Characterization of SteA, an effector of *S. typhimurium*

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Certificate of Examination

This is to certify that the dissertation titled "Characterisation of SteA, and effetor of *Salmonella typhimurium*"submitted by **Ms. Rhythm Shukla** (Reg no. : MS13118) for the partial fulfillment 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.

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(Supervisor)

Dated: April 20, 2018

Declaration

The work carried out 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 institute or university. 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.

Rhythm Shukla

(Candidate)

Dated:
April 20, 2018

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)

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Notations

Abbreviation	Full forms
SPI	*Salmonella* pathogenecity islands
SCV	*Salmonella* containing vacuole
SteA	*Salmonella* translocated effector A
T3SS	Type three secretion systems
PI4P	Phosphatidylinositol-4-phosphate
IκB	Inhibitor of kappa B
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells

Abstract

Salmonella typhimurium causes self-limiting gastroenteritis in humans and typhoid like condition in mice. *S. typhimurium* actively invades and infects intestinal epithelial cells. Various stages of infection are regulated by type three secretion systems encoded on different *Salmonella* pathogenicity islands (SPIs) which translocate effector proteins. They are secreted via the specific secretion systems directly into the host cytoplasm. These effectors are essential for virulence of the bacteria and regulate various host cell responses to help in a successful infection and further dissemination to other tissues. *Salmonella* translocated effector A (SteA) is an effector protein of *S. typhimurium* regulated by both SPI-I and SPI-II. In this report we have found that ΔSteA results in a heightened immune response by affecting IκB.

Introduction

Salmonella enterica is a species of gram-negative, rod-shaped, flagellated, pathogenic, facultative anaerobic bacteria. The species is further divided into various serovars on the basis of their antigenic specificity (1). Many of these serotypes infect various animals including humans. They are majorly subdivided into typhoidal and non-typhoidal serovars. Typhoidal serovars mainly include *S. typhi*, and *S. paratyphi* which cause typhoid in humans, whereas non-typhoidal serovars include *S. typhimurium* and *S. enteritidis* which cause gastroenteritis and diarrhea in humans and *S. typhimurium* results in typhoid-like disease in mice (2). *Salmonella* infection begins by penetrating the epithelial cells of the host's gut (3). To cause a successful infection the bacteria utilizes a specialized needle like apparatus known as the Type three secretion systems (T3SS) to release various effector proteins into the host cell. These effector proteins are encoded on various *Salmonella* Pathogenecity Islands (SPIs) and modulate host cellular machinery to help the bacteria in adhesion, invasion, survival, replication and further dissemination in the host.

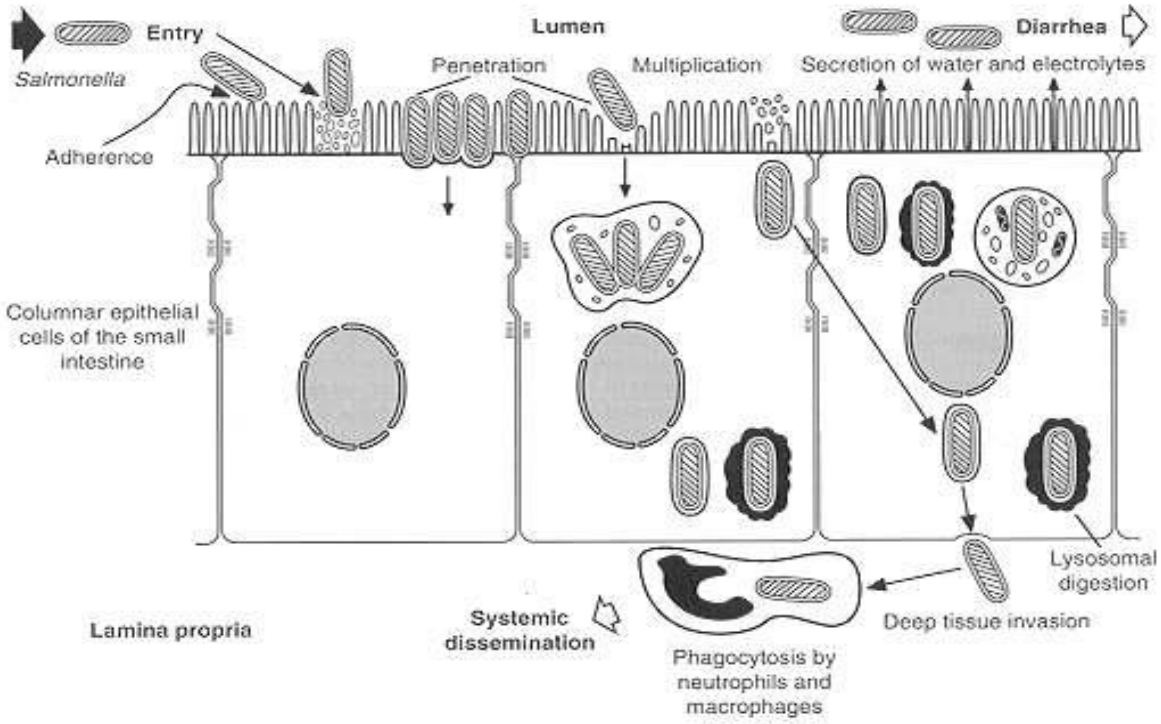

Figure 1: Invasion and spread of infection upon *Salmonella* interaction with the gut epithelial cells. Adapted from *"Salmonella*" Ralph A. Giannella. (http://intranet.tdmu.edu.ua/data/cd/disk2/images/fig21_3)

Type Three Secretion System (T3SS)

The type three secretion system is a specialized injection-like complex through which *Salmonella* releases various effector proteins into the host cell. S. *typhimurium* is known to have two such structures namely, T3SS-I and T3SS-2, which are regulated by SPI-I and SPI-2 respectively and are expressed at different stages of infection (4). The T3SS structure mainly consists of three parts namely, the base, the neck and the needle.

a) The base anchors the entire complex to the outer membrane of the bacteria.
b) The neck consists of a thin rod which connects the base to the needle.
c) The needle penetrates the host cell membrane and helps in the delivery of various effectors. (5)

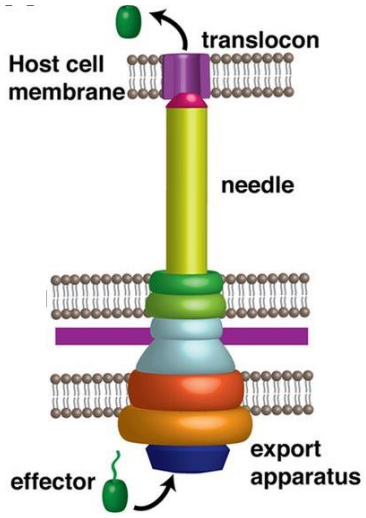

Figure 2: T3SS of *Salmonella.*

Adapted from "The *Salmonella* Type III Secretion System Virulence Effector Forms a New Hexameric Chaperone Assembly for Export of Effector/Chaperone Complexes";
doi : 10.1128/JB.02524-14

Both T3SS serve different purposes during the infection, and in a coordinated manner help in bacterial uptake and replication within the host cells. T3SS-I is expressed during the initial stages of infection and in case of *S. typhimurium* is known to release about 14 different effectors which help in actin modulation of the host cell for bacterial uptake and establishment of infection. It helps in releasing effectors from the bacteria to the cytosol of the host cells. T3SS-II releases about 19 effectors which help in formation of a special niche for the bacteria known as *Salmonella* containing vacuole (SCV), help in bacterial replication and dissemination to further spread the infection to other cells and organs of the host. T3SS-II helps in releasing effectors from the SCV to the cytosol of the host. Some effectors are secreted by both T3SS-I and T3SS-II (4).

Salmonella Pathogenecity Islands (SPIs)

Many of the major components required by *S. typhimurium* for infection are encoded on specific chromosomal regions called *Salmonella* Pathogenecity Islands (SPIs) (6). They are acquired by horizontal gene transfer amongst various serovars (7). Till date about 21 SPIs have been characterized in *S. enterica* of which all are not present in all serovars (8). At present 11 SPIs are characterized in *S. typhimurium* and of these SPI-I and SPI-II are the most widely studied. SPI-I encodes genes which help in bacterial invasion while SPI-II mostly encodes genes which help in bacterial niche formation, survival and replication.

Salmonella Pathogenecity Island – I (SPI-I)

SPI-I is the most well characterized pathogenecity island (8). It codes for about 30 genes which include genes encoding various components of T3SS-I and various effector proteins necessary for invasion by the bacteria. These effectors regulate actin rearrangement, causing membrane ruffling and allowing the bacteria to enter the host cells (9). Other effectors induce various immune responses in the host cells to enable invasion and survival of the bacteria in the host.

SPI-1 Effectors

For invasion in the epithelial cells *S. typhimurium* releases various effectors through the T3SS-I. SopE is one such effector which helps in bacterial entry into the host cells. It has been reported that it interacts with various Rho-GTPases and induce rapid actin rearrangement causing membrane ruffling in the host epithelial cells (9) along with various other effectors like SipA (10). Other SPI-I effectors like SopB, SopA, SipD, AvrA etc help in modulating the host cell responses, inducing various inflammatory responses in the host cells whereas SptP acts antagonistic to these effectors (11). They act on various inflammatory pathways like the NF-κB pathway to regulate host cell responses in order to favour bacterial infection.

SteA Effector of *S. typhimurium*

Many of the effectors of S. *typhimurium* have not been characterized for their functions as of now. SteA (*Salmonella* translocated effector A) is one such effector protein. It is translocated by both T3SS-I and T3SS-II. It was identified and reported for the first time in Kaoru *et al*, 2005(12). It was reported to translocate with trans-golgi network and later in Elena Cardenal-Muñoz *et al*, 2009 it was reported that this gene down-regulates various genes in epithelial host cells thus delaying the cell death through a microarray study (13). Till now it is known that in later stages of infection, SteA regulates the *Salmonella* containing vacuole (SCV) dynamics and interacts with phosphatidylinositol-4-phosphate (PI4P) on the SCV membrane (14)(15).

Since most of SteA's functions were mostly seen in later stages of infection we were curious to see its role as an SPI-I effector and how it down regulates host cell responses to delay cell death, help bacterial invasion and establishment of infection.

Materials and Methods

Bacterial strains

S. typhimurium SL1344 and mutants derived from the same strain were used to perform all the experiments.

Mutants:

1. The replacement of SteA gene with kanamycin cassette was done to construct ΔSteA strain,
2. His-tagged complementary strain in ΔSteA background was contructed,
3. A non-tagged complementary strain in ΔSteA background was constructed.

Bacterial culture used

As SteA is encoded by both SPI-I and SPI-II, we used SPI-I specific conditions to ensure its expression through T3SS-I and check the role of SteA in SPI-I conditions.

SPI-I inducing conditions:

A non-aerated stationary phase bacterium (µaer-ST) was used. S. *typhimurium* strains were grown overnight in Luria Bertani broth containing 0.3M NaCl under static conditions at 37°C.

Antibiotics used:

1. Streptomycin 100 µg/ml is used for wild type S.*typhimurium* cultures.
2. Streptomycin + Kanamycin 100 µg/ml each is used for ΔSteA strain cultures.
3. Streptomycin + Kanamycin + Ampicillin 100 µg/ml each is used for both complementary strain cultures.

Cell culture

HEK 293 cells (NCCS) were cultured in DMEM media supplemented with 10% FBS in a 5% CO_2 incubator at 37°C.

Plasmids used

1. NF-ƘB response element upstream of luciferase gene, containing plasmid
2. Renilla gene containing plasmid
3. pcDNA 3.1(+) and pcDNA 3.1(+) containing HA epitope tagged SteA gene.

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 supplemented with their respective antibiotics. The samples were taken every 30 mins for OD_{600} reading in a Jenway Genova Plus spectrophotometer.

Luciferase reporter assay

HEK 293

1. HEK 293 cells grown in DMEM media with 10% FBS were seeded in a 96-well plate (FALCON) with density of 25,000 cells/well in 100 µl media. These cells were transfected using lipofectamine (PROMEGA) transfecting reagent and plasmids containing Luciferase and Renilla, using OptiMEM media. Transfection mix of 25 µl/well was added and the cells were incubated for 18 h in 5% CO_2 incubator at 37°C. µaer-ST cultures of wild type, ΔSteA and complementary bacterial strains were used to infect HEK 293 cells. *Salmonella* were collected by centrifugation at 6,000xg for 5 mins in a table top centrifuge. The bacteria were then resuspended in PBS and used immediately for infection of HEK 293 with MOI 20. After infection, the plate was immediately centrifuged at 500xg for 2 mins. Then cells were incubated for 30 mins at 37°C for infection. After incubation, extracellular bacteria were removed and cells were washed with PBS buffer. Then they were incubated for another 1 h in DMEM containing 100 µg/ml of gentamicin. After 1 h of gentamicin treatment the cells were kept in 75 µL/well of DMEM containing 1 µg/ml of gentamicin for 5-6 h. To check for invasion differences a well of each treatment was lysed using 0.1% Triton X-100 and viable intracellular bacteria were enumerated using C.F.U. count. Luciferase reagent from the Luciferase kit (PROMEGA) was then added 75 µl/well to the control and test wells and after 10 mins of incubation luminescence reading was taken in POLARSTAR plate reader. After this Renilla reagent from the same kit was added 75 µl/well and incubated for 10 mins. Luminescence reading for Renilla was then taken.
2. HEK 293 cells grown in DMEM media with 10% FBS were seeded in a 96-well FALCON plate with density of 50,000 cells/well in 100 µl media. These cells were transfected using Lipofectamine (PROMEGA) transfecting reagent and plasmids containing Luciferase and Renilla and also with empty pcDNA 3.1(+) vector and pcDNA 3.1(+) containing HA tagged SteA gene at a concentration of 0.2 µg/ ml each, using OptiMEM media. Transfection mix of 25 µl/well was added and the cells were incubated for 18 h in 5% CO_2 incubator at 37°C. The test wells were induced with 1 µg/ ml of TNF-α in 75 µl of media/well. The cells were incubated for 7-8 h in 5% CO_2 incubator at 37°C. Luciferase reagent from the Luciferase PROMEGA kit was then added 75 µl/well to the control and test wells and after 10 mins of incubation at room temperature luminescence reading was taken in POLARSTAR plate reader. After this Renilla reagent from the same kit was added 75 µl/well and incubated for 10 mins at room temperature. Luminescence reading for Renilla was then taken.

Cell lysates

HEK 293 cells

1. HEK 293 cells grown in DMEM media with 10% FBS were seeded in 6-well plate with cell density of $1.5x10^5$/well in 1 ml media. These cells were then infected with µaer-ST cultures of wild type, ΔSteA and complementary bacterial strains. *Salmonella* were collected by centrifugation at 6,000xg for 5 mins in a table top centrifuge. The bacteria were then resuspended in PBS and used immediately for infection of HEK 293 with MOI 50. After infection, the plate was immediately centrifuged at 200xg for 3 mins. Then cells were incubated for 30 mins at 37°C for infection. After incubation, extracellular bacteria were removed and cells were washed with PBS buffer. Then they were incubated for another 1 h in DMEM containing 100 µg/ ml of gentamicin. The cells were then washed with PBS and resuspended in 1 ml of PBS buffer and lysed using whole cell lysis buffer (150mM NaCl; 0.1% Triton X-100; 50mM Tris-HCl pH-8; 0.1% SDS). These lysates were then analysed by western blotting by using primary IκB antibody (1:1000) and secondary rabbit antibody (1:5000).
2. HEK 293 cells were grown in DMEM media with 10% FBS. The entire plates were used for transfection with pcDNA 3.1(+) empty vector and pcDNA 3.1(+) containing HA tagged SteA gene at a concentration of 0.1 µg/ ml. The cells were transfected using PEI (polyethylene imine) in OptiMEM media. After 7 h the media of the plate was changed and the cells were induced with TNF-α after 18 h of transfection for 15 mins and 30 mins time points. The cells were then washed with PBS and resuspended in 1 ml of PBS buffer and lysed using whole cell lysis buffer. These lysates were then analysed by western blotting by using primary IκB and GAPDH antibody (1:1000) and secondary rabbit antibody (1:5000).

Microscopy

HEK 293 cells grown in DMEM media with 10% FBS were plated on 12mm diameter coverslips placed in 24 well plate(FALCON) in 300 µl of DMEM media with 10% FBS at a density of 25,000cells/well. The cells were transfected with pcDNA 3.1(+) empty vector and pcDNA3.1 (+) containing HA tagged SteA gene at 0.5 µg concentration each with PEI transfecting reagent. The media of the plate was changed 7-8 h after transfection. After 18 h of transfection the cells were fixed with 2.5% paraformaldehyde in PBS for 30 mins at room temperature. The cells were then washed with PBS buffer twice. They were treated with primary antibody for 45 mins and then with secondary antibody, in 0.2% Saponin for 30 mins at room temperature and washed with PBS buffer thrice after each antibody treatment. Samples were analyzed using Zeiss 780 Confocal microscope.

Antibodies used:

1. Primary antibody: Anti-rabbit IκB (Santa Cruz), anti-mouse HA (Biolegend).
2. Secondary antibodies: Alexa 488 conjugated goat anti-rabbit-IgG (Life Technologies), Alexa 568 conjugated goat anti-mouse-IgG (Life Technologies).

Growth kinetics of wild type strain of S. *typhimurium* SL1344 and ΔSteA in artificial media

1. Overnight culture of bacterial strains was diluted to OD= 0.01 at 600nm in LB medium. Further the culture was subjected to incubation for 24 h at 37°C with constant monitoring of OD_{600} at every 30 mins for initial 12 h with a final OD at 24 h.

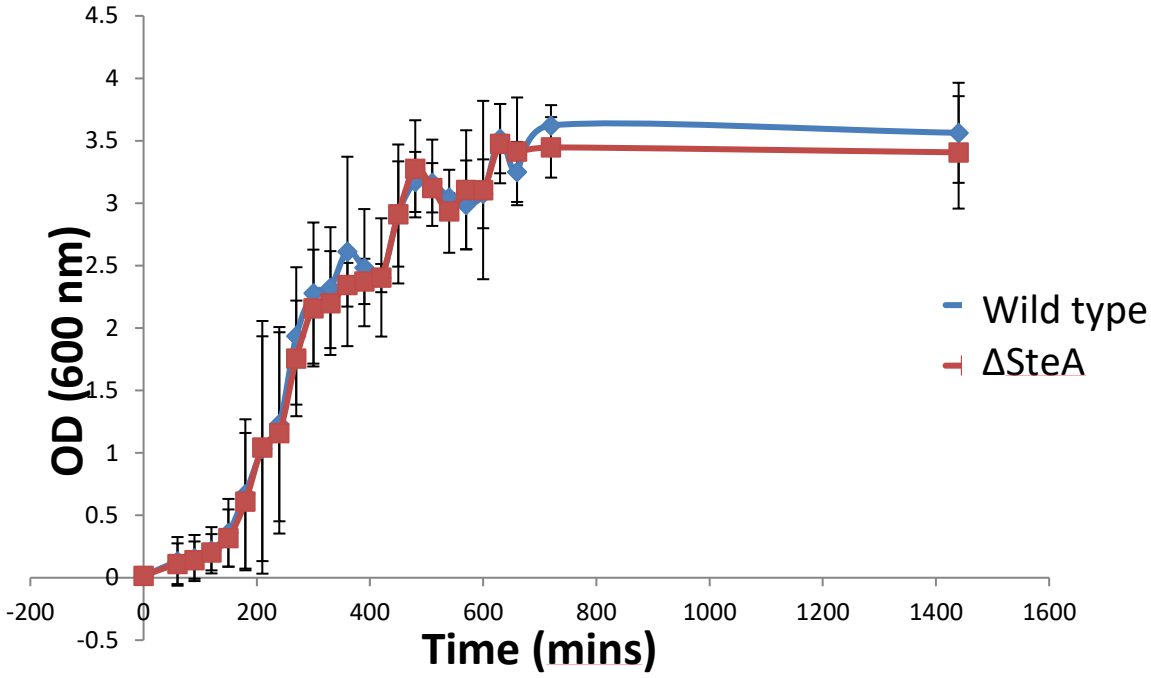

Figure 3: Growth kinetics of wild type and ΔSteA strains in LB broth.

Inference:

As it can be seen that the growth curves of wild type and the deleted mutant for SteA overlap, we can say that the deletion of the gene induced no growth defects in the bacteria in LB medium.

Luciferase reporter assay

2. S. *typhimurium* invades the epithelial layer of cells. For successfully invading the cells, the bacteria release various effector proteins which modulate host cell responses and help in invasion. As the previous experiments performed by Aakanksha Gulati suggested that SteA interfered with the NF-κB pathway in SPI-I inducing conditions, so we performed the Luciferase reporter assay to check for the differences induced due to the effect of our protein of interest, SteA in HEK 293 cell line, which is an epithelial cell line.

   i. HEK 293 cells were plated at density of 25,000 cells/well in a 96-well plate in 100 µl DMEM media with 10% FBS. They were infected with µaer-ST bacteria of wild type, ΔSteA and the complemented strains grown overnight at 37°C in LB + 0.3M NaCl at MOI = 20 for 30 mins of infection time. After 6 h of plating the cells were transfected with Luciferase and Renilla plasmids. The cells were induced with TNF-α after 16 h of trasnfection. The luminescence was then measured after 8 h of induction and the invasion was enumerated by CFU counting.

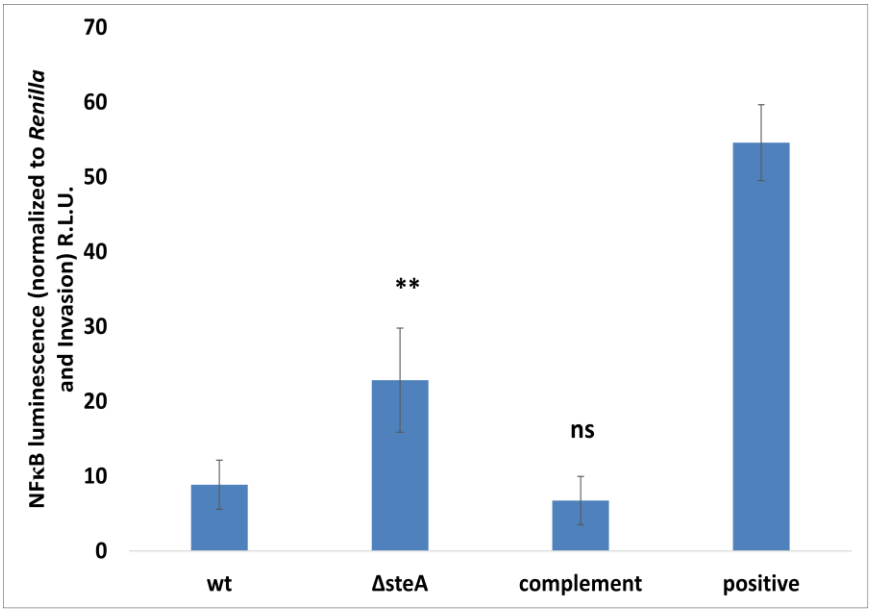

Figure 4: Luciferase reporter assay to check for NF-κB activity when cells were infected with wild type, ΔSteA and complemented strains.

Inference:

We observed that the infection with ΔSteA strain resulted in a significantly higher NF-κB response in HEK 293 cells as compared to wild type strain. Also the complemented strain showed a restoration of the wild type phenotype. Thus, from this we can conclude that the SteA gene results in down regulation of the NF-κB pathway as in its absence the response is higher than the wild type.

ii. To further confirm, we tagged our gene with a single HA tag and cloned it in pcDNA 3.1(+), which is a mammalian expression vector. HEK 293 cells were plated at a density of 25,000 cells/well in a 96-well plate in 100 µl of DMEM media containing 10% FBS. After 6 h of plating the cells were transfected with pcDNA empty vector or vector containing HA-tagged SteA gene along with Luciferase and Renilla plasmids. After induction with TNF-α induction the luminescence was measured.

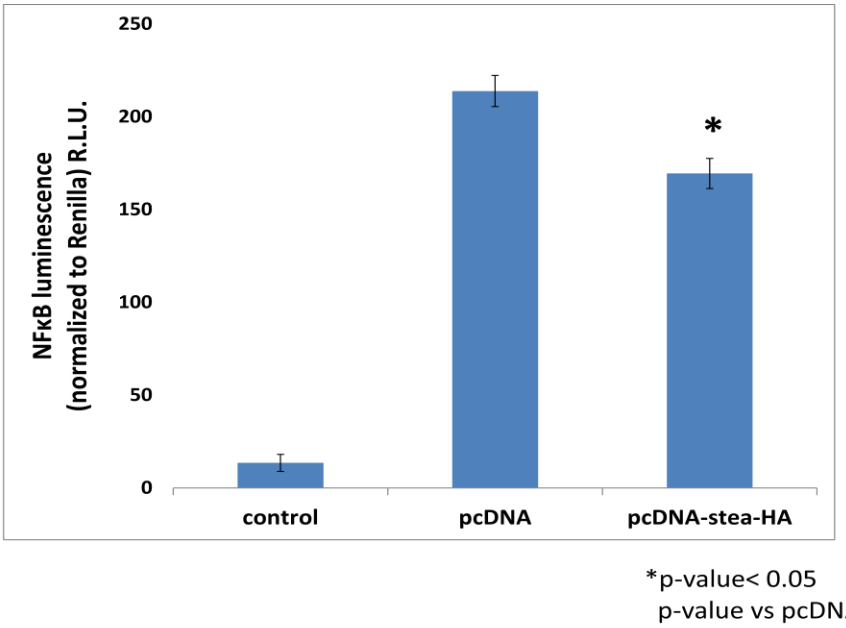

Figure 5: Luciferase reporter assay to check for NF-κB activity upon endogenous expression of SteA against an empty plasmid.

Inference:

From the above result we observed and confirmed that the presence of SteA down regulates the NF-κB pathway, significantly.

Differences in IκB levels due to the effect of SteA

3. From previous experiments and results from Aakanksha Gulati's work on the same project we got a hint that maybe SteA acts on IκB in the NF-κB pathway.
    i. To confirm the same, we plated HEK293 cells at a density of $1.5x10^5$ cells/well in a 6-well plate. The cells were then infected with μaer-ST culture of wild type and ΔSteA bacteria grown in LB + 0.3M NaCl overnight, at MOI=50 for 30 mins. The differences in the IκB levels were checked by western blotting. The invasion of the same was calculated by CFU counting.

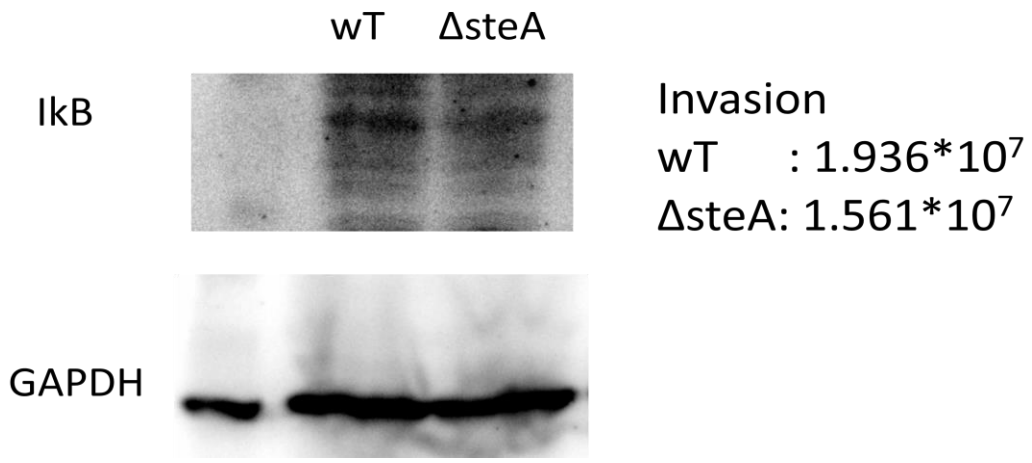

Figure 6: Western blots showing the differences in IκB levels upon infection of HEK 293 cells with wild type and ΔSteA strains.

Inference:

As it can be seen from the above blots, upon infection with ΔSteA strain the IκB levels were considerably lower than as compared to when the cells were infected with wild type bacteria. This implies that SteA gene acts directly or indirectly on IκB in the NF-κB pathway. GAPDH here was used as a loading control.

ii. A similar experiment was performed where instead of *Salmonella* infection we transfected the plated HEK 293 cells with empty pcDNA or pcDNA containing HA tagged SteA with PEI transfecting reagent and the IκB levels were checked by western blotting and induced with TNF-α for 15 and 30 mins.

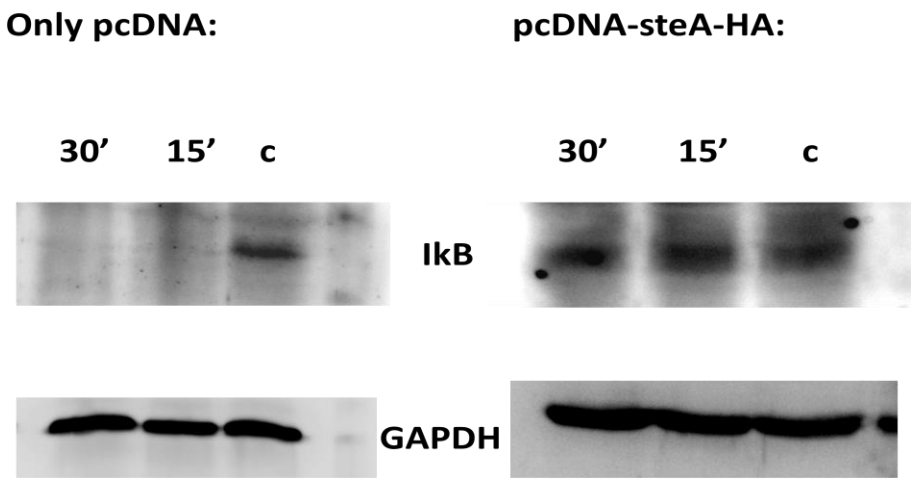

Figure 7: Western blots showing differences in IκB levels upon transfection of HEK 293 cells with empty pcDNA3.1 (+) vector against pcDNA 3.1(+) vector containing HA-tagged SteA.

Inference:

It can be clearly seen from the above blots that upon transfection with pcDNA containing SteA gene the IκB levels were considerably higher as compared to when cells were transfected with the empty vector, where the IκB levels are almost negligible. GAPDH here was used as the loading control.

Microscopy

4. To further check if SteA colocalized with IκB or not we performed confocal microscopy. For this experiment 25,000 HEK 293 cells were plated on coverslips placed in a 24-well plate (FALCON). They were then transfected with SteA containing pcDNA 3.1(+) and empty vector. Eventually they were stained and observed under Zeiss 780 Confocal microscope. In this experiment SteA which is HA tagged was labelled with a red flurophore and IκB was tagged with a green flurophore.

Vector with HA-SteA

Empty vector

Figure 8: Confocal microscopy images showing the colocalisation of HA tagged SteA with IκB in HEK 293 cells.

Inference:

From the above images we can see that when the cells were transfected with an empty vector, the cells showed only green fluorescence. But when the cells were transfected with the vector containing HA tagged SteA the cells appeared to be yellow which could have happened when, red HA-SteA colocalised green IκB. Thus it can be concluded that SteA colocalises with IκB to down regulate the NF-κB pathway.

Discussion

In previous reports people have shown that SteA is translocated via both T3SS-I and T3SS-II and in later stages of infection it plays a role in SCV membrane dynamics by interacting with PI4P. In this report we have found that in early stages of infection when SteA is translocated via T3SS-I

- ΔSteA bacteria result in a higher NF-κB pathway activation in epithelial cells as compared to wild-type bacteria.
- Upon endogenous expression of SteA differences in IκB levels were seen as compared to cells transfected with empty vector.
- No growth defects were found upon its deletion.

Many effectors translocated by both T3SS-I & II regulate the host cell responses by acting on the inflammatory pathways (9). In the initial phases of infection most of effectors are involved in bacterial entry into the cell and down regulation of cells immune responses to successfully invade the cell. In this report we've seen that SteA acts on IκB to down regulate the NF-κB pathway to establish a successful invasion. The results corresponded with the outcomes of experiments when SteA was endogenously expressed with the help of a vector. Through microscopy experiments it was also shown that SteA colocalises with IκB in the cells.

The study can be further taken forward by elucidating the mechanism by which SteA interacts with IκB.

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