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Evaluation of the role of SsaV '*Salmonella* pathogenicity island-2 dependent type III secretion system components on the virulence behavior of *Salmonella enterica* serovar Typhimurium

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Salmonella **is a causative agent of wide range of diseases varying from gastroenteritis to systemic typhoid fever. It uses specialized Type III secretion system (T3SS) by its two compartments to invade and intracellularly survive inside the immune cells. T3SS is expressed in two subsequent phases by two distinct *Salmonella* pathogenicity islands (SP) I and II. Understanding and evaluation of components T3SS-SPI1 and T3SS-SPI2 are very important, not only to evaluate the bacterial virulence but also to develop vaccines. In this study, the effect of mutation on SsaV encoding gene (one of the essential T3SS-SPI2 components) was investigated on the virulence behavior of *Salmonella enterica* serovar Typhimurium. We found a significant reduction in invasion capability and intracellular replication as well.**

Key words: *Salmonella*, T3SS, SsaV, virulence.

INTRODUCTION

Salmonellae are gram-negative pathogens; causing a wide range of diseases, varying from a general mild, self-limiting gastroenteritis, to typhoid fever. Several clusters of virulence genes "pathogenicity islands" are important for adhesion, invasion of enterocytes and for the triggering of symptoms. *Salmonella* depends on its ability to survive and replicate inside host cells, and that's why, virulence trait is linked to its ability to cause systemic infections (Hansen-Wester and Hensel, 2001). *Salmonella* are intracellular bacteria which require large number of genes to cope with nutritional limitations, and to avoid clearance by the host immune cells (Hensel, 2004). The *Salmonella* injectisome "T3SS apparatus" is considered as the most important tool to invade and survive inside immune cells. T3SS injectisome spans the inner and outer membranes of the bacterial envelope secreting translocon and effector proteins. As a consequence, translocon proteins allow effector proteins to access the

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Figure 1. Generation of SPI-2 effector expression cassette. A fusion proteins consisting of SPI-2 effector protein SseJ model antigen hSurvivin with a C-terminal epitope tag HA was constructed on low copy number vector pWSK29.

host cells by forming pores in the host cell membrane, and then forming a channel-like complex which connects between the bacterium and the eukaryotic membrane (Cornelis, 2006).

Salmonella enterica encodes two distinct virulence-associated T3SS with roles in different phases of pathogenesis. Important virulence characters of *S. enterica* are encoded by genes clustered within *Salmonella* Pathogenicity Islands (SPI). These virulence characters include the interaction with enterocytes resulting in diarrhea, the invasion of non-phagocytic cells, the ability to survive phagocytosis and to proliferate within eukaryotic host cells. Two major SPI; SPI1 and SPI2, encode T3SS that translocates bacterial effectors (Cornelis, 2006). The function of SPI2 is essential for the later hallmark of *Salmonella* pathogenesis; the ability to cause systemic infections and to proliferate within host organs (Hegazy and Hensel, 2012).

Salmonella translocates T3SS effector proteins into the host cell cytoplasm, mediated by either SPI1-T3SS at an early stage after entry or SPI2-T3SS from the *Salmonella* containing vacuole (SCV) at later stages during intracellular life (1,2; 5). Efficient immune response mainly requires T-cells induction which depends on the strength of antigen presentation. The antigen presentation strength by its role depends on the antigen-processing efficacy and antigen abundance (Kuhle and Hensel, 2004). *Salmonella* T3SS-mediated translocation can be used to deliver efficiently the heterologous antigens to the cytosol of antigen presenting cells (APC), leading to noticeable CD8 T cell production in orally immunized mice (Yewdell and Bennink, 1999).

Live attenuated strains of *Salmonella* that synthesize and secrete foreign antigens had been used successfully to develop vaccines against infectious bacterial, viral and parasitic diseases and cancer. Recent advances in the understanding the genetics of *Salmonella* virulence led to development of single or multiple mutations in defined virulence genes (Xu et al., 2014). SsaV is an essential component of SPI2-T3SS machinery; it is a component of SPI2-T3SS, required for secretion of most T3SS effectors (Deiwick et al., 2006; Browne et al., 2008). Mutation in *ssaV* gene may lead to significant attenuation, as it may affect the translocation of SPI2-effector protein affecting the intracellular survival of *Salmonella*. In this study we are discussing the effect of mutation in *ssaV* gene on the virulence behavior of *Salmonella*. We investigated the mutation effect on bacterial adhesion, invasion and intracellular replication. Moreover, we tested the efficacy of expression and translocation of SPI2-effector inside the containing vacuole of epithelial cells or macrophage.

MATERIALS AND METHODS

Bacterial strains

Wild-type strain *Salmonella enterica* serovar Typhimurium NCTC 12023 (*S. Typhimurium*) and the mutant Δ*ssaV S. Typhimurium* wild strain (P2D6) were provided kindly from Hensel's lab (Germany). Bacterial strains were routinely cultured in LB broth and on LB agar plates. For selection of recombinant strains, carbenicillin (50 µg/ml) and/or kanamycin (50 µg/ml) were added.

For *in-vitro* analyses of promoters under control of the SsrAB regulatory system, synthetic minimal media with limiting (PCN-P media) or non-limiting (PCN media) amounts of phosphate were used (Deiwick et al., 1999).

Construction of plasmid

In order to test the efficiency of SPI2-T3SS-dependent translocation, plasmid pwsk29 P_{sseJ} *sseJ*::hSurvivin::HA was constructed (Figure 1) (Hegazy et al., 2012). . In brief, the hSurvivin gene was amplified by PCR using hSurv-For-EcoRV (TACGATATCGGTGCCCCGACGTTGCCCCC), hSurvivin-HA-Rev-XbaI(ATTTCTAGATTAAGCGTAGTCTGGGACGTCGTATGGGTAA TCCATAGCAGCCAGCTGCTC) and plasmid pwsk29 P_{sseA} *sscB sseF*::hSurvivin::HA as a template. And then, the amplified hSurvivin was digested with EcoRV and XbaI and ligated to EcoRV and XbaI, digested in low copy plasmid pWSK29. The gene sseJ was PCR amplified using SseJ-Pro-For-KpnI (TACGGTACCTCACATAAAACACTAGCAC) and SseJ-Rev-EcoRV (ACGGATATCTTCAGTGGAATAATGATGAGC) and then digested with KpnI and EcoRV.

SseJ was digested and ligated to digested plasmid pWSK29::hsurvivin with KpnI and EcoRV, resulting in plasmids Pwsk29 P_{sseJ} *sseJ*::hSurvivin::HA. The obtained plasmid was confirmed by colony PCR, diagnostic digestion and sequenced using T7-Seq (TAATACGACTCACTATAGGG) and T3-Seq (AATTAACCCTCACTAAAGG) primers.

Western blotting

Plasmid pwsk29 P_{sseJ} *sseJ*::hSurvivin was transferred to wild type and tested mutant and expression rates were analyzed (Hegazy et al., 2012). Briefly, *S. enterica* serovar Typhimurium wild-type or Δ*ssaV* mutant strains harboring plasmid of fusion proteins, consisting of SPI2 effector protein SseJ, model antigen h-survivin and C-terminal epitope tag HA under control of P*sseJ* promoter were grown in SPI2-inducing minimal media (PCN-P, pH 5.8).

Bacteria were gathered after 6 h of culture under suitable inducing conditions. Equal amounts of bacteria were adjusted at OD600 lysed and subjected to SDS-PAGE and Western blot analyses for the detection of HA epitope tag, which was performed.

Blots were explored with fluorescently labeled secondary antibodies and signal intensities, which were quantified using the Odyssey system (Li-Cor). The cytosolic heat shock protein DnaK was used as loading control, which was detected on the same blot and signals were quantified. The ratios of HA to DnaK signals were calculated in means and standard deviations, the experiment was performed in triplicates.

Adhesion assay

Bacterial strains were cultured in micro-titer plate, incubated at 37°C for 1 h, washed with PBS three times and fixed at 60°C for 20 min. Adhered cells were stained with equal volume of crystal violet (0.1%) for 15 min, washed 5 times with PBS and ethanol was added. Optical densities were measured at 590 nm. The assay was made in triplicate and the means and standard deviations were calculated (Vesterlund et al., 2005).

Evaluation of SPI2-T3SS translocation

In order to quantify the translocation, *Salmonella* wild type or Δ*ssaV* harboring constructed plasmid, for the expression of HA tagged SPI2 effector fusion protein were used to infect HeLa cells or raw macrophage cells at MOI of 100 as previously described (Hegazy et al., 2012).

Infected HeLa cells or macrophages harboring similar numbers of intracellular *Salmonella* were nominated and the signal intensities for the fluorescence channel for HA tagged proteins staining were measured. The Leica laser-scanning confocal microscope was used and the intensities of fluorescence of tagged protein were measured by J-image program. The mean signal intensities and standard deviations for at least 25 infected cells per strains were calculated.

Invasion assay and intracellular replication

Gentamicin protection assay was used to determine the surveillance of *S. Typhimurium* strains in epithelial cells and immune cells (Holzer and Hensel, 2012). Before infection, 24 well plates were seeded either with HeLa cells at a density of 5 x 10^5 cells/ well or RAW264.7 at density of 2 x 10^5 cells/ well. *S. Typhimurium* strains WT and Δ*ssaV* were sub-cultured from an overnight culture and incubated at 37°C for 3.5 h. A master-mix of the inoculum of approximately 1 x 10^5 bacteria per well at multiplicity of infection (MOI 1) was prepared in DMEM and 300 µl were added to each well. Non-internalized *Salmonella* were removed by washing with PBS after 30 min. The remaining extracellular bacteria were killed by 1 h incubation with gentamicin (100 µg/ml).

Hela cells were lysed for invasion assays, with 500 µl per well 0.1% Triton X-100 10 min at room temperature. Serial dilutions of the inoculum and the lysates were plated onto Mueller Hinton (MH) plates. The percentage of invaded bacteria (1 h versus inoculum x 100) was calculated. For intracellular replication assays, the infected cells were washed twice with PBS and lysed with 500µl of 0.1% Triton-X-100 in 10 min at room temperature of 2 h and 16 h post infection. The serial dilutions of the inoculum and the lysates were plated onto MH plates. The percentage of phagocytosed cells/relative untaken cells (2 h versus inoculum x 100) and x-fold intracellular replication (16 h versus 2 h) were calculated.

Statistics

Means and standard deviations shown are deduced from one representative experiment, out of a series of three independent experiments performed in triplicates. Means and standard deviations of triplicate assays are calculated. Statistical significances were determined by Student's t-test using the Graphpad Prism 5 software which is indicated as: n.s. not significant,*P< 0.05, ** P< 0.01 and ***P< 0.001.

RESULTS

Generation and evaluation of SPI-2 effector expression cassettes

To test for efficacy of the live attenuated strain Δ*ssaV* in other to deliver SPI2–effector protein, expression cassettes contain the promoter of *sseJ*. Genes which encode hybrid protein and consist of SPI2-T3SS translocated effector proteins SseJ, tagged with HA was generated (Figure 1). *S. enterica* serovar Typhimurium wild-type or Δ*ssaV* mutant strains harboring the expression cassettes were tested for the expression levels of recombinant SPI2-effector. *In vitro* culture conditions were used to induce the expression of SsrAB regulon and the synthesis of SPI2 effector proteins. The synthesis of SPI2-effector fusion protein tagged with HA was observed in Δ*ssaV* mutant and wild type strains (Figure 2).

To quantify the amounts of recombinant protein produced, Western blots were analyzed using the Odyssey detection system. Signal for model antigens were normalized by the levels of constitutively synthesized control protein DnaK and the level of expression was significantly affected in Δ*ssaV* mutant strain (p = 0.0002).

Adhesion assay

To evaluate the mutation in *ssaV* gene on adhesion, the adhesion of *Salmonella* wild type and Δ*ssaV* mutant strain to abiotic surface was examined. Tested strains were cultured within micro-titer plate, incubated at 37°C for 1 h. The adherent cells were stained with crystal violet, ethanol was added and optical densities were measured at 590 nm (Figure 3). There was no significant reduction in adhesion of Δ*ssaV* mutant than wild type (p = 0.5).

Invasion assay

HeLa cells were infected by *S. enterica* serovar Typhimurium wild-type or Δ*ssaV* mutant strains for invasion assay. The percentage of invaded bacteria (1 h post infected HeLa cells versus inoculum x 100) was calculated (Figure 4). The invasion assay showed a significant reduction of Δ*ssaV* mutant capability, used to invade HeLa cells (p = 0.0073) when compared to *S. enterica* serovar Typhimurium wild-type.

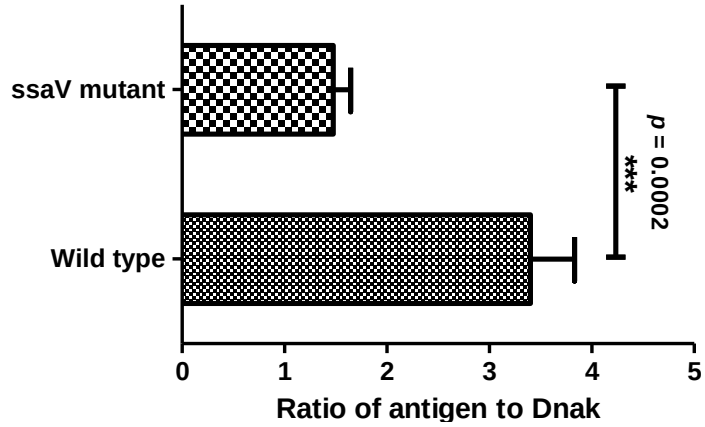

Figure 2. Synthesis of SPI2-effector. *S. enterica* serovar Typhimurium wild-type or Δ*ssaV* mutant strains harboring plasmids expressing SPI2 effector protein tagged with HA under control *in-vivo* inducible promoter were grown in SPI2-inducing minimal media (PCN-P, pH 5.8). The ratios of the HA to DnaK signals were calculated.

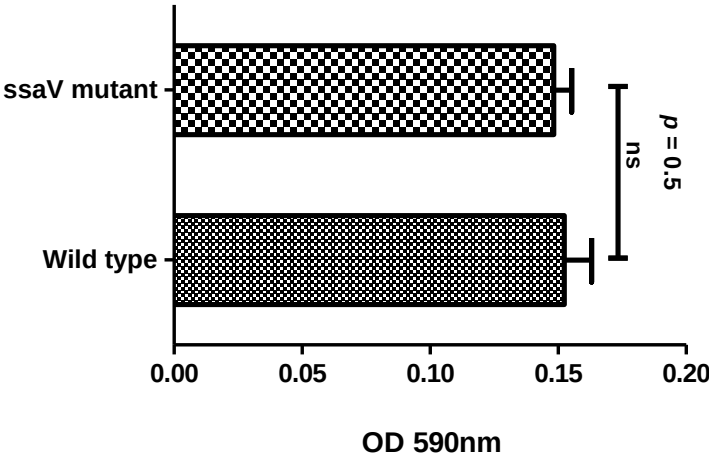

Figure 3. Adhesion assay. *S. enterica* serovar Typhimurium wild-type or Δ*ssaV* mutant strains were cultured in micro-titer plate, incubated at 37◦C for 1 hour, the adhered cells were stained with crystal violet and their optical densities were measured at 590 nm. There was no significance reduction in adhesion between Δ*ssaV* mutant and wild type ($p = 0.5$).

Intracellular replication

Macrophages were infected by *S. enterica* serovar Typhimurium wild-type or Δ*ssaV* mutant strains for intracellular replication assay. The percentage of phagocytosis/relative uptake (2 h post infected macrophage versus inoculum x 100) and x-fold intracellular replication (16 h post infected macrophage cells versus 2 h) were calculated (Figure. 5). The defective secretion system apparatus Δ*ssaV* mutant showed significant reduction ($p < 0.0001$) in replication inside macrophages.

Evaluation of translocation efficiency

The translocation of the HA tagged model antigen by intracellular *Salmonella* in HeLa cells and macrophages was analyzed. The translocation of SseJ-hsurvivin-HA fusion protein was investigated by wild type or Δ*ssaV* mutant strain harboring expression cassettes with P*sseJ* promoter. For quantification, infected HeLa cells or macrophages harboring similar numbers of intracellular *Salmonella* were selected and the signal intensities for the fluorescence channel for HA tagged fusion protein staining were quantified (Figure 6). The comparison of

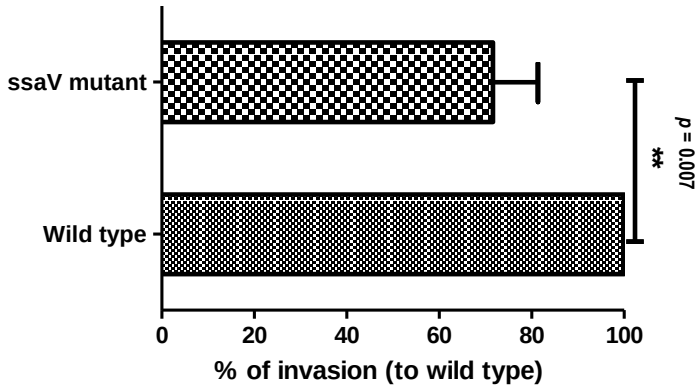

Figure 4. Invasion assay. HeLa cells were infected with *S. enterica* serovar Typhimurium wild-type or Δ*ssaV* mutant strains at an MOI of 1for HeLa cells. After incubation for 25 min at 37°C non-internalized bacteria were removed by washing and the remaining extracellular bacteria were killed by addition of medium containing 100 µg ml-1 gentamicin for 1 h. The internalized cells were counted after cell lysis. Invaded bacteria are expressed as percentage of the inoculum..

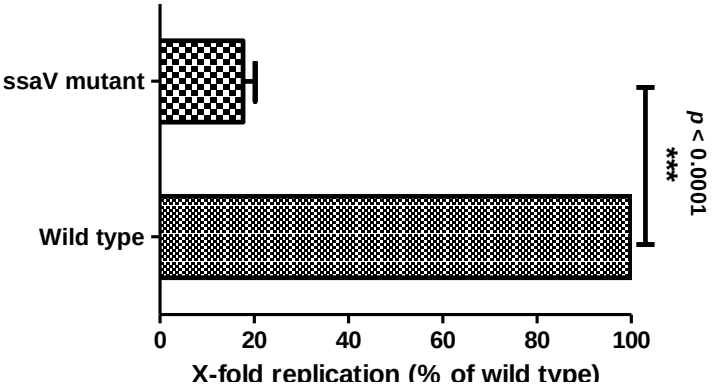

Figure 5. Intracellular replication. RAW264.7 macrophages were infected at an MOI of 1 with *S. enterica* serovar Typhimurium wild-type or Δ*ssaV* mutant strains. Infected cells were lysed 2 h and 16 h after infection and the intracellular bacteria were counted. The x-fold intracellular replication is the ratio of cell forming unit (cfu) recovered at 16 h versus 2 h after infection.

expression cassette indicates a significant variation (p= 0.0002) in translocation by wild type and the Δ*ssaV* mutant in both HeLa cells and raw macrophage.

DISCUSSION

Several important virulence factors of *S. enterica* are encoded by genes within *Salmonella* Pathogenicity Islands (SPI) and two important loci are termed SPI1 and SPI2 (Haraga et al., 2008). SPI1 and SPI2 genes encode distinct T3SS, which will translocate bacterial effectors during different phases of pathogenesis (Hegazy and Hensel, 2012). The invasion capability and intracellular survival are dependent on SPI1-T3SS and SPI2-T3SS machinery, respectively. As a consequence, the defect in this machinery may affect the virulence behavior (Gerlach and Hensel, 2007). Ssav is a component of SPI2-T3SS, and is required for secretion of most effectors, which enable *Salmonella* to cope with the nutritional limitations inside containing vacuoles (Browne et al., 2008).

In this study, we investigated the intracellular behavior of *S. Typhimurium* mutant *ssaV* strain *in vitro*, the invasion of HeLa cells, intracellular replication in RAW macrophages, and most importantly the expression and translocation of SPI2-effector SseJ antigen fusions. For this purpose, fusion protein SPI2-effector SseJ tagged with HA encoding gene was constructed in low copy plasmid pWSK29. As we hypothesized, the expression and translocation levels of SseJ protein fusions were

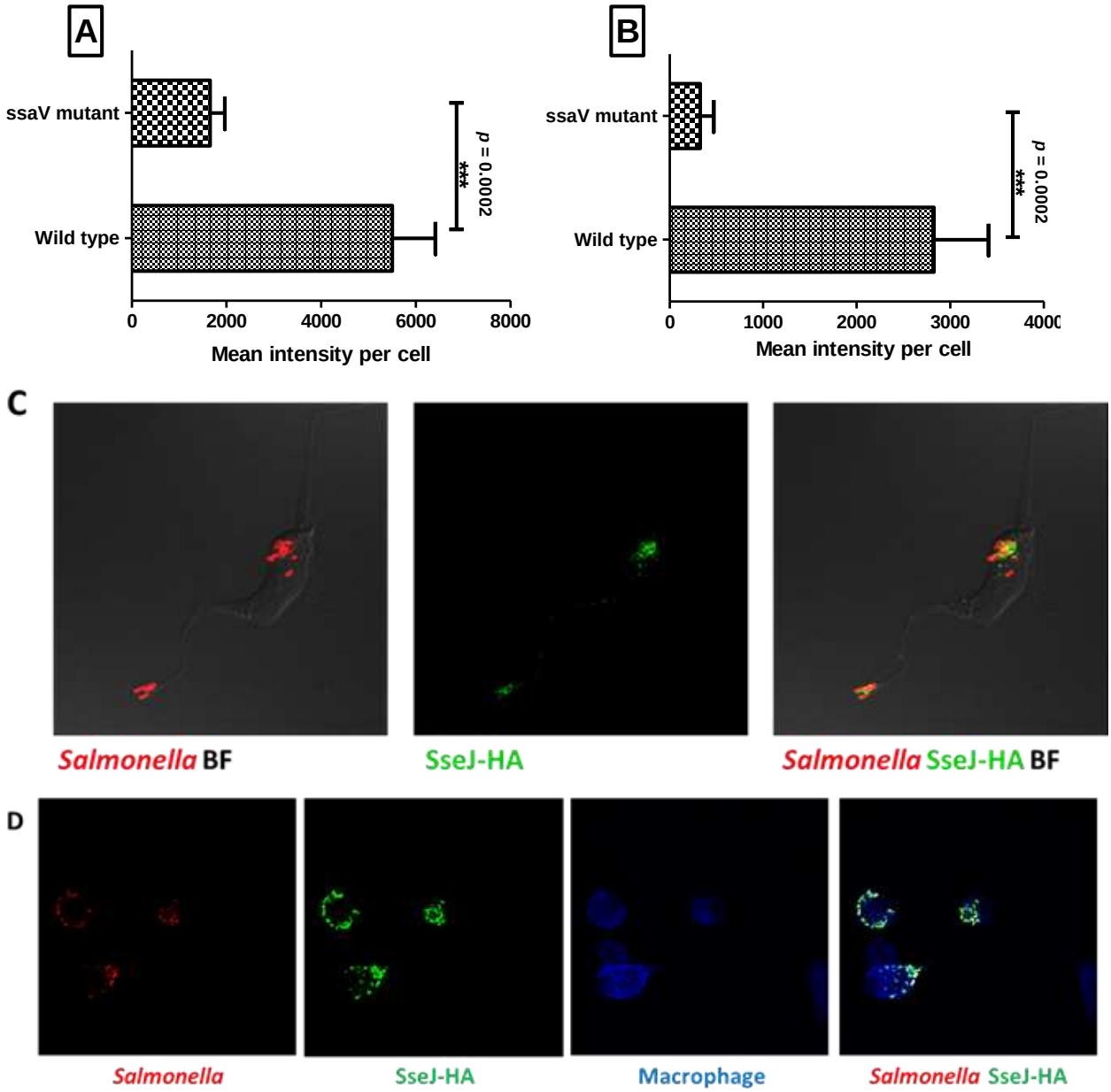

Figure 6. Evaluation of fusion proteins translocation. *S. enterica* serovar Typhimurium wild-type or Δ*ssaV* mutant strains harboring plasmid with cassette for the expression of SPI2 fusion effector protein tagged with HA under control of P*sseJ* promoter were used to infect (A) HeLa cells or (B) RAW macrophages at MOI of 100. The cells were fixed 16 h after infection and processed for immune-staining. Infected cells with similar amounts of intracellular *Salmonella* were selected for the various conditions and the signal intensities of the Cy3 channel for the anti-HA strains were measured with identical exposure times. The mean signal intensities and standard deviations for at least 25 infected cells per strains were shown. (C) HeLa cells (D) Raw macrophages were fixed 16 h after infection and processed for immune-staining of intracellular *Salmonella* (red), translocated fusion protein effector-hsurvivin-HA (green) and macrophage (blue DAPI stained).

significantly reduced in Δ*ssaV* mutant strain. The adhesion of Δ*ssaV* mutant was not significantly reduced in comparison to the wild type, while the invasion capability was significantly diminished. SPI2-T3SS defect by mutation in one of its essential components "SsaV protein" was enough to limit significantly, the expression. Expressions of SPI2-effectors result in significant reduction of intracellular replication of *Salmonella* inside raw macrophage.

Our finding proved that *ssaV* gene mutation affect intracellular replication significantly, in raw macrophage (10 fold) lower than wild type. Inspite *ssaV* gene, mutation decreased the invasion capability which is significantly (3 fold) lower than wild type. These results

are matching with the fact that, SPI2-T3SS is responsible for intracellular life, but its role is not crucial in early stages of invasion in which SPI1-T3SS is the main player. Our *In vitro* findings were compatible with the *in-vivo* observations of other groups (Li et al., 2009a; 2009b; Fierer et al., 2012; Braukmann et al., 2015). Moreover, we tested the antibiotic sensitivity of Δ*ssaV* mutant and wild type strains; we did not observe any change in the sensitivity pattern; the mutation in essential component of SPI2-T3SS did not affect the resistance of *Salmonella* in different antibiotics from different classes (Supplementary Table 1).

The effect of attenuation on *ssaV* gene on virulence and survival of *Salmonella* was also evaluated in this study. The selection of suitable mutant is crucial for designing live recombinant *Salmonella* vaccines. A sufficient attenuation in virulence is required to prevent undesired side effects like bacteremia, diarrhea or fever. However, the balance between attenuation and over-attenuation must be considered as over-attenuation can lead to poor immunogenicity of the vaccine.

Moreover, attenuated *Salmonella* can be used as a carrier to deliver heterologous antigens activating both cellular and humeral responses (Hegazy and Hensel, 2012; Xu et al., 2014). Previous studies showed that T3SS-type 2 deficient *S. Typhimurium* were highly attenuated and conferred protection from further challenges with wild-type, by eliciting serum IgG and secretory IgA against O-antigen in C57BL/6mice (Endt et al., 2010). But *ssaV* mutant was found to be virulent in immune compromised C57BL/6 mice (Periaswamy et al., 2012).

The double mutant of *ssaV* gene and other essential SPI1-T3SS or auxotrophic genes were attenuated enough to be used as vaccines for typhoid (Kirkpatrick et al., 2006; Pati et al., 2013a; 2013b), as a carrier to deliver heterologous antigens (Hegazy and Hensel, 2014; Michael et al., 2010) or to induce chemokines (Li et al., 2009b).

Finally, we can conclude that, mutation in SsaV encoding gene significantly reduced the invasion capability, intracellular replication of *Salmonella. entrica* Typhimurium as a direct result of decreasing the expression and translocation of SPI2-effectors. In the other side, ssaV gene mutation did not affect adhesion and microbial resistance to different antibiotics.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Braukmann M, Methner U, Berndt A (2015). Immune reaction and survivability of *Salmonella typhimurium and Salmonella infantis* after infection of primary avian macrophages. PLoS One 10:e0122540.

Browne SH, Hasegawa P, Okamoto S, Fierer J, Guiney DG (2008). Identification of *Salmonella* SPI-2 secretion system components required for SpvB-mediated cytotoxicity in macrophages and virulence in mice. FEMS Immunol. Med. Microbiol. 52:194-201.

Cornelis GR (2006). The type III secretion injectisome. Nat. Rev. Microbiol. 4:811-825.

Deiwick J, Nikolaus T, Erdogan S, Hensel M (1999). Environmental regulation of *Salmonella* pathogenicity island 2 gene expression. Mol. Microbiol. 31:1759-1773.

Deiwick J, Salcedo SP, Boucrot E, Gilliland SM, Henry T, Petermann N, Waterman SR, Gorvel JP, Holden DW, Meresse S (2006). The translocated *Salmonella* effector proteins SseF and SseG interact and are required to establish an intracellular replication niche. Infect. Immun. 74:6965-6972.

Endt K, Stecher B, Chaffron S, Slack E, Tchitchek N, Benecke A, Van Maele L, Sirard JC, Mueller AJ, Heikenwalder M, Macpherson AJ, Strugnell R, von Mering C, Hardt WD (2010). The microbiota mediates pathogen clearance from the gut lumen after non-typhoidal *Salmonella* diarrhea. PLoS Pathog. 6:e1001097.

Fierer J, Okamoto S, Banerjee A, Guiney DG (2012). Diarrhea and colitis in mice require the *Salmonella* pathogenicity island 2-encoded secretion function but not SifA or Spv effectors. Infect Immun. 80:3360-3370.

Gerlach RG, Hensel M (2007). Protein secretion systems and adhesins: the molecular armory of Gram-negative pathogens. Int. J. Med. Microbiol. 297:401-415.

Hansen-Wester I, Hensel M (2001). *Salmonella* pathogenicity islands encoding type III secretion systems. Microbes Infect. 3:549-559.

Haraga A, Ohlson MB, Miller SI (2008). *Salmonellae* interplay with host cells. Nat. Rev. Microbiol. 6:53-66.

Hegazy WA, Hensel M (2012). *Salmonella enterica* as a vaccine carrier. Future Microbiol. 7:111-127.

Hegazy WA, Xu X, Metelitsa L, Hensel M (2012). Evaluation of *Salmonella enterica* type III secretion system effector proteins as carriers for heterologous vaccine antigens. Infect Immun. 80:1193-1202.

Hensel M (2004). Evolution of pathogenicity islands of *Salmonella enterica*. Int. J. Med. Microbiol. 294:95-102.

Holzer SU, Hensel M (2012). Divergent roles of *Salmonella* pathogenicity island 2 and metabolic traits during interaction of *S. enterica* serovar *typhimurium* with host cells. PLoS One 7:e33220.

Kirkpatrick BD, McKenzie R, O'Neill JP, Larsson CJ, Bourgeois AL, Shimko J, Bentley M, Makin J, Chatfield S, Hindle Z, Fidler C, Robinson BE, Ventrone CH, Bansal N, Carpenter CM, Kutzko D, Hamlet S, LaPointe C, Taylor DN (2006). Evaluation of *Salmonella enterica serovar Typhi* (Ty2 aroC-ssaV-) M01ZH09, with a defined mutation in the *Salmonella* pathogenicity island 2, as a live, oral typhoid vaccine in human volunteers. Vaccine 24:116-123.

Kuhle V, Hensel M (2004). Cellular microbiology of intracellular *Salmonella enterica*: functions of the type III secretion system encoded by *Salmonella* pathogenicity island 2. Cell Mol. Life Sci. 61:2812-2826.

Li S, Zhang MZ, Yan L, Lillehoj H, Pace LW, Zhang S (2009b). Induction of CXC chemokine messenger-RNA expression in chicken oviduct epithelial cells by *Salmonella enterica serovar enteritidis* via the type three secretion system-1. Avian Dis. 53:396-404.

Li S, Zhang Z, Pace L, Lillehoj H, Zhang S (2009a). Functions exerted by the virulence-associated type-three secretion systems during *Salmonella enterica* serovar Enteritidis invasion into and survival within chicken oviduct epithelial cells and macrophages. Avian Pathol. 38:97-106.

Michael A, John J, Meyer B, Pandha H (2010). Activation and genetic modification of human monocyte-derived dendritic cells using attenuated *Salmonella typhimurium*. Sci. World J. 10:393-401.

Pati NB, Vishwakarma V, Jaiswal S, Periaswamy B, Hardt WD, Suar M (2013a). Deletion of *invH* gene in *Salmonella enterica* serovar Typhimurium limits the secretion of Sip effector proteins. Microbes Infect. 15:66-73.

Pati NB, Vishwakarma V, Selvaraj SK, Dash S, Saha B, Singh N, Suar M (2013b). *Salmonella Typhimurium* TTSS-2 deficient mig-14 mutant shows attenuation in immunocompromised mice and offers protection against wild-type *Salmonella Typhimurium* infection. BMC Microbiol. 13:236.

Periaswamy B, Maier L, Vishwakarma V, Slack E, Kremer M, Andrews-Polymenis HL, McClelland M, Grant AJ, Suar M, Hardt WD (2012). Live attenuated *S. Typhimurium* vaccine with improved safety in immuno-compromised mice. PLoS One 7:e45433.

Vesterlund S, Paltta J, Karp M, Ouwehand AC (2005). Measurement of bacterial adhesion-*in vitro* evaluation of different methods. J. Microbiol. Methods 60:225-233.

Xu X, Hegazy WA, Guo L, Gao X, Courtney AN, Kurbanov S, Liu D, Tian G, Manuel ER, Diamond DJ, Hensel M, Metelitsa LS (2014). Effective cancer vaccine platform based on attenuated *Salmonella* and a type III secretion system. Cancer Res. 74:6260-6270.

Yewdell JW, Bennink JR (1999). Immunodominance in major histocompatibility complex class I-restricted T lymphocyte responses. Annu. Rev. Immunol. 17:51-88.

Supplementary Table 1. Antibiotic sensitivity. The sensitivity of *S. enterica* serovar Typhimurium wild-type or Δ*ssaV* mutant strains to different representative antibiotics were tested on Muller Hinton agar plates by the disc diffusion agar method according to Clinical Laboratory and Standards Institute Guidelines (CLSI; 2007). The inhibition zones were measured in mm and defined as Resistant (R), Intermediate (I) or Sensitive (S) according to the CLSI guidlines. The test was made in triplicate.

Antibiotics	**Wild type strain**	***ssaV* strain**
Ampicillin	R	R
Ampicillin / Sulbactam	I	I
Amoxicillin / clavulanic acid	I	I
Piperacillin	S	S
Azetronam	S	S
Imipenem	S	S
Cephardine	R	R
Ceftazidime	R	R
Cefotaxime	R	R
Cefepime	S	S
Ciprofloxacin	S	S
Levofloxacin	S	S
Gatifloxacin	S	S
Tobramycin	I	I
Gentamycin	S	S
Tetracycline	R	R
Chloramphenicol	S	S
Trimethoprim / Sulfamethoxazole	S	S