YshB promotes intracellular replication and is required for Salmonella virulence

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Salmonella virulence requires the initial invasion of host cells followed by the modulation of the intracellular environment for survival and replication. In an effort to characterize the role of small RNAs in Salmonella pathogenesis, we inadvertently identified a 5 kD protein named YshB that is involved in the intracellular survival of Salmonella. We show here that yshB expression is upregulated upon entry into macrophages. When yshB expression is upregulated before bacterial entry, invasion efficiency is inhibited. Lack of YshB resulted in reduced bacterial survival within the macrophages and led to reduced virulence in a mouse model of infection.
IMPORTANCE

Salmonella gastroenteritis is one of the most common causes of food-borne disease, possibly affecting millions of people globally each year. Here we characterize the role of a novel small protein YshB in mediating Salmonella intracellular survival. This elucidation adds another layer to the body of knowledge of how this bacterium confers its intracellular survival.

KEYWORDS

Salmonella, YshB, Small Protein, Invasion, Intracellular Replication

INTRODUCTION

Pathogenic bacteria have evolved complex regulatory circuits to regulate their virulence gene expressions. Commonly, this is achieved by turning on or off a specific repertoire of genes once the pathogen encounters a different nutritional and host immune environment. This is evidently the case in Salmonella enterica Serovar Typhimurium (S. Typhimurium), which first has to actively participate in invasion followed by a lengthy intracellular phase of survival and replication within the host cells. The invasion of the non-phagocytic epithelial cells of intestine is primarily mediated by the effectors of Type III Secretion System-1 (T3SS-1) (1, 2). The invasive phase is succeeded by multiplication inside the host cells within a specialized compartment called the Salmonella Containing Vacuole (SCV). The transformation from the invasive phase to the state in the SCV is mediated by an elaborate network of regulatory proteins regulating the expression of invasion-related genes (3) as well as those required for maintaining bacterial replication inside the SCV (4-9). Many of the effectors of the Salmonella Pathogenicity Island-2 (SPI-2) system are known to be responsible for the formation and biogenesis of the SCV. A few effectors have multi-faceted roles in both the invasion and replication phases (10).
The precise control of gene expression related to invasion and subsequent survival is necessary for *Salmonella* virulence, as gene expression at unwanted times is not only redundant or energy expensive but may also be detrimental. One such example is SipB, a major effector mediating invasion, which is also known to induce apoptosis in macrophages (11). Consequently, there is some degree of inverse regulation between the invasive and survival phases. *Salmonella* is able to sense the cues from its immediate environment as a means to switch the expression of particular sets of genes. For instance, when the bacteria reside within the host macrophage cells, it manoeuvres the regulatory machinery to repress the genes essential for invasion. The PhoP/PhoQ two-component system which promotes intracellular survival also acts as a key genetic switch to mediate repression of invasion-associated genes within macrophage by downregulating the expression of *hilA*, a master regulator of invasion (5, 12).

Recent studies have identified a large number of non-coding small RNAs and many of them have no known functions (13-15). In an effort to assess the roles of these small RNAs in *Salmonella* virulence, we inadvertently found a small protein YshB, and established its role in bacterial virulence. In this report, we show that the small protein YshB is involved in intracellular replication during *Salmonella* infection.

**RESULTS**

Expression of STnc1450 led to lower *S. Typhimurium* invasion. Regulatory factors often exert their functions when their own expressions are upregulated (16-20). In an effort to characterize roles of small non-coding RNAs in *S. Typhimurium* SL1344 mediated invasion of epithelial cells, we induced the expression of sRNAs individually from an arabinose-inducible plasmid in the wild type *Salmonella* strain background. A total of 52 sRNAs (21) were successfully cloned into the arabinose-inducible vector (pZP1137). The invasion efficiency was
then evaluated using the gentamicin protection assay (22) for each of the strain carrying the
sRNA-expressing plasmids with or without L-arabinose induction. We found that induction of
several sRNAs were able to reduce the invasion efficiency compared to that of the wild-type
strain (Fig. 1). Addition of L-arabinose alone did not have any noticeable effect on the invasion
efficiency of Salmonella (data not shown), similar to what was previously reported (23).
Induction of three sRNAs (STnc840, \(p=0.0005\); STnc1420, \(p=0.001\); and STnc1430, \(p=0.02\))
significantly increased the relative invasion rates. Two sRNAs (STnc520, \(p=0.002\); STnc1450,
\(p=0.0001\)) significantly decreased invasion. Induction of sRNA STnc1450 led to the most
drastic reduction in Salmonella invasion, thus was chosen for further studies here.

The small protein YshB encoded within the STnc1450 locus is responsible for the
lowered invasion. Upon further analysis, we found a putative small open reading frame, named
yshB, within the predicted STnc1450 sRNA encoding region (Fig. 2A). Given the presence of
this putative small protein within the STnc1450 sRNA encoding region, the altered invasion
phenotype described above might have been due to the induction of either the protein or the
small RNA. To differentiate these two possibilities, we constructed plasmids encoding the full-
length small protein (p\(_{yshB}\)); the full yshB gene without the start codon (p\(_{yshB\text{noATG}}\)); the full
yshB gene with a frameshift mutation by deleting the first nucleotide (p\(_{yshB\text{fs}}\)); and the full yshB
gene with degenerate nucleotide sequence coding the same 36 amino acids of YshB
(p\(_{yshB\text{dg}}\)). These plasmids were then introduced into the wild type Salmonella and their
invasion efficiency was evaluated using both the gentamicin protection assays and the inside-
outside differential fluorescence staining assay (24). The lower invasion phenotype was
conspicuously absent when no ATG or frameshift mutations were present (Fig. 2B and 2C). To
further demonstrate the presence of the small YshB protein, Western blot was carried out using
bacterial lysates expressing the tagged YshB-TEM1 in-frame fusion proteins (Fig. 2D). A protein
band corresponding to 35 kD YshB-TEM1 fusion protein was observed. Interestingly, the
singular null yshB mutant did not have any significant change in invasion efficiency (Fig. 2B and 2E). Furthermore, the growth rate and motility of the bacteria were unaffected with or without L-arabinose induction. Moreover, the defect in invasion upon YshB induction was not due to any hindrance in the adherence capability of the bacteria to the host cells (Fig. S1). These results indicated that the lower invasion phenotype was due to the expression of the small protein, YshB, rather than the STnc1450 sRNA. From the amino acid sequence, the secondary structure of this small protein was predicted using the PSIPRED online server (Fig. 3), where the second helix constitutes the predicted transmembrane domain (25, 26). The function of YshB remains unknown.

**Induction of YshB lowers the expression of invasion-related genes.** SPI-1 encoded T3SS effectors are known to play key roles in modulating host cell actin cytoskeleton rearrangements to promote bacterial invasion (2, 27). It is known that the expressions of these T3SS effectors are tightly regulated in response to environmental conditions which the bacteria encounter (28, 29). Thus, it is possible that YshB-induced reduction in bacterial invasion is mediated by a change in SPI-1 effector expressions. To test this possibility, we examined the expression of SipA, SipB and SipC in bacterial cell lysates when YshB is induced (Fig. 4A). We found that levels of SipA, SipB and SipC were reduced in strains expressing YshB as compared to the strain carrying the vector only or when there was no YshB induction (Fig. 4). This result is consistent with our notion that the reduced invasion seen in the YshB induced strain is due to a reduction in SipA, SipB and SipC.

Type III effectors are translocated into non-phagocytic epithelial cells to exert their functions during bacterial invasion (2). Therefore, a change in the translocation efficiency could potentially affect bacterial invasion. To test the translocation efficiency of the SPI1-encoded T3SS apparatus, we measured the translocation of SipA into HeLa cells using the β-lactamase translocation assay (30). As shown in Fig. 4, the translocation of SipA remains similar with or
without the induction of YshB. In addition, we also tested the possibility if YshB itself is a T3SS translocated effector by conducting the same translocation assay (Fig. S2). Our result showed that YshB is not translocated.

**YshB is required for efficient intracellular *Salmonella* replication.** Gene expression is tightly regulated by the immediate environment that the pathogen encounters. Previous studies have suggested that *Salmonella* responds to the environmental changes by regulating virulence expression to cope with hostile surroundings. To understand how YshB might function, we therefore conducted experiments to assess the expression of YshB from its native promoter under various conditions. For the *in vitro* conditions, we selected different growth stages when grown in LB broth, with and without 0.3 M NaCl (invasion-inducing media) and SPI-2 media (mimicking intracellular conditions). We also examined the expression level of YshB when the *Salmonella* are inside the macrophage cells. Our results indicate that expression of YshB is considerably higher when grown in the SPI-2 media and when the bacterium is inside macrophage cells (intracellular phase of life cycle) (Fig. 5A).

These data showed that YshB expression is upregulated inside the host cells, suggesting that it might play a role during the intracellular stage of the bacterial life cycle. Once *Salmonella* successfully invades host cells, it switches its priorities to modulating the intracellular microenvironment which consists primarily of a modified phagosome known as the *Salmonella*-containing vacuole (SCV) to promote survival and replication (31). Thus, we tested whether YshB is involved in bacterial survival and replication in macrophages. RAW264.7 macrophages were infected with normal mouse serum opsonized bacteria for 30 min as described in the Materials and Methods. The replication fold was determined by dividing the number of intracellular bacteria at 18 h by the number at 2 h. Our data showed that YshB indeed plays a role in the survival of *Salmonella* inside macrophage cells (Fig. 5B). Hence, this corroborates with the observation that YshB expression is upregulated inside the host cells.
YshB is required for full virulence in mice. Competitive index assay is frequently used to assess the virulence of bacterial pathogens in a mouse model of infection. To assess whether YshB plays a role in Salmonella virulence in the mouse infection model, we employed mixed infections of the wild-type, the ΔyshB mutant, and the ΔyshB strain complemented with a plasmid expressing YshB. This was facilitated by the fact that the ΔyshB strain had a Kanamycin cassette replacing the yshB gene, so that it could be selected on double antibiotics (Streptomycin/Kanamycin) LB plates, as opposed to the wild type strain which is only resistant to Streptomycin and is sensitive to Kanamycin. The competitive index for ΔyshB strain was found to be 0.39 for spleen colonization and 0.52 for liver colonization (Fig. 6). When the small protein was expressed via a plasmid from its native promoter, the virulence was restored to almost wild type level. Our results demonstrate that lack of the yshB gene led to reduced virulence in the mouse model of infection.

DISCUSSION

We initially sought to investigate the roles of Salmonella sRNAs in invading host epithelial cells. Instead, we found a small protein YshB encoded within a putative small RNA molecule that was able to affect bacterial invasion and intracellular survival. Wadler and Vanderpool reported that a 43-amino acid small protein SgrT was encoded within a transcript of a small RNA SgrS (32). In recent years, small proteins have been elucidated as an important group of molecules capable of orchestrating a wide range of cellular processes in bacteria. By convention, small proteins are defined as those with 50 or less amino acids (33). Small proteins mediate a variety of functions in bacteria including but not limited to spore formation; transport of nutrients; regulation of membrane bound enzymes and signal transduction among others. Owing to their small sizes, these proteins more commonly act in mechanical ways via direct interaction with their targets (34). One example of such proteins is MciZ, which inhibits the activity of FtsZ, a main player of bacterial cell division, by binding directly with it (35). Similarly,
MgrB is a small protein which negatively regulates the PhoP/PhoQ system in *E. coli* (36). In this study, the authors were able to demonstrate that MgrB binds and represses PhoQ activity, via a negative feedback loop. Sda is another cytosolic small protein which blocks the interaction between different kinases involved in *B. subtilis* sporulation (37). Interestingly, the interplay between a small protein and its target can also promote the interaction between domains or proteins. In one example, MgtR, a 30-amino acid small protein in *Salmonella* assists the protease activity of FtsH to degrade the virulence factor MgtC (38). MgtR was shown to directly interact with MgtC thereby facilitating its degradation by FtsH. This occurred presumably by changing the conformation of MgtC, hence rendering it sensitive to the protease. Moreover, some small proteins are known to function as chaperones. Smaldone et al suggested that a small protein FbpB may aid the activity of a small RNA FsrA by acting as its chaperone (39).

YshB is a 36 amino acids small protein first annotated in a screening based on the presence of ribosome binding sites (40), and later validated as a protein expressing gene with a predicted transmembrane domain in *E. coli* (25). This protein is ubiquitously present in *Salmonella* species, with 100% sequence identity among the common pathogenic serovars *S. Typhimurium*, *S. Typhi*, *S. Enteritidis* and *S. Choleraesuis*. Further, among the members of Enterobacteriaceae, *Salmonella* YshB sequence is 92% identical (33 out of 36 amino acids) to the *Escherichia coli* (strain K-12), *Shigella* and *Klebsiella oxytoca* protein. Prior to this study, YshB has no known functions nor any described roles in bacterial virulence. We described here that YshB acts as a positive regulator of bacterial intracellular survival. Our data suggest that when induced within the bacteria, YshB may function to downregulate *Salmonella* invasion. It is tempting to speculate that the induction of YshB represents the intracellular phase and consequently triggers a down-regulation of the invasion machinery to facilitate intracellular survival and replication. Although our data demonstrated that the YshB small protein is responsible for coordinating invasion and intracellular survival, we cannot rule out the possibility...
that the originally annotated sRNA STnc1450 serve an unknown independent function. Such a
dual functional example has been described previously (32).

Salmonella encounters various different environmental conditions in which the bacteria have
to compete to survive before, during and after the infection. In order to ensure a successful
transition to a new environment, Salmonella regulates the expression of T3SS-1 genes encoded
in SPI-1 to locations where these gene products are needed. Multiple regulatory factors are
responsible for regulating this process in response to environmental signals. Once the invasion
of the host cell is complete, Salmonella are known to downregulate the expression of T3SS-1
genomes and initiates SCV biogenesis, for which the T3SS-2 induction is essential (41, 42). A
complex network of regulators has been identified that are responsible for coordinating their
expressions. One example of this is the repression of Salmonella SPI-1 genes as the bacteria
reach the proximal sites of the small intestine, where the expression of invasion genes is no
longer necessary (43). Furthermore, the cationic peptides produced inside the macrophages are
also known to silence the expression of SPI-1 genes where invasion is no longer desired (44).

Interestingly, even the SPI-2 system has been known to regulate the expression of SPI-1
genomes involved in bacterial invasion (45-47). PhoP is the response regulator and is known to
repress the expression of the SPI-1 genes (48), while activating the expression of pag genes
including mgtCB, pmrB and SPI-2 genes inside macrophages. The fact that pag genes are
transcriptionally activated when the bacteria is inside the macrophages was observed when as
many as eight proteins were absent in a phoP mutant compared to the wild type following
macrophage uptake (49). This differential regulation corresponds to the spatial localization of
the bacteria in the macrophages where pag genes are needed as opposed to being in the small
intestine, where most SPI-1 genes are needed (12, 46, 50-52). Furthermore, the PhoP/PhoQ
two-component regulatory system needs to remain downregulated for the induction of SPI-1
genomes to promote bacterial invasion (12, 53). A seemingly narrow cation concentration range is
responsible for the activation of most pag genes, with an optimal expression at a concentration
of less than 100 µM while at or above 2 mM concentration, the expression is completely turned
off (51). This corresponds well with the respective sites in the host, where the cation
concentrations are relatively higher in the intestinal environment compared to when the bacteria
are inside the phagosome (52). The PhoP/PhoQ system hence illustrates an exquisite example
of spatio-temporal control of gene expression in Salmonella. Our data showed that YshB was
upregulated when the bacteria were inside the macrophage. Further study is required to
elucidate whether YshB interacts with the PhoP/PhoQ system to regulate the transition from the
extracellular phase to that inside the host cells.

Our data showed that under the conditions in which YshB expression is induced, such as
inside the macrophage, the invasion efficiency is markedly reduced and this is important for
intracellular survival as well. It is known that the expressions of unnecessary genes result in
energy expenditure and sometimes are even detrimental. In a study to examine the temporal
expressions of invasion genes, Boddicker et al found that down regulation of invasion gene
expressions was partly mediated by the Lon protease and this down regulation was essential for
the intracellular survival of Salmonella (54). An uncalled expression of SPI-1 genes inside the
macrophage results in excessive induction of apoptosis, and this is countered by the Lon
protease (54, 55). This was supported by the previous findings from Takaya et al that
transcription of SPI-1 genes in a Δlon mutant was enhanced (56, 57).

We attempted to identify Salmonella proteins that may interact with YshB using a genetic
bacterial two-hybrid screening and biochemical co-immunoprecipitation assays and did not find
any valuable targets. It’s possible that we failed to identify the potential target of YshB in these
screenings. Alternatively, the target for this small protein could be non-proteinaceous, such as a
small RNA. Hence, further analysis of the molecular mechanism of how YshB modulates
bacterial virulence is needed.
MATERIALS AND METHODS

Bacterial strains, plasmids and mammalian cell lines. *Salmonella* and *E. coli* strains were routinely grown in Luria-Bertani (LB) broth. For the induction of SPI-1 machinery, *Salmonella* strains were grown in LB broth with 0.3 M NaCl final concentration. N-salts minimal medium with low Mg\(^{2+}\) concentration (SPI-2 media) was used for the induction of SPI-2 components (46). Where applicable, antibiotics were used at the following concentrations: Streptomycin (150 μg mL\(^{-1}\)), Ampicillin (120 μg mL\(^{-1}\)), Kanamycin (40 μg mL\(^{-1}\)) and Tetracycline (15 μg mL\(^{-1}\)).

Isogenic derivatives of virulent wild-type (WT) SL1344 strain of *Salmonella* Typhimurium were used in this study (58). In-frame chromosomal deletions of genes and chromosomal flag tagged constructs in *Salmonella* strains were generated by the one-step gene disruption strategy using the λ-Red recombination system (59, 60). HeLa (CCL-2) and RAW 264.7 cell lines were purchased from ATCC (Manassas, VA), and maintained in Dulbecco’s modified Eagle’s medium (DMEM; VWR) supplemented with 10% fetal bovine serum (FBS; Gibco).

Primers used in this study are listed in supplemental S1 Table. For inducible expression of various YshB constructs, PCR-generated DNA fragments were cloned into the EcoRI/Xmal sites of an arabinose inducible plasmid (pZP1137) so that it was in frame with the M45 tag at its C-terminus. To facilitate the visualization of YshB by Western blot, a translational fusion with a double M45-TEM1 tag derived from plasmid pM1644 [31] was constructed to the C-terminal end of YshB in pZP1137 to generate plasmid pYshB (pZP3628). The resulting YshB-M45-TEM1 fusion protein expressed from this plasmid had a molecular weight of approximately 35 kD. The same M45-TEM1 tag was also cloned into the empty pZP1137 vector (pVector-TEM1, pZP3635) and used as a control. Another control pVector-EGFP plasmid (pZP3636) was constructed by subcloning the 759 nucleotide fragment generated from Xmal/Xbal digest of pEGFP-N2 into the
same sites of pZP1137. To construct a plasmid in which yshB would be expressed from its native promoter (pyshBnp, pZP3637), a 254 nucleotide fragment was amplified from Salmonella genome including 130 nucleotides upstream of yshB. This fragment was cloned into the XmaI site of the above mentioned vector control plasmid (pVector-EGFP, pZP3636). The resulting plasmid expressed a YshB-EGFP fusion protein with a molecular weight of 31 kD. To construct the plasmid pyshBnoATG (pZP3399), the forward primers were designed so that the ATG for yshB was omitted. The PCR product was amplified and cloned to the EcoRI/XmaI sites of pZP1137. Plasmid pyshBfs (pZP3400) was constructed by designing a primer in which the first adenosine nucleotide for yshB was omitted which resulted in a shifting for the rest of the reading frame. Moreover, plasmid pyshBdg (pZP3634) was constructed by annealed degenerate oligonucleotides coding the same 36 amino acids of YshB into the EcoRI/XmaI sites of pVector-TEM1. Briefly, nucleotide sequences which are degenerate from the wild type sequence of yshB gene were designed such that they had EcoRI and XmaI overhangs. The overlapping oligos were resuspended in annealing buffer (10 mM Tris, pH 7.5-8.0, 50 mM NaCl, 1 mM EDTA) and mixed in equimolar concentrations. The annealing step was performed by heating the mix to 100°C for 5 minutes and slowly equilibrating to room temperature [61]. This insert was then cloned into the EcoRI/XmaI sites of pVector-TEM1.

For expression within the macrophages, yshB was expressed from its native promoter (pZP3637). Furthermore, a plasmid used for co-transformation with pM1644 was constructed by subcloning yshB-EGFP to the XhoI/Notl sites of pACYC-184SK to generate pyshB-EGFP-ACYC (pZP3682). The sipA gene in pM1644 was replaced by yshB by subcloning to generate plasmid pyshB-TEM1 (pZP3639) which was used to assess translocation of YshB into the host cells. All plasmid constructs were verified by restriction digest and sequencing.

**Growth Rate and Motility Assays** For growth rate measurement, overnight LB broth culture of bacteria was subcultured with 1:100 dilution in 10 mL LB broth and grown at 37°C in a...
shaker (200 rpm). Serial dilutions of the culture was then plated every 40 min and bacterial numbers enumerated. Swarm plate assay was performed to assess bacterial motility (62). Briefly, semi-solid LB plates (0.25% agar) with and without 1mM L-arabinose were prepared. From the overnight culture of bacteria, 100 µL was inoculated in 2.5 mL LB and allowed to reach mid-log phase (OD$_{600}$ = ~0.5-0.6). From this culture, 1 µL was spotted on the semi-solid agar plate and incubated at 37°C. The size of the halo around the site of inoculation was measured every 30 min to evaluate the bacterial swarm/motility.

Invasion Efficiency. The invasion efficiencies were assessed by the classical gentamicin protection assay (63). In brief, overnight LB-broth cultures of Salmonella were sub-cultured after a 1:30 dilution in 0.3 M NaCl LB-broth to an OD$_{600}$ of 1.0 at 37°C. HeLa cells were then infected for 15 min at an MOI of 10 in a 24-well plate and incubated at 37°C in a 5% CO$_2$-incubator. After infection, cells were washed thrice with PBS followed by addition of DMEM containing 100 µg mL$^{-1}$ of gentamicin and incubated further for an hour to kill extracellular bacteria. Cells were washed again three times with PBS and lysed with 0.1% sodium deoxycholate. Cell lysates were then serially diluted and plated on LB plates for colony enumeration.

The ability of bacteria to invade host cells was also evaluated with the inside/outside differential staining assay as previously described (24). Briefly, HeLa cells were infected with Salmonella at an MOI of 5 for 15 min. The bacteria which remain outside of the infected cells were identified using rabbit anti-Salmonella O-antigen and visualized with anti-rabbit Alexa Fluor 488 (Molecular Probes). This was followed by permeabilization of the infected cells with 0.2% Triton X-100 and counter-staining both the intracellular and extracellular bacteria with Texas Red (Molecular Probes). Host cell nuclei and bacterial DNA were stained with 4,6-diamidino-2-phenylindole (DAPI, Molecular Probes). The outside bacteria would appear green or yellow and the ones inside will be red when visualized under a double filter for both Alexa Fluor 488 and Texas Red. The invasion efficiency was determined by enumerating percentage of cells with
either 1-5 or more than 5 internalized bacteria per host cell. The experiment was performed in triplicate, with approximately 300 cells counted for each sample.

**Intracellular Survival in Macrophages.** Bacterial infection of RAW 264.7 macrophages and intracellular survival assays were carried out by gentamicin protection assay as described before (47). Briefly, *Salmonella* strains were grown to early stationary phase and diluted to an OD\(_{600}\) of 0.1. The bacteria were opsonized for 20 min in DMEM containing 10% normal mouse serum (Gemini Bio-Products, Woodland, Calif.) at 37°C. Opsonized bacteria were added to RAW264.7 macrophages at an MOI of 10 in 24-well plates and incubated for 30 min at 37°C in 5% CO\(_2\) for infection. This was followed by washing the cells thrice with PBS and incubating further for an hour in DMEM with 10% FBS and 100 μg mL\(^{-1}\) of gentamicin to kill extracellular bacteria. Infected cells were then washed again three times with PBS and incubated for the indicated amount of time in 16 μg mL\(^{-1}\) of gentamicin containing DMEM. At 2 and 18 h post infection, the infected macrophages were washed three times in PBS and lysed with 0.1% sodium deoxycholate. Cell lysates were then serially diluted and plated onto LB plates for bacterial enumeration. The replication fold was determined by dividing the number of intracellular bacteria at 18 h by the number at 2 h.

**Competitive Index Mouse Model of Infection.** Bacteria were grown overnight at 37°C in LB medium. The bacteria were then harvested by centrifugation and resuspended in sterile PBS. Indicated combination of *Salmonella* strains were mixed at a 1:1 ratio. Serial dilutions were prepared for each bacterial mix and plated onto LB + Streptomycin and LB + Streptomycin + Kanamycin plates to calculate the input inoculums. On the LB + Streptomycin plates, both wild type and mutant strains would grow, whereas LB + Streptomycin + Kanamycin would act as selective media for only the mutant strains used in this study. Around 10\(^4\) cfu of bacteria in 200 μL were injected intraperitoneally into 6 to 8-week-old female BALB/c mice (5 mice for each mixture sample). The mice were sacrificed two days post infection by CO\(_2\) euthanasia followed
by cervical dislocation. Spleen and liver from each mouse were harvested and homogenized aseptically in sterile PBS. Serial dilutions were carried out and plated onto LB + Streptomycin and LB + Streptomycin + Kanamycin plates for output enumerations. The Competitive Index (CI) was calculated as the ratio between the mutant strain and the wild type in the output divided by the ratio of the two strains in the input (64).

All animal experiments were conducted in strict accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and following a protocol approved by the Purdue Animal Care and Use Committee (PACUC) (Protocol # 1111000320).

**Adherence and Protein translocation Assays.** To measure bacterial adherence to mammalian cells, HeLa cells were treated with Cytochalasin D (Sigma), an inhibitor of actin filamentation at a final concentration of 1 µg mL\(^{-1}\) for 30 min before infection (65) to stall bacterial internalization. *Salmonella* strains were added to the treated cells at an MOI of 10, and incubated for 15 min to facilitate adherence to the host cells. The cells were then washed thrice with PBS to remove the unattached bacteria followed by subsequent plating for bacterial enumeration.

To measure translocation of bacterial effectors into the host cells, HeLa cells were infected with *Salmonella* strains expressing the corresponding β-lactamase fusions (derived from pM1644) in a 96-well plate at an MOI of 10. CCF4-AM (Invitrogen, Carlsbad, CA) was then added into the wells 15 min post infection and incubated for 2 hours at room temperature. CCF4 which emits green fluorescence is a β-lactamase substrate and upon cleavage emits blue fluorescence. Infected cells were then examined under the fluorescent microscope to quantify the number of green and blue cells. Experiments were performed in triplicate and approximately 300 cells were counted in each sample.

**SDS-PAGE and Immunoblot.** To prepare bacterial samples for SDS-PAGE and
Immunoblot, bacterial strains were grown overnight in LB, followed by subculturing with 1:30 dilutions into respective media and incubated at indicated conditions. The bacterial culture was then centrifuged at 10,000 x g for 5 min and the cell pellets were lysed with 2x Laemmli sample buffer and boiled for 10 min. For intracellular infections, stationary phase LB culture was used to infect macrophage cells at an MOI of 10 for 30 min. Infected macrophage cells were lysed 12 hours post infection with 0.1% sodium deoxycholate. The lysate was centrifuged at 500 x g for 5 min to remove cell debris. The supernatant was then centrifuged at 10,000 x g for 5 min to collect the intracellular bacteria. The pellet was subsequently lysed with 2x Laemmli sample buffer and boiled for 10 min. Samples were run on a 10% SDS-polyacrylamide gel and immunoblotted using respective antibodies. The SipA, SipB, SipC and GFP primary antibodies were rabbit polyclonal antibodies, whereas the anti-TEM1 was mouse monoclonal antibodies. Rabbit anti-ICDH was used to probe ICDH protein as the loading control. The anti-TEM1 mouse monoclonal antibody and rabbit anti-ICDH polyclonal antibody were gifts provided from Dr. Zhao-qing Luo (Purdue University).
**FIGURE LEGENDS**


**Fig. 2. The small protein YshB within the STnc1450 locus is responsible for the lowered invasion.** (A) Genomic location of STnc1450 in *S. Typhimurium* SL1344 showing the presence of an open reading frame encoding a small protein YshB. (B) Relative invasion rates of *Salmonella* strains with induced YshB from an arabinose inducible plasmid (pZP3628) in wild type (SL1344) background evaluated by the gentamicin protection assay. (C) Relative invasion
rates were similarly assessed for YshB encoded from degenerate nucleotide sequences (pZP3634), and with no start codon (pZP3399) or frame-shifted yshB (pZP3400). (D) Immunoblot showing the expression of YshB from pyshB (pZP3628) and pyshBdg (pZP3634). The bacterial cell lysates were subjected to SDS-PAGE followed by immunoblot with anti-TEM1 antibody. Arrow indicates the 35 kD YshB-TEM1 fusion protein. (E) Inside-outside differential staining for the wild type, ΔyshB and YshB induced (pZP3628) strains. Percentage of cells with either 1-5 or more than 5 internalized bacteria was enumerated. Asterisks indicate statistically significant differences with indicated \( p \) values, and three independent experiments were carried out with the mean ± SD represented as error bars.

**Fig. 3. The small protein YshB.** (A) Amino acid sequence information for small protein YshB. (B) Predicted secondary structure for YshB using PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/).

**Fig. 4. Induction of YshB lowers the expression of invasion genes.** (A) Immunoblot analysis of bacterial whole cell lysates for the expression of SipA, SipB and SipC after being grown under SPI-1 inducing conditions with and without YshB induction (pZP3628). Polyclonal rabbit anti-SipA, anti-SipB and anti-SipC primary antibodies were used whereas rabbit anti-ICDH was used to probe the loading control. (B) \( \beta \)-lactamase based translocation assay to assess translocation efficiency of SipA from pM1644 in YshB induced (pZP3682) cells. Wild type Salmonella with pM1644 and plasmid pZP2277, in which the SipA from pM1644 was replaced by the Rfa cassette of Gateway® Vector Conversion System were used as positive and negative controls respectively. The translocation efficiency was evaluated by enumerating blue vs green cells under a confocal laser scanning microscope. (C) Percentage of blue cells in Fig. 3B to quantitate the SipA-TEM1 translocation efficiency. Three independent experiments were carried out and the mean ± SD is represented as error bars. (D) Immunoblot analysis of the bacterial culture used in Fig. 3B to show the SipA expression from pM1644 remains unaffected when YshB is overexpressed (pZP3682).
Fig. 5. YshB expression is upregulated following bacterial invasion and YshB is required for efficient intracellular *Salmonella* replication. (A) Immunoblot analysis to compare the endogenous YshB expression levels from its native promoter (pZP3637) under various *in vitro* conditions and inside RAW 264.7 macrophage cells. Relative band intensity was determined by dividing the intensity of YshB-EGFP band by that of ICDH and normalized to 1 for the LB OD$_{600}$ condition. The normalized relative intensity is the average value from two independent experiments with indicated standard deviation. The arrow indicates the 31 kD YshB-EGFP. (B) Replication fold of ∆yshB *Salmonella* strains along with the complemented strain (pZP3637) and YshB induced strain (pZP3637) by Gentamicin Protection Assay. Data represents mean of three independent experiments with standard deviation from mean and asterisk indicates a statistically significant difference with $p$ value indicated.

Fig. 6. YshB is required for full virulence in mice. Competitive Index Assay was conducted using mixed bacterial cultures for infection. Mixtures containing wild type and ∆yshB or wild type and the complemented strain (pZP3637) were used to infect BALB/c mice intraperitoneally. Liver and spleen from the animals were harvested two days post infection to determine the competitive index of respective strains with respect to the wild type. Asterisks indicate statistical significance with indicated $p$ values.
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The authors declare no competing financial interests.

REFERENCES


Fig. 3

A

MLESIINLVSSGAVDSHTPQTALAAVLCAALVGLFS

B

Conf: [diagram]
Pred: [diagram]
Pred: CHRRHHRRRRRRCCOCOCCHRRRRRRRRRRRRRRCC
AA: MLESIINLVSSGAVDSHTPQTALAAVLCAALVGLFS

Legend:

- helix

- confidence of prediction

- strand

- predicted secondary structure

- coil

AA: target sequence
Fig. 4

A

WT (pVector)  WT (pyshB)  WT (pyshB)

IB: SipA
IB: SipB
IB: SipC
IB: ICDH

0 1 mM
L-arabinose

B

WT (psipA-TEM1)  WT (psipA-TEM1 pyshB)  WT (pVector)

C

Blue Cells (%)

WT (psipA-TEM1)  WT (psipA-TEM1 pyshB)

ns

D

WT PM1644  WT PM1644 pyshB EGFP ACTC

IB: TEM1 (SipA)
IB: GFP (YshB)
IB: ICDH