A *Salmonella* Small Non-Coding RNA Facilitates Bacterial Invasion and Intracellular Replication by Modulating the Expression of Virulence Factors

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**Abstract**

Small non-coding RNAs (sRNAs) that act as regulators of gene expression have been identified in all kingdoms of life, including microRNA (miRNA) and small interfering RNA (siRNA) in eukaryotic cells. Numerous sRNAs identified in *Salmonella* are encoded by genes located at *Salmonella* pathogenicity islands (SPIs) that are commonly found in pathogenic strains. Whether these sRNAs are important for *Salmonella* pathogenesis and virulence in animals has not been reported. In this study, we provide the first direct evidence that a pathogenicity island-encoded sRNA, IsrM, is important for *Salmonella* invasion of epithelial cells, intracellular replication inside macrophages, and virulence and colonization in mice. IsrM RNA is expressed *in vitro* under conditions resembling those during infection in the gastrointestinal tract. Furthermore, IsrM is found to be differentially expressed *in vivo*, with higher expression in the ileum than in the spleen. IsrM targets the mRNAs coding for SopA, a SPI-1 effector, and HilE, a global regulator of the expression of SPI-1 proteins, which are major virulence factors essential for bacterial infection. Mutations in *isrM* result in deregulation of expression of HilE and SopA, as well as other SPI-1 genes whose expression is regulated by HilE. *Salmonella* with deletion of *isrM* is defective in bacteria invasion of epithelial cells and intracellular replication/survival in macrophages. Moreover, *Salmonella* with mutations in *isrM* is attenuated in killing animals and defective in growth in the ileum and spleen in mice. Our study has shown that IsrM sRNA functions as a pathogenicity island-encoded sRNA directly involved in *Salmonella* pathogenesis in animals. Our results also suggest that sRNAs may represent a distinct class of virulence factors that are important for bacterial infection *in vivo*.

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**Introduction**

*Salmonella* (e.g. *S. enterica* serovars Typhimurium and Enteritidis) is the leading cause of food-borne illnesses in the United States, causing diverse diseases ranging from mild, self-limiting gastroenteritis to life-threatening systemic infection [1]. As a facultative intracellular pathogen, *Salmonella* invades non-phagocytic cells such as intestinal epithelial cells and replicates in phagocytes during systemic infection. Two hallmarks of *Salmonella* pathogenesis, i.e. host invasion and intracellular proliferation, correlate with the genes in *Salmonella* pathogenicity islands (SPIs), which are distinct, relatively large chromosomal regions harboring virulence genes and are commonly found in pathogenic strains [2,3]. For example, *Salmonella* pathogenicity island 1 (SPI-1) contains invasion genes, while *Salmonella* pathogenicity island 2 (SPI-2) contains genes required for intracellular survival and replication [4,5,6]. Both SPI-1 and SPI-2 encode type III secretion systems (T3SS), which are specialized organelles that deliver effector proteins to the cytosol of host cells [3,7]. The T3SS apparatus is a needle-like structure that spans the inner and outer membranes of the bacterial envelope and penetrates host cell membranes. Through T3SS *Salmonella* secretes translocon proteins that allow the delivery of effector proteins into eukaryotic cells [3,7], leading to modulation of host cells and immune responses, and promotion of bacterial pathogenesis [4,5,6]. Highly regulated expression of the genes in SPIs and those encoding their effector proteins is observed both *in vitro* and *in vivo* and is required for bacterial pathogenesis and infection [6,8].

The regulation of expression of genes coding for SPI-1 proteins and its effectors is remarkably complex and is yet to be fully characterized [8]. For example, the expression of some genes coding for SPI-1 proteins, including SopA, has been shown to be induced upon invasion of both macrophages and epithelial cells and at the late stage of *Salmonella* infection in animals [9,10,11,12,13]. These results suggest that in addition to their generally recognized roles in invasion, the SPI-1 factors may play an important role post-invasion, possibly in intracellular replication/survival.

Small non-coding RNAs (sRNAs) that act as regulators of gene expression have been identified in all kingdoms of life, including microRNA (miRNA) and small interfering RNA (siRNA) in eukaryotic cells [14,15,16,17]. The bacterial sRNAs are generally...
Expression of isrM during Salmonella infection in vivo

Both immunocompetent BALB/c mice and immunodeficient SCID mice were infected intraperitoneally to study IsrM expression during systemic bacterial infection. Immunodeficient animals, such as the CB17 SCID mice that lack functional T and B lymphocytes, are extremely susceptible to Salmonella infection [12,13]. Analysis of bacterial growth in these mice can be used for comparing the virulence and studying the pathogenesis of different bacterial strains and mutants [12,13]. At different time points postinfection, the spleen and ileum were harvested. Quantitative RT-PCR was carried out to determine the expression of IsrM RNA in Salmonella isolated from the tissues, using the expression of bacterial 16S rRNA as the internal control (Figure 2D). Normalization of samples was also carried out by using total RNAs extracted from the same colony forming unit (CFU) (e.g. 5 x 10^3 CFU) of bacteria. Similar amounts of 16S rRNA were detected from same CFU of bacterial strains and mutants [12,13]. At different time points comparing the virulence and studying the pathogenesis of different Salmonella serovars isolated from the tissues, using the expression of its oral regulator as an internal control (Figure 2A-C).

During enteric infection, Salmonella needs to survive in the stomach, which is acidic, before establishing colonization in the intestine, which is relatively basic. Quantitative RT-PCR assays showed that IsrM had higher expression at pH3.0, pH4.4, and pH7.2, with the highest expression at pH3.0, compared to that at pH7.0 (Figure 2A). These results were further confirmed using Northern blot analyses. For example, using the level of 5S RNA as a control (Figure 2A–C).

Author Summary

Regulated expression of virulence factors is essential for infection by human pathogens such as Salmonella. Small non-coding RNA (sRNA) that act as regulators of gene expression have been identified in all kingdoms of life, and many sRNAs in Salmonella are encoded by genes located at the pathogenicity islands known to be involved in pathogenesis of Salmonella in vivo. Our study suggests that sRNA may function as a distinct class of virulence factors that significantly contribute to bacterial infection in vivo. Furthermore, our results raise the possibility of developing new strategies against bacterial infection by preventing the expression of regulatory sRNAs.
intraperitoneally or intragastrically) or time point postinfection (12–24 hours or 5–7 days) (data not shown), suggesting that the level of 16S rRNA was not significantly different in bacteria from the spleen and ileum.

Salmonella presumably trafficked to the ileum tissue via the lymphatic system in intraperitoneally-infected mice, while in intragastrically-infected mice, the bacteria could directly invade and infect the ileum tissue.

Salmonella isolated from both the spleen and ileum of SCID mice at 18 hours postinfection were found to express IsrM, with its expression in the ileum approximately 2.5-fold higher than that in the spleen (Figure 2D). To rule out the residual expression of IsrM from in vitro bacterial growth, mice were intraperitoneally infected with bacteria for a longer period and tissues were harvested at 7 days postinfection. We also found that IsrM was expressed in both organs, with its expression in the ileum approximately 2.5-fold higher than that in the spleen (Figure 2D). Similar results were also observed in Salmonella isolated from the organs of infected BALB/c mice. Taken together, these results suggest that IsrM is expressed during the early and late stages of Salmonella infection, and that it is differentially expressed, with higher expression in the ileum than in the spleen.

IsrM is dispensable for Salmonella growth in vitro but important for the expression of Salmonella SPI-1 proteins

To investigate the role of IsrM in Salmonella growth, a mutant, ΔIsrM, was derived from the wild type S. typhimurium 14028s strain by deleting the IsrM sequence. Mutant ΔIsrM grew as well as ST14028s in LB broth (Figure S2A)(Supporting Information), indicating that IsrM is dispensable for bacterial growth in vitro.

The unique presence of IsrM in a SPI region and its induced expression in the ileum suggests that IsrM may play a role in the infection acquired by the oral route. Spleens and ileums were collected and the bacteria were recovered at 18 hrs and 7 days postinfection. IsrM was detected in Salmonella isolated from both the spleen and ileum, with its level in the ileum approximately 2.5-fold higher than that in the spleen (Figure 2D). Similar results were also observed in Salmonella isolated from the organs of infected BALB/c mice. Taken together, these results suggest that IsrM is expressed during the early and late stages of Salmonella infection, and that it is differentially expressed, with higher expression in the ileum than in the spleen.

Figure 1. Alignment of isrM sequences of different Salmonella strains including the upstream promoter region, based on BLAST searches of the IsrM sequences in GenBank. The transcription start sites, −10 boxes, −35 boxes, and transcription terminators of isrM genes are boxed and in red [23]. The targeting sequences for SopA and HilE mRNAs that were identified in this study are boxed and in green.

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pathogenesis of *Salmonella* in the intestine. Since SPI-1 proteins are major virulence determinants essential for *Salmonella* invasion [3,7], we reasoned that IsrM may function to regulate the expression of SPI-1 factors, facilitating *Salmonella* infection and pathogenesis. To investigate this possibility, we examined the effect of a deletion of the IsrM sequence on the expression of SPI-1 factors. Bacterial strains T-invJ, T-sopE2, T-sptP, T-spaO, T-sipD, T-sipC, T-sipB, T-sipA, and T-sopB were previously derived from the wild type *Salmonella* strain ST14028s by inserting the FLAG epitope tag sequence into SPI-1 ORFs invJ, sopE2, sptP, spaO, sipD, sipC, sipB, sipA, and sopB, respectively [12,13]. Tagging of the SPI-1 ORFs in these mutants did not impair the invasiveness, growth, or the virulence of the bacteria, and the tagged strains can be used as model strains to study the expression of SPI-1 proteins and effectors [12,13]. We constructed mutants ΔM-T-invJ, ΔM-T-sopE2, ΔM-T-sptP, ΔM-T-spaO, ΔM-T-sipD, ΔM-T-sipC, ΔM-T-sipB, ΔM-T-sipA, ΔM-T-sopB, and ΔM-T-sopA from T-invJ, T-sopE2, T-sptP, T-spaO, T-sipD, T-sipC, T-sipB, T-sipA, T-sopB, and T-sopA, respectively, by deleting the IsrM sequence in each SPI-1 tagged strain (Table 1). These newly constructed isrM-deletion mutant strains grew as well as the parental strains and the wild type ST14028s in LB broth (Figure S2B)(Supporting Information), consistent with our results (Figure S2A)(Supporting Information) that IsrM is not essential for bacterial growth *in vitro*.

We performed Western analyses to determine the expression of the tagged SPI-1 proteins with an anti-FLAG antibody, using the expression of bacterial DnaK protein as the internal control (Figure 3A). Normalization of samples was also carried out by loading total protein extracted from the same CFU (e.g. $5 \times 10^7$ CFU) of bacteria in each lane. The levels of the tagged InvJ, SopE2, SptP, SpaO, SipD, SipC, SipB, SipA, and SopB proteins in the IsrM deletion strains were found to be lower than...
Table 1. Bacterial strains used in this study.

| Bacterial strains | Description | Reference/source |
|-------------------|-------------|------------------|
| ST14028S         | Wild type and parental strain | [50] |
| ΔisrM-P          | ST14028S isrM::kan, polar strain | This study |
| ΔisrM            | ST14028S, isrM deleted, without kanR | This study |
| T-invJ           | ST14028S invJ::1xFLAG | [13] |
| ΔM-T-invJ        | ST14028S invJ::1xFLAG isrM::kanR | This study |
| T-sipA           | ST14028S sipA::1xFLAG | [12] |
| ΔM-T-sipA        | ST14028S sipA::1xFLAG isrM::kanR | This study |
| T-sipB           | ST14028S sipB::1xFLAG | [12] |
| ΔM-T-sipB        | ST14028S sipB::1xFLAG isrM::kanR | This study |
| T-sipC           | ST14028S sipC::1xFLAG | [13] |
| ΔM-T-sipC        | ST14028S sipC::1xFLAG isrM::kanR | This study |
| T-sipD           | ST14028S sipD::1xFLAG | [13] |
| ΔM-T-sipD        | ST14028S sipD::1xFLAG isrM::kanR | This study |
| T-sopA           | ST14028S sopA::1xFLAG | [13] |
| ΔM-T-sopA        | ST14028S sopA::1xFLAG isrM::kanR | This study |
| T-sopB           | ST14028S sopB::1xFLAG | [13] |
| ΔM-T-sopB        | ST14028S sopB::1xFLAG isrM::kanR | This study |
| T-sopE2          | ST14028S sopE2::1xFLAG | [12] |
| ΔM-T-sopE2       | ST14028S sopE2::1xFLAG isrM::kanR | This study |
| T-spaO           | ST14028S spaO::1xFLAG | [12] |
| ΔM-T-spaO        | ST14028S spaO::1xFLAG isrM::kanR | This study |
| T-sptP           | ST14028S sptP::1xFLAG | [12] |
| ΔM-T-sptP        | ST14028S sptP::1xFLAG isrM::kanR | This study |
| T-hiE            | ST14028S hiE::1xFLAG | This study |
| ΔM-T-hiE         | ST14028S hiE::1xFLAG isrM::kanR | This study |
| E.coli Top10     | F- mcrAΔ(mrr-hsdRMS-mcrBC) q80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 gatU galK rpsL (StrR) endA1 napG | Invitrogen |
| E.coli Top 10 F’| F’[pACYC184 (TetrR) mcrAΔ(mrr-hsdRMS-mcrBC) Q80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 gatU galK rpsL endA1 napG | Invitrogen |

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those in the parental strains (Figure 3). In contrast, the protein level of SopA in the IsrM-deletion strain (i.e. ΔM-T-sopA) was found to be approximately 2.5-fold higher than that in the parental T-sopA strain (Figure 3A, compare lanes 1 and 2). Transformation of plasmid pIsrM, which contained the sequence of IsrM under its native promoter and transcriptional terminator sequence, in the deletion mutants restored the levels of the tagged proteins to the wildtype levels (Figure 3, lanes 3 and 6). Using 16S rRNA as the internal control, qRT-PCR assays showed that the deletion of IsrM increased the level of HilE (Figure 5, compare lane 1 and 4). Transformation of plasmid pIsrM carrying the IsrM sequence with the native promoter and transcriptional terminator caused translational repression [18,19,20].

HilE is a negative global regulator of the expression of many SPI-1 genes by sequestering HilD, a major SPI-1 transcriptional activator [8,35]. To determine if IsrM regulates hilE, we constructed a tagged hilE strain T-hiE by inserting the FLAG epitope tag sequence into hilE of ST14028s and furthermore, a corresponding isrM deletion mutant of T-hiE, ΔM-T-hiE (Table 1). Both T-hiE and ΔM-T-hiE grew as well as ST14028s in LB broth, and tagging of hilE did not impair the invasiveness, growth, or the virulence of the bacteria in mice (data not shown).

Using the expression level of DnaK as the internal control, Western blot analyses with an anti-FLAG antibody indicated that deletion of IsrM increased the level of HilE (Figure 3, compare lane 1 and 4). Transformation of plasmid pIsrM carrying the IsrM sequence with the native promoter and transcriptional terminator reduced the HilE expression to the wildtype level in ΔM-T-hiE (Figure 5, lane 2), suggesting that IsrM specifically modulates the protein level of HilE.

To investigate the potential interactions between IsrM and hilE mRNA, we used a two plasmid system [36] that involved co-

Table 1. Bacterial strains used in this study.
expression of the sRNA and a translational target gene with the FLAG fusion at its carboxyl terminus. By transforming both plasmids in *E. coli*, we tested whether the sRNA expression directly affects the protein level of the FLAG-tagged target gene. The 5′ UTR region and the entire coding sequence of *hilE* were cloned into a FLAG fusion vector. Transcription of the *HilE-FLAG* fusion

**Figure 3. Expression of the tagged proteins in *Salmonella***. (A–B) Western blot analyses of the expression of the tagged proteins from bacterial strains T-sopA and ΔM-T-sopA (A), and T-sopE2 and ΔM-T-sopE2 (B). The expression of bacterial DnaK was used as the internal control. Protein samples were separated in SDS-polyacrylamide gels and reacted with antibodies against the FLAG sequence and DnaK. Each lane was loaded with lysate prepared from 5 × 10⁷ CFU bacteria. (C) The effect of the deletion of the *isrM* sequence on the expression of SPI-1 proteins. The values of the relative expression, which are the means of triplicate experiments, represent the ratios of the levels of the tagged proteins in ΔM-T-invJ, ΔM-T-sopE2, ΔM-T-sptP, ΔM-T-spaO, ΔM-T-sipD, ΔM-T-sipC, ΔM-T-sipB, ΔM-T-sipA, ΔM-T-sopA, and ΔM-T-sopA to those in T-invJ, T-sopE2, T-sptP, T-spaO, T-sipD, T-sipC, T-sipB, T-sopA, and T-sopA, respectively.

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**Figure 4. Post-transcriptional targeting of *hilE* and *sopA* by *isrM***. (A–B) Schematic representation of the proposed interactions of *isrM* sRNA to *hilE* (A) and *sopA* mRNAs (B), and of compensatory base-pair changes. Numbers indicate relative position to the translational start site of *hilE* and *sopA* or position downstream of the transcriptional start site of *isrM*. Arrows denote nucleotide substitutions (in box) introduced to *isrM*, and *hilE* and *sopA* mRNAs. The SD and AUG sequences are highlighted in blue and red, respectively. (C–D) Western blot analysis of *E. coli* carrying pLaco-*IsrM*, pLaco-*H-IsrM* and pLaco-*S-IsrM*, in combination with either wild type or mutant target plasmids, as indicated. The expression of the tagged *HilE* (C) and *SopA* (D) proteins was determined using that of GroEL as the internal control.

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was driven by the constitutive PLtetO-1 promoter to specifically assay post-transcriptional regulation. The IsrM sequence was cloned under the control of the IPTG-inducible PLaco promoter, yielding construct pH-IsrM. E.coli transformed with both the hilE-FLAG fusion and pLaco-IsrM plasmid was grown and split into two cultures, only one of which was treated with IPTG. IsrM-dependent regulation was determined by Western blot analysis of the HilE-FLAG expression (Figure 4C). Our results showed that the expression of IsrM significantly reduced HilE-FLAG expression (Figure 4C, lanes 1 and 2).

To confirm the predicted base-pairing interactions between hilE mRNA and IsrM, point mutations were introduced to isRM (U264G265G266) and hilE (G263A264G265) to generate mutant H-IsrM and m-HilE (Figure 4A). The expression of HilE-FLAG was not repressed by mutant H-IsrM (Figure 4C, lanes 3 and 4), while IsrM did not significantly affect the expression of mutant m-HilE-FLAG (lanes 5 and 6). Repression of HilE expression by IsrM was restored using m-HilE-FLAG and H-IsrM, which contained the compensatory mutations (lanes 7 and 8).

To determine the degree of IsrM over-expression from the plasmids used and to correlate the level of IsrM expression with the levels of HilE protein, qRT-PCR assays were performed to determine the level of IsrM. Table S2 (Supporting Information) shows the relative levels of IsrM and the respective levels of SopA protein in the experiments. These results suggest that no significant difference in the expression levels of IsrM in the presence of IPTG was found in these experiments, and that base-pairing interactions between the specific sequences of IsrM (U165G166A167) and SopA mRNA (U264G265A266) were required for IsrM-mediated downregulation of SopA protein expression.

To further validate the interactions between IsrM and sopA mRNA, an additional pair of compensatory mutations was introduced to isRM (U159G160A161) and hilE (G159A160G161) to generate mutants S-IsrM and M-SopA, respectively (Figure 4B). Repression of M-SopA-FLAG protein was restored only by using S-IsrM, which contained compensatory mutations (data not shown). Taken together, these results suggest that IsrM inhibits SopA expression by binding to its mRNA around the SD sequence.

**Independent targeting of hilE and sopA mRNAs by IsrM in Salmonella**

Since HilE globally regulates the expression of most of SPI-1 proteins including SopA [8], targeting of either hilE or sopA mRNAs by IsrM is expected to modulate the SopA protein expression. Consistent with this notion, the expression of SopA in the isRM deletion mutant AM-T-SopA was suppressed to the wildtype level only by transformation of plasmid pSrsM (Figure 5, lane 7), which contained the wild type full length IsrM sequence, but not by pS-IsrM, which contained mutated IsrM sequence at lane 7), which contained the wild type full length IsrM sequence, but not by pS-IsrM, which contained mutated IsrM sequence at lane 7). The over-expression of SopA in AM-T-SopA was also suppressed by transformation of pH-IsrM (Figure 5, lane 10), which contained mutations in IsrM sequence that disrupted the binding of SopA to hilE mRNA (U264G265A266). Interestingly, the SopA level was lower in the pH-IsrM transformed AM-T-SopA than the pSrsM transformed mutant (compare lane 10 to lane 7 of Figure 5). One possible explanation for the observation is that pH-IsrM suppresses HilE while pH-IsrM does not due to mutations in the hilE mRNA binding site, and therefore the HilE level in the pH-IsrM transformed mutant is likely higher than in the pSrsM transformed mutant (compare lane 5 and 2 of Figure 5). Since HilE negatively regulates SopA and other SPI-1
Role of IsrM in Salmonella invasion

It is expected that mutations at IsrM, which result in altered expression levels of HilE, SopA, and other SPI-1 proteins, affect Salmonella invasion since proper expression of SPI-1 factors is required for Salmonella entry to nonphagocytosed cells. To determine whether this is the case, we tested the ability of various mutant Salmonella strains to invade cultured epithelial cells. A reduction of about 90% in invasion of HeLa cells was observed in ΔIsrM, compared to the wild type ST14028s (Figure 6). Transformation with the wild type complementation construct pIsrM restored the ability of ΔIsrM to invade (Figure 6).

Role of IsrM in intracellular survival in macrophages

Intracellular replication in macrophages represents a major aspect of Salmonella pathogenesis [4,5,6]. The ability of various strains to proliferate intracellularly was assayed in mouse J774 macrophages [37]. A reduction of about 85% in intracellular replication was observed in ΔIsrM, compared to the wild type ST14028s (Figure 6). Transformation with the wild type complementation construct pIsrM fully restored the ability of ΔIsrM to replicate in macrophages, while the ability of ΔIsrM to replicate in J774 cells was also restored by about 90% by transformation with construct pH-IsrM that expressed the hilE mRNA binding-defective IsrM mutant (H-IsrM), but not with construct pS-IsrM that expressed the sopA mRNA binding-defective IsrM mutant (S-IsrM) (Figure 6). Meanwhile, these strains, which carried different complementation constructs, appeared to have no growth defect in vitro as they replicated as well as the wild type ST14028s strain in LB (Figure S2(Supporting Information)). These results suggest that IsrM is important for intracellular proliferation of Salmonella in macrophages by modulating the expression of SopA protein.

Role of IsrM in bacterial pathogenesis and virulence in mice

To determine if IsrM is important for pathogenesis in vivo, BALB/c mice were infected intragastrically with the constructed isrM mutants. To study the virulence of Salmonella, the survival rates of the infected animals were determined. When mice were infected intragastrically with 5×10^7 CFU bacteria, all animals inoculated with the wild type ST14028s strain and ΔIsrM carrying complementation construct pIsrM died within 5 days postinfection (Figure 7A). In contrast, mice infected with ΔIsrM, and ΔIsrM carrying construct pH-IsrM and pS-IsrM remained alive until 15, 12, and 10 days postinfection, respectively. Similar results were

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Figure 6. Epithelial cell invasion and intracellular replication in macrophages. Isogenic Salmonella strains carrying different constructs were added to HeLa cells at a MOI of 10, and cell invasion was assayed by determining the ratio of the number of intracellular bacteria at 1 hour postinfection to the number of input bacteria. The ratio for wild-type strain ST14028s at 1 h postinfection was arbitrarily defined as 100%, and the ratios for other samples were expressed as relative values. For the intracellular replication assay, Salmonella was added to J774 macrophages at a MOI of 10. Intracellular growth was assayed by determining the ratio of the number of intracellular bacteria at 8 hour postinfection to the initial number of bacteria at time zero. The ratio for wild-type strain ST14028s at 8 h postinfection was arbitrarily defined as 100%, and the ratios for other samples were expressed as relative values. The data are the averages of three experiments performed in triplicate. The error bars indicate standard deviations. doi:10.1371/journal.ppat.1002120.g006
also observed in SCID mice that were intragastrically infected with these Salmonella strains (Figure S3)[Supporting Information]. These results suggest that the reduced virulence of ΔIsrM is due to the deletion of IsrM and that IsrM is important for Salmonella virulence in vivo.

To further study the pathogenesis of Salmonella mutants in these animals, colonization of the mutants in the spleen and ileum were studied during a 7-day infection period before the onset of mortality of animals infected with the wild type ST14028s strain. At 7 days postinfection, the level of colonization of ΔIsrM carrying construct pIsrM was similar to that of ST14028s in the organs examined (Figure 7B). In contrast, the counts of ΔIsrM in spleens and ileums of the infected animals were lower than those of the ST14028s strain by approximately 15,000 and 70,000 fold, respectively (Figure 7B). The presence of construct pH-IsrM and pS-IsrM, which contained the mutated IsrM sequences with mutations that disrupted the interactions of IsrM RNA with the hilE and sopA mRNAs, respectively, did not completely restore the ability of mutant ΔIsrM to colonize these mice. Similar results were also observed in SCID mice that were intragastrically infected with these Salmonella strains (Figure S3)[Supporting Information]. These results suggest that the attenuated coloniza-
tion of AlsM in these organs is probably due to the disruption of isrM and that IsrM sRNA may be required for optimal colonization and growth of Salmonella in these organs in mice.

Discussion
Pathogenicity island-encoded sRNA as a virulence factor
In this report, we presented the first direct evidence that IsrM modulates the expression of HilE and SopA proteins, and that its function is important for Salmonella invasion of epithelial cells, intracellular replication inside macrophages, and virulence and colonization in mice. A significant number of the sRNAs identified in Salmonella are encoded in the pathogenicity islands, which are not present in commensal bacteria or E.coli [23,24,25]. Only two of these sRNAs, InvR and IsrJ, have been characterized in detail. InvR, encoded in the SPI-1 region, represses outer membrane protein synthesis of Salmonella by regulating chromosomal genes and does not target any genes in the SPIs [24]. IsrJ, whose gene is located between STM2614 and STM2616, is expressed in the presence of the major SPI-1 transcription factor, HilA, and an isrJ deletion mutant is defective in host cell invasion and effector translocation in vitro [23]. However, the targets of InvR sRNA have not been identified. Characterization of the mutants with inactivating InvR and IsrJ RNAs in animals has not been reported. The roles of either InvR or IsrJ sRNAs in bacterial pathogenesis and virulence in mice have not been determined.

Our results showed that IsrM sRNA targets the mRNAs of HilE and SopA independently, leading to down-regulation of expression of these two proteins. IsrM was expressed in vitro under conditions resembling those during the initiation of infection in the gastrointestinal tract (e.g. low pH, oxygen limitation, and high osmolarity). Furthermore, IsrM was found to be differentially expressed in vivo, with higher expression in the ileum than in the spleen. Mutations in IsrM resulted in disregulation of expression of HilE and SopA, as well as other SPI-1 genes whose expression is regulated by HilE. Salmonella with a deletion of isrM was defective in invasion of epithelial cells and intracellular replication/survival in macrophages, and the defect could be corrected by introduction of a complementation construct containing the full length isrM sequence. Moreover, Salmonella with mutations in isrM was attenuated in its virulence in killing animals and was defective in colonization in the ileum and spleen in vivo. These results suggest that IsrM sRNA encodes a virulence factor important for bacterial infection and demonstrate that a pathogenicity island-encoded sRNA is directly involved in Salmonella pathogenesis in mice. Our study raises the possibility that additional pathogenicity island-encoded sRNA may function in different aspects of Salmonella pathogenesis, including invasion of epithelium and intracellular replication in phagocytes.

Differential expression of SPI-1 proteins regulated by sRNA
The regulation of expression of genes encoding SPI-1 proteins and its effectors is remarkably complex. For example, the transcription of many SPI-1 genes can be regulated by HilA, HilC, HilD, HilE, and PhoP/Q [8]. HilE encodes a global regulator that negatively modulates the expression of SPI-1 genes by sequestering HilD, which is a major transcriptional activator required for expression of most of the SPI-1 genes [35,38]. Our results indicated that disruption of IsrM-mediated targeting of hilE mRNA results in increased expression of HilE protein and disregulated expression of SPI-1 proteins, leading to reduced invasion efficiency in cultured cells and attenuation of bacterial virulence and pathogenesis in vivo. Thus, bacterial sRNA presents another mechanism for global regulation of SPI-1 gene expression.

In addition to globally modulating the expression of SPI-1 proteins through HilE, IsrM also interacts with the mRNA of a specific SPI-1 effector, SopA. This interaction is likely to be important as IsrM-mediated modulation of SopA protein expression affects bacterial intracellular replication in macrophages (Figure 6). SopA has been shown to function as an E3 ubiquitin ligase regulating host inflammatory responses [39,40]. The level of this protein is highly regulated in vivo as SopA is found to be expressed during the early and late stage of Salmonella infection in vivo, consistent with its role in invasion as well as in intracellular replication/survival [9,12,13]. Appropriate level of expression of SopA protein in different cells and at specific time points may contribute to defined consequences of pathogenesis. This is consistent with the recent observations that SopA exerts its functions in concert with other effector proteins during Salmonella infection of non-phagocytic cells; however, in the context of systemic infection, its ubiquitin ligase activity may facilitate bacterial replication in phagocytes [39,41,42]. IsrM can potentially negatively regulate SopA protein level at the translation level through its direct binding of the sopA mRNA and positively at the transcriptional level simultaneously through its down-regulation of the negative regulator HilE. This scenario may represent a novel mechanism of precise control of bacterial protein levels in a temporal and tissue/cell specific manner as required for pathogenesis.

It is currently unclear why IsrM is found in some serovars of Salmonella but not others. It is possible that the presence of IsrM in a specific serovar contributes to host specificity in a particular host. This issue can be investigated further by studying the presence of IsrM in different serovars and its role in the pathogenesis of different Salmonella strains in specific hosts in vivo. Our results indicated that IsrM binds to the 5’ UTR regions near the Shine-Dalgarno (SD) sequence of both the hilE and sopA mRNAs and inhibits the expression of these two proteins. While it is generally believed that binding of the SD region by sRNA results in translation repression, the exact mechanism of how IsrM reduces the expression of HilE and SopA proteins is currently unknown. Equally elusive is whether the IsrM-mediated reduction is mediated by bacterial proteins. The Sm-like RNA-binding protein Hfq, which has been found to be potentially associated with more than 40 Salmonella sRNAs [25], acts as a RNA chaperone that modulates the intracellular stability of many small non-coding RNAs and their annealing with target mRNAs for translation repression or stimulation [17,43,44]. It is unclear whether IsrM RNA is associated with or bound to Hfq, based on recent co-immunoprecipitation experiments [23,25]. The SopA mRNA was found to be enriched moderately by co-immunoprecipitation with anti-Hfq antibodies while the HilE mRNA was not detected in these experiments [25]. Further studies are needed to characterize the molecular mechanism of the regulation mediated by IsrM.

It is generally believed that the amounts of various Salmonella SPI-1 proteins expressed in vivo are in a delicate balance as there are hierarchical transports of different effectors during Salmonella entry and extensively ordered synergistic and antagonist relationships between these effectors following their delivery into host cells [3,45,46]. It is conceivable that the ability of the bacteria to establish successful infection and cause pathogenesis in specific tissues may be significantly influenced by the balance of the amounts of these factors during infection. IsrM was found to be expressed in vitro under conditions resembling those during the initial infection in the gastrointestinal tract. Moreover, IsrM was found to be differentially expressed in vivo, with expression levels...
higher in the ileum than that in the spleen. Thus, controlled expression of IsrM is likely a mechanism Salmonella uses to achieve both global and specific regulation of the expression of SPI-1 proteins in vivo at the appropriate time and place in the host. It would be interesting to determine the expression patterns of IsrM as well as other sRNAs and their potential target mRNAs in different tissues and at different stages of infection in vivo, and study how their expression affects Salmonella infection. Further characterization of these sRNAs should provide significant insights into their exact roles in Salmonella infection and pathogenesis.

Materials and Methods

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol for all animal experiments was approved by the Animal Care and Use Committee of the University of California—Berkeley (Protocol #R240 and #R276). All efforts were made to minimize suffering.

Generation of Salmonella mutants and constructs

Table 1 lists the bacterial strains while Table 2 and 3 list the plasmid constructs and the primers used in the study, respectively. Salmonella strain ΔIsrM was derived from S. typhimurium strain ST14028s by deleting isrM with the λ Red recombinase method [47], following the procedures described previously [48]. Briefly, primers P5ΔIsrM and P3ΔIsrM (Table 3) were used to amplify the kanamycin resistance gene sequence in plasmid pKD4. The resulting PCR products were electrophorated into ST14028s carrying plasmid pKD46. Mutants undergoing homologous recombination were selected from electroporated ST14028s and confirmed by PCR using primers P5ΔIsrM and P3ΔIsrM. Once the deletion mutation was confirmed, the mutation was introduced into fresh culture of ST14028s by transduction using phage P22, and P22-free colonies were selected. The non-polar strain ΔIsrM was constructed using plasmid pCP20 [47], selected for its sensitivity to kanamycin, and further confirmed using PCR with the primers P5IsrM and P3IsrM (Table 3) [12,13,48].

Mutant T-hilE was constructed using the λ Red recombinase method [47], following the procedures as described previously [12,13,48]. A pair of primers was designed to amplify the FLAG epitope coding sequence and kanamycin resistance gene using pUC-H1PF1 as the template [49]. The resulting PCR products were electrophorated into Salmonella ST14028s carrying plasmid pKD46. The non-polar strain T-hilE was constructed using plasmid pCP20 [47], selected for its sensitivity to kanamycin, and further confirmed using PCR with the primers P5IsrM and P3IsrM (Table 3) [12,13,48].

Validation of IsrM targets in E.coli

The TargetRNA and RNAhybrid programs were used to predict the target mRNAs of IsrM against the entire genome of Salmonella typhimurium, the potential interaction of IsrM to the hilE and sopB mRNAs. To generate construct pLaco-IsrM expressing IsrM, a 2.2 kb DNA with the IsrM sequence generated by PCR with primers P5comp and P3comp (Table 3) that annealed to a 20-nt sequence located at 208 nucleotides upstream from the transcription initiating site and 51 nucleotides downstream from the transcription terminator of IsrM, respectively.

In vitro growth analysis of Salmonella

Growth analysis of bacteria in LB broth was carried out by first inoculating a single colony from a fresh plate in 2 ml LB broth and shaking at 250 RPM and 37°C for 4 hours. The bacterial cultures were centrifuged at 5,000×g for 5 minutes. To study the effect of pH, the pelleted bacteria were re-suspended in 1 ml fresh LB broth (control, pH 7.0) or 1 ml LB broth at pH 3.0, 4.4, 5.4, 6.4, 7.2, or 8.4, respectively, shaken at 250 RPM and 37°C for an additional 3 hours, and then collected. To study the effect of osmolarity, the pelleted bacteria were re-suspended in 1 ml NaCl-free LB broth supplemented with 0, 42.5, 85, 160, 340, and 680 mM sodium chloride, respectively, shaken at 250 RPM and 37°C for an additional 3 hours, and were collected. Regular LB broth, which contained 170 mM NaCl, was used as the control. To study the effect of oxygen limitation, the pelleted bacteria were re-suspended in 1.5 ml fresh LB broth. One group of bacteria was shaken at 250 RPM and 37°C for an additional 3 hours with good aeration (control) while another group of bacteria was transferred into 1.5 ml microcentrifuge tubes with their covers closed tightly and wrapped with paraffin, and incubated at 37°C without shaking for an additional 3 hours. Bacteria were pelleted by centrifugation (2 min, 13,000 rpm, 4°C), and RNA samples were isolated with the Trizol method (Invitrogen, Carlsbad, CA), treated with DNase I (TURBO DNA-free, Ambion, Austin, TX), and used for quantitative RT-PCR analysis, following the procedures described previously [13,48,50]. The analyses were repeated three times and the average of three experiments are presented. For Northern blot analysis, the Salmonella RNA samples were separated in 2% agarose gels that contained formaldehyde, transferred to nitrocellulose membranes, hybridized with the [32P]-radiolabeled DNA probes that contained the DNA sequence coding for Salmonella IsrM and 5S RNA, and analyzed with a STORM840 Phosphor-imager [49].
Table 2. Plasmid constructs used in the study.

| Plasmids            | Description                                                                 | Reference/source   |
|---------------------|-----------------------------------------------------------------------------|--------------------|
| pKD4                | Containing a kanamycin resistance cassette and the flipase recognition sites | [47,48]            |
| pKD46               | ApR, containing the Red recombining sequence for λ phage                     | [47]               |
| pCP20               | ApR, containing the expression cassette of flipase                          | [47]               |
| pUC-H1PF1           | ApR and KmrR, template plasmid for 1xFLAG epitope tag                       | [49]               |
| pBR322              | ApR, cloning vector, backbone plasmid for constructing sRNA expression complementation plasmids | New England Biolab |
| pUC18               | ApR, cloning vector                                                         | Invitrogen         |
| pHG101              | ApR; pBR322 with the multiple cloning sequence replaced by that of pUC18    | This study         |
| pIsrM               | ApR, pHG101 containing isrM sequence for complementation of ΔisrM mutant   | This study         |
| pHisrM              | ApR, pIsrM with base substitutions (T257C258A259 -> A257G258G259) at the HiIE mRNA binding site of the ism sequence | This study         |
| pS-IsrM             | ApR, pIsrM with base substitutions (T165C166A167 -> A165G166T167) at the SopA mRNA binding site of the ism sequence | This study         |
| pZE12-luc           | ApR, cloning vector for sRNA expression in E.coli                           | Expressys (Germany) |
| pLaco-IsrM          | ApR, IsrM expression plasmid for validating the target genes of IsrM in E.coli | This study         |
| pLaco-H-IsrM        | ApR, pLaco-IsrM derivative containing nucleotide substitutions (T257C258A259 -> A257G258G259) at the HiIE mRNA binding site of the ism sequence | This study         |
| pLaco-H2-IsrM       | ApR, pLaco-IsrM derivative containing nucleotide substitutions (A257G258C259 -> T257C258G259) at the HiIE mRNA binding site of the ism sequence | This study         |
| pLaco-S-IsrM        | ApR, pLaco-IsrM derivative containing nucleotide substitutions (T165C166A167 -> A165G166T167) at the SopA mRNA binding site of the ism sequence | This study         |
| pLaco-S2-IsrM       | ApR, pLaco-IsrM derivative containing nucleotide substitutions (T159C160A161 -> A159G160T161) at the SopA mRNA binding site of the ism sequence | This study         |
| pXG10               | Chlr, expression plasmid for candidate target genes of IsrM in E.coli       | [36]               |
| pFG10HiEp1          | Chlr, pXG10-derivative expression plasmid with the FLAG epitope fused to the 3’ end of the full-length HiIE from the promoter 1 | This study         |
| pFG10HiEp2          | Chlr, pXG10-derivative expression plasmid with the FLAG epitope fused to the 3’ end of the full-length HiIE from the promoter 2 | This study         |
| pFG10HiEp3          | Chlr, pXG10-derivative expression plasmid with the FLAG epitope fused to the 3’ end of the full-length HiIE from the promoter 3 | This study         |
| pFG10-m-HiIE        | Chlr, pFG10HiEp1 derivative containing nucleotide substitutions (G3A2A1 -> T3T2T1) at the Ism binding site of the hiIE sequence | This study         |
| pFG10-m2-HiIE       | Chlr, pFG10HiEp1 derivative containing nucleotide substitutions (G2C2T6 -> C2G2A6) at the Ism binding site of the hiIE sequence | This study         |
| pFG10SopA           | Chlr, pFG10-derivative expression plasmid with the FLAG sequence fused to the 3’ end of the sopA sequence from -61 nt to +774 nt. | This study         |
| pFG10-M-SopA        | Chlr, pFG10SopA derivative containing nucleotide substitutions (T159G20A13 -> A159C20T13) at the Ism binding site of the sopA sequence | This study         |
| pFG10-M2-SopA       | Chlr, pFG10SopA derivative containing nucleotide substitutions (T159G20A13 -> A159C20T13) at the Ism binding site of the sopA sequence | This study         |

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Expression of tagged proteins in bacteria

To study the expression of tagged proteins in E.coli, the plasmids for expressing HiIE- and SopA-FLAG proteins (e.g. pFG10Hi-

fragment, which contained a PLaacO promoter (from the position -1), an ampicillin resistance cassette, a ColE1 replicon, and a strong rnb terminator, was amplified from the high-copy plasmid, pZE12Luc (EXPRESSYS, Ruelzheim, Germany), with primers P5b-Laco-IsrM and P3b-Laco-IsrM. This DNA fragment was used as the backbone to ligate with the DNA fragment (generated in PCR with primers P5i-Laco-IsrM and P3i-Laco-IsrM) containing the entire IsrM coding sequence and its transcription terminator sequence, generating construct pLaco-IsrM.

To generate constructs expressing HiIE and SopA proteins in E.coli, the low-copy plasmid, pXG10 (a gift from Dr. J. Vogel, Germany) [36], was used as the backbone to insert the hiIE and sopA sequence for expression of 1xFLAG fusion proteins. The entire coding sequence of hiIE was included in the FLAG-fusion protein. As hiIE has three initiating sites of transcription, three plasmid constructs (pFG10HiEp1, pFG10HiEp2, and pFG10Hi-

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lEp1, pFG10HilEp2, pFG10HilEp3, and pFG10SopA) were co-transformed into *E. coli* Top10F with pLaco-IsrM and its derivatives, which contained the wild type and mutated *isrM* sequence, respectively. The transformants expressed HilE- and SopA-FLAG proteins in the presence of 20 μg/ml tetracycline and expressed *isrM* and its related RNA in the presence of 1 mM IPTG. Plasmid pZE12lu (Table 2), which served as the backbone of RNA expression plasmids for target validation, was used as the control plasmid.

To determine the effect of the wild type and mutated *IsrM* RNA on the expression of HilE and SopA, 60 μl of overnight culture of *E. coli* Top10F that contained both an expression plasmid for *IsrM* and an expression plasmid for SopA- or HilE-FLAG protein was inoculated in 3 ml of LB broth containing ampicillin (100 μg/ml) and chloramphenicol (20 μg/ml), and shaken at 37°C, 250 RPM for 4 hours. Tetracycline and IPTG were added into bacterial cultures to a final concentration of 20 μg/ml and 1 mM, respectively, and shaken at 37°C, 250 RPM for an additional six hours. Bacterial pellets were collected and protein samples were isolated. Western blot analysis was performed to determine the levels of the tagged proteins with the expression of GroEL as the internal control [13,48,50].

To study protein expression in *Salmonella*, the SPI-1 protein-tagged strains carrying the deletion mutation of *isrM* were inoculated in 2 ml of LB broth and grew at 37°C and 250 RPM overnight. Sixty microliters of overnight bacterial culture was
inoculated in 3 ml of LB broth and grew at 37°C and 250 RPM for 6 hours. Protein samples were prepared and Western blot analysis was performed using the expression of DnaK as the internal control, following the procedure described previously [13,49,50]. The analyses were repeated three times.

Western blot analyses

Polyepitides from bacterial lysates were separated on SDS-containing 10-12% polyacrylamide gels, transferred to nitrocellulose membranes, and reacted with anti-mouse IgG conjugated with alkaline phosphatase in addition to the antibodies against DnaK protein (StressGen, Victoria, BC, Canada), GroEL protein (Sigma, St Louis, MO), or the FLAG sequence (Sigma, St Louis, MO), following the procedure described previously [13]. The membranes were subsequently stained with a chemiluminescent substrate with the aid of a Western chemiluminescent substrate kit (GE Healthcare, Waukesha, WI) and quantified with a STORM840 phosphorimagier. Normalization of samples was also carried out by loading total proteins extracted from the same CFU (e.g. 5×10^7 CFU) of bacteria in each lane. Quantitation was performed in the linear range of protein detection.

Invasion and intracellular replication assays

Assays for bacterial invasion in HeLa cells and intracellular survival/repllication in J774 macrophages were performed as described previously [37,49,50]. Briefly, Salmonella was added to HeLa cells at multiplicities of infection (MOI) of 5:1 to 10:1, and intracellular bacteria were quantified after 1 h of incubation for invasion, which was followed by incubation in the presence of 50 μg/ml of gentamicin to kill extracellular bacteria. The invasiveness of Salmonella was measured by determining the ratio of intracellular bacteria, which was calculated as follows: (number of intracellular bacteria/number of input bacteria) ×100 [49,50].

To study intracellular growth of Salmonella in macrophages, mouse J774 macrophages were infected with Salmonella for 1 h at an MOI of 10, washed with phosphate-buffered saline (PBS), and then incubated in fresh medium containing 50 μg/ml of gentamicin for 1 hour. A set of cells was washed and lysed to quantify the intracellular bacteria and used as the zero-time (0-h) sample. Additional sets of cells were harvested after an additional 8 h of incubation to quantify intracellular Salmonella (8-h samples). The growth of Salmonella inside J774 cells was measured by determining the increase in the number of intracellular Salmonella, which was calculated by dividing the number of intracellular bacteria in the 8-h sample by that in the zero-time sample [49,50]. All analyses were repeated three times.

Animal studies

Female BALB/c and SCID mice (6-8 weeks old) were obtained from Jackson Laboratory (Bar Harbor, ME). Overnight bacterial cultures were serially diluted to suitable colony forming unit (CFU)/ml in phosphate-buffered saline (PBS) for infection. To assess the virulence of the Salmonella strains, mice (5 animals per group) were either inoculated intragastrically with 5×10^7 CFU/ml of bacteria in phosphate-buffered saline (PBS) for infection. To determine the level of IsrM RNA, the homogenates of the spleen and ileum samples were centrifuged at 9,000 x g for 10 minutes [13,48,50]. Pellets were incubated in lysis buffer (120 mM NaCl, 4 mM MgCl2, 20 mM Tris/HCl, pH 7.5, 1% Triton-X100) at 4°C for 1 hour, and released bacteria were collected by centrifugation at 18,000 x g for 10 minutes. Harvested bacteria were then resuspended in PBS, and adjusted for the bacterial CFU. RNA samples were prepared from isolated Salmonella and analyzed by qRT-PCR to determine the expression of IsrM RNA [13,48,50]. The analyses were repeated three times.

Supporting Information

Figure S1 The levels of IsrM in Salmonella grown in vitro at pH4.4, pH7.0, and pH7.2, as determined by Northern blot analyses. The levels of Salmonella 58 RNA were used as the internal control. The experimental procedures are described in Materials and Methods. The Salmonella RNA samples were separated in 2% agarose gels that contained formaldehyde, transferred to nitrocellulose membranes, hybridized with the [32P]-radiolabeled DNA probes that contained the DNA sequence coding for Salmonella IsrM and 58 RNA, and analyzed with a STORM840 Phosphorimagier. (TIF)

Figure S2 Growth analysis of Salmonella strains in LB broth. (A) The growth of the wild type ST14028s, mutant ΔIsrM, and ΔIsrM transformed with different plasmids. (B) The growth of the wild type ST14028s and the SPI-1 protein-tagged Salmonella strains. The experimental procedures are described in Materials and Methods. The results are the means of three experiments performed in triplicate. The error bars indicate standard deviations. (TIF)

Figure S3 Virulence and colonization of Salmonella in mice. (A) Mortality of the SCID mice infected with isogenic strains carrying different constructs. SCID mice (5 animals per group) were infected intragastrically with Salmonella (1×10^7 CFU). (B) The numbers of bacteria (CFU) in spleen and ileum of the infected animals. Groups of SCID (5 animals per group) mice were infected intragastrically (IG) with 1×10^7 CFU of isogenic strains carrying different constructs, and bacteria were recovered from the organs at 7 days post inoculation. Each sample was analyzed in triplicate and the analysis was repeated at least three times. The CFU of the sample was expressed as the average of the values obtained. The concentrations of bacteria were recorded as CFU/ml of organ homogenate. The limit of bacteria detection in the organ homogenates was 10 CFU/ml. (TIF)

Table S1 Relative levels of IsrM in E. coli transformed with different constructs, as compared to those in E. coli that expressed HilE-FLAG and IsrM in the presence of IPTG. Relative levels of HilE protein in E. coli transformed with different constructs, as compared to those in the plIsrM-containing E. coli that expressed HilE-FLAG and m-HilE-FLAG in the absence of IPTG, respectively. (DOC)
Table S2  Relative levels of IsrM in E. coli transformed with different constructs, as compared to those in E. coli that expressed SopA-FLAG and IsrM in the presence of IPTG. Relative levels of SopA protein in E. coli transformed with different constructs, as compared to those in the pB-M-containing E. coli that expressed SopA-FLAG and M-SopA-FLAG in the absence of IPTG, respectively.

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