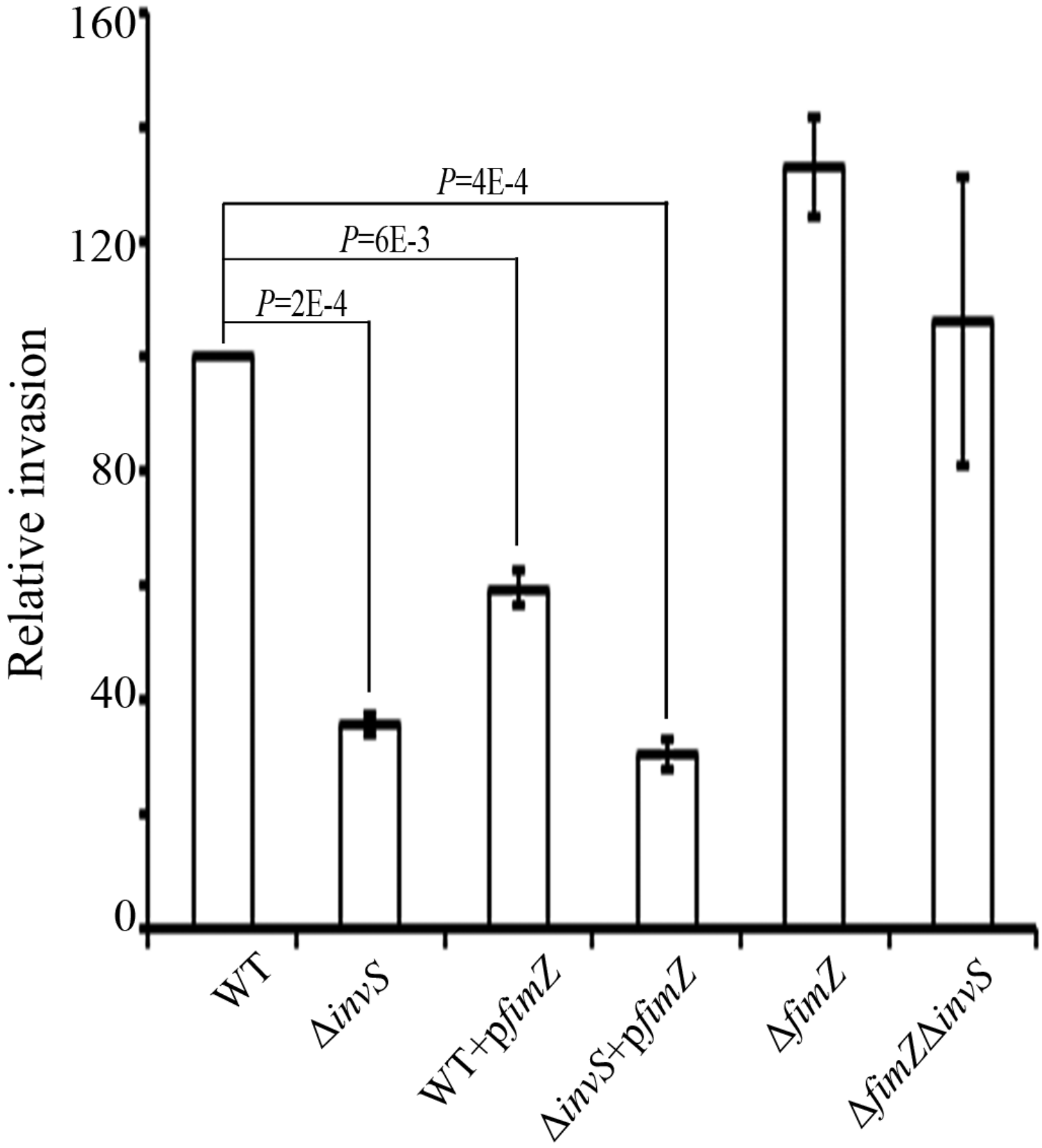
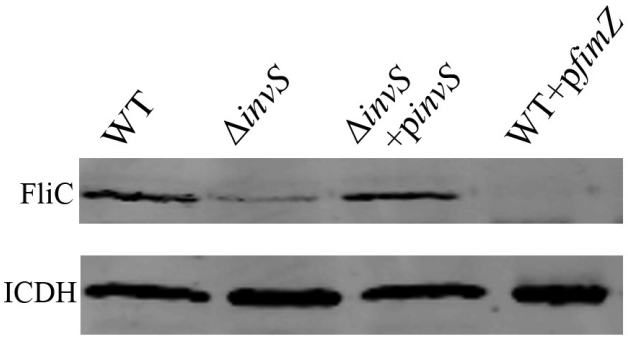
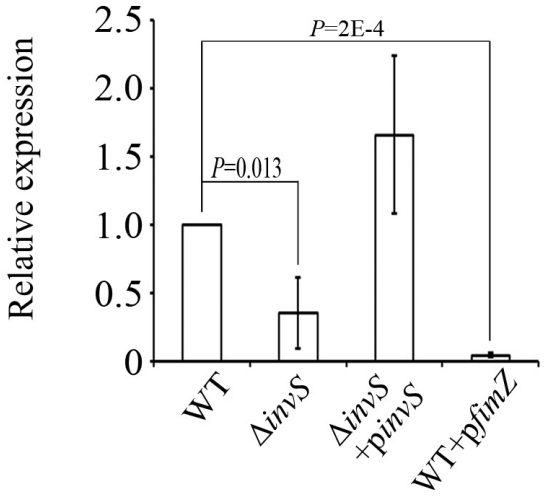

160
120
80
40
0
Relative invasion
P=4E-4
P=6E-3
P=2E-4
WT
ΔinvS
WT+pfimZ
ΔinvS+pfimZ
ΔfimZ
ΔfimZΔinvS

Fig.8

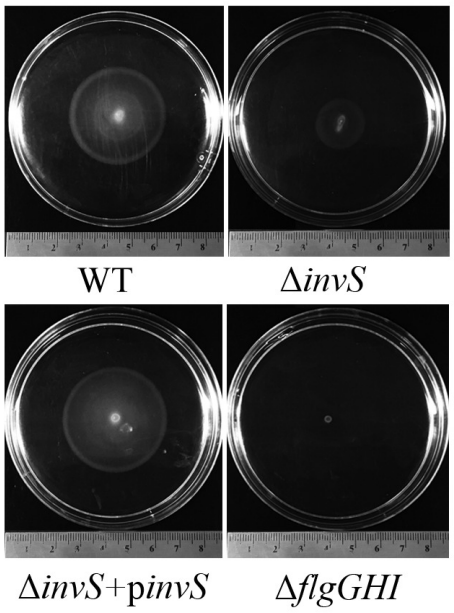
A


WT
ΔinvS
ΔinvS +pinvS
WT+pfimZ
FliC
ICDH

B


Relative expression
2.5
2.0
1.5
1.0
0.5
0
P=2E-4
P=0.013
WT
ΔinvS
ΔinvS +pinvS
WT+pfimZ

C


WT
ΔinvS
ΔinvS+pinvS
ΔflgGHI

D

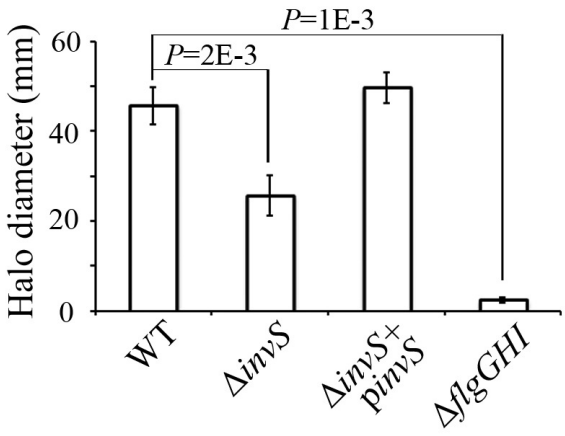

Halo diameter (mm)
60
40
20
0
P=1E-3
P=2E-3
WT
ΔinvS
ΔinvS+ pinvS
ΔflgGHI

Fig.9

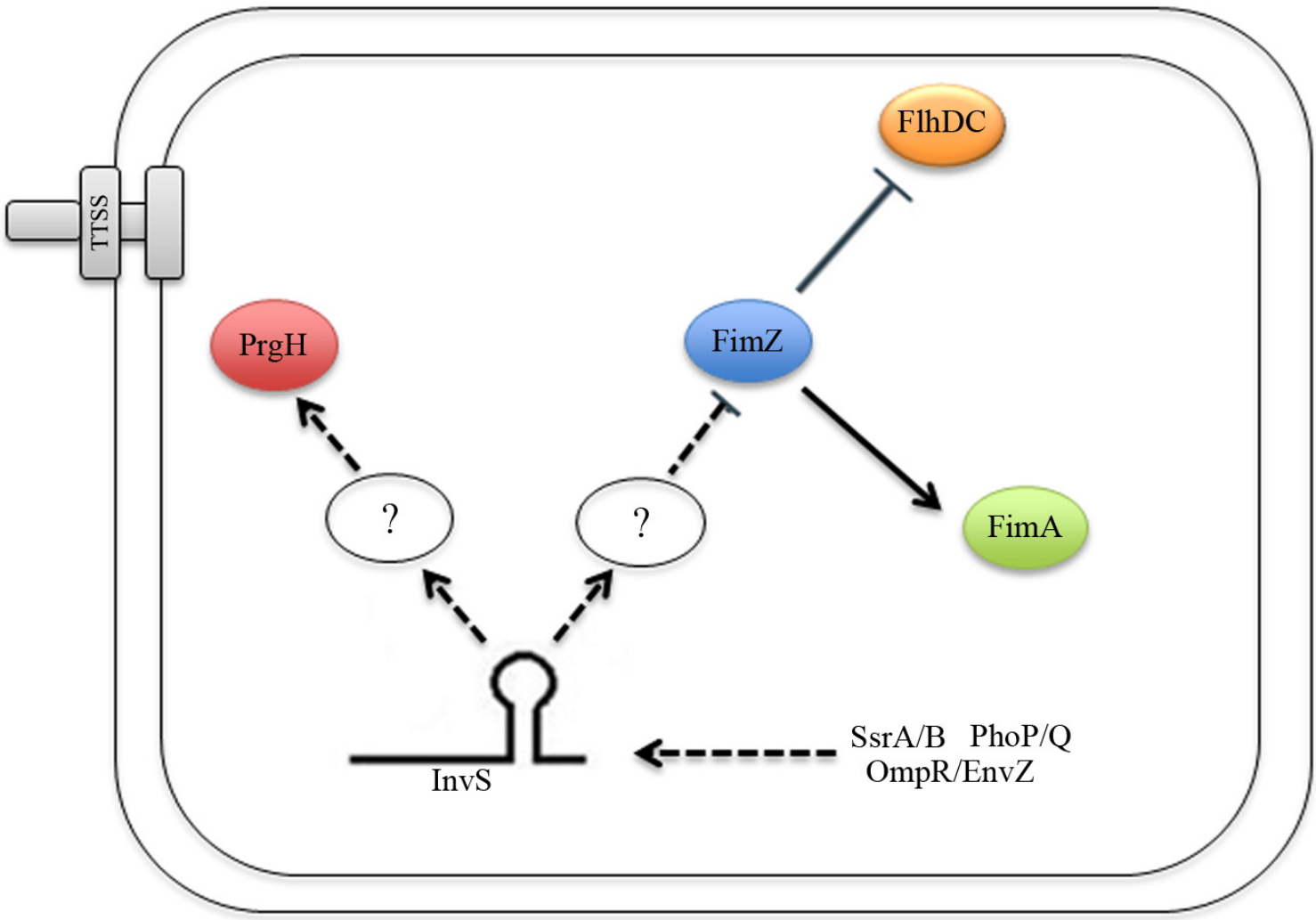


Fig. S1

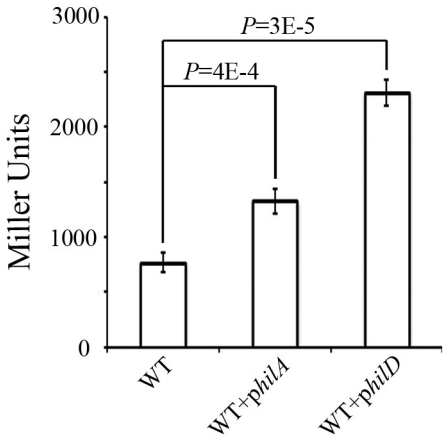


Fig. S1. HilD activates invF transcription more profoundly than HilA. The lacZ reporter gene was placed under the transcriptional control of invF promoters into different Salmonella strains. β-galactosidase activity assay was measured following the description in the experimental procedures. Data were obtained from three independent experiments. Results were presented as the mean in Miller units. Error bars indicate standard deviations.