Role of *Salmonella* Typhimurium Effector Protein SpvD in Host Cell Infection

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Certificate of Examination

This is to certify that the dissertation titled **"Role of *Salmonella* Typhimurium Effector Protein SpvD in Host Cell Infection"** submitted by **Mr. Raminder Singh** (Reg. No. MS11052) for the partial fulfilment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr. Kausik Chattopadhyay Dr. Mahak Sharma Dr. Arunika Mukhopadhaya
(Supervisor)

Dated: April 22, 2016

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Dr. Arunika Mukhopadhaya at the Indian Institute of Science Education and Research Mohali. This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Raminder Singh
(Candidate)

Dated: April 22, 2016

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr. Arunika Mukhopadhaya
(Supervisor)

Acknowledgement

I would like to thank my supervisor, Dr. Arunika Mukhopadhaya, for accepting me as a master's student, for all the helpful discussion about this project and for the constant motivation throughout this project.

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MS11052

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Notation

Abbreviation	Full form
LAMP	Lysosome associated molecular patterns
N-WASp	Wiskott–Aldrich syndrome protein
RILP	Rab-interacting lysosomal protein
SPI	*Salmonella* pathogenicity island
SCV	*Salmonella* containing vacuole
SpvD	*Salmonella* plasmid virulence factor D
SIF	*Salmonella*-induced filaments
T3SS	Type-three secretory system
WAVW2	(aka WASF2) Wiskott-Aldrich syndrome protein family member 2.

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Abstract

Salmonella Typhimuriun causes self-limiting gastroenteritis in humans and typhoid like condition in mice. *S.* Typhimurium actively invade intestinal epithelial and macrophage cells and replicate inside the host cell within a modified phagosome known as *Salmonella* containing vacuole (SCV). Invasion and replication is regulated by two different type-three secretion system (T3SS) that translocate effector proteins, encoded by both *Salmonella* pathogenicity island-1 (SPI-1) and SPI-2, into the host cell cytoplasm. T3SS-1 regulates invasion and T3SS-2 regulates intracellular replication. *Salmonella* plasmid virulence factor D (SpvD) is an effector protein encoded by both SPI-1 and SPI-1. In this report, we have found that ΔSpvD shows replication defects only in macrophage cell but not in epithelial cell lines. From our conclusion we hypothesize that ΔSpvD is a macrophage sensitive mutant.

Introduction

Introduction

Salmonella spp. is a gram-negative enteric pathogen. A large number of animal species including humans can be infected by a different type of *Salmonella* serotypes. *Salmonella enterica* serovar Typhimurium is the leading cause of acute bacterial gastroenteritis in humans. The main reason for its success as an enteric pathogen is its ability to actively invade non-phagocytic cells like intestinal epithelial cells through a specialised needle-like apparatus known as type III secretion system (T3SS). *Salmonella* uses type III secretion system encoded within *Salmonella* pathogenicity island-1 (SPI-1) and SPI-2 to translocate effectors across the host cell plasma membrane and to subvert the function of the host cell. The SPI-1 encodes T3SS-1 and is activated extracellularly in the lumen of the gut and translocates effector proteins needed for the invasion of non-phagocytic cells, as well as, inflammation and biogenesis of intracellular niche for internalized *Salmonella* such as *Salmonella*-containing vacuole (SCV). The SPI-2 encoded T3SS-2 gets activated inside SCV and translocates effectors across the SCV membrane into the host cell cytoplasm and SP1-2 effectors are involved in intracellular replication of *Salmonella* and systemic infection.

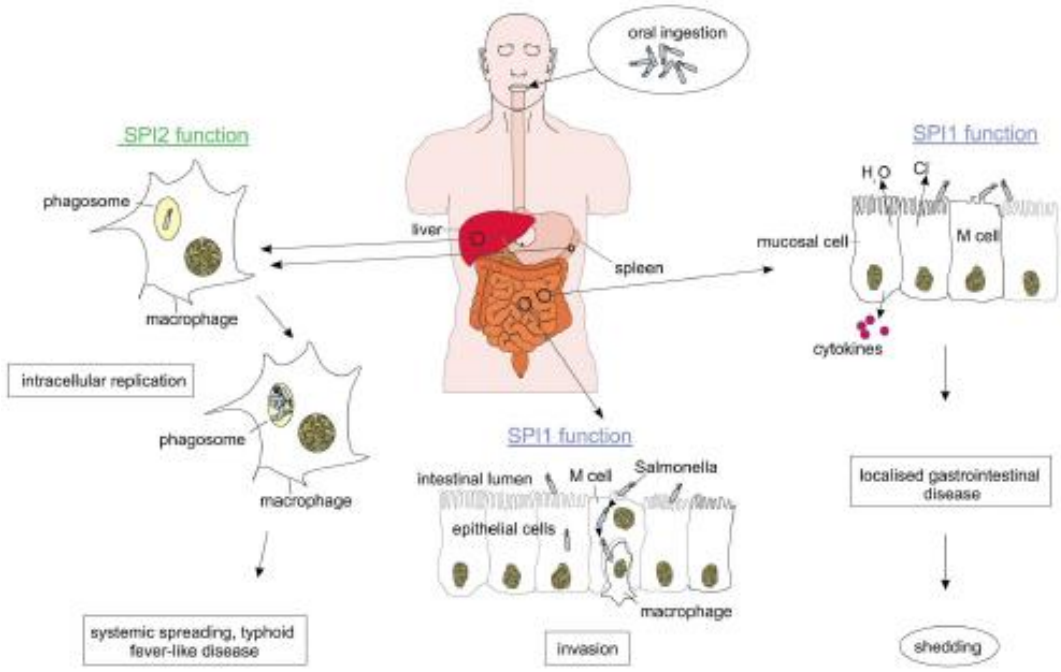


Figure 1. Host-pathogen interaction during *Salmonella* infection. Adapted from (Hansen-Wester and Hensel 2001).

Type III Secretion System (T3SS)

T3SS is needle-like apparatus present in several pathogenic bacteria (Troisfontaines and Cornelis 2005), specialized in translocating bacterial effector proteins into the host cell (Galán and Wolf-Watz 2006). T3SS can be divided into three parts: (i) basal body, a transmembrane export apparatus; (ii) extracellular needle; and (iii) a translocon that punches pore in the host cell membrane (Cornelis 2006). For the proper functioning of the T3SS, some additional elements are also required, such as, chaperons that bind to and asserts the secretion of effector proteins and an ATPase present at the cytosolic part of the basal body. The ATPase catalyse the dissociation of the effector-chaperon complex before the secretion of the effector proteins (Akeda and Galán 2005).

Both T3SS play an equally important roles in different phases of bacterial infections. SPI-1 encoded-T3SS is activated extracellularly inside the lumen of the gut having SPI-1 inducing conditions like anaerobic environment, basic pH and high osmolarity (Galan and Curtiss 1989). SPI-1-T3SS is required for bacterial invasion, inflammatory responses of intestinal cells (Wood, Jones et al. 1998) and innate immune responses (Bruno, Hannemann et al. 2009). SPI-2 encoded T3SS is expressed inside the host cell having SPI-2 inducing conditions, like low pH and low phosphate ion post-infection. SPI-2 T3SS is majorly required for intracellular bacterial replication and survival (Deiwick, Nikolaus et al. 1998; Bruno, Hannemann et al. 2009).

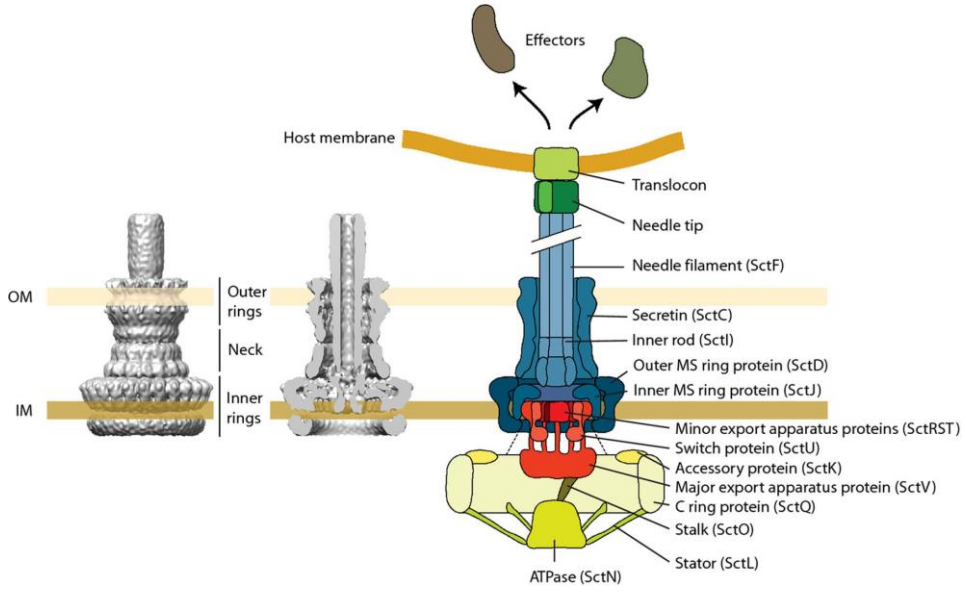


Figure 2. Overview of T3SS and its components. Adapted from (Diepold and Wagner 2014).

Salmonella Virulence

Virulence of *Salmonella* is dependent on various factors and most of these virulence factors are encoded within *Salmonella* pathogenicity islands (SPI). SPIs are horizontally acquired stretch of genes. Many of these SPIs are common in all *S. enterica* serovars while some serovars have their own unique SPIs as well. Other than SPIs some *Salmonella* serovars have virulence plasmid as well, but *S.* Typhi and *S.* Para Typhi do not have virulence plasmids. The expression of almost all of these virulence determinants are regulated through a two-component regulatory systems. Some of the major members of the two-component regulatory systems are OmpR/EnvZ, PhoP/PhoQ and SsrA/SsrB.

Salmonella Pathogenicity Islands (SPIs)

Salmonella pathogenicity islands (SPIs) are a stretch of DNA distributed all over the genome that encodes majority of the virulence genes of the bacteria. They encode determinants responsible for the host-pathogen interaction like host cell invasion and replication (Ochman, Soncini et al. 1996; Groisman and Ochman 1997). *Salmonella* acquired these pathogenicity islands through horizontal gene transfer, which helps bacteria to gain complex virulence functions relatively quick (Hensel 2004). Until now, 21 different SPIs have been identified but not all of them are present in all the serovars. *S.* Typhimurium has 11 SPIs in common with *S.* Typhi (SPI-1 to 6, 9, 11, 12, 13 and 16). SPI-14 is specific to *S.* Typhimurium (Sabbagh, Forest et al. 2010). Out of all these SPIs, SPI-1 and SPI-2 are the most important and most studied SPIs.

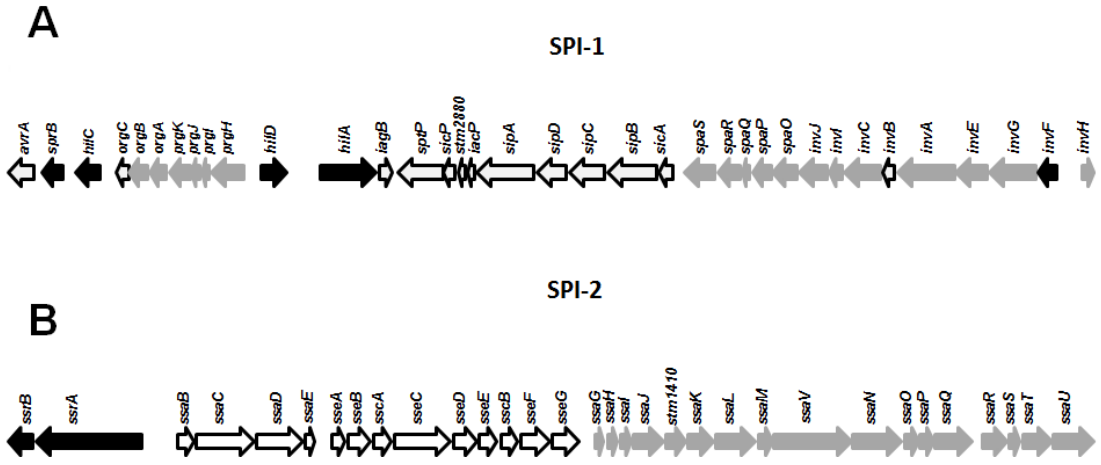


Figure 3. Genetic map of SPI-1 and SPI-2. The genes encoding structural proteins are in grey, and the genes that code for transcriptional regulators are in black. Modified from (Dieye, Ameiss et al. 2009).

Salmonella Pathogenicity Island-1 (SPI-1)

SPI-1 is approximately 40 Kb in size. It is located at the 63 centisome of *S.* Typhimurium. It encodes for a T3SS-1 that translocates effector protein across the plasma membrane into the host cell cytoplasm. A subset of SPI-1 effector proteins are involved in the invasion of the non-phagocytic cells by rearranging the actin cytoskeleton (Patel and Galan 2005). Another subset of effectors are involved in the inflammation of intestinal epithelium (Wood, Jones et al. 1998). SPI-1 effector proteins are encoded both inside as well as outside of the pathogenicity island like SopB a translocated effector of SPI-1 is encoded within SPI-5.

SPI-1 and Invasion

For epithelial cell invasion SipA, SipC, SopB, SopE and SopE2 are the major players and SptP act as an antagonist of these effector proteins. SopB, SopE and SopE2 act as activators of small Rho GTPases Rac1 and RhoG which activates N-WASp and WAVE2, which leads to the recruitment of Arp2/3 complex to the site of internalization and helps in actin polymerization to induce membrane ruffling and reorganization (Criss and Casanova 2003; Unsworth, Way et al. 2004; Shi, Scita et al. 2005). Mutation in these genes leads to defects in invasiveness of the bacteria (Zhou,

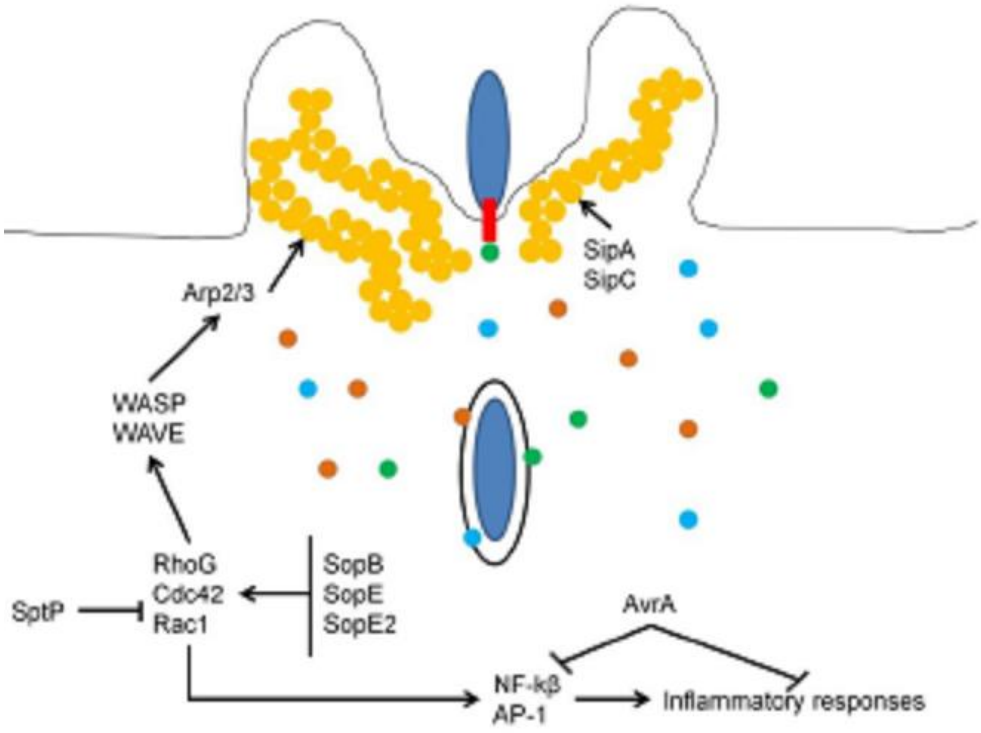


Figure 4. SPI-1 induced invasion of non-phagocytic cells. Effector proteins (coloured dots), actin (yellow dots). Adapted from (Braun and Brumell 2010).

Chen et al. 2001). After internalization SptP, another effector protein which acts as a GTPase activating protein (Fu and Galán 1998) oppose the activity of SopE and SopE2 and helps in regaining the normal architecture of actin cytoskeleton.

After entry into the host cell, *Salmonella* resides in a membrane-bound compartment known as *Salmonella*-containing vacuole (SCV). SCV is a modified phagosome. SopB, SptP and SopE are shown to be important for SCV maturation. As *Salmonella* replicates within the mature SCV, the function of SopB, SptP and SopE is necessary for *Salmonella* replication. SCV maturation includes (i) remodelling of the negative charge of SCV membrane, which prevents SCV fusion with lysosome (Bakowski, Braun et al. 2010), (ii) recruitment of small GTPase Rab5 (Mallo, Espina et al. 2008).

SPI-1 and Inflammation

Salmonella internalization through SPI-1 T3SS also leads to the activation of pro-inflammatory signals. SopB, SopE and SopE2 act on the small GTPase Cdc42 which in turn activate AP-1 and NF-κB (Winter, Thiennimitr et al. 2010; Lopez, Winter et al. 2012). Both AP-1 and NF-κB are transcription factors for many genes involved in the inflammatory responses. Another SPI-1 effector, SipB are known to activate caspase-1and modulate intestinal inflammatory responses (Hersh, Monack et al. 1999). However, this inflammatory response is down-regulated by another effector protein, AvrA that inhibits NF-κB activity and pro-inflammatory cytokines secretion and induces cell death leading to the elimination of infected cells and prevention of systemic spread (Collier-Hyams, Zeng et al. 2002).

SPI-2 and Intracellular Replication

SPI-2 effector proteins are known to be involved in SCV membrane dynamics and positioning of SCV in the infected cells (Hensel, Shea et al. 1995; Helaine, Thompson et al. 2010). After SCV formation, this modified phagosome move towards the perinuclear/Golgi region of the cell (Ramsden, Mota et al. 2007). This centripetal movement of SCV accompanied by its maturation which occurs through the interaction of SCV with components of the endocytotic pathway. Fusion with early endosome enrich SCV in markers like EEA-1, Rab5 and Tfr and these markers replaced shortly by markers of late endosome like LAMPs, vacuolar ATPase, Rab7, Rab11 and decrease in the luminal pH of the SCV (Garcia-del Portillo and Finlay

1995; Steele-Mortimer, Méresse et al. 1999; Drecktrah, Knodler et al. 2007). Rab7 together with RILP recruit dynein to the SCV (Harrison, Brumell et al. 2004). SCV enriched in late endosomal/lysosomal markers do not have lysosomal hydrolases in their lumen. However, there are reports which shows that SCV interacts with the lysosome (Drecktrah, Knodler et al. 2007) but *S.* Typhimurium interfere with mannose-6-phosphate receptor trafficking and depletes lysosomes of hydrolases (McGourty, Thurston et al. 2012). SCV maturation and peri-nuclear movement are thought to be important for *Salmonella* intra-cellular replication.

After reaching perinuclear region *Salmonella* induces the formation of a tubular structure known as *Salmonella*-induced filament (SIF). SIF is a very dynamic structure enriched in a subset of late endosomal and lysosomal markers. SIF

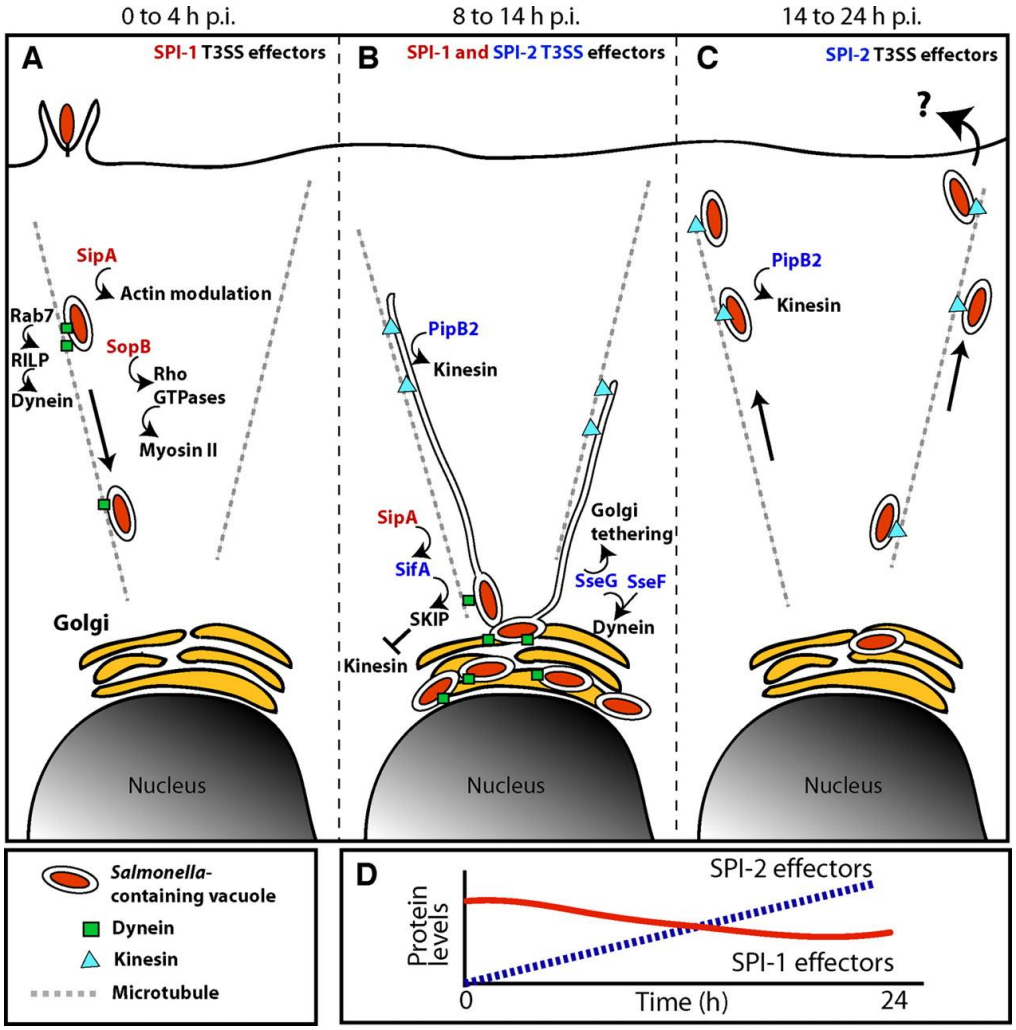


Figure 5. SCV positioning and SIF formation in infected cell. Role of different effectors in SCV maturation and SIF formation during infection. Adapted from (Szeto, Namolovan et al. 2009).

formation is SPI-2 dependent and effectors involved in SIF formation are: SifA, PipB2, SopD2, SseF, SseG, SseJ and SpvB (Jiang, Rossanese et al. 2004; Birmingham, Jiang et al. 2005; Knodler and Steele-Mortimer 2005). The maintenance of SCV at the perinuclear region requires SifA, SseF and SseG. They recruit dynein to the SCV which is necessary for SCV positioning and intracellular replication. SifA perform multiple functions to control the SCV membrane dynamics. SifA mutant loses the SCV membrane-integrity and shows defects in replication and SIF formation. SseJ exhibits glycerophospholipid-cholesterol acyltransferase (GCAT) activity (Lossi, Rolhion et al. 2008; Nawabi, Catron et al. 2008) and its function is to esterify cholesterol in infected cells. Absence of SseJ results in rigidity of the SCV membrane which eventually leads to rupture of the SCV.

Both SPI-1 and SPI-2 can be assigned distinct function in the host cell infection, but there is no strict temporal distinction between the two. For example, InvA, an SPI-1 effector helps in invasion and ΔInvA *Salmonella* Typhimurium is unable to grow inside the host cell as well. This shows that SPI-1 effectors help in intracellular replication as well. In addition a number of effectors can be secreted by both SPI-1 and SPI-2.

Virulence Plasmid – spv operon

The spv operon is encoded on a virulence plasmid found on non-typhoidal *Salmonella* serovars (Guiney, Fang et al. 1994). It consists of five genes, SpvRABCD. These genes enhance the virulence of the *S.* Typhimurium. Transcription of spv genes depends on both SpvR and rpoS (stationary phase sigma factor) (Chen, Buchmeier et al. 1995; Wilson and Gulig 1998). Till now the function of only one spv gene, SpvB, is known. The C-terminal of SpvB contains NAD-binding sequence which contributes to its ADP-ribosyl transferase activity (Lesnick, Reiner et al. 2001). Its primary substrate is monomeric actin. SpvD is encoded by both SPI-1 and SPI-2 (Niemann, Brown et al. 2011). ΔSpvD mutant shows attenuated replication in mouse bone-marrow-derived macrophage cells (Figueira, Watson et al. 2013). Here in this report, we tried to establish the replication defect of the ΔSpvD mutant in the host cell and reflected upon the mechanism behind it.

Material and methods

Bacterial strains

S. enterica serovar Typhimurium LT2 (MTCC of IMTECH, Chandigarh) and mutant derived from the same strain were used. The replacement of SpvD gene with kanamycin cassette (ΔSpvD::Kan) was constructed using the method as describe in (Datsenko and Wanner 2000).

Bacterial culture used and rationale behind it

As SpvD is encoded by both SPI-1 and SPI-2, we used different conditions to ensure expression of SpvD through a single SPI and then to check the role to SpvD in that particular condition.

SPI-1 inducing condition:

1. μaer-ST (non-aerated stationary phase bacteria) were used. *S.* Typhimurium strains were grown overnight at 37°C in LB–0.3 M NaCl medium under static conditions.
2. Aer-LL (areated late-log phase bacteria with OD_{600} = 3.0): Bacteria were grown for 16 h at 37 °C with shaking and then sub-cultured (1:33) in Luria-Bertani broth for 3 h.

SPI-2 inducing condition:

1. Bacteria from cultures in LB were washed and diluted in 1:100 with low-phosphate, low-magnesium minimal medium (LPM) at pH 5.8 and incubated overnight at 37°C with shaking.

Low-phosphate, low-magnesium minimal medium (LPM) contained: Filter sterilized with 0.22μm filter (Millipore Millex).

1. 80 mM 2-(*N*-morpholino) ethanesulfonic acid (pH 5.8)
2. 5 mM KCl
3. 7.5 mM $(NH_4)_2SO_4$
4. 0.5 mM K_2SO_4
5. 0.1% Casamino Acids
6. 38 mM glycerol

7. 337.5 μM K_2HPO_4-KH_2PO_4 (pH 7.4)
8. 8 μM $MgCl_2$

Antibiotic used: Kanamycin (Kan) form HIMEDIA, 40 μg/ml is used for ΔSpvD cultures.

Cell culture

RAW 264.7 cells (ATCC) were cultured in RPMI and HeLa cells (ATCC) in DMEM supplemented by 10% FBS in a 5% CO_2 incubator at 37°C.

Measurement of bacterial growth

For the measurement of bacterial growth, *Salmonella* was cultured in 100 ml round bottom conical flask with 50 ml LB media. The samples were taken at every 30 min for OD_{600} readings in a Jenway Genova Plus spectrophotometer.

Invasion assay (Gentamicin protection assay)

In RAW 264.7

Late-log phase bacteria (SPI-1 condition) were used for the invasion of RAW 264.7 cells. RAW 264.7 cells grown in RPMI media with 10% FBS were seeded on 24-well plate with cell density of 10^6 cells/ml. *Salmonella* were collected by centrifugation at 10,000 rpm for 4 min, resuspended in complete RPMI and used immediately for infection of RAW 264.7 cells with M.O.I of 10. After infection, monolayer were centrifuged immediately at 1000 xg for 1min. Then incubation for 15 min, 30 min and 60 min at 37°C for infection. After incubation, extracellular bacteria were removed and monolayer were washed twice with PBS. Then incubated for another 1 h in RPMI with 100 μg/ml gentamicin. After 1 h of gentamicin treatment cells were lysed with 0.1% Triton X-100 and viable intracellular bacteria were enumerated by C.F.U. count.

In HeLa cells

μaer- ST bacteria (SPI-1 condition) were used for the invasion of HeLa cells. HeLa cells grown in DMEM media with 10% FBS were seeded on 24-well plate with cell density of $6x10^5$ cells/ml. *Salmonella* were collected by centrifugation at 10,000 rpm

for 4 min, resuspended in complete DMEM and used immediately for infection of HeLa cells with M.O.I of 150. After infection, monolayer were centrifuged immediately at 1000 xg for 1min. Then incubated for 30 min, 60 min and 120 min at 37°C for infection. After incubation, extracellular bacteria were removed and monolayer were washed twice with PBS. Then incubated for another 1 h in DMEM with 100 μg/ml gentamicin. After 1 h of gentamicin treatment cells were lysed with 0.1% Triton X-100 and viable intracellular bacteria were enumerated by C.F.U count.

Replication assay

In RAW 264.7 cells

Stationary phase bacteria grown in LPM (SPI-2 condition) were used for the replication of RAW 264.7 cells. RAW 264.7 cells grown in RPMI media with 10% FBS were seeded on 24-well plate with cell density of 10^6 cells/ml. *Salmonella* were collected by centrifugation at 10,000 rpm for 4 min, resuspended in complete RPMI and used immediately for infection of RAW 264.7 cells with an M.O.I of 150. After infection, monolayer were centrifuged immediately at 1000 xg for 1 min. Then incubated for 60 min at 37°C for infection. After incubation, extracellular bacteria were removed and monolayer were washed twice with PBS. Then incubated for another 1 h in RPMI with 100 μg/ml gentamicin. Following that RPMI with 15 μg/ml of gentamicin were added for the rest of the experiment. After 2 h and 16 h post infection cells were lysed with 0.1% Triton X-100 and viable intracellular bacteria were enumerated by C.F.U count.

In HeLa cells

μaer-ST bacteria (SPI-1 condition) were used for the replication of HeLa cells. HeLa cells grown in DMEM media with 10% FBS were seeded on 24-well plate with cell density of 0.6 * 10^6 cells/ml. *Salmonella* were collected by centrifugation at 10,000 rpm for 4 min, resuspended in complete DMEM and used immediately for infection of HeLa cells with an M.O.I of 150. After infection, monolayer were centrifuged immediately at 1000 xg for 1 min. Then incubated for 60 min at 37°C for infection. After incubation, extracellular bacteria were removed and monolayer were washed twice with PBS. Then incubated for another 1 h in DMEM with 100 μg/ml gentamicin. After that DMEM with 15 μg/ml of gentamicin were added for the rest of

the experiment. After 2 h and 16 h post infection cells were lysed with 0.1% Triton X-100 and viable intracellular bacteria were enumerated by C.F.U count.

Lactate dehydrogenase (LDH) release assay for measuring cytotoxicity

LDH (Lactate dehydrogenase) release is an assay for estimating the viability of cells. LDH is an oxidoreductase present in the cell. When toxic material damages a cell, cell releases LDH into the surrounding medium. LDH is a very stable enzyme and it is widely used as a marker for cell death. To evaluate the cytotoxicity level of wild type and ΔSpvD infection in SPI-2 condition, RAW 264.7 ($1x10^6$ cells/ml) and HeLa ($0.6x10^6$ cells/ml) were infected with bacteria with an M.O.I of 150 and LDH release was estimated using LDH release assay kit (Promega). Briefly, Solution containing cells debris and media were collected 16 h post infection. Cells lysed with 1X lysis solution were used as positive control (100% cell death). Supernatants were collected and to estimate the cytotoxicity level supernatants were mixed with the substrate in 1:1 v/v ratio. Further, incubated for 5-10 min at room temperature till the orange colour developed. OD was measured at 490 nm using an ELISA reader (BIO-RAD)

Cytotoxicity was calculated using the following formula:

$$\boldsymbol{Cytotoxicity} = \frac{(\boldsymbol{Test} - \boldsymbol{Blank})}{(\boldsymbol{Positive\ triton\ control} - \boldsymbol{Blank})} \times \mathbf{100}$$

Estimation of nitric oxide production

Nitric oxide (NO) was measured as its stable end product nitrite using Griess reagent as describe in (Green, Wagner et al. 1982). Culture supernatants were collected 16 h post infection (using same condition as replication) and mixed with Griess reagent in 1:1 v/v. After 15 min, absorbance was measured at 540 nm in 96 well plate. The concentration of nitrite was measured in the sample with reference to the standard curve made with sodium nitrite.

Salmonella-induced filament formation

Formation of *Salmonella*-induced filament requires SPI-2 encoded T3SS. HeLa cells grown in DMEM media with 10% FBS were seeded on 12-mm-diameter glass coverslips for 6–10 h before experiments in 24-well plate with a cell density of 50,000 cells/ml. *Salmonella* were collected by centrifugation at 10,000 rpm for 4 min, resuspended in complete DMEM and used immediately for infection of HeLa cells with an M.O.I of 150. After infection, monolayer were centrifuged immediately at 1000 xg for 1min. Then incubated for 60 min at 37°C for infection. After incubation, extracellular bacteria were removed and monolayer were washed twice with PBS. Then incubated for another 1 h in DMEM with 100 μg/ml of gentamicin. Followed by incubation for 8 h in the presence of 15 μg/ml of gentamicin. Fixation was performed with 2.5% paraformaldehyde for 30 min at 37°C. Fixed cells were washed twice with PBS and permeabilized by treatment with 0.2% saponin in PBS. Primary and secondary antibodies were added on coverslips in 0.2% saponin with PBS for 45 min and 30 min respectively at room temperature, followed by three washes with PBS. Samples were analysed using Zeiss 780 Confocal Microscope.

Antibodies used

Primary antibodies used: Anti-rabbit *Salmonella* (BD Biosciences), Anti-mouse LAMP-1 (BD Biosciences).

Secondary antibodies used: Alexa 488 conjugate goat anti-rabbit-IgG (Life Technologies), Alexa 568 conjugated goat anti-mouse-IgG (Life Technologies).

Results

1. Growth kinetics of wild-type (WT) and mutant (ΔSpvD) *Salmonella* Typhimurium LT2 in artificial medium (LB)

Overnight culture of wild-type and mutant bacteria were diluted to OD 0.01 at 600nm in LB medium. Further the diluted culture was incubated for 24 h while subjected to monitoring of the growth (OD_{600}) at every 30 min for the initial 12 h with final OD at 24 h (Figure 6).

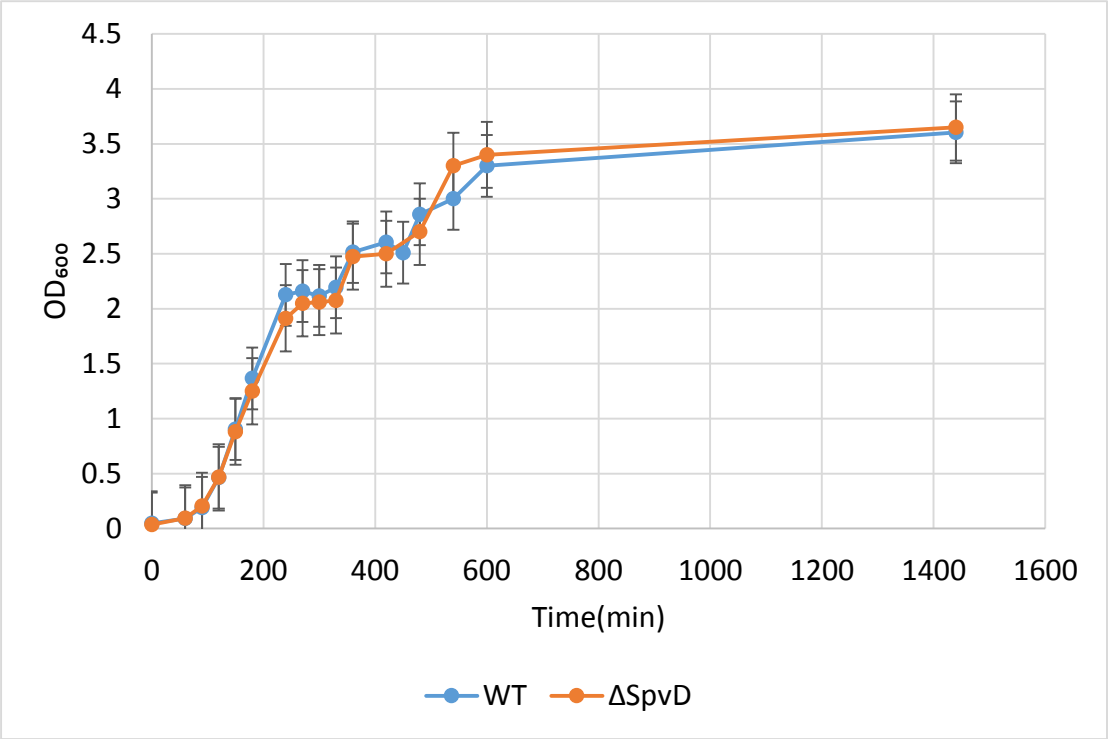


Figure 6. Growth curve of WT and ΔSpvD *Salmonella*.

Inference: As the growth curves of the wild-type and the mutant bacteria are almost super-imposing on each other we can conclude that there is no difference in growth of WT and ΔSpvD in artificial LB media.

2. Assay for bacterial invasion into the host cell

Salmonella Typhimurium actively invades intestinal epithelial cells. Following invasion, the epithelial cell is destroyed and the bacteria gain access to sub-epithelial lymph tissue and the lamina propria, where they encounter macrophages and other immune cells and leads to systemic spread. Here, we have used RAW 264.7 (macrophage cell line) and HeLa (epithelial cell line) for our study. As mentioned previously, SpvD is encoded by both SPI-1 and SPI-2. Therefore, at first we wanted to check its role as an SPI-1 effector. Active invasion of host cell is a function regulated by SPI-1 T3SS. So, we did gentamicin protection assay to check whether there is any difference in the active invasiveness of the bacteria during the infection. For invasion assay we infect the cells with SPI-1 induced *Salmonella.*

i. HeLa cells were plated in 300 µl with cell density of $0.6x10^6$ cells/ml in 24 well plate and were infected with overnight bacterial culture grown in LB media containing 0.3 M NaCl in static condition with M.O.I of 150. A number of bacteria invade the cell was determined by C.F.U enumeration at 30 min, 60 min and 2 h post infection (Figure 7).

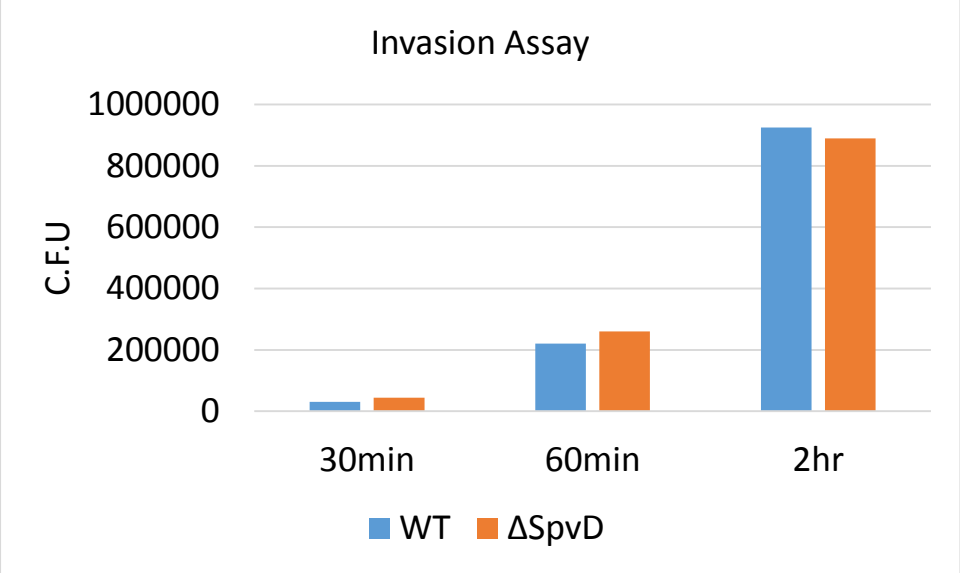


Figure 7.Gentamicin protection assay to compare the invasiveness of the WT and ΔSpvD in HeLa cells.

ii. RAW 264.7 cells were plated in 500 µl with cell density of 10^6 cells ml^{-1} in 24 well plate and Infected with late-log phase bacteria with M.O.I of 10. Number of bacteria invade the cell was determined by C.F.U enumeration at 15 min, 30 min and 60 min post infection. In RAW264.7 the total number of bacteria invaded into the host cell is the sum of active invasion through SPI-1 T3SS and phagocytic activity of macrophage (Figure 8)

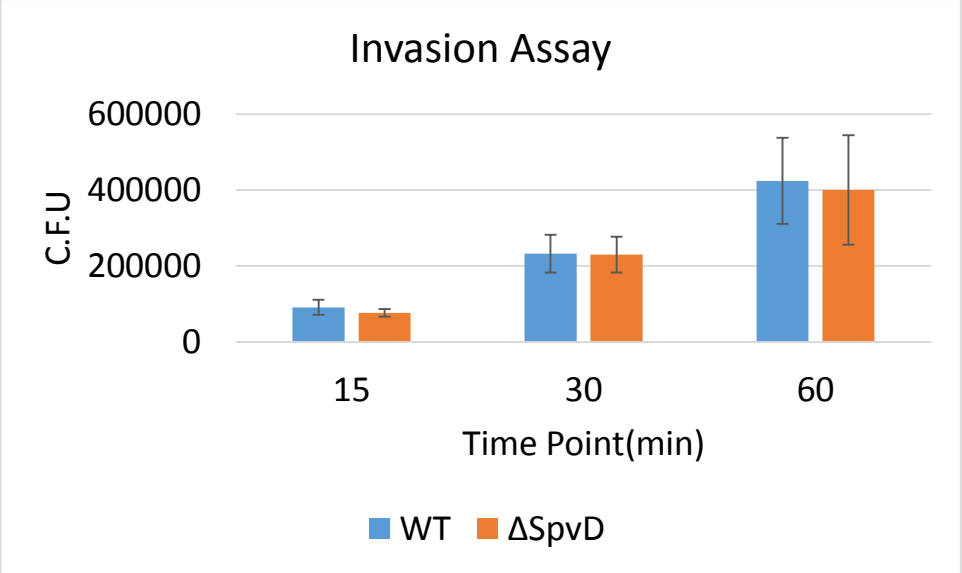


Figure 8. Gentamicin protection assay to compare the invasiveness of the WT and ΔSpvD in RAW 264.7 cells.

Inference: We observed that there is not much difference in the C.F.U count at a given time point in both WT and ΔSpvD in epithelial as well as macrophage cell lines. Therefore, from this observation we can conclude that there is no difference in the invasiveness of the wild-type and mutant bacteria.

3. Replication of WT and ΔSpvD *Salmonella* Typhimurium LT2 inside host cells

Further, we wanted to check the intracellular replication in WT and ΔSpvD bacteria. As macrophages are also equipped with phagocytosis we used SPI-2 condition in case of infection and for HeLa cells we used SPI-1 condition. Followed by infection we evaluated intra-cellular replication of both types of bacteria.

i. RAW 264.7 cells were plated in 500 µl with a cell density of 10^6 cells/ml/well in a 24 well plate and infected at an M.O.I of 150 with the WT or ΔSpvD, grown overnight in LPM (SPI-2 inducing condition) . Fold change in intracellular bacterial load was calculated by enumeration of C.F.U at 2 h and 16 h post infection (Figure 9).

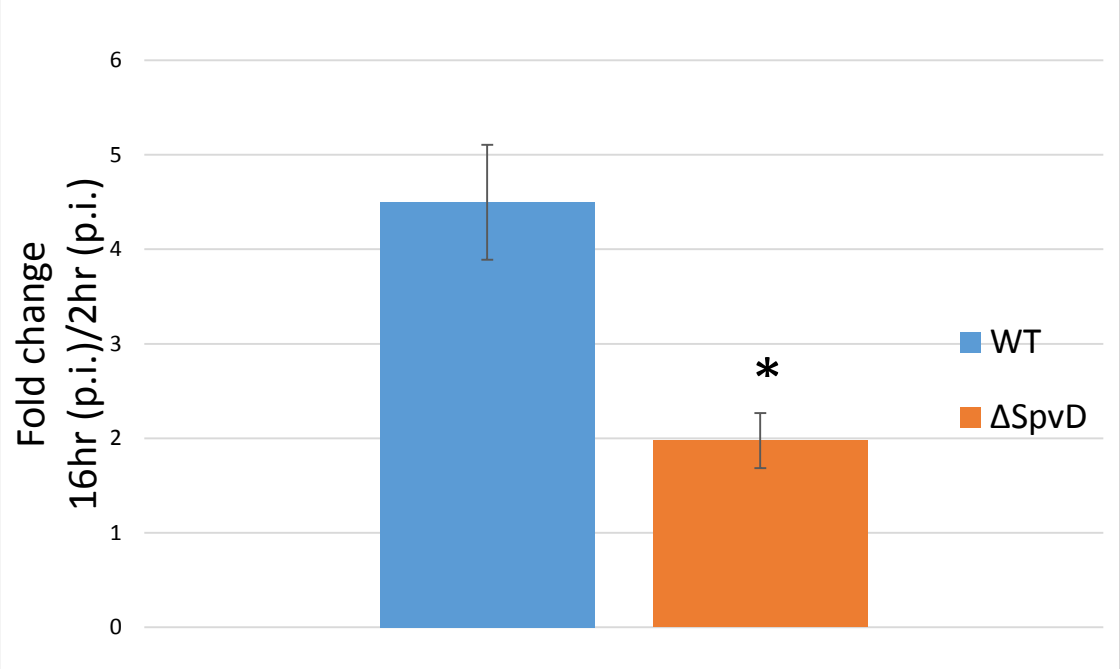


Figure 9. Intra-macrophage replication assay with WT and ΔSpvD in RAW 264.7 cells.

Inference: We observed that in case of ΔSpvD infection fold change in bacterial load (between 2 h to 16 h (p.i.)) was significantly low as compared to WT inside macrophage (p-value of 0.02). Therefore, from this observation we can conclude that ΔSpvD shows intra-macrophage replication-defect.

ii. HeLa cells were plated in 300 µl with cell density of $0.6x10^6$ cells/ml in a 24 well plate and infected with overnight culture of WT and ΔSpvD *Salmonella* in LB media containing 0.3 M NaCl in static condition with M.O.I of 150. Fold change in intracellular bacterial load was calculated by enumeration of C.F.U at 2 h and 16 h post infection. Here we have used SPI-1 condition to actively invade HeLa cells (epithelial cells) (Figure 10).

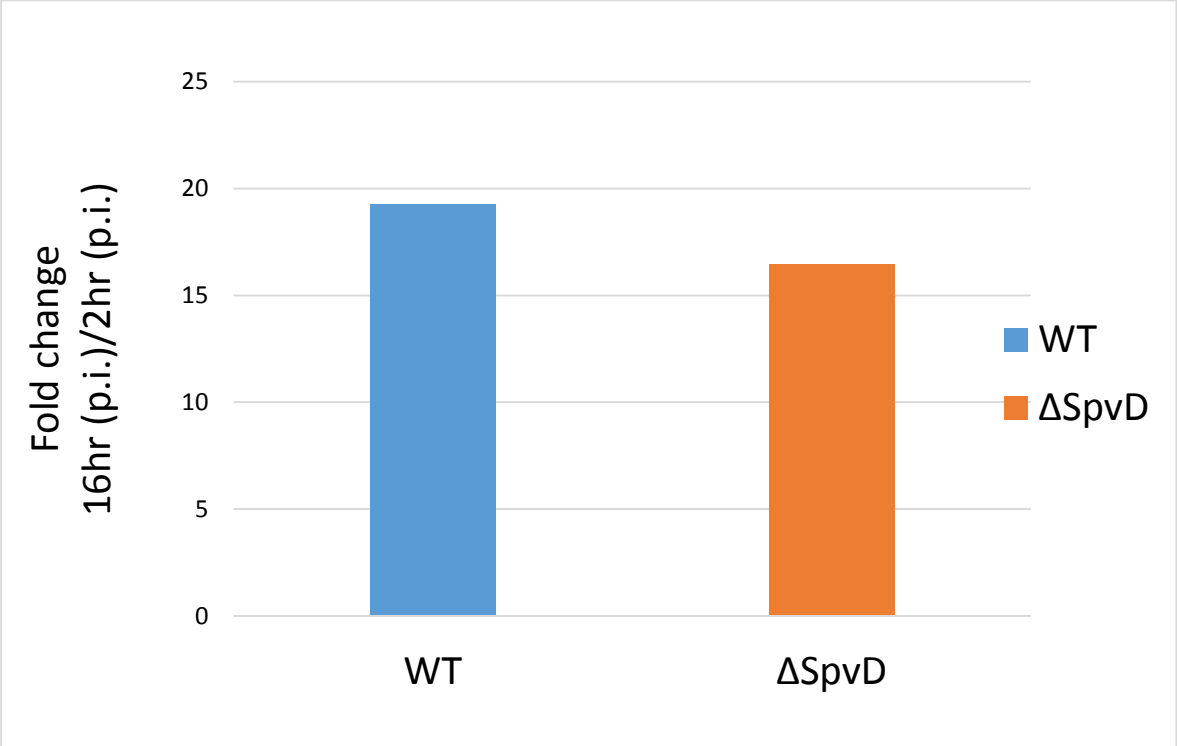


Figure 10. Intra-cellular replication assay with WT and ΔSpvD in epithelial (HeLa) cells.

Inference: We observed that fold change in bacterial load (between 2 h to 16 h p.i.) was almost similar in ΔSpvD and WT. Therefore, from this observation we can conclude that there is no replication defect inside epithelial cells.

4. Assessment of host-cell death following infection with WT and ΔSpvD *Salmonella* Typhimurium LT2

As in the previous section we observed more than four folds increase in wild-type *Salmonella* in macrophage which is higher than the reported fold change (normally 2 fold is observed) in macrophage (Lathrop, Binder et al. 2015). The reason for this might be that we have used SPI-2 condition for infection and normally people use SPI-1 T3SS induced *Salmonella* for infection which is responsible for early cell death and inflammation in host cell during infection. So, in our case there is no initial cell death and inflammation induced in macrophage cells which gave the *Salmonella* an opportunity to grow. Yet we wanted to check whether, in SPI-2 condition there is any effect on the cell health in both WT and mutant infection. Therefore, we used LDH (lactate dehydrogenase) release assay for estimating the cell cytotoxicity due to infection.

To check the cell death induced in response to WT and ΔSpvD in SPI-2 condition LDH release assay was performed with the cell culture supernatant from the same infected cells which were used for the replication assay (Figure 11 & 12).

In RAW 264.7 cells

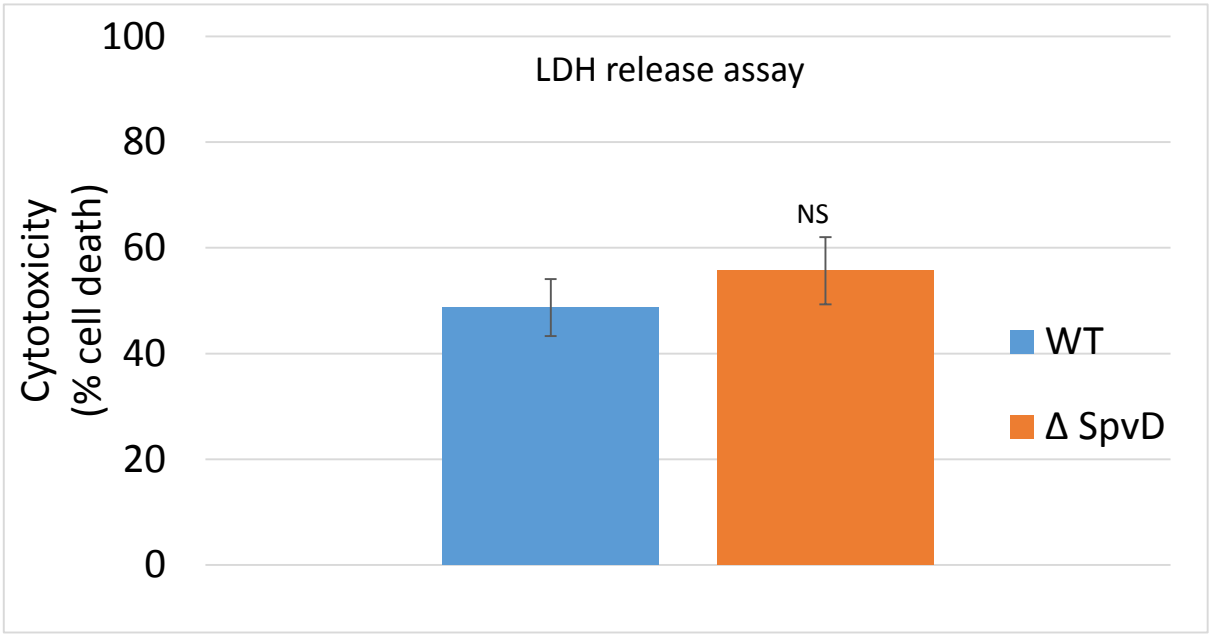


Figure 11. LDH release assay with WT and ΔSpvD infected RAW 264.7 cells. 16 h (p.i.).

In HeLa cells

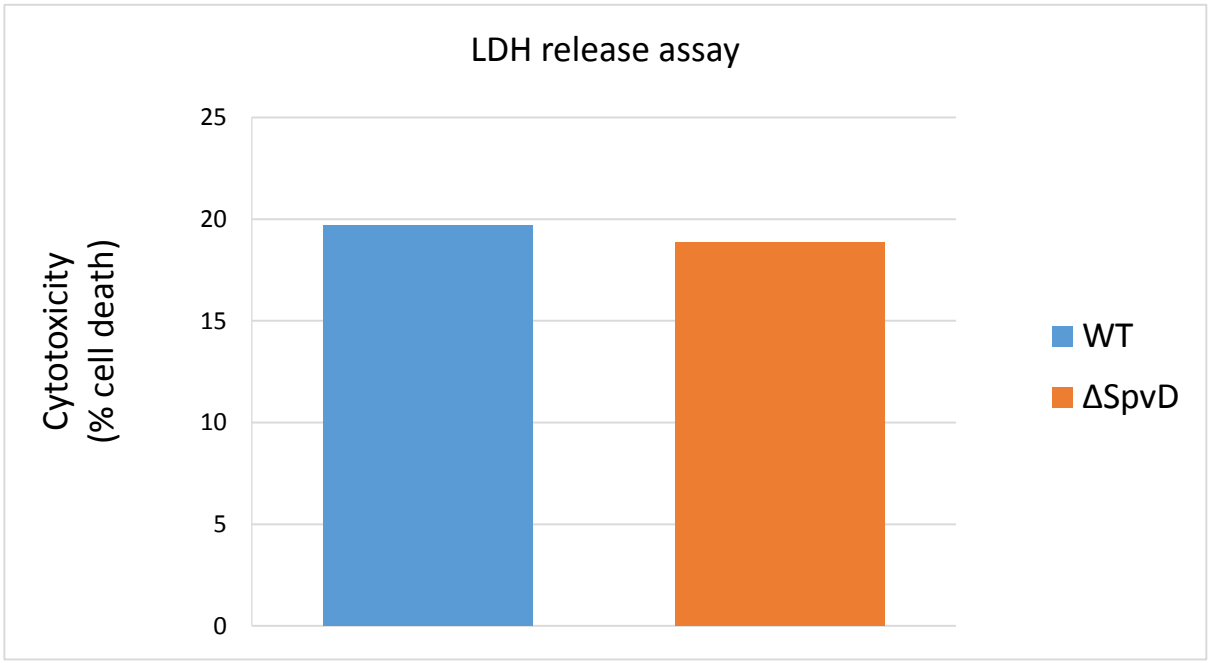


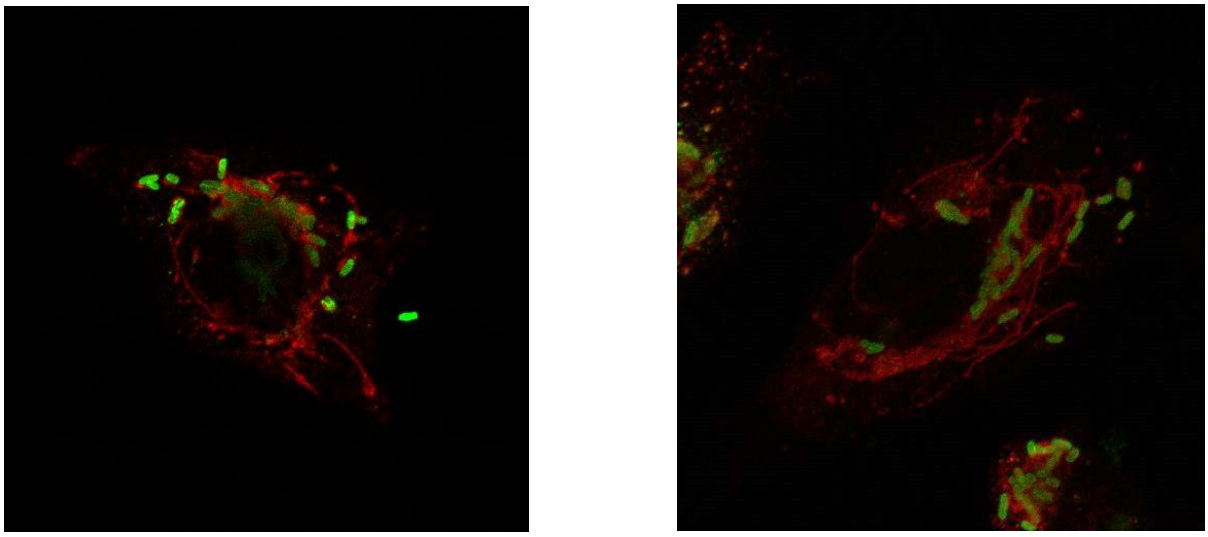
Figure 12. LDH release assay with WT and ΔSpvD infected HeLa cells. 16 h (p.i.).

Inference: We observed that there is no difference in the cell death induced by the WT and ΔSpvD *Salmonella*, both in macrophage as well as in epithelial cell lines which confirms that the results found in intracellular replication assay is not due to the difference in the cell death induced by WT and ΔSpvD.

5. *Salmonella*-induced filament (SIF) formation inside host cell

Salmonella-induced filament are formed at later stage of SCV maturation (4-6 h post infection). Its maximum expression occurs around 8-10 h post infection and declining thereafter. Almost all the mutants with mutation in gene involved in SIF shows replication defects in epithelial cells, but some mutant like ΔSteA do not have replication defects but they shows defects in SIF formation in epithelial cells. To check whether SpvD involved in SIF formation we have checked the SIF formation at 8 h post infection.

i. HeLa cells were plated in 500 µl with the cell density of 50,000 cells/ml in 24 well plate having cover slip at the bottom. Infected with late-log phase bacteria with M.O.I of 150 (Figure 13).



WT (8hr p.i.) **ΔSpvD (8hr p.i.)**

Figure 13. *Salmonella*-induced filament formation inside WT and ΔSpvD infected HeLa cells. *Salmonella* (Green), LAMP-1 (Red) late endosome marker.

Inference: We observed that there is no difference in the morphology of the SIF. Therefore, we concluded that there is no structural difference in SIFs.

6. Nitric oxide (NO) production in RAW 264.7 cells by wild-type and mutant *Salmonella.*

Nitric oxide is produced by macrophage in response to phagocytosis to kill the internalized bacteria. From the above results, we got a hint that our mutant might be a macrophage sensitive mutant. Those mutant are unable to survive the oxidative burst of the macrophage but they induce similar nitric oxide production as compared to wild type. To probe this phenotype, we did nitric oxide estimation with wild-type and mutant *Salmonella*. We have used SPI-2 conditions i.e. Salmonella grown in LPM, because we are getting the replication defects in RAW 264.7 macrophage using SPI-2 conditions (Figure 14).

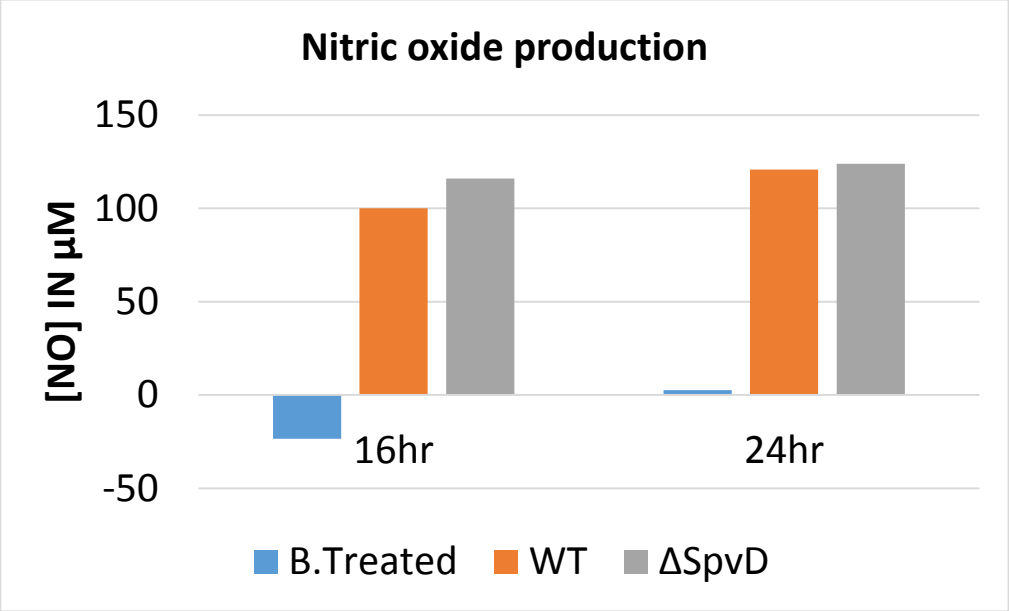


Figure 14. Nitric oxide production by WT and ΔSpvD infected RAW 264.7 cells 16 h post infection.

Inference: We observed that there is no difference in nitric oxide production by WT and ΔSpvD infected cells.

Discussion

In previous reports people have shown that SpvD is encoded by both SPI-1 and SPI-2 and ΔSpvD shows replication defects in mouse bone-marrow-derived macrophage cells. Here, in this report, our main findings are: (i) ΔSpvD shows replication defects in RWA 264.7 macrophage cells but not in HeLa cells which is the epithelial cell line, (ii) No defects were found in invasion and SIF formation. The only difference between macrophage and the epithelial cell is that macrophage have phagocytic and microbicidal activity. There are mutants which show replication defects only in macrophage and these mutants are known as macrophage sensitive mutants. These mutants are unable to survive the oxidative burst generate due to phagocytosis in the macrophage (Gallois, Klein et al. 2001). After entering into the macrophage bacteria resides in phagosome which has a nutrient limiting environment. Vesicles containing NADPH-oxidase and iNOS constantly fuses with the phagosome (Chakravortty and Hensel 2003) and assembles NADPH-oxidase and iNOS complexes on the phagosome membrane, these complexes pumps electrons into the compartment to reduce oxygen to produce superoxide anion (o_2^-). *Salmonella* uses SPI-2 to survive in the macrophage. SPI-2 is required to prevent the localization of NADPH-oxidase and iNOS to SCV of the infected host cell, an activity leading to the evasion of intracellular *Salmonella* from oxidative damage. Macrophage sensitive mutants have four characteristic features (Vazquez-Torres, Xu et al. 2000), (i) replication defect only in macrophage, (ii) increased localization of NADPH-oxidase and iNOS around SCV, (iii) no defects in nitric oxide production, (iv) rescue of the replication defect using inhibitors for NADPH-oxidase and iNOS. Here in this report, we have checked the replication defects and nitric oxide production. From this two observation we hypothesize that ΔSpvD is a macrophage sensitive mutant. To prove our hypothesis we have to check the localization of NADPH-oxidase and iNOS and rescue the replication phenotype with the help of specific inhibitors. we hypothesize that ΔSpvD mutant is a macrophage sensitive mutant and SpvD helps in evading the oxidative burst generated in macrophage due to phagocytosis.

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