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YshB is a positive regulator for Salmonella intracellular survival and facilitates the spatio-temporal regulation of bacterial pathogenesis

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YSHB IS A POSITIVE REGULATOR FOR *SALMONELLA* INTRACELLULAR SURVIVAL AND FACILITATES THE SPATIO-TEMPORAL REGULATION OF BACTERIAL PATHOGENESIS

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For my Family.

and also to all the rational human beings who are trying to make this world a better place to live in.

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ABBREVIATIONS

ANOVA	Analysis of Variance
ATR	Acid Tolerance Response
bp	Base Pairs
CI	Competitive Index
DAP	Diaminopimelic acid
DAPI	4, 6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's Medium
DTT	Dithiothreitol
EGFP	Enhanced Green Fluorescent Protein
EMSA	Electrophoretic Mobility Shift Assay
GAP	GTPase Activating Protein
GEF	Guanine nucleotide Exchange Factor
IB	Immunoblot
ICDH	Isocitrate Dehydrogenase
iTRAQ	Isobaric Tags for Relative and Absolute Quantification
LB	Luria-Bertani
LCFA	Long Chain Fatty acid
MLN	Mesenteric Lymph Node
MOI	Multiplicity of Infection
NAP	Nucleoid-Associated Proteins
nt	Nucelotides
NTS	Non-Typhoidal *Salmonella*

OD_{600}	Optical density at 600 nm
PBS	Phosphate Buffered Saline
PUFA	Poly-Unsaturated Fatty Acid
RB-TnSeq	Random Bar-code Transposon-site Sequencing
rpm	Revolutions Per Minute
SCFA	Short Chain Fatty Acid
SCV	*Salmonella* Containing Vacuole
SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SGEF	SH3-containing Guanine nucleotide Exchange Factor
SIF	*Salmonella* Induced Filaments
SPI	*Salmonella* Pathogenicity Island
sRNA	Small RNA
STM	Signature Tagged Mutagenesis
T3SS	Type 3 Secretion System
TCS	Two Component System
WT	Wild Type

ABSTRACT

Bomjan, Rajdeep Ph.D., Purdue University, May 2018. YshB is a Positive Regulator for *Salmonella* Intracellular Survival and Facilitates the Spatio-Temporal Regulation of Bacterial Pathogenesis. Major Professor: Daoguo Zhou.

Salmonella pathogenesis primarily involves invasion of host cells followed by modulation of the intracellular environment for survival and replication. While tremendous progress has been made toward understanding the pathogenesis, the picture is far from complete. In an effort to characterize the role of small RNAs (sRNAs) in *Salmonella* pathogenesis, we identified a previously undefined small protein YshB as a positive regulator for intracellular bacterial survival and virulence. Essentially, we were able to show YshB was important for survival within macrophages and contributed significantly in mouse virulence. Further, when induced within the bacteria, YshB would lower the invasion efficiency. Notably, *yshB* expression was found to upregulate when the bacteria were inside macrophage cells. Moreover, we demonstrate that YshB mediates upregulation of PhoP, a key modulator of *Salmonella* virulence. We therefore propose a scenario where the induction of YshB mimics the intracellular phase and therefore triggers a down-regulation of the invasion machinery.

Interestingly, during the process of elucidating the mechanism of regulation mediated by this small protein, we came across a link between bacterial pathogenesis and fatty acid oxidation pathway. In particular, we found that FadB, an enzyme involved in breakdown of long chain fatty acids was upregulated in the *Salmonella* cells with induced YshB. FadB was previously implicated to be one of the *in vivo* induced (ivi) genes, which lead us to speculate that this gene might be important to

maintain intracellular survival of *Salmonella*. We were able to demonstrate that the *fadB* knockout mutants were indeed compromised in their intracellular life cycle.

The upregulation of PhoP and FadB in the YshB induced cells might therefore trigger the spatio-temporal switch from invasion-competent phase of infection to a survival-competent phase.

Based on the results we gathered, we therefore postulate a role for this small protein as a molecular liaison to mediate a cross-talk between the invasion competent extracellular and the survival competent intracellular phase of *Salmonella*.

1. INTRODUCTION

1.1 *Salmonella enterica* and the Global Burden

Salmonella enterica is a Gram negative, facultatively intracellular bacterium consisting of as many as 2,600 serovars belonging to six subspecies [1]. Almost all human pathogens belong to subspecies *enterica* (Subspecies I), whereas the other five are mainly found to infect cold blooded animals [2]. Moreover, some serovars cause infection among a broad-range of hosts, including humans, animals and even plants [3]. *Salmonella enterica* serovar Typhi however has restricted host selectivity as it can only infect humans [4]. This unique serovar causes enteric fever (typhoid) in the human host, which is a systemic illness characterized by fever along with enlargement of spleen and liver [5] causing significant morbidity, mortality and global burden worldwide, particularly in the developing world [6, 7]. In contrast, the non-typhoidal *Salmonella* (NTS) strains, primarily belonging to serovars Typhimurium and Enteritidis typically cause self-limiting inflammatory diarrhea or enterocolitis in the host [8, 9]. However, immunocompromised individuals are particularly vulnerable to the systemic form of this disease [10, 11]. According to an estimate, there are approximately 90 million cases of non-typhoidal Salmonellosis every year worldwide with 1 million cases in the United States [12]. Importantly, this is the leading cause of hospitalization and death among cases arising from all food-borne illnesses with a mortality rate of upto 0.2% [13, 14]. Consequently, to reduce and manage this global burden, development of efficient therapeutic strategies against this illness is crucial, chiefly because of lack of effective vaccines [15, 16] and also due to ever-increasing incidence of multidrug resistance [17, 18].

1.2 Pathogenesis Model

Since *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium henceforth) is an enteric pathogen, its host is infected upon ingestion of food or drinking water contaminated with fecal or urinary particles from carriers [19]. This is followed by the invasion of intestinal epithelium, subsequently leading to dissemination into deeper tissues [20].

After ingestion, the immediate adverse environment *Salmonella* encounters is the acidic pH of stomach. This results in the activation of several inducible genes which constitutes for the acid tolerance response (ATR). This protects the bacteria from the acid stress by maintaining the intracellular pH at higher values [21, 22].

In *Salmonella*, as in other pathogenic flagellated bacteria, motility is key for virulence [23]. An unimpaired motility along with an intact chemotactic ability is necessary for efficient colonization of the intestinal epithelia [24]. Interestingly, motility is essential only during the early phases and is dispensable once the infection is established. Hence, upon contact with the host cells, motility is switched off in favor of activation of the invasion machinery [25].

Once they reach the small intestine, they cross the mucus layer and adhere to the intestinal epithelial cells. The attachment to host cell is facilitated by specialized adhesive structures called adhesins that protrude from the cell surface [26]. The most common ones are the fimbrial adhesins encoded by multiple different operons such as *lpf*, *pef*, *fim* and *agf* [27]. Moreover, SiiE, a giant non-fimbrial adhesin (at 595 KDa also is the largest protein encoded by *Salmonella* genome) mediates the initial contact of the bacteria to the polarized epithelial cells of host, thus facilitating the eventual entry [28].

Following adhesion, *Salmonella* then preferably enters the M-cells of Peyer's Patches [29], although they are also seen to trigger internalization into other non-

phagocytic enterocytes [30]. The bacteria actively mediate their internalization by triggering a cascade of signaling events in the host cell characterized by massive actin rearrangement [31]. Phagocytic cells are recruited as a result of this event into the intestinal lumen followed by production of several pro-inflammatory cytokines, which eventually leads to colitis [32]. The entry process also known as the macropinocytosis results in the formation of a specialized compartment known as the *Salmonella* Containing Vacuole (SCV), where the bacteria survives and eventually replicates [33]. The SCV matures as it integrates with the host endocytic pathway; however at the later stages, it co-opts the trafficking to avoid fusion with lysosomes [34]. This is also accomplished by migration towards the Golgi apparatus to facilitate acquisition of nutrients and membrane components [35]. In few hours, the bacteria egress from the epithelial cells and are consequently captured by the phagocytes. Majority of them are macrophages followed by neutrophils, and are recruited by the ensuing inflammatory signals [36, 37]. Also among the population of phagocytic cells are the dendritic cells, which expedite the transport of bacterial population to the mesenteric lymph nodes (MLN) [38]. Moreover, these specialized cells have also been reported to directly snatch the bacteria from intestinal lumen [39]. This is followed by systemic dissemination, where the phagocytes carry the bacteria, residing within the SCV, into deeper tissues such as liver and spleen [40].

1.3 *Salmonella enterica* serovar Typhimurium Infection and Virulence Strategies

In order to successfully establish a niche in its host, bacterial pathogens need to adapt to new environments, which it does mainly by regulating the spatio-temporal expression of genes responsible for virulence [41].

1.3.1 *Salmonella* Pathogenicity Islands

As is common with most bacterial pathogens, the *Salmonella enterica* chromosome also has large regions behaving as discrete molecular units accommodating clusters of genes dedicated to encode a panoply of virulence factors [42, 43] known as the *Salmonella* Pathogenicity Islands (SPIs) [44]. These are horizontally acquired from unrelated bacteria during the course of evolution and typically have a low G+C content than the rest of the chromosome [45, 46].

There are 23 such pathogenicity islands characterized so far in *S. enterica*, and among these, five (SPI-1 through 5) are found in all serovars [47]. In *S.* Typhimurium, the 40 kb island comprising at least 39 genes flanked by the *mutS* and the *flhA* genes and located at centisome 63 of the chromosome is designated as SPI-1 [48]. This is primarily responsible for regulating the invasion machinery of this bacterium, whereby facilitating its entry into the non-phagocytic intestinal epithelium of the host [49]. Acquisition of SPI-1 into the genome has therefore been considered as the most significant event in the evolution of this bacterium towards becoming pathogenic [50]. Whereas another island, the SPI-2 is located at centisome 31, flanked by the *ydhE* and the *pykF* genes and is responsible for intracellular survival within the host macrophages and virulence in mice [44,51]. Therefore, these two islands contribute to specific phases during bacterial disease progression. Owing to its complex life cycle, the virulence in *Salmonella* is thus determined by multiple genes dispersed around the chromosome [50]. In terms of their distribution, SPI-1 was found to be present in all lineages, while SPI-2 was conspicuously absent in *Salmonella bongori* [45].

Furthermore, a number of genes within the SPI-1 have homologs in other pathogenic enterobacteria [52, 53], whereas those belonging to the SPI-2 are limited only in *Salmonella* [54].

1.3.2 Virulence Plasmid

S. Typhimurium strains also harbor a plasmid called the pSLT, which is approximately 95kb in size and consists of a conserved *spv* locus which encodes for five proteins [55] essential for virulence in mice. Among the proteins, SpvR is the regulator which activates the *spv* locus [56]. SpvA is an outer membrane protein [57], whereas SpvB, SpvC and SpvD are effectors [58] whose functions are briefly outlined in the next section.

1.3.3 *Salmonella* Type III Secretion Systems

Most bacterial pathogens employ a macromolecular system to secrete proteins across its membranes into the extracellular milieu or inject directly into the cytosol of host cells [59]. These secreted proteins, also known as the substrates mainly function in enhancing bacterial virulence by modulating a wide array of host cellular processes [60]. There are six such dedicated secretion systems in Gram negative bacteria numbered I through VI [61]. Among these, the Type III secretion system (T3SS), also commonly known as the "injectisome" or the "needle complex" is a nanosyringe which efficiently secretes the substrates across the bacterial membranes [62]. Moreover, this system is specialized in delivering the substrates directly into the eukaryotic cytosol, in a process called "translocation". In such instances, the substrate proteins are called "effectors" [63]. The T3SS therefore has to traverse through three phospholipid membranes and the peptidoglycan layer of the bacteria, in order to directly deliver the effector proteins into the target host cells [64]. Characteristically, the T3SS substrates have their secretion signal in the N-termini and are transported in an unfolded state [60].

Structure-wise, the T3SS has three main subunits [60]: the "basal body" spans both the bacterial membranes and the periplasm and also contains the cytoplasmic components; the "needle" with a hollow core acting as a conduit protrudes externally from the basal body; and the "tip complex", at the distal end of the needle which regulates the secretion process. The translocation pore or the "translocon" is the crucial element of the tip complex for the delivery of effector proteins into the host cell [65].

S. Typhimurium employs two type III secretion systems, namely the T3SS-1 and T3SS-2, encoded within the SPI-1 and SPI-2 pathogenicity islands respectively. The T3SS-1 is spatio-temporally coordinated to ensure that it is synthesized only when the bacteria needs to invade the host cells [66]. The ensuing contact with the host cell acts as an activating signal for the secretion of the T3SS substrates [67, 68].

There are two gene clusters within SPI-1 responsible mainly for coding the structural components of the secretion machinery, namely the *inv-spa* and the *prg-org* clusters. Between these two clusters lies the *sic-sip* gene cluster which encode the major SPI-1 effector proteins and their chaperones [66].

The first gene of the *inv-spa* cluster, *invH*, encodes a lipoprotein which is critical for positioning InvG in the outer membrane [69,70]. InvG is one of the major component of T3SS-1 basal body [71] and forms the outer ring, and hence is essential for the secretion machinery to operate, as no needle structure was detected in a $\Delta invG$ strain [72]. InvE which is homologous to *Yersinia* YopN, was shown to control the secretion of substrates by interacting with the SipB-SipC complex [73]. InvA is an integral membrane protein which forms the export gate, and its C-terminus is responsible for recognizing species-specific substrates for secretion [53]. InvB has been characterized as a T3SS associated chaperone for the effector proteins SipA, SopE, SopE2 and SopA [74–77], demonstrating the same protein can function as chaperone for multiple

substrates. InvC is the conserved ATPase of the T3SS-1 system providing the energy necessary for secretion [78]. InvJ is a regulatory protein which acts as the molecular ruler [79] of the T3SS-1 needle complex and along with PrgJ mediates substrate switching [80]. In the $\Delta invJ$ mutant, the needles are abnormally long and non-functional [81]. The SpaP, SpaQ, SpaR and SpaS proteins form the export machinery along with the InvA and InvC [82].

The *prg* (PhoP-repressed genes) genes were first identified as a group of genes repressed in a mutant strain with constitutively active PhoP (*pho-24* mutant) of the *phoP/phoQ* two component system (TCS) [83]. Among them, PrgH and PrgK form the inner ring of the basal body [84]. PrgJ is the inner rod protein which links the needle to the base, and regulates substrate switiching and the needle length [80], while PrgI is the needle protein [81]. The *org* (oxygen-regulated genes) genes *orgA* and *orgB* are transcribed from the upstream *prgH* promoter and code for proteins OrgA and OrgB which are key elements of the cytoplasmic components of the basal body [85].

Although the components of T3SS-2 system are not as well characterized as those of T3SS-1 [86], role of several structural proteins are defined. SsaV is integral component of the inner ring of the secretion system [87], whereas SseB, SseC, and SseD are the substrates of T3SS-2 and form the translocon [88]. SsaN is the ATPase of T3SS-2 system [89].

1.3.4 Effectors of Type III Secretion Systems

As mentioned previously, the effectors of the T3SS-1 mediate the entry of the bacterium into the non-phagocytic host cells, marking the initiation of infection process; whereas those translocated via the T3SS-2 are crucial in the intracellular survival of the bacterium. Most T3SS-1 effectors are encoded within the SPI-1, whereas

some that are translocated via the T3SS-1 are encoded in the regions of chromosome outside of SPI-1 [90]. Collectively, these effectors trigger a cascade of biochemical reactions in the host cell which ultimately lead to the internalization of bacteria by the non-phagocytic epithelial cells [91]. Interestingly, their function is largely limited to mediating the entry into epithelial cells, as the SPI-1 mutant infected intraperitoneally is still able to attach to the cultured cells and cause systemic disease in mice [92].

Among the effectors of the T3SS-1, the Sip (*Salmonella* invasion protein) proteins SipA, SipB, SipC and SipD are encoded by the *sip* operon and are among the most crucial proteins mediating invasion [93]. These proteins have a high degree of sequence homology with the Ipa proteins from *Shigella* [94, 95]. SipA modulates actin filament polymerization by directly binding to this host protein, reducing the critical concentration and inhibiting the depolymerization [96]. Moreover, SipA facilitates the bacterial entry by increasing the actin-bundling activity of the host protein T-plastin [97]. SipC is a component of the T3SS-1 translocon and is able to insert into the host membrane as soon as 15 minutes post infection [98]. In addition to this, it also has a role as a *bona fide* translocated effector, where it mediates actin bundling and nucleates actin polymerization [99, 100]. SipB is critical for the formation of the translocon complex along with SipC [98, 101]. SipD is the tip protein which interacts with the needle protein PrgI to form the needle complex to influence effector secretion [102, 103].

SopE is the guanine nucleotide exchange factor (GEF) for the host small GTPases Cdc-42 and Rac-1 [104], while the closely related SopE2 is the GEF only for Cdc42 [105] thus activating these G-proteins to initiate the biochemical cascade ultimately resulting in the formation of specialized membrane structures called "ruffles" which mediates the bacterial uptake. SopB is encoded by a gene located in SPI-5 [106], and

is a phosphatidylinositol phosphatase which promotes membrane ruffling and thus the bacterial internalization [107, 108]. This property of SopB is also enhanced by its activation of SH3-containing guanine nucleotide exchange factor (SGEF), an upstream exchange factor for RhoG [109]. SptP is a tyrosine phosphatase and also acts as the GTPase activating protein (GAP) for Cdc42 and Rac1, reversing the biochemical effects initiated by the invasion proteins and hence mediating the recovery of normal actin architecture [110, 111].

SopA is an E3 ligase for ubiquitination [112] and along with other major invasion proteins, also plays a role during bacterial entry [113]. Further, this multifunctional protein has also been demonstrated to promote neutrophil infiltration [114].

Apart from the above mentioned effectors which have roles in the invasion process, the SPI-1 encodes effectors which function in other aspects of *Salmonella* pathogenesis. For instance, AvrA inhibits the host immune response, and has been proposed to mediate bacterial survival within macrophages by suppressing apoptosis and thus preventing early bacterial dissemination [115].

Once *Salmonella* successfully enters into the host cell, it switches its attention to maintaining a replicative niche inside the host in the form of a specialized membrane bound compartment known as the *Salmonella* containing vacuole (SCV) [116]. As indicated previously, this phase of the life cycle is mediated by the action of a panel of effectors secreted through the T3SS-2 [117]. The T3SS-2 is known to translocate at least 28 effectors from within the SCV into the host cytosol. The primary functions of these are to modulate intracellular growth, and are essential for intestinal as well as disseminated infection [118]. As in the case of T3SS-1 secreted effectors, these have diverse functions. Most are known to have enzymatic activities, whereas a subset of them behave as adaptors influencing the functions of their target host proteins [118].

Although there are multiple effectors being translocated through T3SS-2, SseF and SseG are the only two effectors which are encoded by the SPI-2 [119]. These are present as core effector proteins in all serovars and mediate the tethering of SCV to the Golgi network [35] by forming a complex with the host protein ACBD3 [120]. The localization of SCV near to the Golgi network is key as it contributes to acquisition of nutrients and host membrane components during the maturation of the bacteria containing vacuole [121]. Moreover, these two effectors play a critical role during intracellular growth inside host macrophages [119], or *in vivo* mice infection [35]. Another core effector, SifA, is multifunctional and maintains vacuolar integrtiy [122], as well as reduces the toxic lysosomal activity for which binding with its target SKIP is required [123]. Consequently, a *sifA* mutant releases to the cytosol from the SCV. In an epithelial cell, the *sifA* mutant can hyper-replicate [124], whereas in the macrophages, it induces pyroptosis [125]. SifA also contributes to the formation of long specialized tubules originating from the SCV originally termed SIFs for *Salmonella* Induced Filaments [126, 127]. These structures along with other similarly induced interconnected tubular systems have since been renamed as SITs or *Salmonella* Induced Tubules [128]. The proposed function for these systems is aiding nutrient acquisition for the intracellular bacteria owing to the increased surface area due to tubulation [129].

SseJ and SopD2 appear to antagonize the membrane stabilizing role of SifA [130, 131] as these were responsible for destabilizing the vacuole membrane in the absence of SifA. SseJ is an acyltransferase which is located at the cytosolic face of SCV and esterifies cholesterol to modulate the lipid content and hence the stability and fluidity of the SCV membrane [132]. This also presumably results in the accumulation of lipid droplets in the host cell cytosol [133]. The deubiquitinase activity of SseL mediates clearance of these lipid droplets by either degrading them or inhibiting their

formation [134]. Moreover, SseL also inhibits the autophagy pathway by removing ubiquitin from the host cytosolic aggregates formed post-infection [135]. SopD2 binds to and inhibits the nucleotide exchange of host small GTPase Rab7 [136] and is a GAP for Rab32 preventing its accumulation on the SCV thus contributing to restricting vesicular transport and endosome to lysosome trafficking [137]. PipB2 tethers kinesin-1, a host microtubule motor protein to the SCV membrane, which is activated by SifA, thus resulting in anterograde extension of SCV and formation of SITs [138]. SteA is known to bind to host phosphatidylinositol 4-phosphate (PI[4]P) [139], and is responsible for the partitioning of SCV membrane [140], which results in an individual vacuole enclosing a single bacterium. SpiC, which is also a component of the T3SS-2, inhibits phagolysosome formation [141] by targeting Hook3 protein of the host [142]. *Salmonella* therefore dedicates multiple effectors, mainly translocated via T3SS-2, in modulating the stability of the SCV membrane and also co-opting the host trafficking system.

SteC is a kinase, and mediates the assembly of F-actin meshwork around the SCV [143], whereas SpvB, a virulence plasmid encoded protein ADP-ribosylates G-actin to prevent actin polymerization [144].

GtgE is a specific cysteine protease that targets Rab32 as well as Rab29 and Rab38 [145]. This effector is absent in serovar Typhi, and thus is attributed to the strict requirement of human host for this serovar, since Rab32 is shown to be the host factor responsible for preventing the growth of serovar Typhi in murine macrophages [146].

There are several effectors, interestingly most of which are secreted both via the T3SS-1 and T3SS-2 which play anti-inflammatory roles by suppressing host innate immunity [118]. PipA, GogA, GogB, GtgA, SpvD and SseK2 have been shown to inhibit host NF-κB signaling [147–149], whereas SpvC inhibits the MAPK signaling

pathway [150]. SseK1 and SseK3 in addition to inhibiting the NF-κB signaling also inhibits necroptosis in the host [151, 152].

1.4 Regulation of Virulence Determinants

1.4.1 Regulators of Bacterial Invasion

Various factors including bacterial proteins and environmental signals are responsible for regulating the process of bacterial invasion [153].

HilA is the master regulator of bacterial invasion and belongs to the OmpR/ToxR family of transcriptional activators [154]. The central role played by this protein in bacterial invasion is emphasized by an observation that a *hilA* deletion phenotype is similar to deletion of whole SPI-1 locus [155]. HilA directly binds to the promoters of the *prg/org* and *inv/spa* operons to activate them [156]. Furthermore, it is shown to transcriptionally activate the expression of SopB, a key T3SS-1 effector [157].

InvF belongs to the AraC family of regulators [71]. Its expression is primarily activated by HilA [158], although HilA-independent mechanisms have also been reported [159]. InvF and a chaperone protein SicA acts together to mainly activate effectors of the *sic/sip* operon [160].

HilC and HilD are encoded within the SPI-1 [161] and along with RtsA, control the expression of HilA [162]. All three regulators belong to the AraC/XylS family and bind to the *hilA* promoter as well as their own respective promoters for self activation [155]. HilD is the major activator of HilA, and along with the other two, amplifies the signal for *hilA* transcription [163]. Moreover, HilD and HilC can also activate *invF* expression by binding to an alternative promoter [159].

FliZ is primarily associated with regulation of flagella expression, but is also known to post-transcriptionally increase HilD level in the cell, thus acting as a positive regulator of invasion [164].

The BarA-SirA two component system also has an input in bacterial invasion [165]. SirA is the response regulator which activates *hilA* transcription in the presence of HilD [155]. SirA mediates its action by upregulating the expression of *csrB* and *csrC*, which code for small RNA molecules that are inhibitory to an RNA binding protein CsrA [166]. CsrA is a negative regulator of invasion as it binds to *hilD* mRNA to block its translation [167].

HilE is the negative regulator of bacterial invasion, and it acts by directly binding to and hence blocking the HilD activity which results in the negative regulation of HilA [168].

FimZ is another negative regulator of the invasion machinery as it directly binds and activates the *hilE* promoter [169]. Further, this protein is also know to negatively regulate bacterial motility by repressing the flagellar *flhDC* operon [170].

H-NS is the major nucleoid-associated protein (NAP) which represses several genes involved in bacterial virulence by binding to DNA at or near the promoter region [171]. Among the regulators of invasion, H-NS represses expression of HilA, HilD, HilC, and RtsA [172]. Moreover, H-NS is also known to repress genes other than those involved in invasion. Interestingly, among these other genes are some SPI-2 genes such as *ssaB*, *ssaG*, *ssaM*, and *sseA* [173]. Apart from H-NS several other NAPs also contribute to regulation of diverse virulence genes. Hha synergistically aids H-NS mediated repression of *hilA*, *hilC* and *rtsA* [172, 174]. Similarly, YdgT negatively regulates the SPI-2 genes by acting synergistically with Hha [175]. Integration host factor (IHF) is an NAP that acts as an activator of invasion during the logarithmic growth phase at the level of HilD [176]. Similarly, Fis activates invasion genes by influencing *hilD*

transcription [177]. Moreover, this global regulator also activates multiple genes from the SPI-2, SPI-4, and SPI-5 loci [178, 179].

There are still other proteins that play regulatory functions during invasion. RtsB influences SPI-1 gene expression by negatively regulating FliZ [180]. Lrp is a negative regulator of most virulence genes such as *hilA*, *invF* and *ssrA* [181]. Lon protease is a heat shock protein that negatively regulates *hilA* transcription [182]. Whereas the chaperone complex of DnaK/DnaJ antagonizes the Lon activity and promotes invasion [183]. Fur is a positive regulator of invasion due to its repression of *hns* gene expression [184].

1.4.2 Regulators of Intracellular Survival

The SsrA-SsrB two component system is the master regulator of the SPI-2 gene expression responsible for intracellular bacterial survival and mouse virulence [185]. SsrA is the sensor kinase and SsrB is the response regulator of this system [186]. SsrB facilitates the induction of SPI-2 genes by relieving transcriptional repression mediated by H-NS [173]. Interestingly, in a splendid example of cross talk between the SPI-1 and SPI-2 systems, HilD binds to the regulatory region downstream of *ssrAB* promoter to displace H-NS and induce expression of multiple SPI-2 genes [187, 188]. Moreover, SsrB has been recently shown to repress SPI-1 expression through its actions on *hilA* and *hilD* transcription [189].

The OmpR regulator from the EnvZ/OmpR two component system activates the *ssrAB* transcription by directly binding to the promoter following entry into the macrophages [190]. Furthermore, SlyA is another regulator that modulates intracellular survival and virulence by stimulating *ssrA* transcription [191].

1.4.3 *phoQ/phoP* Two Component System

The *phoP* gene was originally described as a regulator of nonspecific acid phosphatase (PhoN) expression [192]. However, its role in bacterial virulence was first characterized in 1989 as the one encoding a protein that protects the bacteria from the action of host antimicrobial peptide defensin [193]. In the same year, Miller *et al.*, showed that this is a two-component regulon comprising of the periplasmic domain containing sensor kinase PhoQ which phosphorylates and activates the response regulator PhoP. Moreover, they identfied in this study a gene *pagC* which was required for virulence in mice and intra-macrophage survival [194]. Still in 1989, the two-component nature of the system and its role in transcriptional regulation of *psiD* gene in response to phosphate availability was demonstrated independently by Groisman *et al.*, [195].

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 as opposed to the wild type following macrophage uptake [196]. Further, it was observed that the activation of certain *pag* genes occurred maximally when the environment inside the macrophage became acidic (pH<5) and not when the bacteria were inside the vacuoles in epithelial cells [197]. However, a later study indicated the signals activating *pag* genes might be similar in both cell types [198]. Moreover, it was known later that the pH mediated induction of these genes is independent of the *phoP/phoQ* system, since the activation was noted even in *phoP* and *phoQ* mutants [199, 200]. Nevertheless, this suggested that the change in the microenvironment encountered by the bacteria can induce a switch in gene expression profile.

In an interesting finding, Miller *et al.*, observed that constitutive expression of PhoP (PhoP^C phenotype) owing to the *pho-24* mutation [192] in the *phoP/phoQ* locus, would have a detrimental effect on *Salmonella* virulence to a similar extent to

a *phoP* deletion mutant. They observed the level of multiple proteins being repressed in the PhoP^C phenotype and thus termed the genes encoding them collectively as *prg* (*phoP*-repressed genes) [83]. Further, this virulence phenotype associated with the PhoP^C strain was seen to require the presence of plasmid encoded *spv* gene products [201]. However, it was later demonstrated that the PhoP^C phenotype does not involve the *phoP* gene *per se* nor it results in constitutive expression, as the phenotype was attributed to a single amino acid substitution (T48I) in PhoQ resulting in increased kinase activity [202]. Further, this mutant form of PhoQ required much higher concentration of Ca^{2+} in the environment in order to repress the transcription of target genes [203].

In a landmark study, Behlau and Miller showed the *phoP/phoQ* locus oppositely regulates bacterial invasion of epithelial cells and survival inside macrophages. Specifically, they found that one of the *prg* gene products PrgH which was repressed considerably in the PhoP^C phenotype was essential to induce bacterial mediated endocytosis in the MDCK epithelial cells. Expression of genes in the *prg* loci also appeared to be repressed when grown under conditions inducible for *pag* gene activation [204]. This further illustrated that appropriate regulation of a set of genes is key to virulence. Two years later, the same group showed activated PhoP negatively regulates the *prgHIJK* operon essential for effector secretion, resulting in the significant difference in the secretion profile and hence the consequential defect in the invasion phenotype of these cells [205]. In 1996, Bajaj *et al.*, found that the PhoP-mediated modulation of invasion was mediated by regulating the expression of HilA [156].

The PhoP-PhoQ regulon has multiple effects on bacterial physiology, as they are known to regulate more than 1% of open reading frames of *Salmonella* genome [206]. These cellular processes include transport of Mg^{2+} [207], modification of bacterial

lipopolysaccharide layer [208, 209], resistance to host derived cationic antimicrobial peptides [210–212], resistance to bile [213] and peptidoglycan remodeling [214].

The virulence defect attributed to *phoP/phoQ* mutant is partially mediated by one of the PhoP activated genes *mgtC*. This gene is located within the SPI-3 locus and the encoded protein MgtC primarily functions in acquisition of Mg^{2+} by the cell. This protein therefore assures the availability of Mg^{2+} to the intravacuolar bacteria [215].

PhoP is a 224-amino acid protein with sequence similarity to other DNA-binding transcriptional regulators of OmpR subfamily [216]. Whereas PhoQ is a 487-amino acid sensor kinase that phosphorylates PhoP at the N-terminus in response to relevant environmental signals by transferring a phosphate group from its autophosphorylated histidine [217]. The regulon is autogenously regulated by its components PhoP and PhoQ in a positive manner [218], facilitating the amplification of signals sensed by the sensor kinase. There are two promoters for the operon, one of them $phoP_{p2}$ is constitutive, whereas the activation of the other one $phoP_{p1}$ is triggered by low Mg^{2+} and is PhoP-PhoQ dependent. Seemingly, the PhoP proteins that are present at basal levels are phosphorylated by PhoQ following environmental stimulation. The phospho-PhoP would then initiate a positive regulatory loop by activating transcription of $phoP_{p1}$, leading to increased expression of *pag* gene products [199]. This two component system primarily responds to the periplasmic levels of the divalent cations Mg^{2+} and Ca^{2+}, leading to regulation of various cellular processes [199]. Notably, this system is activated by the very low (micromolar) levels of Mg^{2+}, whereas even millimolar concentration is high enough to repress the transcription of the operon [219], presumably by triggering the dephosphorylation of phosphorylated PhoP by PhoQ [220]. The sensing of Mg^{2+} concentration by this system aids the bacteria to determine its spatial location, where the intracellular microenvironnment is characterized by a substantially low concentration of this divalent cation [221]. This causes the dif-

ferential expression of genes required at different phases of the bacterial life cycle corresponding to different stages of pathogenesis [222, 223].

The PhoP/PhoQ system hence illustrates an exquisite example of spatio-temporal control of gene expression essential in pathogenic bacteria.

1.5 Influences of Long Chain Fatty Acids (LCFAs) in Bacterial Survival

External fatty acids of different chain lengths can affect the metabolism of bacteria in diverse ways. For decades, the antimicrobial properties of fatty acids have been demonstrated. The germicidal action of fatty acids on fungal growth was reported as early as 1899 [224]. Burtenshaw in 1942 proposed an association between the self-disinfecting nature of skin with the free fatty acids of long chain length [225]. The inhibitory action of fatty acids can be attributed to the physico-chemical mechanisms. In particular, the unsaturated fatty acids can alter the surface tension if accumulated around bacterial cells thus significantly affecting the permeability of the surface [226]. It is observed in general that longer chain length and higher degree of unsaturation of the fatty acid chains tend to increase this detergent-like inhibitory properties [227].

Lipids of different forms are also known to activate the mononuclear phagocyte system. Specifically, triglycerides of various fatty acids [228] and polyunsaturated fatty acids (PUFAs) [229] stimulate the phagocytic activity. Further, incorporation of unsaturated fatty acids supplied exogenously into the macrophage membrane enhances its phagocytic activity by altering the membrane fluidity [230, 231]. Supplementation of macrophage membrane with PUFAs also alters the physico-chemical properties of lipid raft membrane domains and subsequently enhances the antibacterial properties [232]. Unsaturated fatty acids also trigger the differentiation of macrophages into the M2 subtype which are more efficient phagocytes [233, 234]. Some specific long chain fatty acids play critical immunomodulatory roles. Arachidonic acid is

one such polyunsaturated fatty acid which is a precursor for eicosanoid mediators Prostaglandin and Leukotriene [235]. These metabolites are pro-inflammatory in nature and serves to eradicate pathogenic invaders [236].

The fact that presence of long chain fatty acids in the immediate vicinity can have detrimental effects on bacteria should prompt these pathogens to try and breakdown these biomolecules. This is accomplished via the fatty acid breakdown or the β-oxidation pathway which maintains the lipid homeostasis [237]. In *Salmonella* and in *E. coli*, the breakdown is catalysed by the enzymes of the *fad* regulon [238]. The steps involved can be summarized as transport of long chain fatty acids into the cell interior followed by activation and enzymatic cleavage generating molecules of acetyl coenzyme A [239]. Transport of LCFAs from the external milieu across the bacterial outer membrane towards the periplasm is facilitated by a membrane protein FadL [240]. The fatty acids are next transported to the cytosol across the inner membrane by FadD, an acyl coenzyme A synthase [241]. The long chain acyl-CoA is then converted to enoyl-CoA by the dehydrogenase FadE [242]. The enoyl-CoA is hydrated followed by oxidation (dehydrogenation) mediated by FadB, and the final step of thiolytic cleavage is catalyzed by FadA. The FadB and FadA proteins form a tetrameric complex consisting of two copies of each molecule [243]. This complex displays five different enzymatic activities. FadB alone mediates the epimerase, isomerase, hydratase and dehydrogenase activity while FadA possesses the thiolase activity [244]. This cycle is then repeated with the removal of one acetyl CoA from longer acyl CoA molecule in each cycle [237].

Regulation of fatty acid degradation and its biosynthesis is mediated by FadR protein Fig.1.1. This molecule acts as a transcriptional repressor of the degradation pathway [238] by binding to the operator of *fad* genes; while acting as an activator of the biosynthesis pathway [246]. The long chain acyl CoA molecules are known

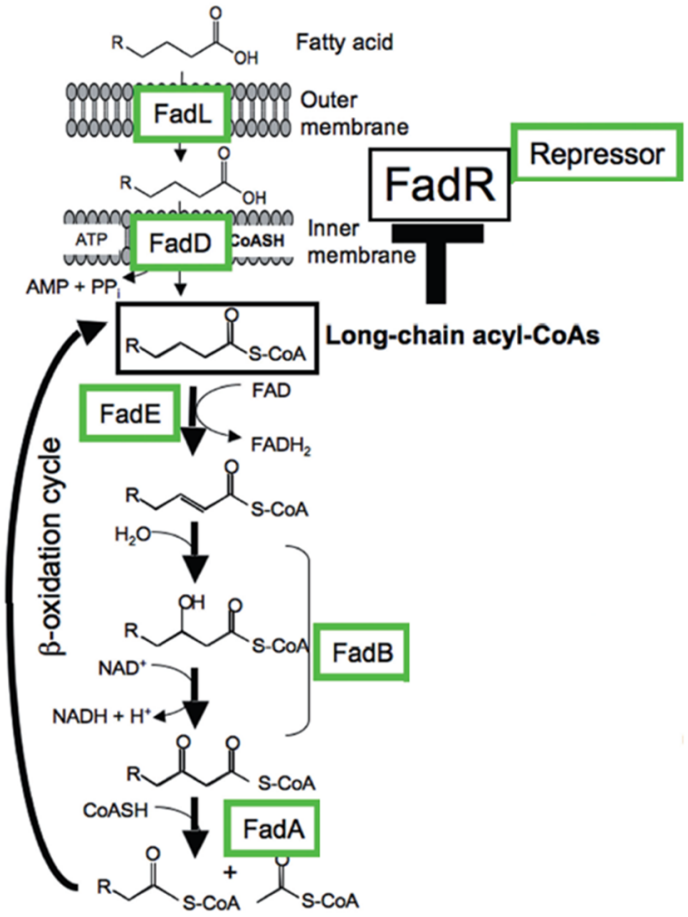

Fig. 1.1. **Fatty acid metabolism and regulation through FadR. (Reprinted with permission from [245]).**

to bind to the FadR protein, causing their release and hence the derepression of *fad* genes [247, 248]. Overall, the regulation of fatty acid degradation and biosynthesis pathways are sophisticated and finely tuned.

1.6 Small RNAs in Bacterial Virulence

Small non-coding RNAs (sRNAs) often also referred to as small regulatory RNAs are molecules heterogenous in size (ranging from 50 to 500 nucleotides) and known to post-transcriptionally regulate gene expression in bacteria [249]. These charac-

teristically belong to a specific regulon and are typically able to modulate multiple targets resulting in a global control mechanism [250]. Commonly, the mode of action for these molecules is to base pair with the target mRNA entities, causing an altered expression by affecting the stability or translation of the target molecules [251]. The antisense activity can be either *cis*- (when it is encoded from the opposite strand) or *trans*- when the target loci are physically unlinked [252]. The pairing, specially in *trans*-acting molecules is usually discontinuous and relatively short, consequently its formation is fast. Further, different regions within a single sRNA molecule can pair with multiple targets [253]. The response mediated by these molecules are therefore rapid and are particularly suited to respond to sudden environmental changes the bacteria might encounter during the various phases of its life cycle [254]. For most virulent bacteria this underscores the transition between saprophytic and pathogenic lifestyles [255]. These molecules therefore add another layer to the complex interplay between various factors involved in gene regulation [256].

For many years, the roles of regulatory RNA in bacterial pathogenesis went unappreciated. RNAIII in *Staphylococcus aureus* was considered a paradigm for RNA species modulating bacterial pathogenesis, where it was known to act as antisense regulator of *Staphylococcus* virulence factors [257]. In recent years, owing largely to the rapid development of biocomputational methods for prediction and screening technologies such as tiling arrays and RNA-seq, there has been intensive studies and discoveries linking sRNAs and bacterial pathogenesis [258]. Since sRNAs are particularly useful in mediating rapid response in gene expression, one instance where these are proven to be crucial is when bacteria are subjected to various stress conditions or environmental insults when they enter the host [259]. Also of special importance is the role of RNA-binding proteins such as Hfq and CsrA, which work in close conjunction with numerous sRNAs to regulate diverse aspects of bacterial pathogenesis [260, 261].

Hfq-associated small RNAs play a vital role in *Salmonella* pathogenesis. In a study, it was found that more than half of all known *Salmonella* secreted proteins could be targets for these small RNAs [262]. Some well characterized sRNAs in *Salmonella* relevant to pathogenesis include InvR, SgrS, IsrJ, IsrM, PinT and CsrB/C [263]. InvR was the first small RNA discovered from a pathogenicity island (SPI-1), and functions to regulate porin synthesis [264]. IsrM is another island encoded sRNA that regulates both the invasion and intracellular survival phase of virulence by targeting *hilE* and *sopA* mRNA [265]. SgrS is a dual-functioning core-genome encoded conserved sRNA which represses the expression of *sopD* mRNA [266]. The prophage encoded IsrJ mediates host cell invasion by regulating translocation of SPI-1 effector SptP [267]. PinT is a PhoP-activated sRNA that represses the expression of *sopE*/*sopE2* encoding the GEFs responsible for bacterial invasion, and also *crp* (cyclic AMP receptor protein) which activates several virulence genes during the infection process [268]. CsrB and CsrC are redundant regulators and antagonize the action of an RNA-binding protein CsrA which has pleotropic effects in *Salmonella* virulence [166, 167].

Given the diverse role they have in bacterial gene regulation, an in depth study could well elucidate novel mechanism of virulence control by these molecules.

1.7 Small Proteins

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 [269]. By convention, small proteins are defined as those with 50 or less amino acids. They are also different from small peptides resulting from the cleavage of larger precursor proteins [270]. Although ubiquitous, these molecules have traditionally been under-studied, due to limitations in biochemical, genetic and bioinformatic studies owing to

their size [271]. These can be broadly grouped into the non-secreted ones which may be cytoplasmic or membrane-associated and mostly play regulatory roles in cell physiology and the secreted ones which mainly function in interbacterial communication such as quorum sensing autoinducer peptides or competition such as bacteriocin [272].

The diverse cellular processes modulated by the non-secreted small proteins typically involve spore formation (in *Bacillus subtilis*) [273, 274]; cell division [275–277]; signal transduction [278, 279]; transport [280–282]; regulation of enzymatic activity of membrane bound proteins [283–285] and chaperone function [286, 287].

Due to the small size of these proteins, the number of activities they can have is limited. For instance, these are very unlikely to have any enzymatic properties. These molecules therefore preferably function in mechanical manner. One example of such protein is MciZ (mother cell inhibitor of FtsZ), which inhibits the activity of tubulin like protein FtsZ, a major component of the protein complex mediating cell division in bacteria [288]. From a yeast two-hybrid screening, MciZ was found to directly bind to and thus block the GTP-binding domain of FtsZ, hence inhibiting FtsZ polymerization. This resulted in prevention of unwanted cell division during sporulation [275]. Similarly, MgrB is a small protein which negatively regulates the PhoQ/PhoP system in *E. coli* [289]. In this study, the authors noted that deletion of *mgrB* increased the transcription activity of its own promoter, whereas overexpressing MgrB from a plasmid would repress the transcription. Since the PhoQ/PhoP system was known to regulate *mgrB* expression [290], they tested whether MgrB would interact with the two-component system. They were able to demonstrate that MgrB would indeed bind and repress PhoQ activity, as a result of the negative feedback loop. In 2016, Salazar *et al.*, observed that MgrB modulates the bifunctional activity of *E. coli* PhoQ to regulate the partial adaptation of PhoP dependent genes [279]. Sda is another cytosolic small protein which blocks the interaction between different

kinases involved in *B. subtilis* sporulation and even between different domains of a single protein [291].

Interestingly, interplay between a small protein and its target can promote interaction between domains or proteins. In one example, MgtR, a 30-amino acid small protein in *Salmonella* assists the protease activity of an AAA+ protease FtsH to degrade the virulence factor MgtC [285]. MgtR was shown to directly interact with MgtC thereby facilitating its degradation by FtsH. This occurs presumably by changing the structure of MgtC, hence rendering it sensitive to the protease. Similarly MntS, a 42-amino acid small protein in *E. coli* is known to optimize the Mn^{2+} concentration inside the cell by inhibiting the export of this cation by the transporter MntP [286]. Morever, some small proteins are proposed to function as chaperones for small RNAs. In a study, Smaldone *et al* suggested that a small protein FbpB may aid the activity of a small RNA FsrA by acting as a chaperone [292].

In the following chapters of this dissertation, we provide evidence for a small protein YshB playing a regulatory role in different aspects of *Salmonella* virulence. This ubiquitous small protein constitutes of 36 amino acids and was annotated in a screen based on presence of ribosome binding sites [293], and later validated as a protein expressing gene in *E. coli* [294].

2. A SMALL PROTEIN YSHB WITHIN A PREDICTED SMALL RNA STNC1450 LOCUS MODULATES *SALMONELLA* VIRULENCE

2.1 Abstract

Bacterial small non-coding RNAs (sRNAs) are known to modulate expression of virulence genes to withstand host defenses during infection. Therefore, we hypothesized that the small non-coding RNAs in *Salmonella* play a key role in its pathogenesis. In order to test our hypothesis, we first constructed a deletion mutant library of putative *Salmonella* sRNAs in strain SL1344, and characterized the mutants by conducting assays to assess their efficiency on invasion of host epithelial cells. Altogether, 52 knockout strains were assessed for their invasion efficiency in HeLa cells. However, none of the deletion mutants displayed any significant phenotype, therefore, we exogenously expressed some of the sRNAs from an arabinose inducible plasmid in the wild type strain. From this, we found that induction of sRNA STnc1450 significantly reduced the invasion efficiency. However, the genomic location of STnc1450 showed that there was a small open reading frame within the predicted sRNA encoding gene named *yshB*. This led us to suspect that the phenotype might have been due to the induction of either the protein or the small RNA. We were able to demonstrate that the phenotype was in fact due to the induction of small protein. More importantly, we showed that YshB is important during the intracellular phase of the bacterial life cycle and plays a significant role in mice virulence. Furthermore, we found *yshB* expression was higher when the bacteria were grown under SPI-2 inducing conditions or when inside host macrophages. Consequently, we propose the induction of this small

protein in *Salmonella* mimics intracellular conditions and hence acts as a signal to switch the down-regulation of the invasion machinery.

2.2 Introduction

Pathogenic bacteria have evolved complex regulatory circuits to tightly regulate the expression of virulence genes. Commonly, this is achieved by turning on or off a certain repertoire of genes once the pathogen reaches either a specific site in the host, or after a certain period of time post infection. This is evidently the case in *Salmonella*, which first has to actively participate in the 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 principally mediated by the effectors of Type III Secretion System-1 (T3SS-1). Apart from the direct role of these secreted effectors in mediating invasion, an elaborate network of regulatory proteins also play a vital role in regulating the expression of invasion-related genes [153]. Essentially, the expression of these genes is governed by the signals from external environment; in this case, the distal small intestine of the infected host [295]. The invasive phase is succeeded by sustenance and multiplication inside the host cells within a specialized compartment called the *Salmonella* Containing Vacuole (SCV). This is mediated primarily by the components of the *Salmonella* Pathogenicity Island-2 (SPI-2) system. This spatio-temporal regulation of virulence gene expression is critical for *Salmonella* mainly because most of the effectors are committed to either the invasive or the replicative phase of the life cycle. Although it is known that some effectors have a multi-faceted role in modulating these diverse phases [296].

Salmonella is able to sense cues from its immediate environment as a means to switch the expression of particular sets of genes. Depending on where the bacteria

are located, these signals might vary. For instance, the site of invasion into the host cells is the distal intestine. Therefore, the immediate environment at this site which is low oxygen and high osmolarity triggers the induction of SPI-1 genes. This results in the synthesis of the invasion machinery, which renders the bacteria competent for actively entering host enterocytes. Once the bacteria successfully enter the host cells and reside within the vacuole, the chemical composition of the microenvironment encountered is different as opposed to when they are extracellular. Particularly when the bacteria are engulfed by the macrophages, the intraphagosomal environment is predominantly one with low cation concentration and mildly acidic pH. These cues are necessary to switch the preference towards the intracellular adaptation, so that different sets of genes are induced to suit the needs.

More importantly, this fine-tuning for the switch is necessary from multiple stand points, as gene expression at unwanted times is not only redundant or energy expensive but also detrimental. One example is SipB, a major protein mediating invasion, which is also known to induce programmed cell death in macrophages [297]. Consequently, when the bacteria reside within the host macrophage cells, it maneuvers the regulation machinery to repress the genes essential for invasion. Hence, there is some degree of inverse regulation between the invasive and survival phases.

Interaction of *Salmonella* with its host cells has consistently been a fascinating subject of inquiry for many decades now. Owing to some recent exciting discoveries due mainly to the technological advancements in the field of RNA sequencing and *in silico* approaches [265, 298, 299], we made an effort to assess the role of small RNAs in *Salmonella* virulence. While doing so, in a rather exhilarating case of serendipity, we functionally characterized a small protein YshB to establish its role in bacterial virulence.

In this chapter, we present evidence for the role of a novel small protein YshB as a modulator of the switch to trigger adaptation to the intracellular phase of the bacterial life cycle.

2.3 Materials and Methods

2.3.1 Bacterial strains and mammalian cell lines

Isogenic derivatives of virulent wild-type (WT) SL1344 strain of *Salmonella* Typhimurium were used in this study [300]. In-frame chromosomal deletions of genes and chromosomal flag tagged constructs in *Salmonella* strains were generated by the one-step gene disruption strategy [301, 302] using the λ-Red recombination system. *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 [303]. Antibiotics when needed were used at the following concentrations: Streptomycin (150 μg/ml), Ampicillin (120 μg/ml), Kanamycin (40 μg/ml) and Tetracycline (15 μg/ml).

The mammalian cell lines HeLa (CCL-2) and RAW 264.7 were purchased from ATCC 120 (Manassas, VA), and maintained in Dulbecco's modified Eagle's medium (DMEM; VWR) supplemented with 10% fetal bovine serum (FBS; Gibco).

2.3.2 Plasmids

For the induction plasmids, PCR-generated amplicons were cloned into *Eco*RI/*Xma*I sites of an arabinose inducible plasmid (pZP1137) so that it was in frame with the M45 tag at the C-terminus. Plasmid pM1644 expressing SipA fused to TEM-β-lactamase was used for translocation assay [304]. Since YshB is a 5

kD protein, in order to facilitate its visualization by Western blot, a translational fusion with an M45-TEM double tag fragment derived from pM1644 was made to the C-terminal end of YshB in pZP1137 by subcloning to generate plasmid p*yshB* (pZP3628). The resulting YshB-M45-TEM protein expressed from this plasmid had a molecular weight of 35 kD. The same M45-TEM tag was also subcloned into the empty vector (pVector-TEM; pZP3635) and used as a control. Another vector control pVector-EGFP (pZP3636) was constructed by subcloning the 759 nucleotide fragment generated from *Xma*I/*Xba*I digest of pEGFP-N2 into the same sites of pZP1137. To construct a plasmid in which *yshB* would be expressed from its native promoter (p*yshBnp*; pZP3637), a 254 nucleotide fragment was amplified from *Salmonella* genome including 130 nucleotides upstream of *yshB*, which included its native promoter. This fragment was cloned to the *Xma*I site of the vector control plasmid (pVector-EGFP). The resulting YshB-EGFP protein expressed from this plasmid had a molecular weight of 31 kD. To construct the plasmid p*yshBnoATG* (pZP3399), the forward primers were designed so that the ATG for *yshB* was omitted. The PCR product thus amplified was then cloned to the *Eco*RI/*Xma*I sites of pZP1137. Plasmid p*yshBfs* (pZP3400) was constructed by designing a primer in which the first adenosine nucleotide for *yshB* was omitted which resulted in a shifting of the reading frame. Moreover, plasmid p*yshBdg* (pZP3634) was constructed by annealed oligo cloning of a degenerate oligonucleotide sequence coding the same 36 amino acids of YshB into the *Eco*RI/*Xma*I sites of pVector-TEM. For expression within the macrophages, the respective genes were subcloned to a plasmid with a SPI-2 induced promoter Pro*sseA*, pZP545 [305]. Furthermore, a plasmid used for co-transformation with pM1644 was constructed by subcloning the 2308 nucleotide fragment from p*yshB* containing YshB-M45-TEM along with the *araC* and P*araBAD* regions for arabinose induction to the *Cla*I/*Hind*III sites of pACYC-184SK plasmid

to generate plasmid p*yshB*-TEM ACYC (pZP3638). The *sipA* gene of pM1644 was replaced with *yshB* by subcloning to generate plasmid p*yshB-TEM'* (pZP3639) which was used to assess translocation of YshB into the host cells.

2.3.3 Invasion Efficiency

The invasion efficiencies were assessed by classical gentamicin protection assay [306]. In brief, overnight LB-broth cultures of *Salmonella* were sub-cultured after a 1:30 dilution at 37^0C in 0.3M NaCl LB-broth to an OD_{600} of 1.0. HeLa cells were then infected at an MOI of 10 in a 24-well plate and incubated for 15 minutes at 37^0C in a 5% CO_2-incubator. After infection, cells were washed thrice with PBS followed by addition of DMEM containing 100 μg/ml of gentamicin to each well and further incubated 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 next day.

2.3.4 Intracellular Survival Efficiency

Bacterial infection of RAW264.7 macrophages and intracellular survival assays were carried out by gentamicin protection assay as described previously [307]. Briefly, the bacterial cultures grown to an early stationary phase were 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 37^0C. Opsonized bacteria were added to RAW264.7 macrophage cells at an MOI of 10 in a 24-well plate and incubated for 30 min at 37^0C 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 of gentamicin to kill extracellular bacteria. After one hour, cells

were washed again three times with PBS and incubated for the required amount of time in 16 μg/ml of gentamicin containing DMEM. At 2 and 18 h post infections, the infected macrophages were washed three times in PBS and lysed with 0.1% sodium deoxycholate. Samples were then serially diluted and plated onto LB plates for enumeration. The replication fold was determined by dividing the number of intracellular bacteria at 18 h by the number at 2 h.

2.3.5 Competitive Index Assay

Bacteria were grown overnight at 37^0C in LB medium. The bacteria were then harvested by centrifugation and resuspended in sterile PBS. The wild type and the mutant or the complemented strains were mixed at a 1:1 ratio based on their OD_{600} values. Serial dilutions were prepared for each bacterial mix and plated onto LB+streptomycin and LB + streptomycin + kanamycin plates to calculate the input titre. 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 mutants. Around 10^4 cfu of bacteria in 200μl 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 using Dounce homogenizer aseptically in sterile PBS. Serial dilutions of the organ suspensions were prepared and plated onto LB+streptomycin and LB + streptomycin + kanamycin plates for output titre. The colonies were enumerated the next day and Competitive Index (CI) 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 [308].

2.3.6 Differential inside/outside staining

The ability of bacteria to invade host cells was also evaluated with the inside/outside differential staining assay as previously described [309]. Briefly, HeLa cells were infected with *Salmonella* at an MOI of 5 for 15 minutes. The bacteria which remain outside the cells were identified using rabbit anti-*Salmonella* O-antigen and visualized with anti-rabbit Alexa Fluor 488. This was followed by permeabilization of cells with 0.2% Triton X-100 and staining all bacteria, including the ones inside, 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 be differentiated as they appear green or yellow and the ones inside as 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.

2.3.7 Fluorescent F-actin staining

For the purpose of studying ruffle formation, F-actin staining assay was conducted as described previously [310]. Briefly, invasion primed *Salmonella* was used to infect HeLa cells at a multiplicity of infection (MOI) of 10. After 15 minutes post infection, the cells were washed with PBS and fixed with 3.7% formaldehyde in PBS for 15 minutes. The mammalian cells were then permeabilized with 0.2% Triton X-100 in PBS. *Salmonella* was stained using anti-*Salmonella* O-antigen (Difco), and visualized with anti-rabbit Alexa Fluor 488 (Invitrogen), whereas F-actin was visualized by staining with Texas-Red conjugated Phalloidin (Molecular Probes, Eugene, OR). Zeiss LSM 700 confocal microscope was used to take images.

2.3.8 Growth/Motility

For growth rate measurement, overnight LB broth culture of bacteria was subcultured with 1:100 dilution onto 10ml LB broth and grown at 37^0C in a shaker with 200rpm. Serial dilution of the culture was then plated every 40 minutes and colonies enumerated. Swarm plate assay was performed to assess bacterial motility [311]. 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 onto 2.5ml LB grown until mid-log phase (OD_{600} = 0.5-0.6). From this culture, 1 μl was plated into the semi-solid agar plate and incubated at 37^0C. The size of the halo around the site of inoculation was measured every 30 minutes.

2.3.9 Adherence Assay

HeLa cells were treated with Cytochalasin D (Sigma), an inhibitor of actin filamentation at a final concentration of 1 μg/ml for 30 minutes before infection [312] to prevent bacterial internalization. *Salmonella* strains were added to thus treated cells at an MOI of 10, and allowed for 15 minutes to adhere to the host cells. The cells were then washed thrice with PBS to remove the unattached bacteria followed by subsequent plating for enumeration.

2.3.10 Protein Translocation Assay

HeLa cells were infected with *Salmonella* strains carrying plasmids expressing the β-lactamase fusions 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 Zeiss LSM 700 confocal microscope to quantify the number of green and blue cells. Experiments were performed in triplicate and approximately 300 cells were counted in each sample.

2.3.11 Immunoblotting

Strains were grown overnight in LB, followed by subculture the next day in respective media. The *in vitro* culture was then centrifuged at 10,000 x g for 5 minutes and the cell pellets were lysed with 2x Laemmli sample buffer and boiled for 10min. For the intracellular infection samples, the macrophage cells were lysed 12 hours post infection with 0.1% sodium deoxycholate. This lysate was centrifuged at 500 x g for 5 minutes to remove cell debris. The supernatant was then centrifuged at 10,000 x g for 5 minutes to collect the intracellular bacteria. The samples were run on a 10% SDS-polyacrylamide gels and immunoblotted using respective antibodies. The SipA, SipB, SipC and GFP primary antibodies were polyclonal rabbit, whereas the anti-TEM and anti-Flag (Sigma) were mouse monoclonal. Rabbit anti-ICDH was used to probe ICDH protein as the loading control.

2.4 Results

2.4.1 Induction of a small protein YshB positioned within a predicted small RNA STnc1450 locus causes lowered invasion efficiency in *Salmonella* Typhimurium

In order to characterize the role of small non-coding RNAs in *Salmonella* Typhimurium SL1344 mediated invasion of epithelial cells, we constructed several knock-out mutants of these sRNAs in the SL1344 wild type background and analyzed the effects on invasion of epithelial cells. Altogether, 52 sRNA mutants were constructed

following one-step gene disruption strategy [301] using the λ-Red recombination system, and were assessed for their invasion efficiencies by the classical gentamicin protection assay (Fig.2.1).

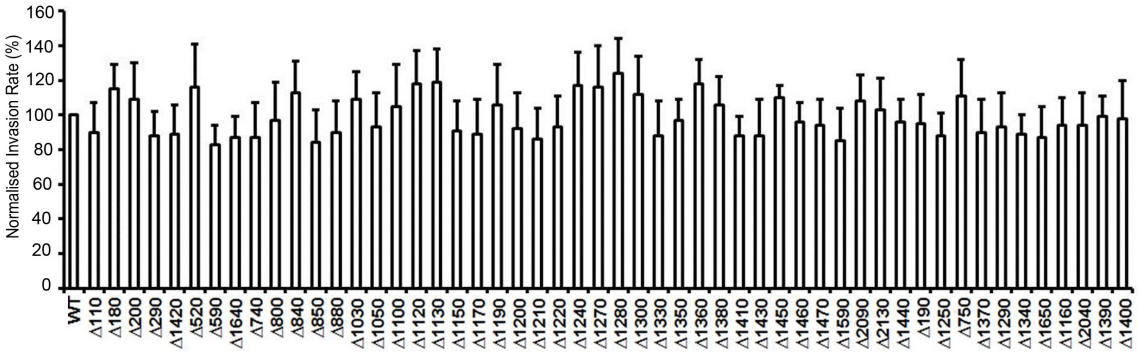

Fig. 2.1. **Invasion efficiencies of various sRNA knock out mutants**: The sRNA mutants were assessed for their invasion efficiencies by gentamicin protection assay. The error bars indicate standard deviations from mean of three independent experiments.

None of the 52 deletion mutants displayed any significant difference in the invasion phenotype (Fig.2.1). Since regulatory factors often exert their functions upon "over"-expression, we induced these sRNAs from an arabinose inducible plasmid in the wild type strain. On conducting the gentamicin protection assay for invasion among these strains, we found that induction of sRNA STnc1450 was able to significantly reduce the invasion efficiency of the strain compared to that of the wild type (Fig.2.2).

It is important to note here that upon addition of L-arabinose alone, we did not see any debilitating effect on the invasion efficiency of *Salmonella* (Fig.2.3), as was reported previously [313].

Upon further analysis, the genomic location of STnc1450 showed a small open reading frame within the predicted sRNA encoding gene named *yshB* (Fig.2.4).

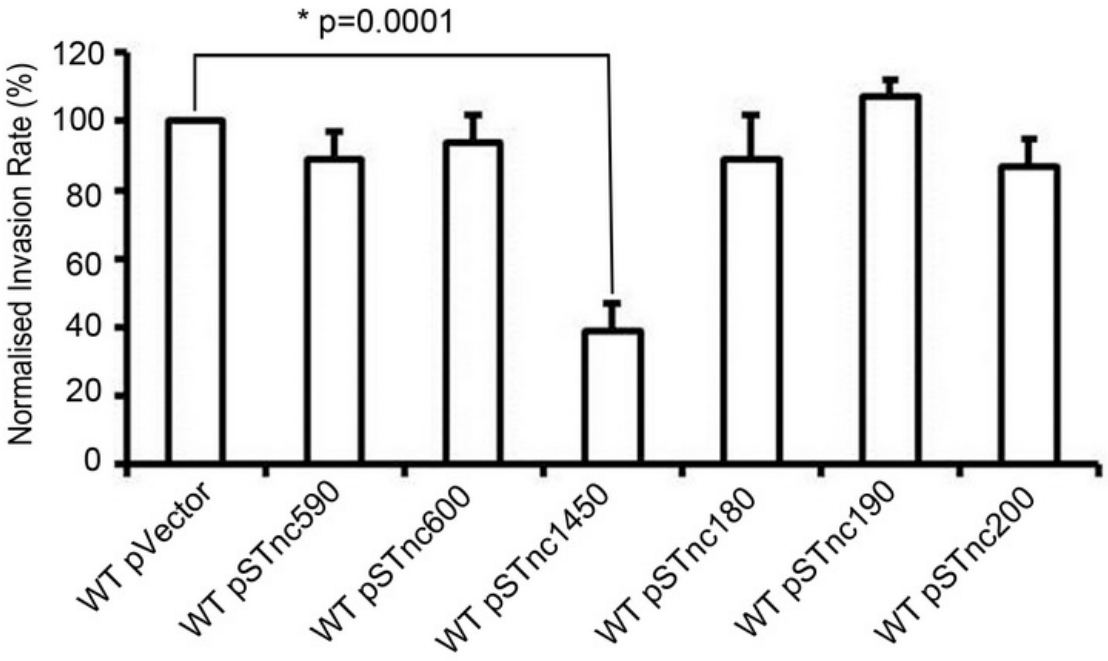

Fig. 2.2. **Induction of STnc1450 causes lowered invasion efficiency in *S.* Typhimurium**: Relative invasion rates of *Salmonella* strains with induced small RNAs from an arabinose inducible plasmid. Gentamicin Protection Assay was performed on HeLa cells infected with *Salmonella* strains with the corresponding small RNAs. The invasion efficiency of wild type strain with an empty vector was normalized as 100. The data are the averages of three independent experiments with error bars indicating standard deviations from mean. Asterisk indicates a statistically significant difference compared to the wild type with empty vector using the Students t-test with p value indicated.

Given the presence of the small protein encoding open reading frame within the small RNA tested, the phenotype might have been due to the induction of either the protein or the small RNA. To test this, we constructed plasmids with full length of the small protein (p*yshB*); without the start codon (p*yshBnoATG*); with frameshift mutation introduced by deleting the first nucleotide (p*yshBfs*) and with degenerate nucleotide sequence coding the same 36 amino acids of YshB (p*yshBdg*) (Fig.2.5).

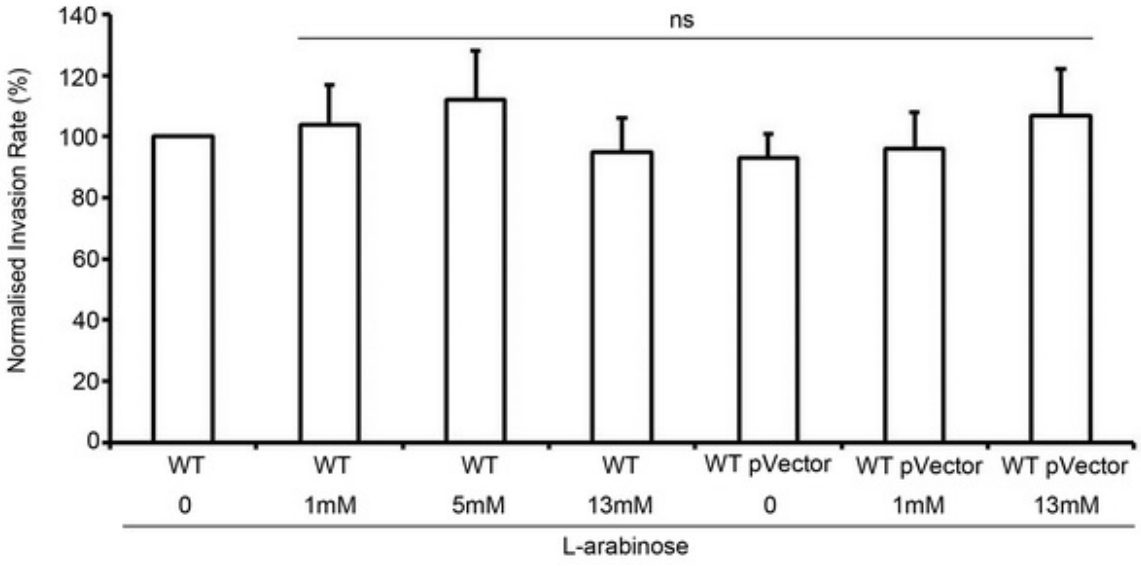

Fig. 2.3. **Addition of L-arabinose has no significant effect on *Salmonella* invasion**: Effects of L-arabinose on invasion efficiency of wild type *Salmonella* and wild type with empty vector was checked by gentamicin protection assay. (ns: not significant)

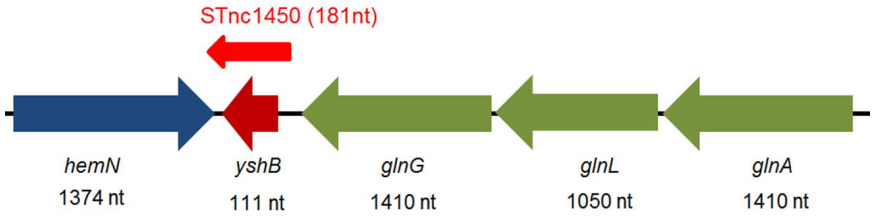

Fig. 2.4. **Genomic location of STnc1450 in *S.* Typhimurium SL1344 showing the presence of an open reading frame encoding a small protein YshB.**

These plasmids were introduced into the wild type *Salmonella* and their invasion were evaluated by gentamicin protection assay. We observed the deficient phenotype in the strains with induction of full length proteins, both with the wild type and degenerate nucleotide sequences. The phenotype however was conspicuously absent when the plasmids with no ATG or frameshift mutation were introduced (Fig.2.6A

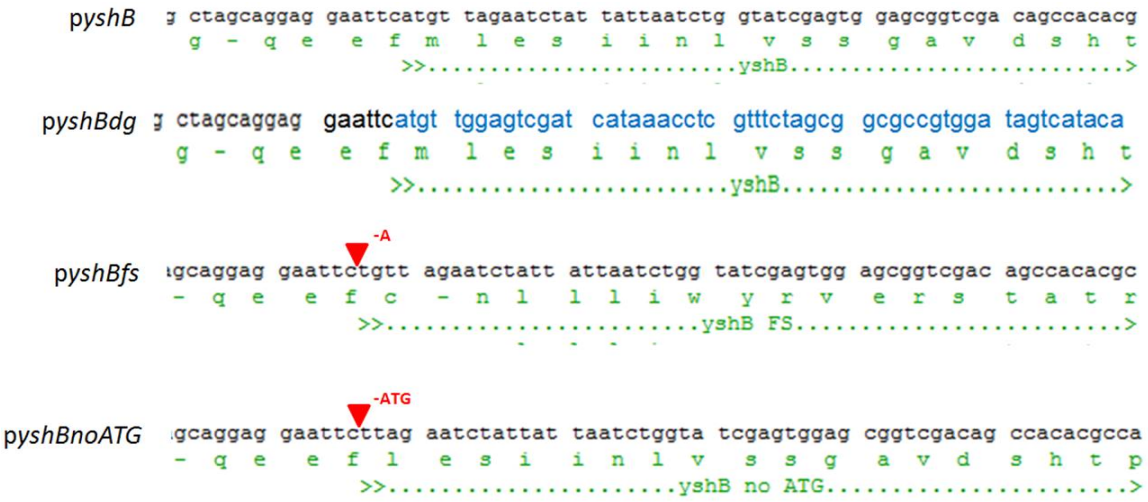

Fig. 2.5. **Sequence information of modified *yshB* showing variations in wild type, degenerate, frame-shifted and no ATG constructs.**

and B). The expression of the protein was demonstrable by Western blot (Fig.2.6C). This strongly indicated that the phenotype was due to the induction of small protein.

The relative invasion efficiencies upon YshB induction were also assessed by carrying out the inside-outside differential fluorescence staining [309]. The results from this further supported the invasion deficiency of the YshB induced strain. Interestingly, the mutant strain alone did not have any significant change in invasion levels (Fig.2.7).

One of the hallmark events of *Salmonella* invasion of epithelial cells is the formation of ruffles as a consequence of actin polymerization, actively mediated by the bacterial effectors [96]. Since we observed a decrease in invasion efficiency from the invasion assays, we further analyzed whether YshB induction has any effects on inducing ruffle formation by *Salmonella*. As evident from Fig.2.8, when YshB was expressed exogenously, the bacteria induced formation of weaker ruffles as compared to the wild type or the mutant strain.

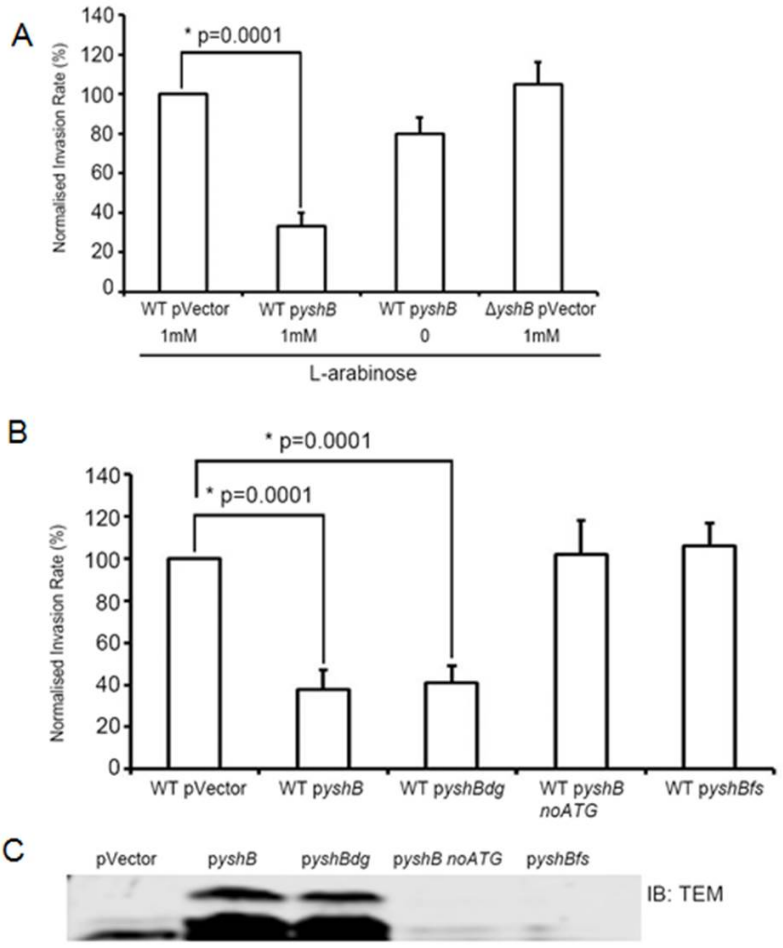

Fig. 2.6. **Induction of a small protein YshB positioned within the predicted small RNA STnc1450 locus is responsible for the lowered invasion efficiency**: A) Relative invasion rates of *Salmonella* strains with YshB induced from an arabinose inducible plasmid in wild type (SL1344) background evaluated by Gentamicin Protection Assay. (B) Relative invasion rates were similarly assessed for YshB encoded from degenerate nucleotide sequences, and with no start codon or frame-shifted *yshB*. (C) Immunoblot showing the expression of YshB from p*yshB* and p*yshBdg*. The bacterial cell pellets were subjected to SDS-PAGE followed by immunoblot with anti-TEM antibody. For all the graphs, asterisks indicate a statistically significant difference with indicated p values, and three independent experiments were carried out with the mean $\pm$ SD represented as error bars.

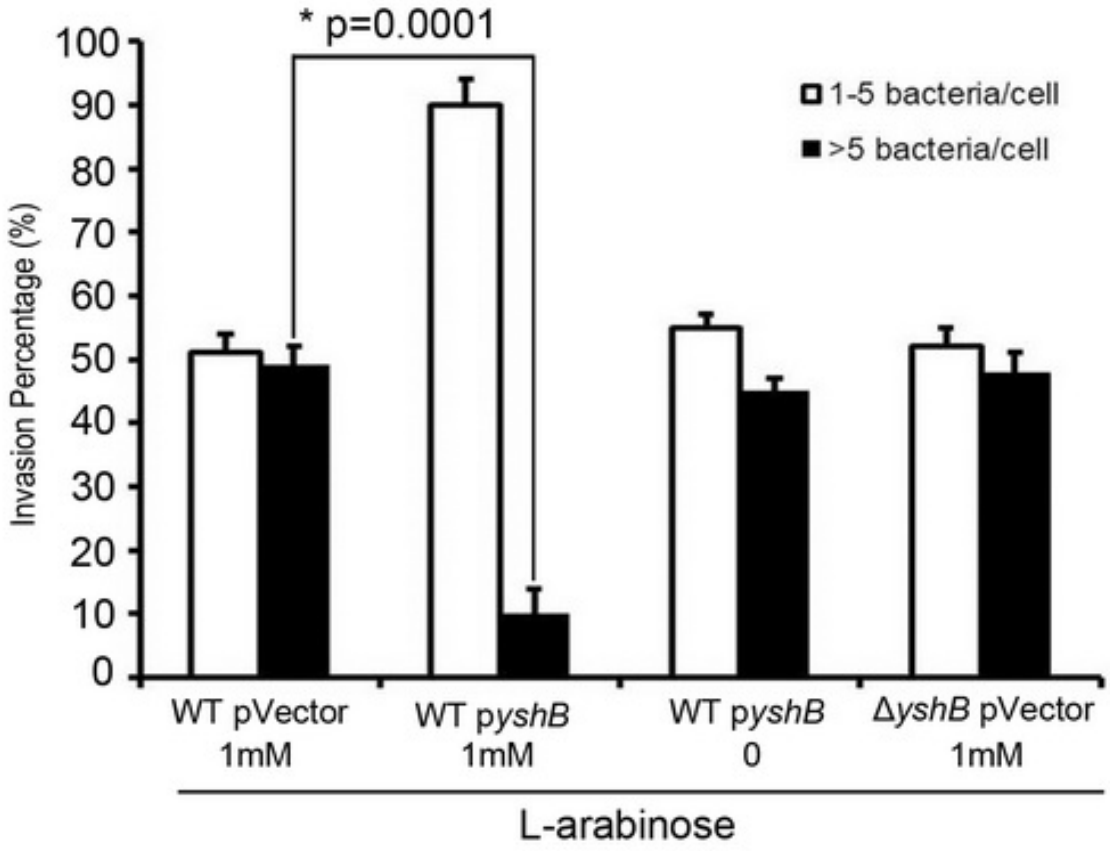

Fig. 2.7. **Quantification of Inside-outside differential staining showing repression of invasion in YshB induced strain**: Percentage of cells with either 1-5 or more than 5 internalized bacteria was enumerated. Asterisk indicates a statistically significant difference with indicated p value. Three independent experiments were carried out with the mean $\pm$ SD represented as error bars.

2.4.2 Induction of YshB has no effect in adherence to host cells or the growth and motility of the bacteria

Salmonella infection is initiated by attachment to the host cells. So any deficiency in invading non phagocytic cells could be due to flaw in the adhesion mechanism. To test this, we conducted adherence assay with the YshB induced strain. In this, bacterial entry into host cells is blocked by adding an inhibitor of actin polymerization.

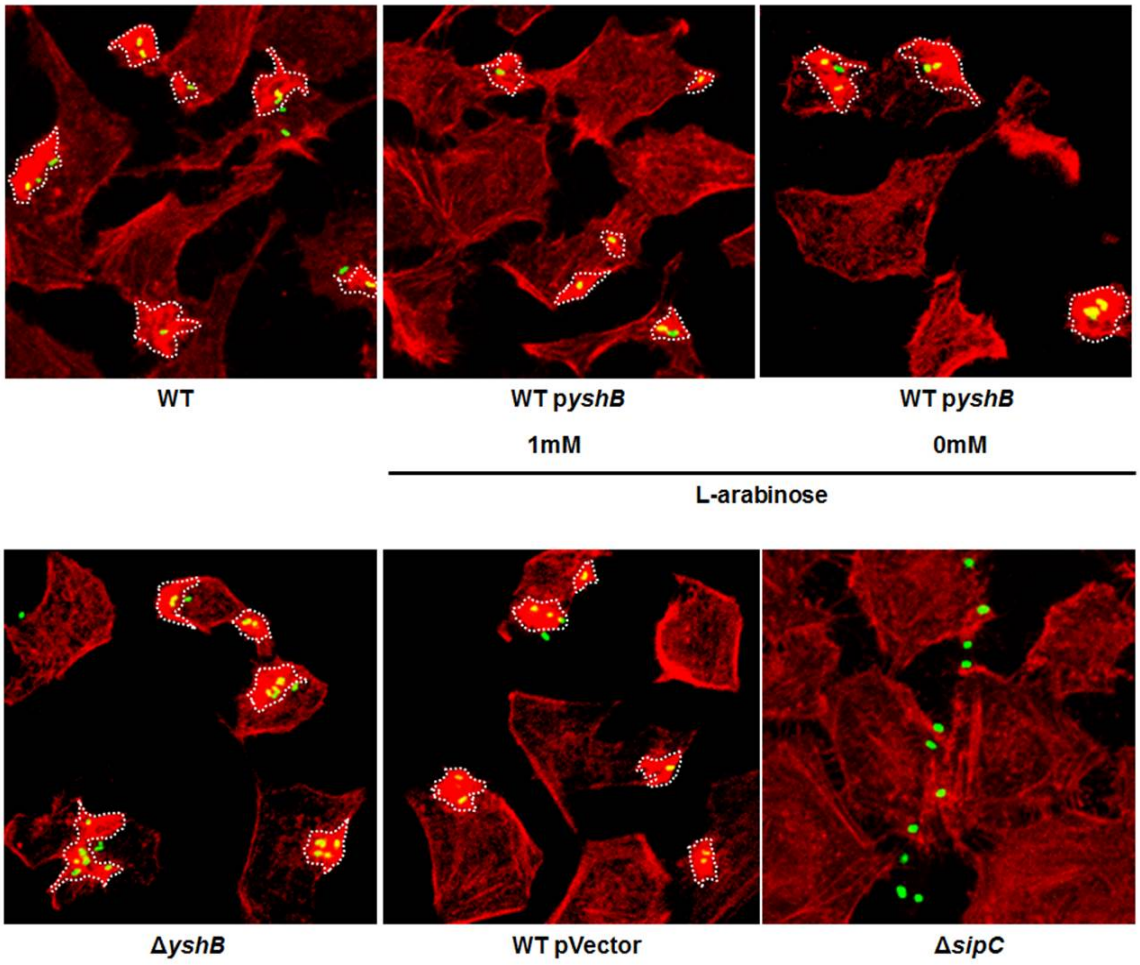

Fig. 2.8. **YshB induction causes the formation of weaker ruffles on host cells**: Following infection of HeLa cells with indicated *Salmonella* strains at an MOI of 10 for 15 min, Texas Red (Red) conjugated Phalloidin was used to visualize F-actin accumulation whereas bacteria were stained using rabbit anti-*Salmonella* O and a secondary anti-rabbit AF488 conjugate (green) and images taken on a Zeiss LSM 700 confocal microscope. Ruffles are shown by encircling white dotted lines.

The bacteria would therefore attach to the surface, but are unable to enter the host cells. We found the defect in invasion on YshB induction was not due to any hindrance in the adherence capability of bacteria to the host cells (Fig.2.9).

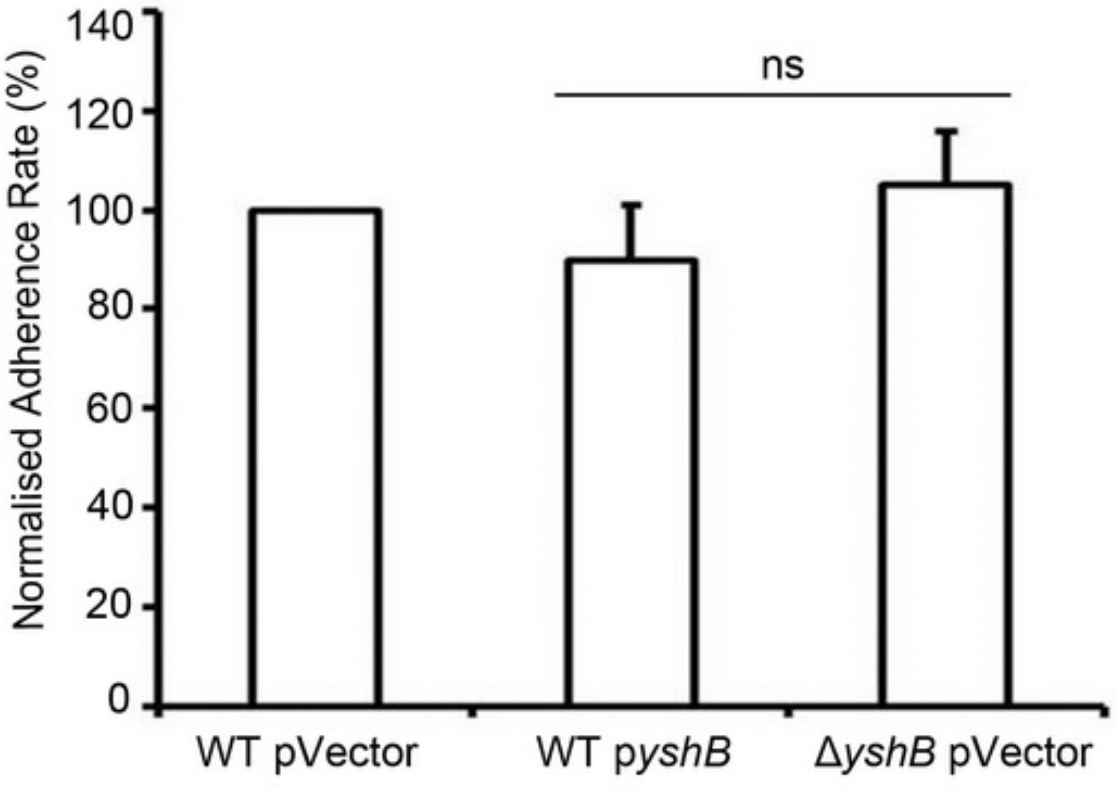

Fig. 2.9. **Induction of YshB has no effect in adherence to host cells**: HeLa cells were treated with Cytochalasin D (1μg/ml final concentration) for 30 minutes before infection. Bacteria were added to cells at an MOI of 10 and after 15 minutes were washed and subsequently plated for enumeration (ns: not significant). Error bars indicate the standard deviation from mean of three independent experiments.

Furthermore, the growth rate and motility of bacteria were also unaffected upon YshB induction (Fig.2.10).

2.4.3 Predicted secondary structure for YshB

The 36 amino acids YshB was described as a small protein with a predicted transmembrane domain with unknown function in previous studies [294, 314]. From the

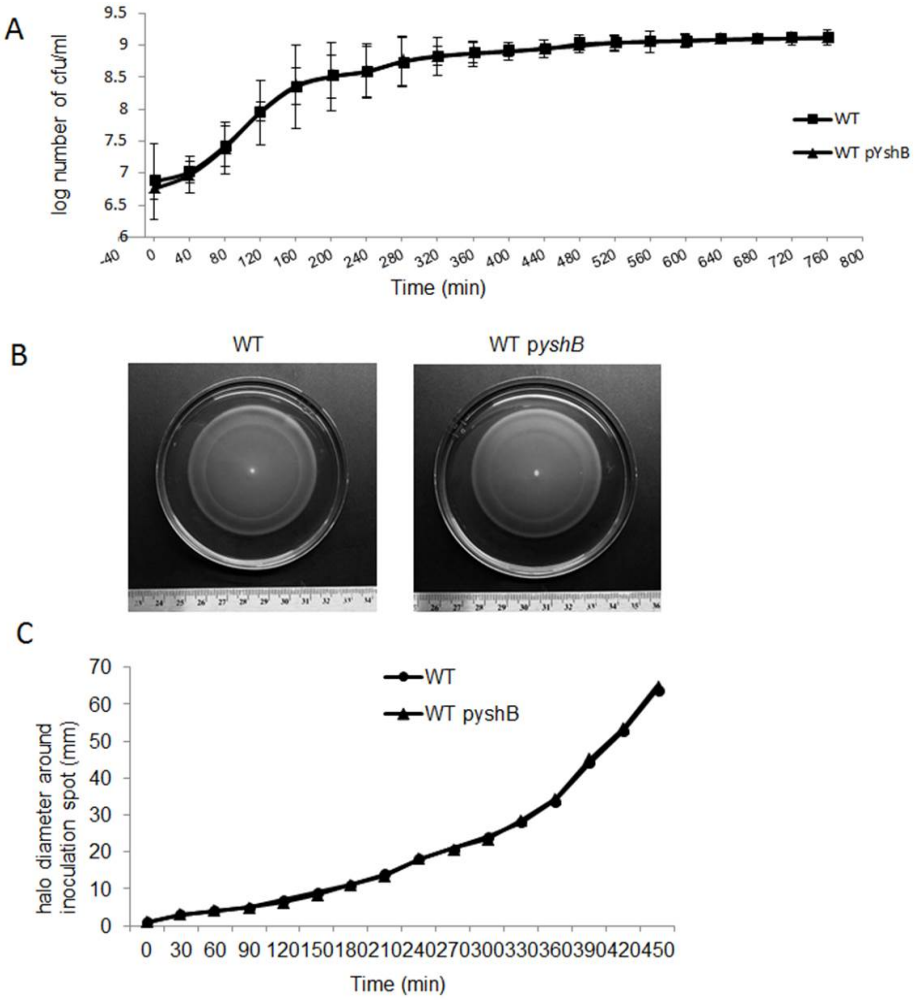

Fig. 2.10. **YshB induction does not affect *S.* Typhimurium growth and motility**: (A) Growth rate of wild type and YshB induced *Salmonella* strains in LB medium. (B) Swarming assay to detect motility rates of *Salmonella* strains with induced YshB in LB with 0.25% agar. Images were taken 7 hours post inoculation. (C) Quantitative representation of the motility rates between wild type and YshB induced strains as shown by diameter of the halo around the spot of inoculation with time.

amino acid sequence, the secondary structure of this small protein was predicted using PSIPRED online server (Fig.2.11), where the second helix constitutes the predicted transmembrane domain.

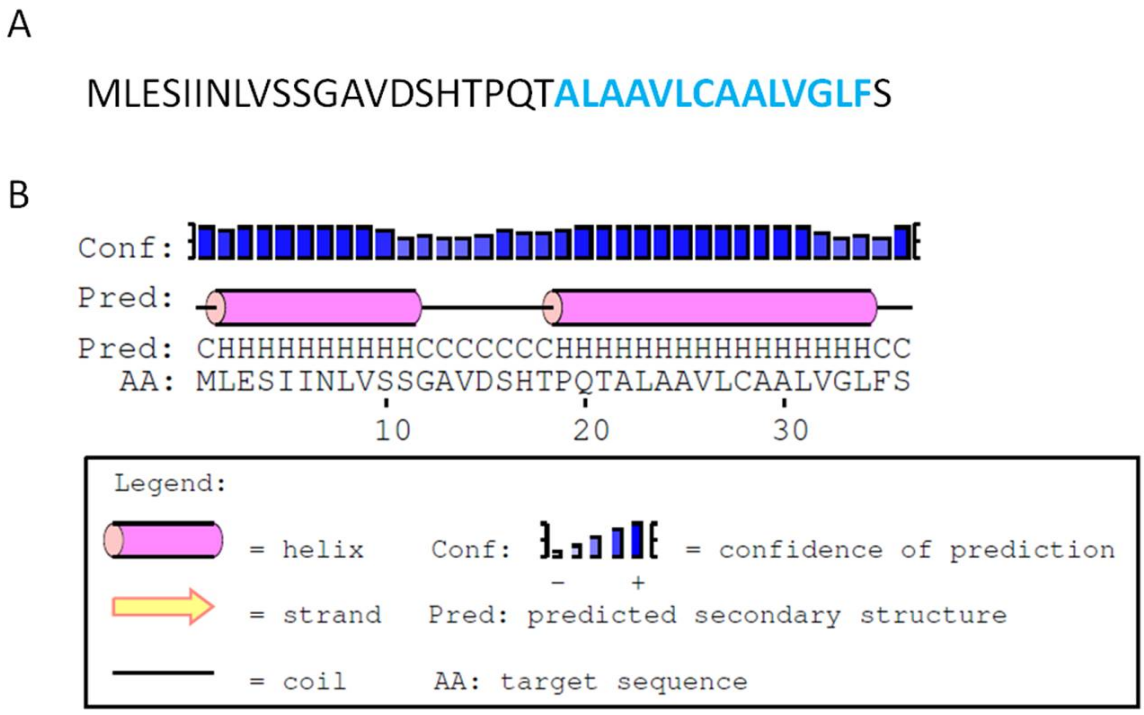

Fig. 2.11. **Predicted secondary structure for YshB**: (A) Amino acid sequence information for small protein YshB.The residues in blue constitute the hydrophobic stretch of the putative transmembrane domain. (B) Predicted secondary structure for YshB using PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/).

2.4.4 Induction of YshB reduces the levels of invasion proteins

The SPI-1 effectors play key roles in mediating bacterial invasion. In order to assess whether the level of major SPI-1 effectors is altered in the cell due to YshB induction, we tested the levels of SipA, SipB and SipC (major SPI-1 effectors mediating invasion) by Western blot analysis on bacterial cell lysates.

A reduction can be seen in the level of these effectors in the induced strain as compared to wild type strain with empty vector (Fig.2.12). This result is consistent to invasion phenotype seen in the YshB induced strain.

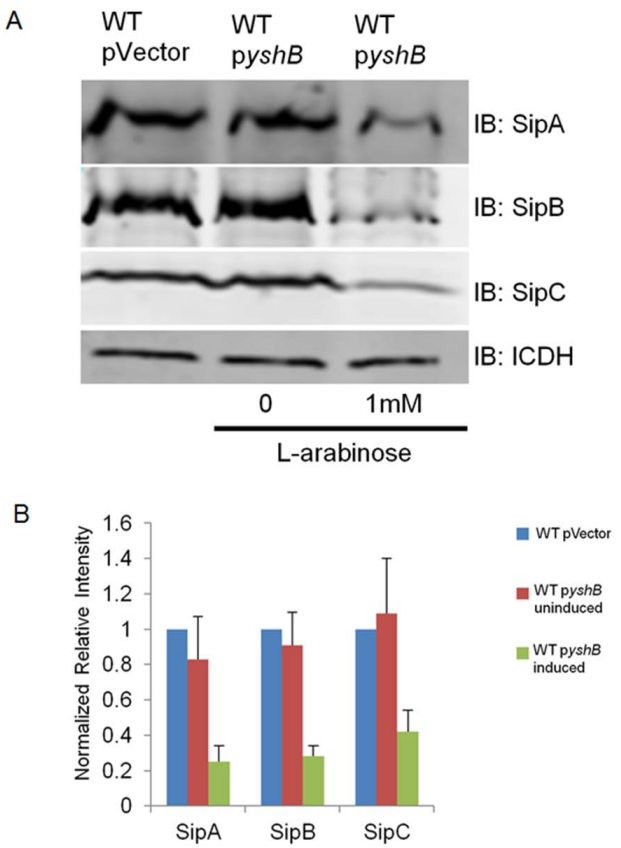

Fig. 2.12. **YshB induction reduces the expression of invasion genes**: (A)Immunoblot analysis of bacterial whole cell lysates for the expression of SipA, SipB and SipC after being grown under SPI-1 inducing conditions. Polyclonal rabbit anti-SipA, anti-SipB and anti-SipC primary antibodies were used whereas rabbit anti-ICDH was used to probe the loading control.(B) Quantitative representation of normalized relative intensity for reduced protein levels.

2.4.5 Translocation rate of effector protein is not affected on YshB induction

Multiple bacterial effectors participate in the internalization of *Salmonella* into the non-phagocytic epithelial cells. These effectors are translocated into the host cell through the Type III secretion system [295].

Therefore, a change in the translocation efficiency could potentially affect the bacterial invasion. To test the translocation of SipA into HeLa cells in the YshB-induced strain, we conducted the β-lactamase assay. In this, we co-transformed the SL1344 strain with a plasmid expressing *yshB* (from a pACYC backbone) and plasmid pM1644, which has the *sipA* gene fused to the β-lactamase TEM [304] to infect HeLa cells. The fusion protein (SipA-TEM) from pM1644 translocates from bacteria to the host cell through the T3SS, and upon addition of the substrate CCF4, which is cleaved by the β-lactamase, the color of the host cell changes from green to blue.

As shown in Fig.2.13A and B, the translocation rate of SipA remains unaffected on YshB induction. The SipA level in the cell expressed from plasmid pM1644 also remained unaffected upon YshB induction (Fig.2.13C).

2.4.6 YshB is not a translocated effector

Furthermore, we showed that YshB itself is not a translocated effector by conducting the same translocation assay by using a plasmid construct in which *sipA* from pM1644 is replaced with *yshB* (Fig.2.14).

2.4.7 YshB is required for efficient intracellular *Salmonella* replication

Once *Salmonella* successfully invades non-phagocytic 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 [315]. A specialized panel of proteins mediates the maintenance of SCV integrity and thus the intracellular survival.

Since YshB affected the invasion process, we assessed the role of this small protein in intra-macrophage survival. The data in Fig.2.15 indicates that YshB indeed plays

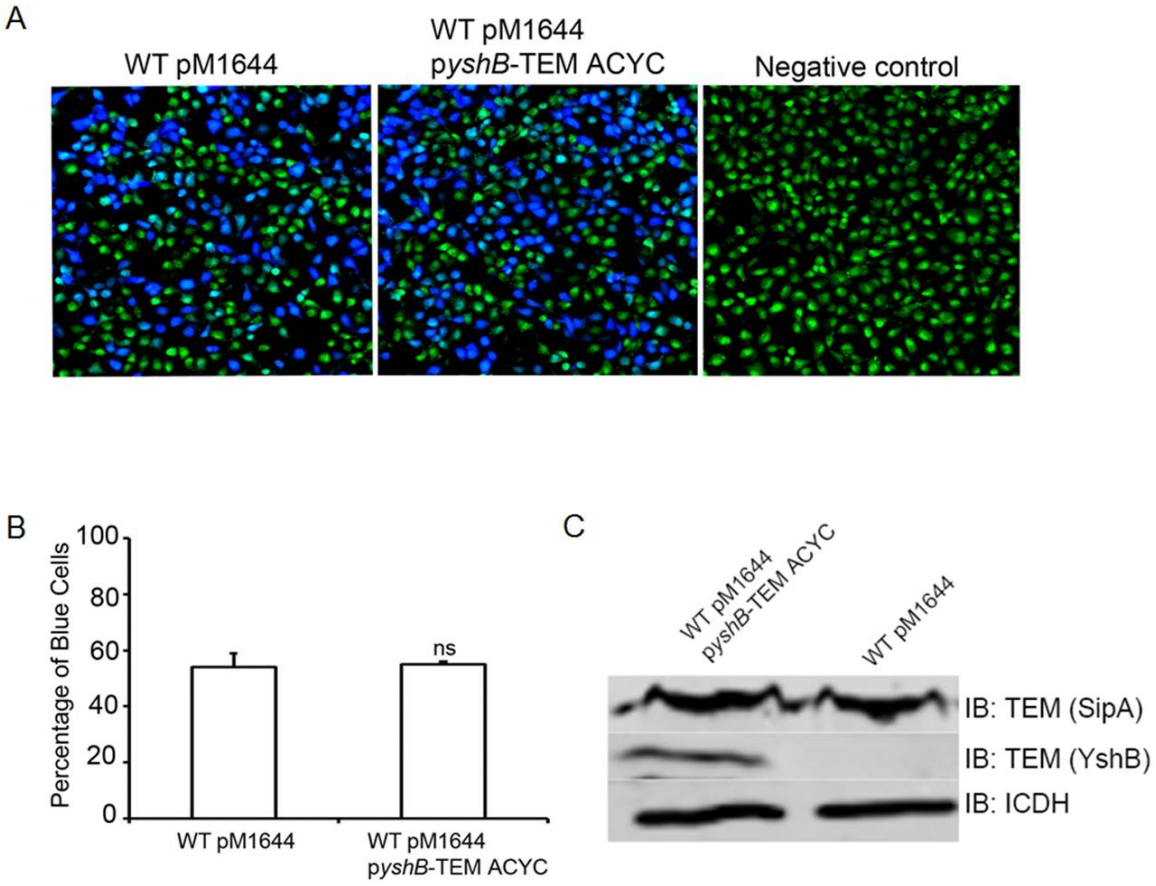

Fig. 2.13. **Translocation rate of a major SPI-1 protein remains unaltered upon YshB induction**: (A) β-lactamase based translocation assay to assess translocation efficiency of SipA from pM1644 in YshB induced cells. Wild type *Salmonella* with pM1644 and plasmid pZP2277, in which the *sipA* from pM1644 is 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. (B) Percentage of blue cells to quantify the SipA-TEM translocation efficiency. Three independent experiments were carried out and the mean $\pm$ SD is represented as error bars. (C) Immunoblot analysis of the bacterial culture used in Figure 2.12A to show the SipA expression from pM1644 remains unaffected when YshB is induced.

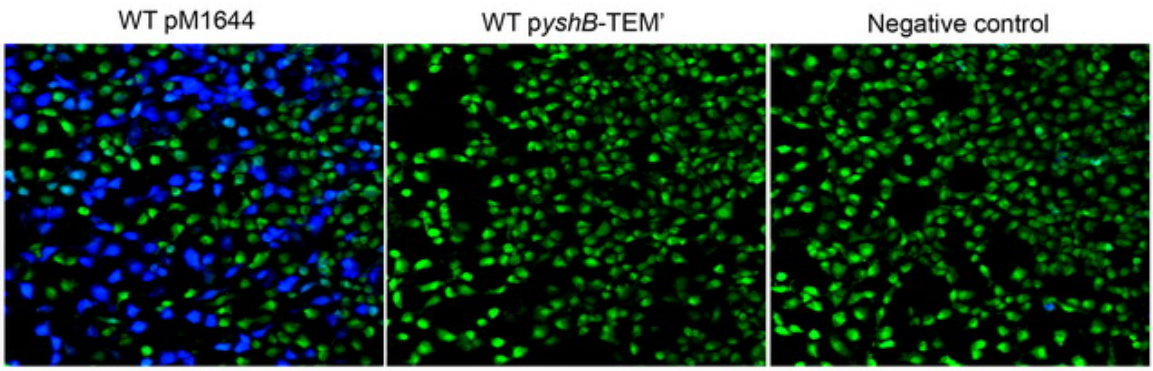

Fig. 2.14. **YshB is not a translocated effector**: A β-lactamase translocation assay was conducted by using a plasmid construct in which *sipA* from pM1644 was replaced with *yshB* (p*yshB-TEM'*). Wild type *Salmonella* with pM1644 and plasmid pZP2277 were used as positive and negative controls respectively.

a role in the survival of this bacterium inside macrophage cells. This demonstrates that YshB plays a substantial role in modulating diverse phases of bacterial infection.

2.4.8 *yshB* expression is induced under the SPI-2 inducing *in vitro* conditions and when inside macrophage cells

Gene expression is tightly regulated by the immediate environment the pathogen encounters. Our data indicated that *Salmonella* might be responding to the environmental changes by regulating *yshB* expression.

We therefore conducted experiments to assess the expression of *yshB* from its own promoter under various environmental conditions. For the *in vitro* conditions, we selected different growth stages when grown in LB broth, along with growth in 0.3M NaCl LB broth (invasion inducing media) and SPI-2 media (mimicking intracellular conditions). We also examined the expression level of *yshB* when the cells were inside the macrophage.

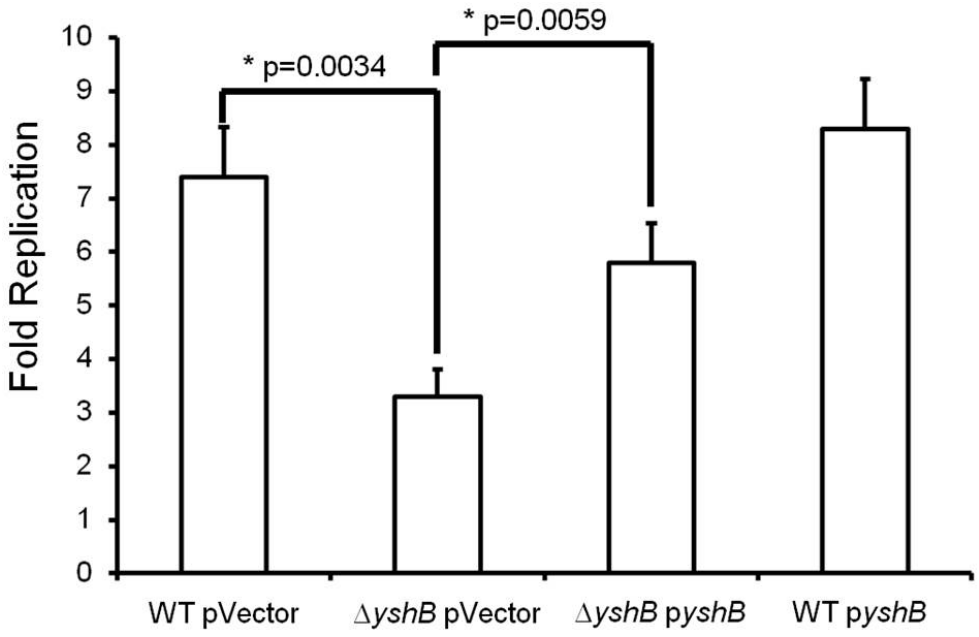

Fig. 2.15. **YshB is required for efficient intracellular *Salmonella* replication**: Replication fold of Δ*yshB* *Salmonella* strains along with the complemented strain and YshB induced strain by Gentamicin Protection Assay. Data represents mean of three independent experiments with standard deviation from mean and asterisks indicate a statistically significant difference with p values indicated.

The results indicate that, *yshB* expression is considerably high when grown in the SPI-2 media and even higher when the bacterium is inside macrophage cells (intracellular phase of life cycle) (Fig.2.16). This shows that *yshB* expression is upregulated during the distinct physiological phase of intracellular stage of the bacterial life cycle. Hence, this corroborates the observation that YshB plays a significant role in *Salmonella* intracellular survival.

2.4.9 YshB has significant effect on *Salmonella* mouse virulence

Competitive index assay is frequently used to assess virulence of bacterial pathogens in mouse model. For this, we employed mixed infections with wild

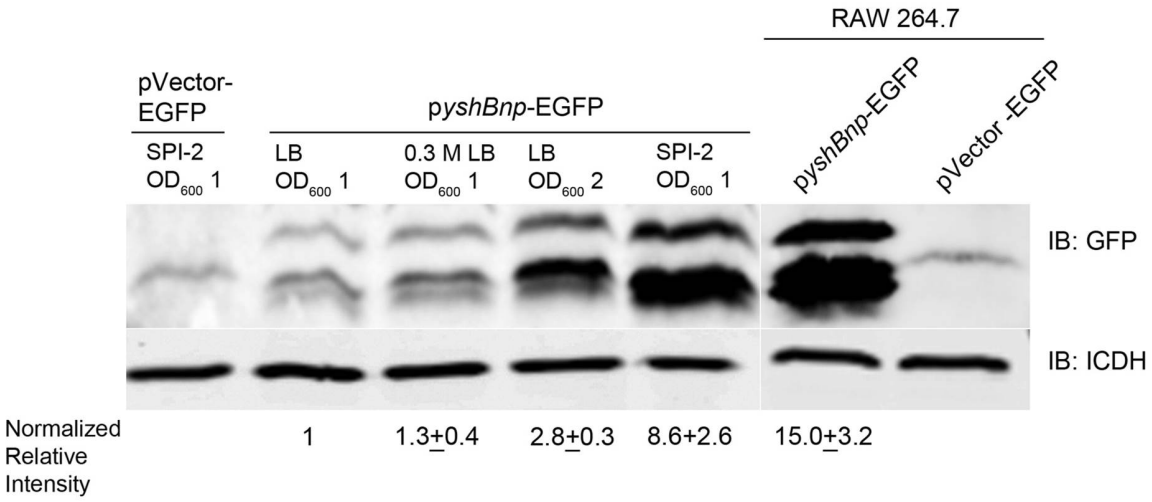

Fig. 2.16. ***yshB* expression is significantly higher under the SPI-2 inducing *in vitro* conditions and when inside macrophage cells**: Immunoblot analysis to compare the endogenous *yshB* expression levels from its native promoter under various *in vitro* conditions and inside RAW 264.7 macrophage 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} of 1 condition. The normalized relative intensity is the average value from two independent experiments with indicated standard deviation.

type, mutant and the complemented strains to assess attenuation of virulence *in vivo*. This was facilitated by the fact that the Δ*yshB* 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 only had the Streptomycin marker.

Our results demonstrate that YshB is significant not only in the *in vitro* macrophage model for intracellular survival but also during mouse virulence. The competitive index for Δ*yshB* strain was 0.39 for spleen colonization and 0.52 for liver colonization (Fig 2.17). When the small protein was expressed via a plasmid

from its native promoter, the virulence phenotype was restored to almost wild type level in both cases.

This strongly indicated that YshB mediates a significant role in *Salmonella* virulence.

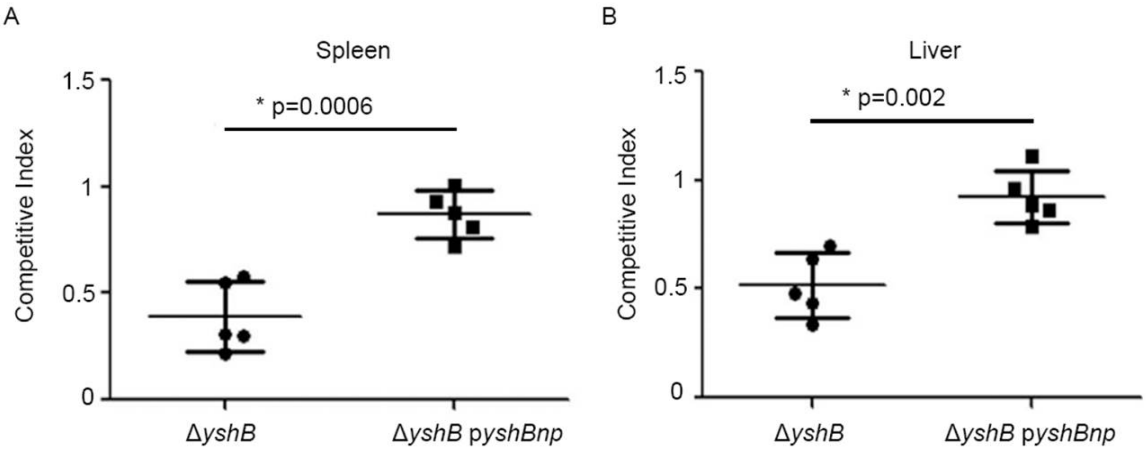

Fig. 2.17. **YshB has significant effect on *Salmonella* mouse virulence**: Competitive Index Assay was conducted using mixed bacterial cultures for infection. Mixtures containing wild type and Δ*yshB* or wild type and the complemented strain were used to infect BALB/c mice intraperitoneally. Liver and 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.

2.5 Discussion

In this chapter, we have characterized a previously undefined small protein YshB and established its role in regulating discrete aspects of bacterial pathogenesis.

This work was initiated to assess the roles of *Salmonella* small RNAs in invading host epithelial cells. Remarkably though, we found that a small protein YshB encoded through an open reading frame within a small RNA molecule locus was

able to significantly affect the regulation of bacterial pathogenesis. In a similar case, where a novel small protein was serendipitously found to be encoded within a small RNA, Wadler and Vanderpool reported that a 43-amino acid small protein SgrT was encoded within a transcript of a small RNA SgrS [316].

YshB is a 36-amino acid small protein first annotated in a screen based on presence of ribosome binding sites [293], and later validated as a protein expressing gene with a predicted transmembrane domain in *E. coli* [294]. 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 Enterobacteriacae, *Salmonella* YshB sequence is 92% identical (33 out of 36 amino acids) to the *Escherichia coli* (strain K12), *Shigella* and *Klebsiella oxytoca* protein. Moreover, prior to this study, this protein has not been described as having known functions or any roles in bacterial virulence.

Essentially, we describe here that YshB and not the encompassing small RNA STnc1450 could positively regulate bacterial survival within macrophages and also virulence in mice. Moreover, we propose that the induction of YshB represents the intracellular phase, and consequently is sufficient to downregulate *Salmonella* invasion to facilitate intracellular survival and replication. This repression of invasion machinery resulted in the decrease in the levels of major invasion proteins SipA, SipB and SipC, and consequently formation of weaker ruffles compared to the wild type. We further characterized YshB as a regulator protein as it did not translocate into the host cell via the Type III secretion system.

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 the locations where these are needed. A

complex network of regulators has been identified that are responsible for controlling the expressions of these genes. Once the invasion of host cell is complete, *Salmonella* downregulates T3SS-1 and initiates SCV biogenesis, for which the T3SS-2 induction is essential [185].

Many environmental cues are known to regulate gene expression in *Salmonella*. One example of this is the repression of SPI-1 genes by bile. This occurs in the proximal sites of small intestine, where the bacteria do not invade the host cells [317]. 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 [318]. Here, we show *yshB* expression was increased when grown in a low Mg^{2+} *in vitro* medium, an environment mimicking the intracellular conditions. Moreover, we could also detect an increase in YshB level when the bacteria were inside the macrophage. Importantly, we found YshB plays a role in *Salmonella* intracellular survival, as the knockout mutant had a defective phenotype.

Not only does the expression of unnecessary genes results in energy expenditure, but could also be detrimental. In a study to examine the temporal expressions of invasion genes, Boddicker *et al.* found that down-regulation of invasion gene expression was partly mediated by Lon protease and this was essential for the intracellular survival of *Salmonella* [319]. An uncalled for expression of SPI-1 genes inside the macrophage results in excessive induction of programmed cell death, and this is controlled by the Lon protease [320]. This was supported by the previous findings from Takaya *et al.* that in a Δlon mutant, the transcription of SPI-1 genes were dramatically enhanced [182, 321]. Consistent to this, we show in our study that conditions in which YshB expression is induced, such as inside the macrophages, the invasion efficiency is markedly reduced.

Murine salmonellosis can result in access to the deeper tissues followed by colonization of diverse host niches by the bacteria. After the bacteria invade the intestinal epithelial cells, they eventually establish themselves in the organs of reticuloendothelial system such as liver and spleen. Virulence contributed by a particular bacterial factor thus can be assessed also by its efficiency to colonize these organs. We show here that YshB significantly affects the ability of bacteria to successfully establish a niche in organs such as liver and spleen. YshB thus is a positive regulator for the intracellular phase and *Salmonella* virulence.

The next chapter of this dissertation explores the molecular bases of YshB mediated regulation.

3. YSHB MODULATES *SALMONELLA* INTRACELLULAR SURVIVAL AND VIRULENCE BY POSITIVELY REGULATING THE EXPRESSION OF PHOP AND FADB PROTEINS

3.1 Abstract

The phenotypic consequences of *yshB* knock out and induction pointed to the direction of regulation of genes involved in *Salmonella* intracellular survival and virulence. We therefore examined the effects on the master regulators of survival and virulence. Moreover, we conducted a global proteomics profile to comprehensively analyze any variations in the protein levels in the cell upon YshB induction. We found YshB positively regulates the expression of *phoP* and *fadB* genes to account for the observed phenotype. The effects on these targets however seem to be indirect as we did not see direct interaction between YshB and these proteins.

3.2 Introduction

The essential role of *phoP* gene for intracellular survival in macrophages and mouse virulence was demonstrated in a study in 1989 [193]. This gene along with *phoQ* comprises a two-component system and regulates multiple aspects of bacterial metabolism and virulence [195]. Miller *et al.* proposed environmental signals such and starvation would trigger the PhoQ mediated phosphorylation of PhoP, resulting in the downstream activation of *pag* (for *phoP*-activated gene) genes [194].

Interestingly, the PhoP/PhoQ two-component system also acts as a key genetic switch to mediate repression of invasion associated genes within macrophage by down-regulating the expression of master regulator of invasion, *hilA* [156, 204]. A fine tuned balance primarily mediated by the environmental signals to regulate the expression of the operon is therefore critical to sustain the virulent properties of this bacterium [322].

The chemical environment which the bacteria encounters within the host has a robust influence in the progress of infection. Notably in the host gastrointestinal tract, the interaction between the resident population of microbes and the host largely determines the chemical constituents of the immediate neighborhood. Initially believed to be commensals, these resident flora significantly manipulate the surroundings of their habitat via interactions with the intestinal mucosal surface and epithelium [323].

The fatty acid composition of the micro-environment is one such aspect which depends on the host-microbiome interplay. The direct and indirect detrimental effects of long chain fatty acids on bacterial growth and survival have been studied extensively. Therefore, it is likely that the bacteria would employ strategies to protect themselves from the toxic effects elicited by the surrounding fatty acids molecules. One possibility is the induction of pathways to break down the long chain fatty acids to shorter lengths. It is known that the long chain fatty acid act as ligands to derepress the expression of genes mediating fatty acid breakdown. This can be viewed as an adaptation to use these substrates as a signal to prime the bacteria for accomplishing the eventual breaking down and reducing the toxic effects of the longer chain fatty acids. Under such scenario, the upregulation of fatty acid degradation pathway would be favored during the intramacrophage stage. Interestingly, one of the key proteins of the fatty acid breakdown pathway FadB, was seen to be induced under *in vivo* conditions [324]. This suggested that induction of fatty acid breakdown may assist the

bacteria to inhibit and survive the local inflammatory response encountered when they are inside an animal host. This observation also hints at the possibility that the proteins of the β-oxidation pathway could play important role in intracellular survival.

In this chapter, we demonstrate that YshB regulates the intracellular and virulent phases by modulating the levels of PhoP and FadB proteins. YshB induction upregualted the expression of *phoP* and *fadB* genes. Interestingly, we further show here that FadB plays an important role in bacterial intramacrophage survival. Notably, FadB also acts as a negative regulator of bacterial invasion. This is consistent with the premise that a positive regulator for survival can inhibit the invasion machinery. Hence, we propose that the switch mediated by YshB from invasion competent to survival competent phase demonstrated in previous chapter is orchestrated largely via PhoP and FadB proteins.

3.3 Materials and Methods

3.3.1 Plasmids

Induction plasmids were generated as described in Chapter 2, by cloning the PCR-generated amplicons into vectors pZP1137 or pZP545. The plasmid carrying *fadB* point mutation was constructed by QuikChange site-directed mutagenesis kit (Stratagene) using the Pfu Turbo DNA polymerase (Agilent Technologies, Inc.) following manufacturers instructions.

3.3.2 β-galactosidase assay

The promoter region of the respective genes was cloned into the *Xma*I/*Xba*I site of a promoterless *lacZ* possessing suicide vector pSB1039. The resulting plasmids

were then introduced into wild-type *Salmonella* via conjugation and integrated into the chromosome by homologous recombination. The resulting strains were subjected to β-galactosidase assay as previously described [325].

3.3.3 Immunoblotting

For assessing the PhoP-Flag levels, overnight LB culture was washed twice in sterile PBS and subcultured in the SPI-2 inducing media with a 1:30 dilution for 6 hours. One ml of this culture was centrifuged and the bacterial pellet lysed in the Laemmli buffer and subjected to Western blot. Monoclonal anti-Flag antibody (Sigma) was used to probe the PhoP protein while polyclonal anti-ICDH antibody was used to probe the loading control.

3.3.4 Proteomic analysis

Global proteomic analysis of YshB induced strain was carried out in collaboration with Dr. Ernesto Nakayasu at the Bindley Bioscience Center, Purdue University.

The wild type *Salmonella* containing an empty vector control and p*yshB* plasmid were cultured under SPI-1 inducing conditions to OD_{600} of 1.0. The cell pellets of the respective strains were then subjected to protein digestion, labeling with 4-plex isobaric tags for relative and absolute quantification (iTRAQ) reagent and peptide fractionation by high pH reverse phase liquid chromatography.

Briefly, bacterial cells were lysed with silica beads in 50 mM NH_4HCO_3 buffer by vortexing. Cell lysate proteins were then denatured in 8 M urea in 50 mM NH_4HCO_3 containing 5 mM dithiothreitol (DTT) for 30 min at 37^0C. This was followed by alkylation of samples with iodoacetamide to a final concentration of 10 mM by incubating for 30 min at room temperature in dark. For digesting the samples, the

reaction was diluted 8-fold with 50 mM NH_4HCO_3 and incubated with trypsin at an enzyme/protein ratio of 1/50 (m/m)for 4 h at 37^0C. Samples were desalted with C18 SPE cartridges as previously described [326]. Peptides derived from cell lysates were labeled with 4-plex iTRAQ reagent (Applied Biosystems) according to the manufacturers instructions. This was followed by fractionation by high-pH reverse-phase liquid chromatography as previously described [327]. Peptides were collected first into 60 fractions, then concatenated into 15 fractions, and dried in a vacuum centrifuge.

For quantitative proteomic analysis, peptides were dissolved in 0.1% formic acid and loaded into a C18 trap column connected to a nanoHPLC system (Ekspert nanoLC 400; Eksigent). The separation was performed in a capillary C18 column. Eluting peptides were directly analyzed in an electrospray ionization mass spectrometer (5600 TripleTOF; AB Sciex). Full mass spectrometry spectra were collected in the range of 400 to 2000 m/z, and the top 20 most intense parent ions were submitted to fragmentation using rolling-collision energy. The identification and quantification of peptides were performed with Paragon software as part of the ProteinPilot package (AB Sciex) by searching tandem mass spectra against *Salmonella enterica* serovar Typhimurium SL1344 sequences downloaded from Uniprot KnowledgeBase. Database searches were performed considering trypsin digestion, cysteine residue alkylation with iodoacetamide, and biological modifications as factors. Peptides were filtered with a confidence score above 95. The iTRAQ channel intensities were extracted using ProteinPilot and intensities from different peptide-spectrum matches and peptides from the same protein were summed together. Sample load was then normalized by total channel intensity and significance was tested by analysis of variance (ANOVA) using InfernoRDN [328].

3.3.5 Bacterial Two-Hybrid assay

Bacterial two-hybrid assay for screening putative targets of the small protein was carried out using the BACTH-system [329, 330].

yshB was cloned into pEB1029 (Kan^R) [330] so that it was translationally fused to the T25 fragment of *Bordetella pertussis cya* gene to get plasmid pEB1029-*yshB* (pZP3649). This plasmid was transformed to *cya* deficient *E. coli* strain BTH101 [329]. pEB1029 has a Flag epitope enabling detection of the recombinant proteins. Genomic DNA from *S.* Typhimurium SL1344 was extracted and partially digested with *Sau*3AI. Fragments corresponding to 500bp to 3kb was purified and ligated into *Bam*HI digested pUT18C (Amp^R), the plasmid with T18 fragment of *cya* gene. This was transformed to DH5α, and the colonies pooled to assemble the library. Plasmid was extracted from the library and transformed into BTH101 with pEB1029-*yshB*. This was plated onto double antibiotics LB plates with 40μg/ml X-Gal or M63 medium with 1% lactose as sole carbon source.

For detecting interaction of YshB with individual targets, each putative target was cloned into pEB1029 and pEB1030 [330]. This was then co-transformed with pEB1029-*yshB* or pEB1030-*yshB* into BTH101 strain and plated onto LB plates with 40μg/ml X-Gal or M63 medium with 1% lactose as sole carbon source. Interaction between the two proteins would reconstitute adenylate cyclase in the BTH101 strain which could be visualized as a Lac^+ phenotype [331].

3.4 Results

3.4.1 YshB positively regulates PhoP level in *Salmonella*

Since YshB was found to be important in successfully establishing the intracellular lifestyle and also virulence in mice, we checked if this small protein affects the

expression of the *phoP/phoQ* operon. We constructed the transcriptional (*lacZ*) fusion to the operon and a translational (Flag-tagged) fusion to the PhoP protein in the chromosome in wild type SL1344 as well as Δ*yshB* background. We then monitored the role of YshB on expression of *phoP/phoQ* operon and PhoP protein level in these constructs. We found that upon exogenous expression of *yshB*, the transcription activation of the *phoP-phoQ* operon increased by around 2 fold, as determined by a β-galactosidase assay (Fig.3.1A). Moreover, in the Δ*yshB* strain, the transcription activity was lower compared to the wild type strain.

This also translated with the observation that the PhoP level was increased in the YshB induced cells by around 2 fold (Fig.3.1B), whereas the level was relatively low in the Δ*yshB* background. Since the PhoP/PhoQ two-component system is essential for survival in macrophage as well as known to mediate SPI-1 repression, we propose that the phenotype associated with YshB is mediated partly via this signaling pathway.

3.4.2 YshB does not affect the regulation of the *ssrAB* operon

The SsrA/SsrB two component system is a major regulator of SPI-2 gene expression. We next checked if YshB regulates the expression of these by using transcriptional and translational fusion constructs of these genes.

The results in Fig.3.2 indicate that YshB induction does not significantly affect the transcription or translation of the *ssrAB* operon.

3.4.3 YshB induction causes upregulation of proteins involved in fatty acid oxidation pathway

In order to compare the global protein profile between the wild type and YshB induced strains, a comprehensive quantitative proteomic analysis using isobaric tags

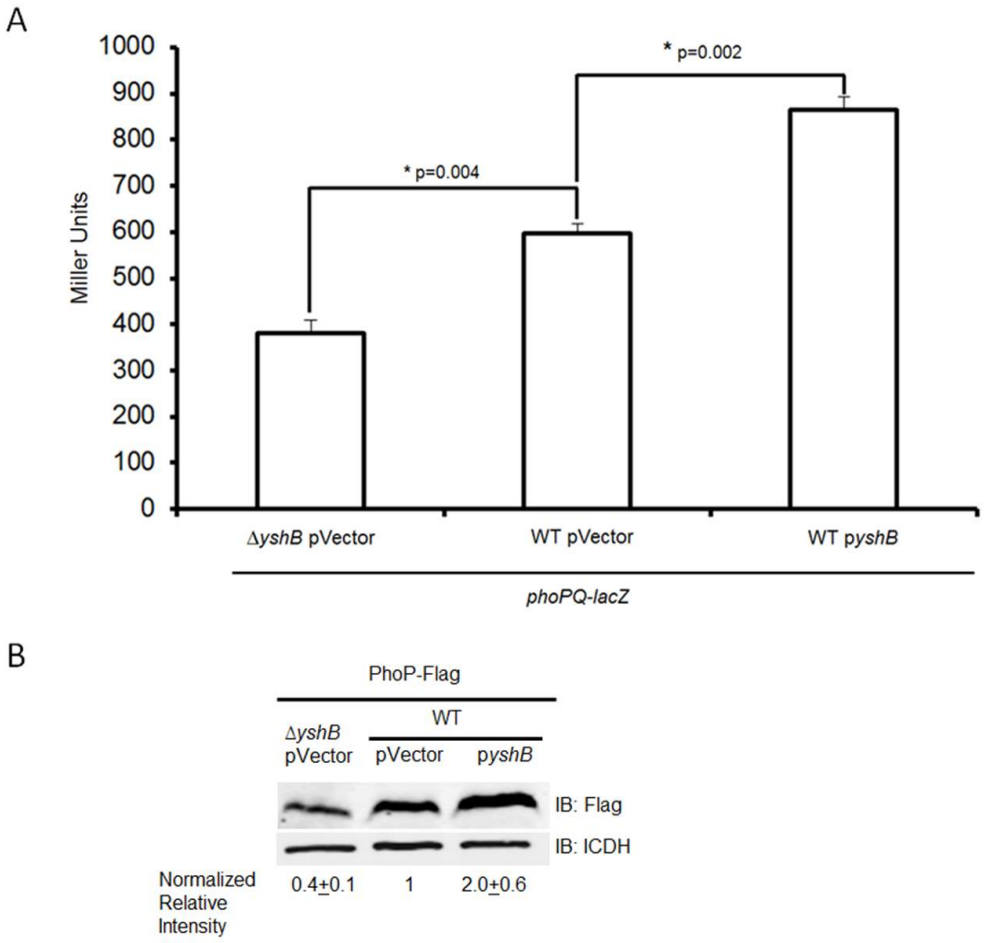

Fig. 3.1. **YshB positively regulates PhoP level in *Salmonella***: (A) β-galactosidase assay to demonstrate that YshB induction upregulates the transcription of the *phoPQ* operon, whereas in the Δ*yshB* strain, the transcription activity is lower. The transcriptional *lacZ* fusion strains were subjected to β-galactosidase assay, and the readouts expressed as Miller units. Three independent experiments were carried out and the mean $\pm$ SD is represented as error bars. Asterisks indicate a statistically significant difference with indicated p values. (B) Immunoblot analysis showing increase in the PhoP level upon YshB induction and reduced PhoP level in Δ*yshB* strain. Relative band intensity was determined by dividing the intensity of PhoP band by that of ICDH and normalized to 1 for the strain with empty vector. The normalized relative intensity is the average value from three independent experiments with indicated standard deviation.

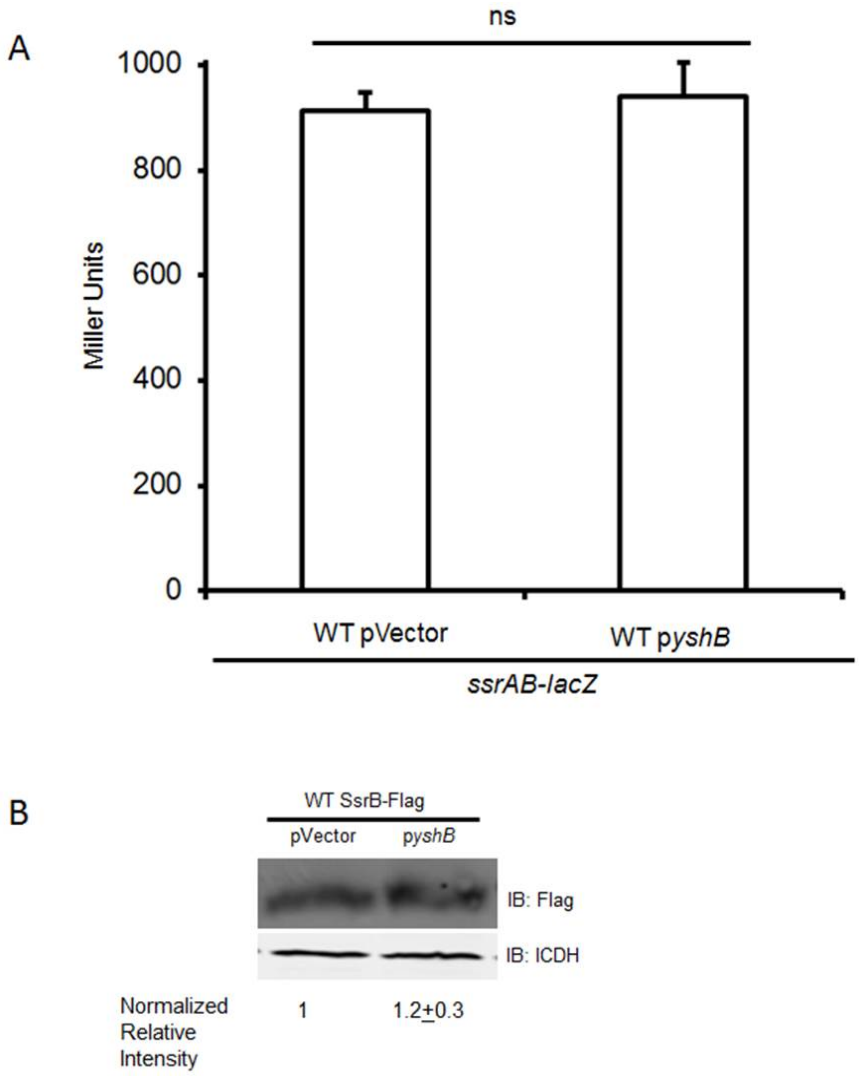

Fig. 3.2. **YshB does not affect the regulation of the *ssrAB* operon**: (A) β-galactosidase assay was carried out which showed that YshB induction does not significantly alter the transcription of the *ssrAB* operon. Three independent experiments were carried out and the mean $\pm$ SD is represented as error bars. (ns: not significant). (B) Immunoblot analysis showing levels of SsrB protein upon YshB induction. Relative band intensity was determined by dividing the intensity of SsrB band by that of ICDH and normalized to 1 for the strain with empty vector. The normalized relative intensity is the average value from three independent experiments with indicated standard deviation.

for relative and absolute quantification (iTRAQ) method was carried out [332, 333]. Briefly, the SL1344 wild type strain with empty vector (WT pVector) and the one expressing YshB from an arabinose inducible plasmid (WT p*yshB*) were sub-cultured from overnight LB culture in SPI1 inducing condition and the presence of 1mM final concentration of L-arabinose, till OD_{600} reached 1.0. The bacterial cells were spun to separate supernatant fraction and cell pellet. Cell lysates were then subjected to iTRAQ labeling and data analysed to generate a quantitative profile of protein expression level. The level of a number of proteins were found to alter in the YshB induced strain (Table C.1). Interestingly, this data led us to investigate a link between YshB induction and fatty acid oxidation pathway. The levels of key proteins of the pathway, namely FadA and FadB were found to increase significantly in the YshB induced strain (Fig.3.3).

Among these, FadB was selected for further analysis based on its role we witnessed in *Salmonella* virulence.

3.4.4 YshB positively regulates FadB expression in a FadR dependent manner

In order to validate the effect of YshB induction on FadB level, we first constructed strains with a Flag encoding tail on the *fadB* chromosomal locus of the wild type, Δ*yshB*, Δ*fadR* and Δ*yshB*Δ*fadR* backgrounds [302]. We also constructed the transcriptional (*lacZ*) fusion to the *fadBA* operon in both wild type and Δ*yshB* background. We then examined the effects of *yshB* knockout and YshB induction on these backgrounds to assess how it affected the transcription of the operon and the protein level of FadB in the cell by conducting β-galactosidase and Western blot analysis.

From Fig.3.4A, it is evident that upon *yshB* knockout, the transcription activity of the *fadBA* operon is reduced while it is higher upon YshB induction. Similarly,

A

Protein of Interest	Fold Change on YshB induction
FadA (3-ketoacyl-CoA thiolase)	1.62
FadB (Fatty acid oxidation complex subunit alpha)	1.98

B

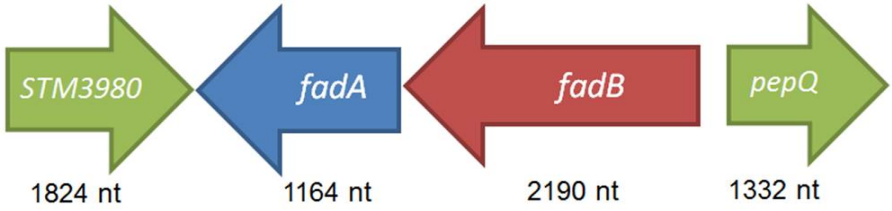

Fig. 3.3. **YshB induction causes upregulation of proteins involved in fatty acid oxidation pathway**: : (A) Average fold change of FadA and FadB proteins on YshB induction as determined by quantitative proteomic analysis (iTRAQ). (B) Genomic orientation of *fadBA* operon in *Salmonella* chromosome.

FadB level decreases in the cell in the Δ*yshB* strain, while it increases upon induction of YshB (left panel) and this is dependent on the presence of FadR regulator, since YshB fails to do so in a Δ*fadR* background (Fig.3.4B).

This told us that YshB is a positive regulator of the *fadBA* operon.

3.4.5 FadB induction causes significant decline in invasion rate

Since we validated that FadB levels are increased upon YshB induced, we hypothesized that FadB would mimic the phenotype displayed by YshB.

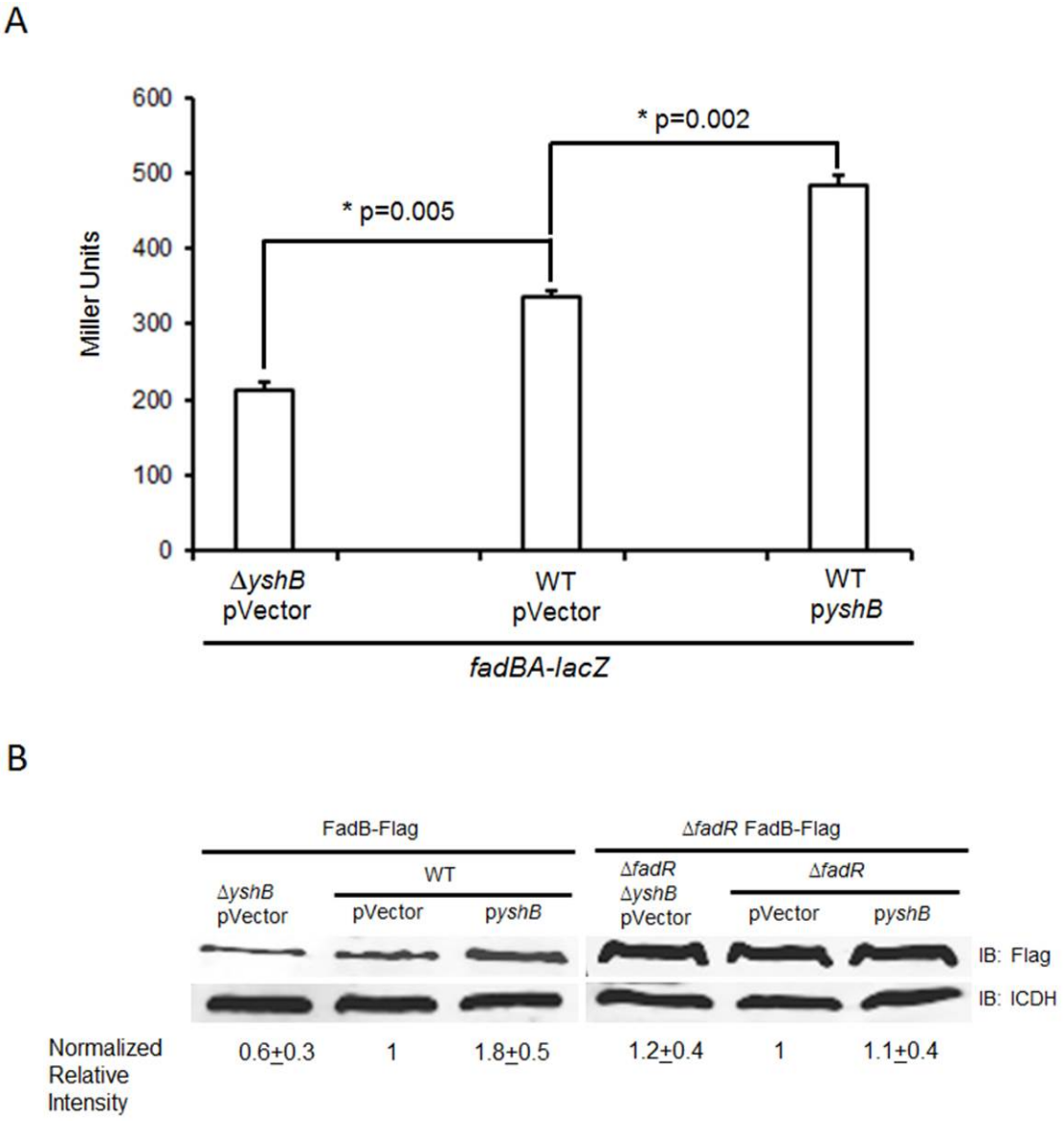

Fig. 3.4. **YshB positively regulates FadB level in *Salmonella***: (A) β-galactosidase assay to demonstrate that YshB positively regulates the transcription of the *fadBA* operon. The transcriptional fusion strains were subjected to β-galactosidase assay, and the readouts expressed as Miller units. Three independent experiments were carried out and the mean $\pm$ SD is represented as error bars. Asterisks indicate a statistically significant difference with indicated p values. (B) Immunoblot analysis showing increase in the FadB level upon YshB induction and reduced FadB level in $\Delta yshB$ strain.

FadB encodes a fatty acid oxidation complex subunit which converts enoyl-CoA to 3-ketoacyl-CoA. In the same operon is a gene encoding FadA which catalyses the last step of the pathway resulting in shortening of the input acyl-CoA by two carbon atoms to give acetyl-CoA [245]. The His^{450} is the catalytic residue for the dehydrogenase activity of FadB [334].

We analyzed the effects of exogenously expressing wild type and the catalytically inactive $FadB^{H450Q}$ protein for the dehydrogenase activity in *Salmonella* and assessed the invasion rate. To our surprise, we found the invasion phenotype seen upn FadB induction was independent of its dehydrogenase activity (Fig.3.5).

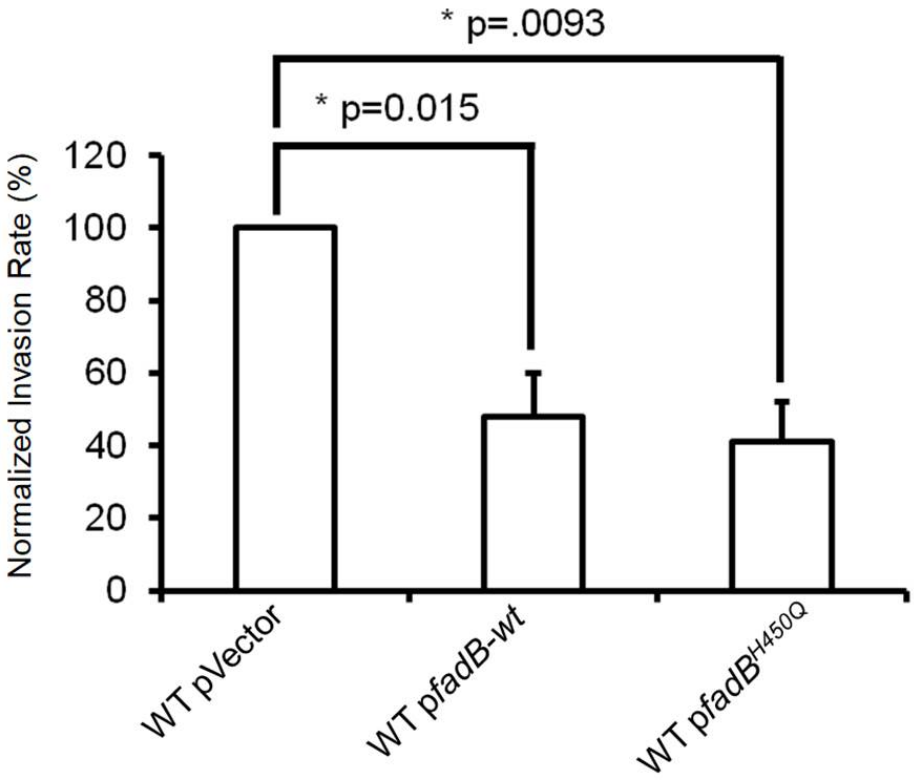

Fig. 3.5. **FadB induction causes significant decline in invasion rate**: Gentamicin protection assay was conducted to assess the invasion efficiency upon FadB induction. It is notable that the phenotype is independent of the dehydrogenase activity of FadB.

Furthermore, this phenotype was also seen in the Δ*fadL* background (Fig.3.6B), where FadL is a membrane protein responsible for transporting external long chain fatty acids (LCFAs) into the periplasm [245].

This suggested that FadB accumulation in the cell may be sufficient to act as a signal, independent of its enzymatic activity or the presence of its substrates in the vicinity. The scenario we thus propose is upon induction of YshB, there is an increase in FadB level in the cell, which mimics the intracellular phase of the bacterial life cycle and therefore a switch to the toned-down invasion machinery.

3.4.6 *fadB* deletion compromises intracellular survival

FadB was implicated to be one of the *in vivo* induced (ivi) genes when mice were challenged with wild type *Salmonella* strain [324]. It was proposed that FadB would contribute to *in vivo* survival, since the FadB-mediated fatty acid degradation would possibly reduce the multiple potential adverse effects of high concentration of fatty acids. This led us to hypothesize that FadB would also be important for the intracellular survival of *Salmonella* within macrophage cells. Consequently, FadB induction would mimic the intracellular phase of bacterial life cycle, hence repressing the invasion machinery.

To test this, we conducted gentamicin protection assay for intracellular survival of *fadB* knockout mutant. Briefly, RAW 264.7 cells were infected (MOI: 10) by respective *Salmonella* strains followed by lysis and plating 2 and 24 hours post infection. The fold replication was determined by colony counts on plates after each time point.

From the survival assay, it was evident that FadB plays a role in the intracellular phase of bacterial life cycle (Fig.3.7). Similar to what we detected in the invasion phenotype, even the FadB^{H450Q} protein which is defective in its dehydrogenase ac-

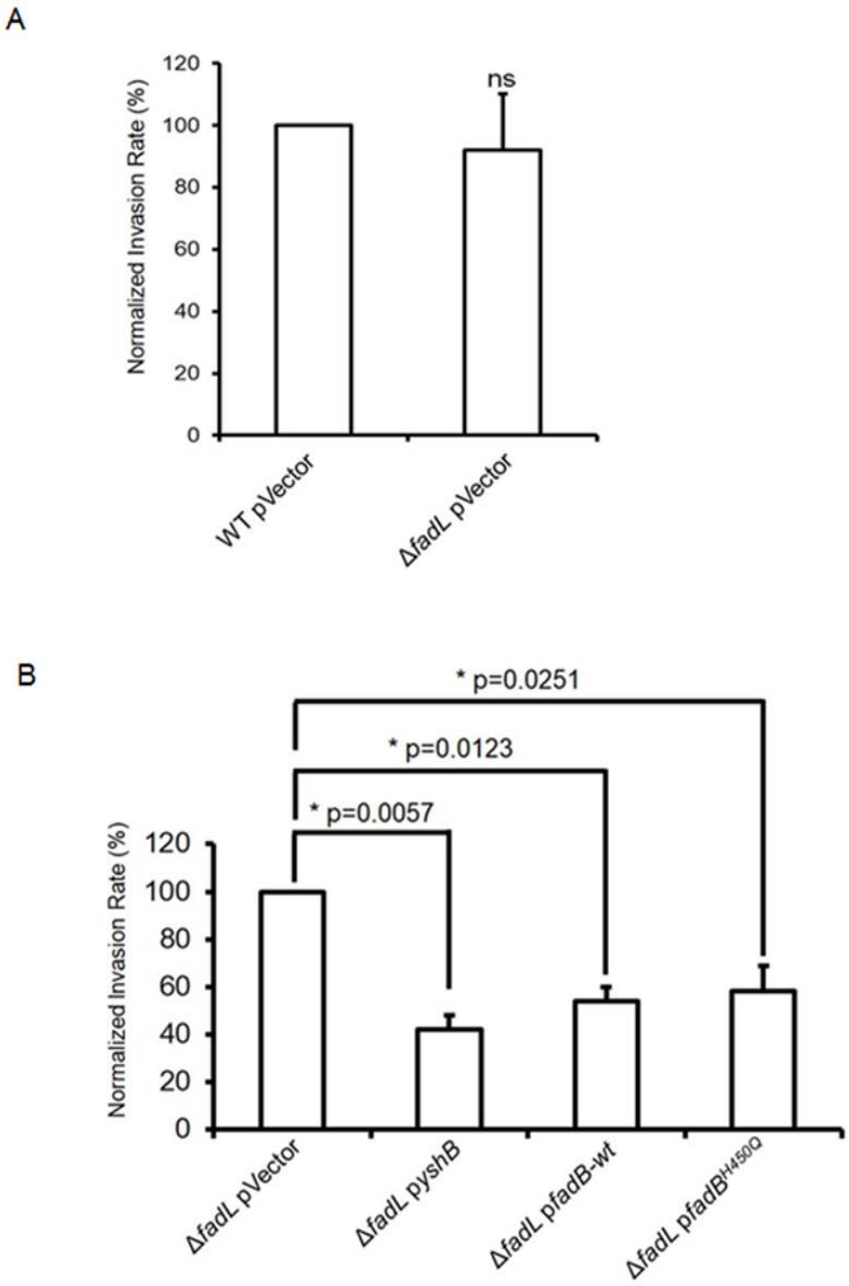

Fig. 3.6. **FadB invasion phenotype is independent of presence of its long chain fatty acid substrates**: (A) Deletion of *fadL*, the transporter of long chain fatty acid has no significant effect on the invasion efficiency of *Salmonella* (B) Inducing FadB in $\Delta fadL$ strain causes reduced invasion efficiency.

tivity was able to rescue the phenotype. This supported our proposition that FadB molecules could act as signals to trigger a switch to intracellular mode of life.

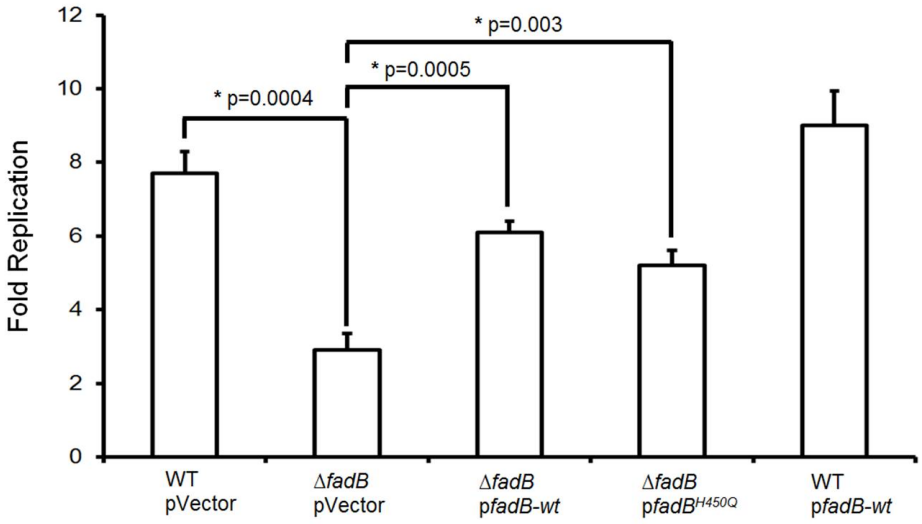

Fig. 3.7. **FadB plays a role in *Salmonella* intracellular survival**: Gentamicin protection assay was carried out to assess the survival efficiency of Δ*fadB* strain. Plasmids expressing the wild type FadB and FadB^{H450Q} proteins were used to complement the phenotype. Asterisks indicate statistical significant difference with p value indicated.

Surprisingly, the role of FadA protein in the bacterial virulence could not be established, as neither the Δ*fadA* nor the FadA induced strain displayed any significant phenotype in the survival or the invasion assay Fig.3.8. This is peculiar since both FadB and FadA are expressed through the same operon.

3.4.7 PhoP and FadB may not be the only targets of YshB

In order to assess if PhoP and FadB are the only targets of YshB, the effects of YshB induction in the Δ*phoP*, Δ*fadB* and a double knock out Δ*phoP*Δ*fadB* backgrounds on invasion rate were assessed. From the result in Fig.3.9, it can be seen that inducing YshB in the Δ*phoP* or the Δ*fadB* does reduce the invasion rate, however

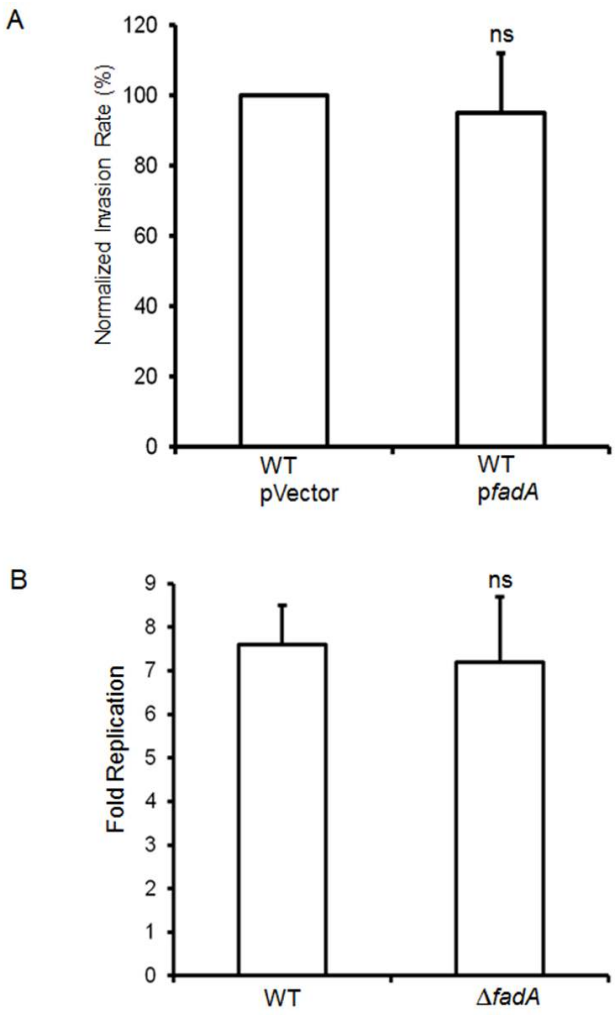

Fig. 3.8. **FadA has no significant role in *Salmonella* virulence**: Effects of FadA induction on invasion efficiency (A) and *fadA* knock-out deletion in intracellular survival (B) were assessed by gentamicin protection assay. (ns: not significant)

not to an extent that it does in a wild type background. Interestingly, deleting both *phoP* and *fadB* didn't show any additive effect on the invasion phenotype mediated by YshB. This suggested that YshB may be modulating bacterial invasion through other targets as well.

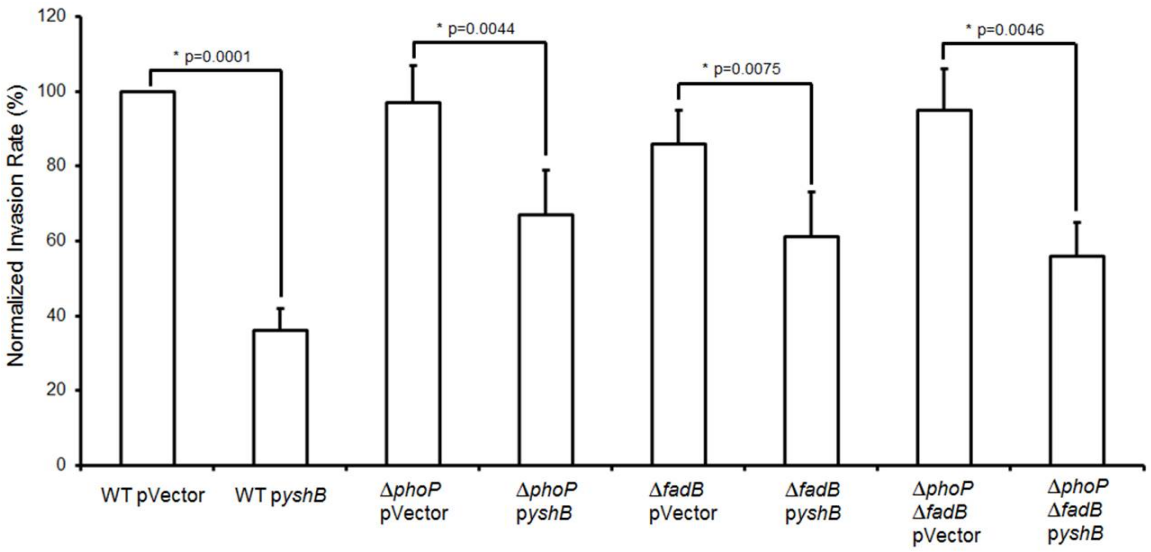

Fig. 3.9. **PhoP and FadB may not be the only targets of YshB**: Relative invasion rates were assessed by Gentamicin Protection Assay. Invasion efficiency of wild type strain with an empty vector was normalized as 100. The data are the averages of three independent experiments with error bars indicating standard deviations from mean. Asterisks indicate a statistically significant difference compared to the empty vector using the Students *t*-test with p value indicated.

3.4.8 FadR plays a significant role in *Salmonella* virulence

One of the key proteins of the fatty acid metabolism is FadR, the negative regulator of fatty acid degradation pathway. Since we demonstrated that FadB plays a significant role in the invasion and intracellular survival of *Salmonella*, we therefore constructed a Δ*fadR* strain which should have an increased FadB level in the cell, and evaluated the invasion and survival phenotype of this strain Fig.3.10A and B. The results were in accordance with our previous data, as Δ*fadR* strain had a significantly reduced invasion and enhanced survival phenotype. Moreover, we were able to show that upon *fadR* deletion or FadB induction, the protein level of the major

SPI-1 effectors was considerably reduced, thus accounting for the reduced invasion phenotype (Fig.3.10C and D).

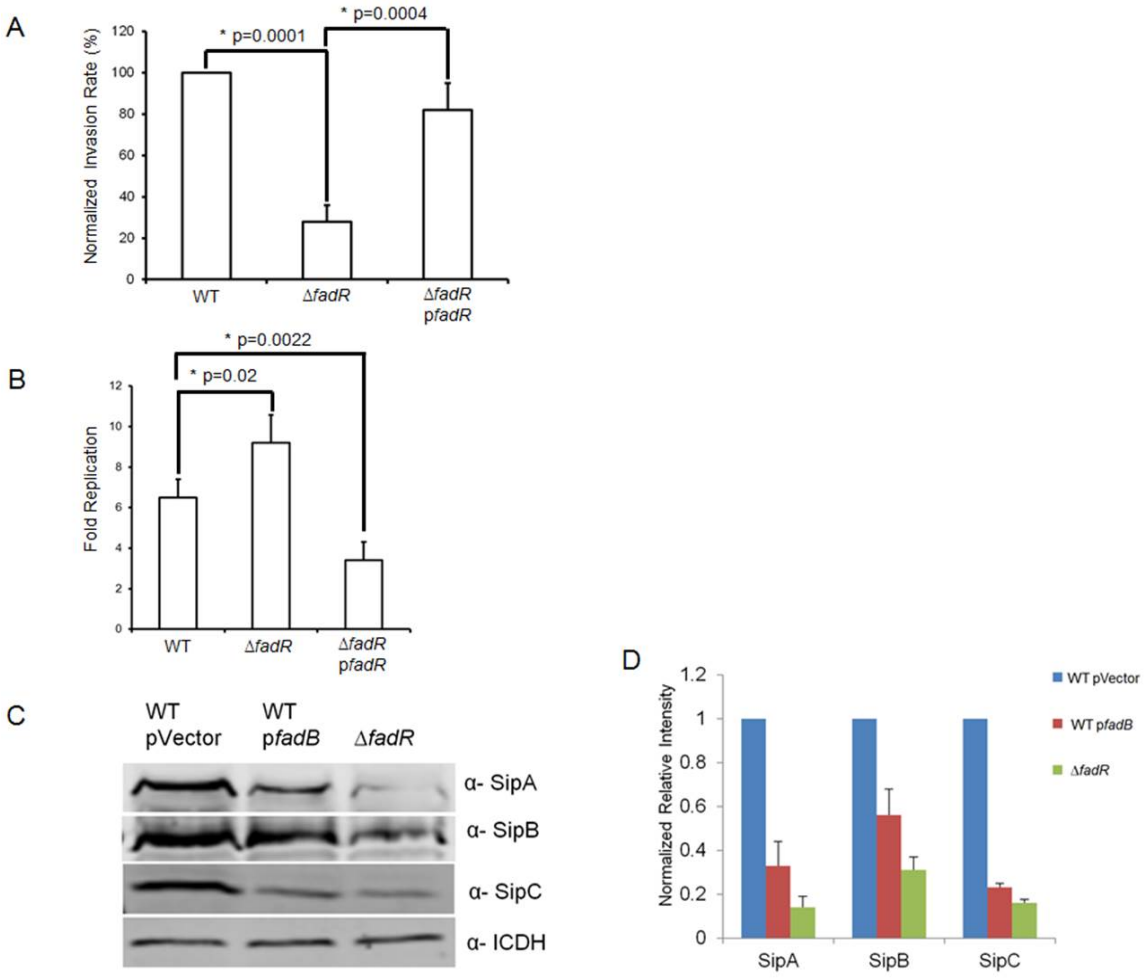

Fig. 3.10. **FadR plays a significant role in *Salmonella* virulence**: (A) Gentamicin protection assay for invasion with a *fadR* knock-out mutant along with a complemented strain. (B) Gentamicin protection assay for intracellular survival with a *fadR* knock-out mutant strain. Asterisks indicate a statistically significant difference. (C) Immunoblot analysis to check the protein levels of SipA, SipB and SipC proteins in a Δ*fadR* strain along with the FadB induced strain.(D) Quantitative representation of normalized relative intensity for reduced protein levels.

3.4.9 YshB does not interact with PhoP, PhoQ, FadB or FadR

Small proteins preferably act by directly interacting with their targets. We therefore checked whether or not YshB interacts with its putative targets using the bacterial two-hybrid system [329, 330].

Briefly, the putative interacting proteins were translationally fused to either the T25 or the T18 fragments which constitute the catalytic domain of *Bordetella pertussis* adenylate cyclase. In case of an interaction between the two hybrid proteins, there is a functional complementation between T25 and T18 fragments which leads to cAMP synthesis. The generation of cyclic AMP then triggers the transcriptional activation of operons that yield a characteristic phenotype, in this case, the β-galactosidase activity. However, using this system, we were unable to detect any interaction between YshB and its putative target proteins (Fig.3.11).

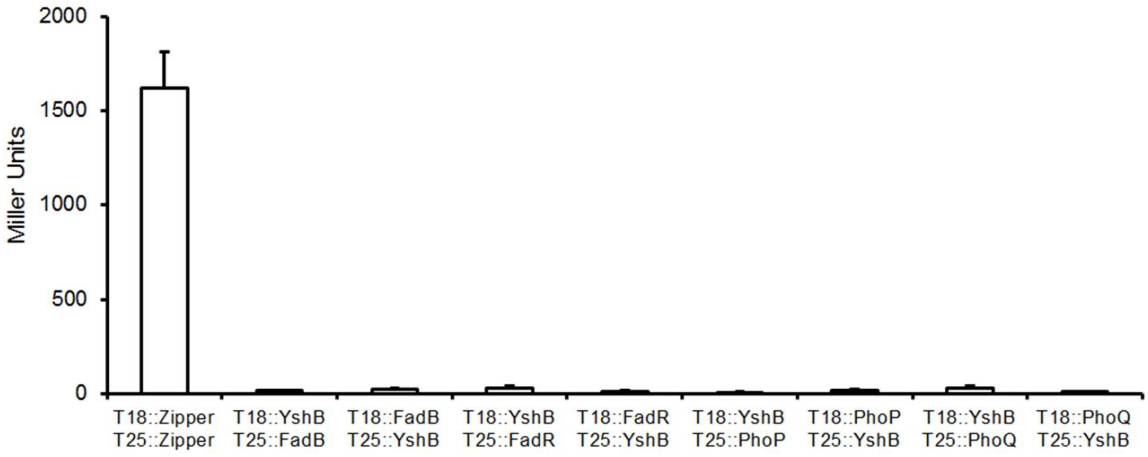

Fig. 3.11. **YshB does not interact with PhoP, PhoQ, FadB or FadR**: A bacterial two hybrid assay was conducted to examine any interaction between YshB with PhoP, PhoQ, FadB or FadR proteins using BACTH system. Miller Units was used as the read out following β-galactosidase assay.

3.4.10 PhoP and FadB act independently in mediating virulence phenotype

Since FadB was shown to be involved in intracellular survival and repressed bacterial invasion, we tested if this protein regulates or is regulated by PhoP. For this, we exogenously expressed FadB in the cell to measure the level of PhoP. Similarly, we assessed the effects of PhoP induction on FadB levels in the cell. From Fig.3.12, it is evident that inducing either PhoP or FadB in the cell would not significantly alter the level of FadB or PhoP respectively. Therefore, it is more likely that these two proteins mediate similar function in the cell independently.

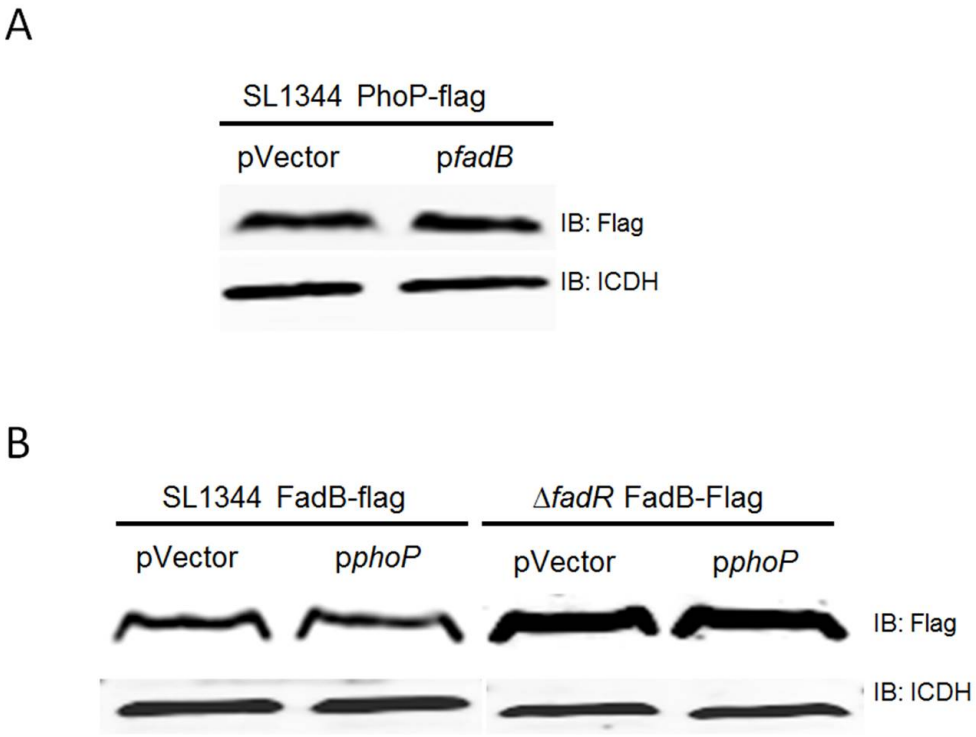

Fig. 3.12. **PhoP and FadB act independently in mediating virulence phenotype**: (A) Immunoblot analysis to examine the effects of inducing FadB on the level of PhoP in *Salmonella* (B) Immunoblot analysis to examine the effects of inducing PhoP on the level of FadB in wild type and *fadR* knock-out *Salmonella*.

Based on the cumulative results assembled in this study, we propose a working model as outlined in Fig.3.13. In this, the small protein YshB is shown to modulate the invasion and replication phases by positively regulating the expression of PhoP and FadB proteins.

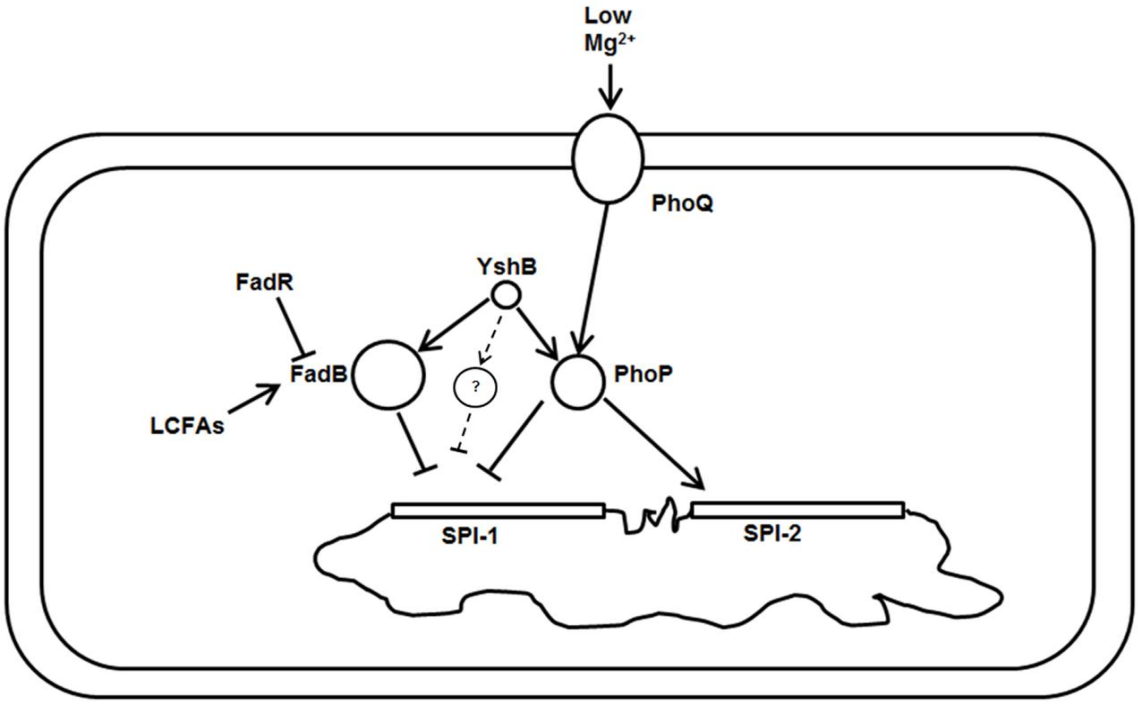

Fig. 3.13. **A working model - YshB facilitates the switching from invasion to subsequent intracellular survival of *Salmonella***: YshB modulates the discrete virulence phases of *Salmonella* by positively regulating PhoP and FadB levels in the cell. PhoP is a known activator of the SPI-2 system necessary for intracellular survival and virulence. Furthermore, PhoP is known to repress the SPI-1 system essential for invasion of host cell. Here we also show that FadB, whose level increases upon YshB induction, is also able to repress the invasion machinery. FadR is the negative regulator for *fadB* expression, whereas presence of long chain fatty acids increases FadB level in the cell. Presumably, PhoP and FadB are likely not the sole targets of YshB mediated regulation of *Salmonella* pathogenesis and other molecule(s) (denoted as "?") may well be involved. Arrowhead indicates activation while bar-head indicates inhibition. Dotted lines indicate probable signaling pathway.

3.5 Discussion

In this chapter, we show the phenotypes displayed on *yshB* knockout or YshB induction are mediated largely via two downstream proteins PhoP and FadB.

The SPI-2 system has been known to regulate the expression of SPI-1 genes involved in bacterial invasion [335]. Particularly, the PhoP/PhoQ two component system needs to remain down-regulated for the induction of SPI-1 genes to promote bacterial invasion [336]. PhoP is the response regulator and is known to repress the SPI1 genes, while activating the expression of *pag* genes. This differential regulation corresponds to the spatial localization of the bacteria in the macrophages where *pag* genes are needed to be expressed as opposed to being in the intestine, where most SPI-1 genes are expressed [205]. In the case of PhoP, 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 100μM while at or above 2mM concentration, the expression is completely turned off [199]. 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 is inside the phagosome [337]. In our study, we found that inducing YshB increases PhoP level in the cell, and thus we propose that this could be one of the factors mediating the corresponding virulence phenotype. Our study adds another layer of regulation upstream of PhoP-PhoQ to regulate the transition from the extracellular phase to that inside the host cells.

The fatty acid composition present in the intestinal tract may vary depending on the diet, host metabolism and microbiota of different regions of the tract [338]. In this regard, the role of short chain fatty acids in acting as environmental cues has been reported previously. Essentially, they can function as signals on where the bacteria are in the intestinal tract, hence modulate the regulation of virulence, especially the SPI-1 gene expression in different ways [339]. Therefore, while acetate and formate are

known to activate the SPI-1 genes [340,341]; propionate and butyrate act by repressing the SPI-1 system [342, 343]. This discrepancy amounts to the fact that diverse fatty acids are abundant at different sites in the intestinal tract and accordingly play a role in either activating or repressing the SPI-1 system. For instance, propionate and butyrate are abundant in the large intestinal regions, which are not optimal sites for inducing bacterial invasion. Hence, these molecules facilitate the down-regulation of the SPI-1 gene expression. Propionate does this by interfering with HilD activity by destabilizing this regulator of bacterial invasion [343]. On the other hand, acetate and formate act as inducers of invasion since their concentration is higher in the distal ileum [344]. The activation of SPI-1 gene expression by these fatty acids is mediated via the BarA/SirA two component system which eventually upregulates *hilD* expression [340].

Interestingly, not only SCFAs but LCFAs have also been recently reported to directly modulate the invasion phenotype by binding to HilD and affecting its DNA binding ability. In this work, they showed that LCFAs acted as direct signals to regulate a virulence phenotype [345].

In our study, the proteomics data and subsequent immunoblot showed that induction of YshB in *Salmonella* led to the increased level of FadB in the cell. Moreover, we showed that inducing FadB leads to reduced invasion efficiency. FadB is a hydroxyacyl-coA dehydrogenase that catalyzes the break-down of fatty acids during β-oxidation [346]. This is a multifunctional protein with the His^{450} as the catalytic residue for the dehydrogenase activity [334]. Interestingly, this protein was shown to be one of the *in vivo* induced (ivi) genes in *Salmonella* infected mice [324].

Fatty acids have a profound influence on macrophage function [347]. In particular, unsaturated fatty acids have been shown to enhance the antibacterial activity of macrophages [348, 349]. For instance, one of the poly-unsaturated fatty acids

produced during infection, arachidonic acid has proinflammatory metabolites [350]. Moreover, most fatty acids have a detergent-like property which is toxic to bacteria [351]. Therefore induction of *fadB* inside a phagocyte should prompt the bacteria to break down the long chain fatty acids (LCFAs), and might assist in suppressing these adverse effects.

Transcription of *fadB* is tightly regulated by the repressor of fatty acid oxidation, FadR. Interestingly, Golubeva *et al.* in 2016 reported that a Δ*fadR* strain had a repressed SPI-1 gene expression [345]. In our work, we also show that the protein levels of the invasion proteins are reduced in the Δ*fadR* as well as the FadB induced strain (Fig.3.10). Since FadR is the negative regulator of *fadB* transcription, a Δ*fadR* strain will have increased FadB levels. Furthermore, they showed that this phenotype is independent of the presence of free LCFAs as this was not suppressed in a Δ*fadL* strain. FadL is a membrane protein responsible for transporting external LCFAs into the periplasm. Similar to this finding, we demonstrated in our study that inducing FadB even in the Δ*fadL* strain will result in the reduced invasion phenotype. This suggested that FadB mediated downregulation of SPI-1 system is independent of the immediate presence of the LCFA substrates in the cytosol. This is further supported by our data showing a catalytically dead FadB^{H450Q} is still able to demonstrate the invasion phenotype. This indicates that the enzymatic activity of FadB *per se* is not necessary for the induction associated phenotype. We hence propose that FadB accumulation in the cell is sufficient to act as a signal mimicking the induction of SPI-2 system and consequently triggering the repression of the SPI-1 genes.

Small proteins mediate a variety of functions in bacteria and owing to their small size, these proteins more commonly act in mechanical ways via direct interaction with their targets [271]. We attempted to identify *Salmonella* proteins that interact with YshB using a bacterial two hybrid screening, but did not find any direct targets.

Although we cannot rule out the possibility that the potential target of YshB could have been missed in the process. Alternatively, the target for this small protein might be a small RNA where it may function as a chaperone. Hence, further analysis on the molecular mechanism of how YshB modulates bacterial virulence is necessitated.

4. CONSTRUCTION OF GENOME-WIDE *SALMONELLA* TYPHIMURIUM SL1344 MUTANT LIBRARY USING RANDOMLY BAR-CODED TRANSPOSONS

4.1 Abstract

Functional characterization of a gene is commonly assessed by identifying a mutant phenotype, which can be achieved by either forward or reverse genetics. Even though several of the environmental stresses that *Salmonella* encounters during the course of infection is known, the information on the genetics behind the successful establishment of an infection is still incomplete. Considering the impracticality of analyzing innumerable mutants with targeted single gene deletions, most study engage in investigating a pool of mutants. Signature Tagged Mutagenesis (STM) provides a means to differentiate between different mutants. Hence, we planned to utilize the powerful tool of Random Bar-code Transposon-site Sequencing (RB-TnSeq) to characterize the hitherto unknown bacterial virulence factors which might play significant roles in the infection process. With this goal, we constructed a comprehensive transposon-based genome-wide *Salmonella* Typhimurium SL1344 mutant library covering 90% of non-essential genes.

4.2 Introduction

Identification of an uncharacterized gene responsible for a phenotype can be accomplished by performing a genetic screening [352]. For the purpose of conducting such screening by constructing a pool of diverse mutants, transposon mutagenesis

has been a very reliable tool for decades. In this, a vector system delivers a transposon cassette to a recipient strain so that there are random insertions within the genome, whereby complete loss of gene function can be achieved on appropriate insertion [353]. However, one caveat with the earlier versions of this tool was inability to identify particular mutants bearing a phenotype of interest from a pool of thousands of mutagenized bacteria. Therefore, any functional screening to assess fitness of a particular mutant in a pooled population was profoundly facilitated by the introduction of signature DNA tags, in a system called Signature Tagged Mutagenesis [354]. The transposons responsible for mutagenesis carry these unique DNA tags, which can later be detected by downstream high throughput sequencing assays from the pool, each containing a unique sequence as the tag [355]. These tags can thus be considered as a molecular barcode which imparts a unique identification to each mutant in the pool. STM thus utilizes the potential of insertional mutagenesis and *in vivo* negative selection to screen for a desired phenotype from a population of mutants [356]. Ideally, the desired pool of mutants should have maximal coverage of the genome with minimal complexity [357].

Discrete virulence factors are involved in growth and survival of bacterial pathogens under different stages of infection. To characterize their roles, a functional screen on a genomic level can be conducted by employing comprehensive mutant libraries and next-generation sequencing. The rationale behind this would be to identify factors, the loss of which results in altered fitness of the bacterium under a particular condition or when subjected to infection of mammalian cell lines or animal models. In each case the tags amplified from genomic DNA isolated from the input and the output pool can be compared to assign the fitness profiles for the mutagenized strains.

4.3 Materials and Methods

4.3.1 Bacterial strains, plasmid library and media

Salmonella Typhimurium strain SL1344 was used as the recipient strain for transposon mutagenesis. The vector library used was strain APA766 [358]. This library consists of the randomly barcoded pKMW7 vector library in *E. coli* WM3064. The pKMW7 is a Tn5 class transposon vector that confers resistance to kanamycin and is DNA barcoded with a 20mer nucleotide. The WM3064 cells are derivative strains of β2155, which are auxotrophic for Diaminopimelic acid (DAP) [359]. The final concentration of DAP was maintained at 300μM in the media. Kanamycin was used at 50μg/ml final concentration.

4.3.2 Conjugation

The recipient *Salmonella* SL1344 strain was grown in LB, whereas the donor APA766 cells were grown in LB+Kan+DAP until late log phase. The donor culture was washed twice by centrifuging for 5 minutes at 5000 x g to get rid of the antibiotic. Equal amounts (as determined by OD_{600}) of the recipient *S.* Typhimurium and donor APA766 cells were mixed together. The mixture was centrifuged to remove the supernatant, and resuspended in 100μl LB with DAP and conjugated on LB-agar supplemented with DAP. After 10 h of conjugation at 37^0C, the cells were resuspended in LB-broth and plated onto freshly made LB-agar plates containing 50μg/ml kanamycin (without DAP). Following growth overnight, the kanamycin-resistant colonies were scraped together into LB-broth with 50μg/ml kanamycin. This pool of mutant library was then diluted to an OD_{600} of 0.25 in 100 ml of LB-broth with 50μg/ml kanamycin, and outgrown at 37^0C to a final OD_{600} of 1.5.

4.3.3 DNA Extraction and Tn-Seq

A 1ml aliquot from the mutant pool was subjected to genomic DNA extraction with the DNeasy kit (Qiagen). The extracted DNA was sent to the Lawrence Berkeley National Lab for Tn-Seq. To the remaining volume, glycerol was added to a final concentration of 10% (v/v), and multiple 1ml aliquots were made in cryovials to prepare -80^{0}C stocks.

4.4 Results

We constructed a randomly bar-coded genome-wide transposon mutant library in *Salmonella enterica* serovar Typhimurium SL1344 by conjugating the wild type SL1344 strain with a pool of donor *Escherichia coli* strain carrying the Tn5 vector library (Fig.4.1) [358].

Following the TnSeq, a comprehensive profile of our genome-wide mutant library was generated, the information for which can be obtained from the following link: $http://morgannprice.org/tmp/FEBA/Salmon1\_ML1\_barseqtest/$

The summary and coverage of the mutant library is expressed in the following table:

Table 4.1.
Summary of *S.* Typhimurium SL1344 randomly barcoded transposon mutant library

Category	**Name**
Mutant library name	SL1344_APA766
Transposon used	*Tn5*
Method of delivery	Conjugation
Number of strains with unique barcodes	74971
Total number of protein coding genes in the genome	4929
Number of genes with insertions	4228

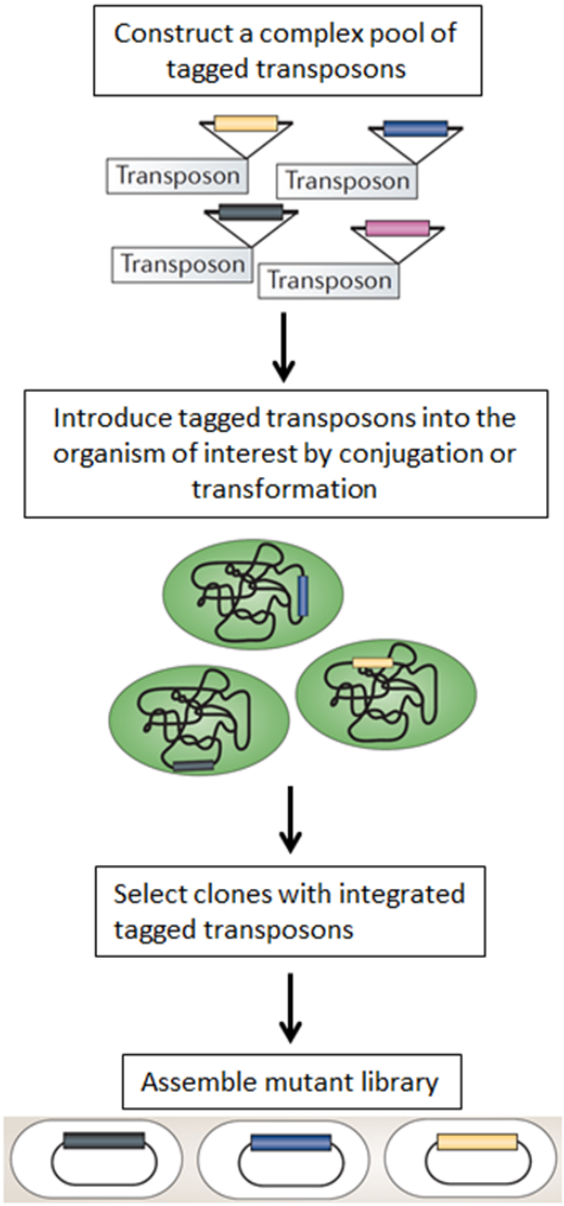

Fig. 4.1. **Scheme for random *in vivo* transposition to generate pools of tagged mutants**: A complex pool of tagged transposons is generated and used to mutagenize bacteria of interest, which are assembled into a pooled library. Different colored segments represent unique DNA tags. (Modified with permission from [360])

We therefore constructed a comprehensive genome-wide mutant library consisting of a pool of mutants with unique random insertions in more than 90% of non-essential

open reading frames of *Salmonella* genome. DNA can be extracted from this pool before and after a selective process and sequenced to identify unique tags linked to each mutation. The sequence information generated from pre- and post-selection conditions can be compared to distinguish the strains with altered fitness. This library can thus be used to carry out myriads of experiments by tracking thousands of unique strains in parallel with an objective to characterize the role of proteins or small RNA entities with as of yet unknown function.

4.5 Discussion

STM is a robust and powerful screening technique to analyze non-essential genes and their roles in various aspects of an organism's life cycle. A critical advantage of any STM strategy is that each individual mutant carries a unique signature sequence which helps to figure out their relative abundance in the pool. Moreover, these individual mutants are derived from an isogenic background.

However there are some limitations of working with a randomly mutagenized library. Essentially, a very large number of unique insertions are required to make sure a maximal coverage of genes of interest. This very high complexity due to the presence of thousands of insertion mutants creates a "biological bottleneck", particularly when using animal models of infection. For instance, during an infection, there are several stages such as invasion of intestinal epithelial cells or entry into phagocytic cells, where several mutants of the pool can be stochastically lost rather than due to a true defect. This will result in the identification of significant number of false negatives. There is also a probability of a truly defective mutant being complemented by other mutants of the pool leading to identification of false positives. Another disadvantage is the insertion is more likely to cause polar effects in the neighboring genes, which makes the validation of phenotype tricky.

Despite having these few limitations, the STM strategy has been successfully employed to identify multiple novel virulence factors in a large number of pathogens [361]. Entirely different sets of virulence factors can be involved at discrete stages of infection, which necessitates the need of different models of infection, preferably infecting different host species with the same library. Furthermore, since no single strategy could be comprehensive enough by itself, multiple approaches could be adapted that complement each other to get a broad understanding of virulence determinants of any pathogen.

5. CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Research Summary

Salmonella gastroenteritis is one of the most common causes of food-borne disease, possibly affecting millions of people globally each year.

Here we characterized the role of a novel small protein YshB in mediating a spatio-temporal switch between discrete virulence phases of this bacterium. Small proteins of less than 50 amino acids are very difficult to identify and characterize, even when they have many important functions in diverse cellular processes.

In this dissertation, we revealed the functional role of YshB as a positive regulator for bacterial intracellular survival and virulence. Deletion of *yshB* gene in wild type *Salmonella* resulted in defective intracellular replication and mouse virulence. YshB was shown to be induced in *Salmonella* under physiological conditions mimicking intracellular stages, such as *in vitro* growth media containing low Mg^{2+} concentrations (SPI-2 inducing) or inside RAW264.7 macrophage cells. Moreover, we displayed YshB induction could repress the invasion machinery, as its exogenous expression in the cell caused significant defect in the bacteria's ability to enter non-phagocytic epithelial cells. YshB therefore acts as a positive regulator of intracellular survival and negative regulator of bacterial invasion. It is well known that in *Salmonella*, a switch from infective stage to the intracellular stage is accompanied by repression of the mechanisms facilitating invasion. This saves the bacteria from excess energy expenditure and also protects from triggering of multiple detrimental downstream events, such as induction of host cell death and activation of host innate immune responses.

Further, we demonstrated that this regulation by YshB is largely mediated via two downstream proteins PhoP and FadB, presumably acting independently of each other. The role of PhoP in *Salmonella* pathogenesis is well established. Essentially, a fine-tuned regulation of the *phoPQ* operon is critical to the maintenance of bacterial physiology and virulence, as this operon is known to regulate a multitude of functions. FadB is a key enzyme in the β-oxidation pathway which facilitates the break down of long chain fatty acids into shorter ones. This act could prove to be crucial in escaping from the adverse effects which the long chain fatty acids owing to their detergent-like properties can have on bacterial survival. In addition to playing a role in intracellular survival, FadB had an inhibitory effect on SPI-1 gene expression. Interestingly, we found that this property of FadB was independent of its catalytic activity or the presence of long chain fatty acid substrates. This led us to propose that aggregation of FadB molecules in the cell was sufficient to initiate the down regulation of invasion genes. Presumably this accumulation acts as a signal mimicking intracellular phase which triggers the downstream events to subsequently repress the invasion related genes.

Hence, we provide evidence to a hitherto unknown cross talk between the two critical phases of the pathogens life cycle mediated by a small protein. We thus consider YshB as a key molecular liaison in the overall picture of *Salmonella* virulence. This elucidation adds another layer in our understanding of how this bacterium orchestrates the transformation once it is inside the host cell.

Understanding the dynamics associated with the coordinated regulation of virulence is fundamental in developing therapeutic strategies. This eventually can lead to paving ways for controlling the severity of the infection and hence contribute in reducing the burden of the *Salmonella* associated ailments among the affected public domains.

5.2 Future Directions

In this work, we showed PhoP and FadB as targets of YshB, however we could not detect any interaction between YshB and these proteins with the BACTH Bacterial Two Hybrid system. This suggests that these targets may be indirect. Furthermore, the data indicated that YshB could have pleiotropic effects and may modulate the regulation of other targets along with these two proteins. In our study, a screening method based on the same two hybrid system was unable to find any potential targets which interacted with YshB. One possible scenario for this could be the target for YshB could have been missed during the screening process. For addressing this issue, a co-immunoprecipitation can be carried out to capture the target. Another possibility is that YshB could be an RNA binding protein and the target could be an RNA molecule instead of a protein. For the purpose of identifying any putative RNA target, RNA capturing technique such as RNA immunoprecipitation can be carried out.

The role of fatty acid degrading enzymes in mediating bacterial virulence is quite uncommon. Here we showed that FadB and its regulator FadR play a role in bacterial intracellular survival and also invasion of host cells. Interestingly, FadB and not FadA was seen to have a role in virulence. Moreover, it seemed the enzymatic activity of FadB is not the reason for the associated phenotype. However, FadR is a global regulator of fatty acid degradation and biosynthesis, and considering its significance in *Salmonella* virulence, one interesting avenue of research could be to compare the lipid profiles of the wild type with the mutants of these proteins or the overexpressed strains.

The proteomics study generated a list of proteins whose levels were significantly changed upon YshB induction. These proteins are mostly associated with various

metabolic pathways. Nevertheless, it would be interesting to test whether these proteins could affect *Salmonella* virulence.

Small proteins most commonly interact with their targets via direct binding. Even though small proteins are not known to bind to DNA because of the lack of distinct protein domains, there is always a possibility. Assays can be performed such as electrophoretic mobility shift assay (EMSA) with purified YshB using the regulatory DNA regions of putative targets to examine any potential interaction.

Since YshB is only 36 amino acids long, we could also mutagenize each relevant amino acids and examine the effects on protein stability and subsequently the phenotype associated with this protein.

These studies could provide further insights and help understand the molecular details and functional characterization of YshB.

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APPENDICES

A. PLASMIDS USED IN THE STUDY

Table A.1.: List of plasmids used in the study

Plasmid Name	Description
pZP1137	pBAD vector with M45 tag
pSB1039	promoterless lacZ possessing suicide vector
pACYC-184SK	low copy plasmid with p15A origin of replication
pEGFP-N2	Mammalian Expression vector as source of EGFP tag
pEX0233	pM1644
pEX0316	pKD4
pEX0317	pCP20
pEX0320	pKD46
pEX0342	pKT25zip pUT18C zip
pEX0345	pKT25
pEX0346	pUT18C
pEX0347	pEB1029
pEX0348	pEB1030
pZP545	plasmid with a SPI-2 induced promoter Pro*sseA*
APA766	randomly barcoded pKMW7 library in WM3064
pZP2277	pM1644 with *sipA* replaced by the RfA cassette
pZP3398	STnc1450 in pZP1137
pZP3399	*yshB* with no ATG in pZP1137

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Table A.1.: *continued*

Plasmid Name	Description
pZP3400	*yshB* with frameshift in pZP1137
pZP3628	*yshB* in pZP1137 expressing YshB–M45–TEM
pZP3629	STnc590 in pZP1137
pZP3630	STnc600 in pZP1137
pZP3631	STnc180 in pZP1137
pZP3632	STnc190 in pZP1137
pZP3633	STnc200 in pZP1137
pZP3634	*yshB* with degenerate sequence in pZP1137
pZP3635	pVector with M45–TEM in pZP1137
pZP3636	pVector with EGFP from pEGFP-N2 in pZP1137
pZP3637	*yshB* expressed from its native promoter in pZP3636
pZP3638	*yshB* in pACYC–184SK expressing YshB–M45–TEM
pZP3639	*yshB* in pM1644 expressing YshB–M45–TEM
pZP3640	*phoPQ*-lacZ in pSB1039
pZP3641	*ssrAB*-lacZ in pSB1039
pZP3642	*fadA* in pZP1137
pZP3643	*fadB*-wt in pZP1137
pZP3644	$fadB^{H450Q}$ in pZP1137
pZP3645	*fadB*-wt in pZP545
pZP3646	$fadB^{H450Q}$ in pZP545
pZP3647	*fadR* in pZP1137
pZP3648	*fadR* in pZP545
pZP3649	*yshB* in pEB1029

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Table A.1.: *continued*

Plasmid Name	Description
pZP3650	*yshB* in pEB1030
pZP3651	*fadB* in pEB1029
pZP3652	*fadB* in pEB1030
pZP3653	*fadR* in pEB1029
pZP3654	*fadR* in pEB1030
pZP3655	*phoP* in pEB1029
pZP3656	*phoP* in pEB1030
pZP3657	*phoQ* in pEB1029
pZP3658	*phoQ* in pEB1030
pZP3659	*phoP* in pZP1137

B. STRAINS USED IN THE STUDY

Table B.1.: List of strains used in the study

Strains	Description
SB300	SL1344 WT
ZP18	Δ*ssaV*
ZP128	Δ*sipC*
EX100	*E. coli* BTH101
ZP331	SB300 with pKD46
ZP413	Δ*STnc1450*
ZP601	Δ*STnc180*
ZP602	Δ*STnc200*
ZP603	Δ*STnc290*
ZP604	Δ*STnc1420*
ZP605	Δ*STnc520*
ZP606	Δ*STnc590*
ZP607	Δ*STnc1640*
ZP608	Δ*STnc740*
ZP609	Δ*STnc800*
ZP610	Δ*STnc840*
ZP611	Δ*STnc850*
ZP612	Δ*STnc880*

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Table B.1.: *continued*

Strains	Description
ZP613	Δ*STnc1030*
ZP614	Δ*STnc1050*
ZP615	Δ*STnc1100*
ZP616	Δ*STnc1120*
ZP617	Δ*STnc1130*
ZP618	Δ*STnc1150*
ZP619	Δ*STnc1170*
ZP620	Δ*STnc1190*
ZP621	Δ*STnc1200*
ZP622	Δ*STnc1210*
ZP623	Δ*STnc1220*
ZP624	Δ*STnc1240*
ZP625	Δ*STnc1270*
ZP626	Δ*STnc1280*
ZP627	Δ*STnc1300*
ZP628	Δ*STnc1330*
ZP629	Δ*STnc1350*
ZP630	Δ*STnc1360*
ZP631	Δ*STnc1380*
ZP632	Δ*STnc1410*
ZP633	Δ*STnc1430*
ZP634	Δ*STnc1400*
ZP635	Δ*STnc1460*

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Table B.1.: *continued*

Strains	Description
ZP636	Δ*STnc1470*
ZP637	Δ*STnc1590*
ZP638	Δ*STnc2090*
ZP639	Δ*STnc2130*
ZP640	Δ*STnc1440*
ZP641	Δ*STnc190*
ZP642	Δ*STnc1250*
ZP643	Δ*STnc750*
ZP644	Δ*STnc1370*
ZP645	Δ*STnc1290*
ZP646	Δ*STnc1340*
ZP647	Δ*STnc1650*
ZP648	Δ*STnc1160*
ZP649	Δ*STnc2040*
ZP650	Δ*STnc110*
ZP651	Δ*STnc1390*
ZP652	Δ*yshB*
ZP653	SB300 with pZP3398
ZP654	SB300 with pZP3399
ZP655	SB300 with pZP3400
ZP656	SB300 with pZP3628
ZP657	SB300 with pZP3629
ZP658	SB300 with pZP3630

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Table B.1.: *continued*

Strains	Description
ZP659	SB300 with pZP3631
ZP660	SB300 with pZP3632
ZP661	SB300 with pZP3633
ZP662	SB300 with pZP3634
ZP663	SB300 with pZP3635
ZP664	SB300 with pZP3636
ZP665	SB300 with pZP3637
ZP666	SB300 with pZP3638
ZP667	SB300 with pZP3639
ZP668	SB300 with pZP3642
ZP669	SB300 with pZP3643
ZP670	SB300 with pZP3644
ZP671	SB300 with pZP3645
ZP672	SB300 with pZP3646
ZP673	SB300 with pZP3647
ZP674	SB300 with pZP3648
ZP675	Δ*yshB* with pZP3628
ZP676	Δ*yshB* with pZP3635
ZP677	Δ*yshB* with pZP3636
ZP678	Δ*yshB* with pZP3637
ZP679	SB300 with pM1644 and pZP3638
ZP680	SB300 with pZP3639
ZP681	*phoPQ-lacZ* in SB300 with pZP3628

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Table B.1.: *continued*

Strains	Description
ZP682	*phoPQ-lacZ* in SB300 with pZP3635
ZP683	*phoPQ-lacZ* in Δ*yshB* with pZP3635
ZP684	*ssrAB-lacZ* in SB300 with pZP3628
ZP685	*ssrAB-lacZ* in SB300 with pZP3635
ZP686	PhoP-Flag in SB300 with pZP3628
ZP687	PhoP-Flag in SB300 with pZP3635
ZP688	PhoP-Flag in Δ*yshB* with pZP3635
ZP689	SsrB-Flag in SB300 with pZP3628
ZP690	SsrB-Flag in SB300 with pZP3635
ZP691	*fadBA-lacZ* in SB300 with pZP3628
ZP692	*fadBA-lacZ* in SB300 with pZP3635
ZP693	*fadBA-lacZ* in Δ*yshB* with pZP3635
ZP694	FadB-Flag in SB300 with pZP3628
ZP695	FadB-Flag in SB300 with pZP3635
ZP696	FadB-Flag in Δ*yshB* with pZP3635
ZP697	FadB-Flag in Δ*fadR* with pZP3628
ZP698	FadB-Flag in Δ*fadR* with pZP3635
ZP699	FadB-Flag in Δ*fadR* Δ*yshB* with pZP3628
ZP700	Δ*fadL* with pZP3628
ZP701	Δ*fadL* with pZP3635
ZP702	Δ*fadL* with pZP3643
ZP703	Δ*fadL* with pZP3644
ZP704	Δ*fadB* with pZP3628

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Table B.1.: *continued*

Strains	Description
ZP705	Δ*fadB* with pZP3635
ZP706	Δ*fadB* with pZP3645
ZP707	Δ*fadB* with pZP3646
ZP708	Δ*fadA*
ZP709	Δ*phoP* with pZP3628
ZP710	Δ*phoP* with pZP3635
ZP711	Δ*phoP*Δ*fadB* with pZP3628
ZP712	Δ*phoP*Δ*fadB* with pZP3635
ZP713	Δ*fadR*
ZP714	Δ*fadR* with pZP3647
ZP715	Δ*fadR* with pZP3648
ZP716	BTH101 with pEX0342
ZP717	BTH101 with pZP3649 and pZP3652
ZP718	BTH101 with pZP3650 and pZP3651
ZP719	BTH101 with pZP3649 and pZP3654
ZP720	BTH101 with pZP3650 and pZP3653
ZP721	BTH101 with pZP3649 and pZP3656
ZP722	BTH101 with pZP3650 and pZP3655
ZP723	BTH101 with pZP3649 and pZP3658
ZP724	BTH101 with pZP3650 and pZP3657
ZP725	PhoP-Flag in SB300 with pZP3643
ZP726	FadB-Flag in SB300 with pZP3659
ZP727	FadB-Flag in Δ*fadR* with pZP3659

C. QUANTITATIVE PROTEOMICS PROFILE OF ALTERED PROTEINS UPON YSHB INDUCTION

Table C.1.: List of proteins whose levels were significantly altered upon YshB induction (FC: Fold change in YshB induced strain)

Protein Name (Description)	FC
NapA (Periplasmic nitrate reductase)	0.54
NapC (Cytochrome c-type protein)	0.57
YbhL (Hypothetical membrane protein)	0.6
GlpC (Anaerobic glycerol-3-phosphate dehydrogenase subunit C)	0.63
FlgD (Flagellar hook formation protein)	0.65
CcmG2 (Cytochrome C-type biogenesis protein)	0.65
CcmE (Cytochrome C-type biogenesis protein)	0.66
DcuA (Anaerobic C4-dicarboxylate transporter)	0.66
FadA (3-ketoacyl-CoA thiolase)	1.62
RhlE (ATP-dependent RNA helicase)	1.65
CspJ (Cold shock-like protein)	1.66
CycA (D-serine/D-alanine/glycine transporter)	1.84
FadB (Fatty acid oxidation complex subunit alpha)	1.98
CspA (Cold shock protein)	3.14
RtcB (RNA-splicing ligase)	6.93

VITA

VITA

[Rajdeep was born and brought up in the beautiful city of Kathmandu, Nepal. He completed his Bachelor of Science (B.Sc) and Master of Science (M.Sc) degrees in Microbiology from Tribhuvan University, Nepal. He then worked as a lecturer of Microbiology in a private campus in Nepal for four years. After being long fascinated by aspects of how pathogens and their hosts have co-evolved to engage in a state of one-upmanship, he decided to pursue a Ph.D and got enrolled in the PULSe program at Purdue University in 2010. In August 2011, he joined Dr. Daoguo Zhou's lab in the Department of Biological Sciences and conducted research in regulation of *Salmonella* pathogenesis. His further plan is to continue research in the field of Cellular and Molecular Microbiology, specifically deciphering the intriguing facets of host-pathogen interface.]