

# Acidic pH induced STM1485 gene is essential for intracellular replication of Salmonella

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**Abbreviations:** STM, Salmonella Typhimurium; SCV, Salmonella containing vacuole; SPI-2, Salmonella pathogenicity island-2; T3SS, type three secretion system; ATR, acid tolerance response; iNOS, inducible nitric oxide synthase; NADPH, nicotinamide adenine dinucleotide phosphate-oxidase; BAF, bafilomycin A1; WT, wild-type

During the course of infection, Salmonella has to face several potentially lethal environmental conditions, one such being acidic pH. The ability to sense and respond to the acidic pH is crucial for the survival and replication of Salmonella. The physiological role of one gene (STM1485) involved in this response, which is upregulated inside the host cells (by 90- to 113-fold) is functionally characterized in Salmonella pathogenesis. In vitro, the  $\Delta$ STM1485 neither exhibited any growth defect at pH 4.5 nor any difference in the acid tolerance response. The  $\Delta$ STM1485 was compromised in its capacity to proliferate inside the host cells and complementation with STM1485 gene restored its virulence. We further demonstrate that the surface translocation of Salmonella pathogenicity island-2 (SPI-2) encoded translocon proteins, SseB and SseD were reduced in the  $\Delta$ STM1485. The increase in co-localization of this mutant with lysosomes was also observed. In addition, the  $\Delta$ STM1485 displayed significantly reduced competitive indices (CI) in spleen, liver and mesenteric lymph nodes in murine typhoid model when infected by intra-gastric route. Based on these results, we conclude that the acidic pH induced STM1485 gene is essential for intracellular replication of Salmonella.

## Introduction

*Salmonella enterica* comprises a group of Gram-negative bacteria capable of causing clinical syndromes ranging from self-limiting diarrhea to severe fibrinopurulent necrotizing enteritis and life threatening systemic diseases.<sup>1,2</sup> During the course of infection, Salmonella encounters several potentially lethal conditions such as the extremely low pH of the stomach, bile salts, reactive oxygen intermediates etc.<sup>3</sup> One of the most frequently encountered hostile conditions by Salmonella is acid stress and hence the ability to sense and respond to the acid stress is crucial for its survival. Salmonella responds to the acidic pH changes through complex acid survival systems collectively called acid tolerance response (ATR).<sup>4</sup> When *S. Typhimurium* cells are grown at sublethal pH (pH 4.5–5.5) in vitro for one generation, Salmonella gain the ability to survive at extreme acidic conditions (pH 3); this is known as ATR.<sup>5</sup> ATR is characterized by synthesis of approximately 38–60 acid shock proteins (ASPs) upon exposure to moderate acidic conditions; in turn these ASPs appear to promote bacterial survival under extreme acidic stress conditions.<sup>6</sup>

Another crucial aspect of Salmonella pathogenesis is tolerance of episodes of low pH within the macrophage phagosome or phagolysosome.<sup>7</sup> Following phagocytosis of Salmonella and its compartmentalization into the modified phagosome known as the Salmonella containing vacuole (SCV), a series of defense systems are initiated inside the host macrophages. In macrophages, phagosome acidification is considered as one of the major killing mechanism for clearing intracellular bacteria. Moreover, phagosomal acidification enhances the efficiency of other bactericidal mechanisms such as free radical formation (superoxide and nitric oxide radicals), increased fusion of phagosomes with lysosomes and activation of lysosomal acid hydrolases.<sup>8</sup> Phagosomal pH is maintained between 4 to 5 by combined action of v-ATPases and chloride channels present in the vacuolar system.<sup>9</sup> Salmonella are of particular interest as they reside in modified phagosomes called SCV and resist killing by modulating phagosomal maturation and also altering the vesicular trafficking of iNOS and NADPH oxidase vesicles.<sup>10–12</sup> Furthermore, Salmonella uses acidic pH (-pH 4.5) for transcriptional regulation of a subset of PhoP regulated genes<sup>13</sup> and assembly of the Salmonella pathogenicity

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island-2 (SPI-2) type three secretion system (T3SS),<sup>14-16</sup> which is essential for survival inside the macrophages.

Several transcriptomic analyses of intracellular *Salmonella* showed that a set of acid inducible genes such as *mgtC*, *cadB*, *cysB*, *adiY*, *pagC* and *marAB* are upregulated inside the macrophage vacuole, which is consistent with the acidic nature of the SCV. However, none of the ATR regulators such as *ompR*, *phoP* or *rpoS* are significantly upregulated.<sup>17-19</sup> STM1485 encoding putative acid shock protein of 82 amino acids was significantly upregulated inside the macrophages<sup>17,19</sup> and to a very lesser extent in epithelial cells.<sup>18</sup> The microarray data by Eriksson et al., depicted an interesting biphasic expression level of STM1485 in macrophages being 90-fold at 4 h, 26-fold at 8 h and 113-fold at 12 h after infection.<sup>17</sup> Though the roles of *mgtC*, *pagC*,<sup>20</sup> *adiY*<sup>21</sup> and *cadB*<sup>18</sup> acid inducible genes in *Salmonella* pathogenesis are known, the physiological role of one of the most highly induced genes STM1485 in *Salmonella* pathogenesis remains undetermined.

Therefore, the present study was undertaken to understand the possible role of STM1485 in the survival of *Salmonella* during acid stress, inside the host cells and in the mouse model. We report here that the STM1485 gene is fully dispensable for its in vitro growth. On the other hand, STM1485 plays a key role in intracellular survival of *Salmonella* by altering the phagolysosomal fusion of SCV through SPI-2 T3SS. This study is the first of its kind to show that STM1485, an acidic pH induced gene in *Salmonella* is required for its intracellular survival. It further fuels the possibility of an intricate relationship between STM1485 and SPI-2 and its importance in determining the course of *Salmonella* pathogenesis.

## Results

**STM1485 is not required for the log and stationary phase acid tolerance response in *S. Typhimurium*.** It was reported that *E. coli asr* gene is highly expressed at low pH (pH 5.0)<sup>22</sup> and is required for growth at moderate acidity (pH 4.5) and also for the log phase ATR of *E. coli*. Since *Salmonella* putative acid shock protein (STM1485) is having ~77% similarity at nucleotide level and ~68% identity at amino acid level compare with *E. coli* Asr protein, initially we have studied the role of STM1485 under acidic conditions.

STM1485 gene was deleted from *Salmonella enterica* ser Typhimurium (WT) using lambda red recombination system<sup>23</sup> and the mutant was confirmed by colony PCR with confirmatory primers against the sequence flanking the STM1485 gene and with a reverse primer specific for the *CAT* gene of pKD3 (Table S1 and Fig. S1A and B). We then compared the growth of  $\Delta$ STM1485 strain with that of the WT in rich medium (LB) defined medium (minimal media) at neutral pH 7.4, minimal medium F and ISM medium at pH 5.0. The growth of the WT and the  $\Delta$ STM1485 strains was comparable at pH 7.4 (LB and minimal media) and 5.0 (F- and ISM media) (Fig. S1C–F).

The WT and  $\Delta$ STM1485 were tested for their ability to grow in LB and LPM media buffered with sodium citrate buffer at pH 4.5, 4.0 and 3.5 for a period of 12 h as described in materials

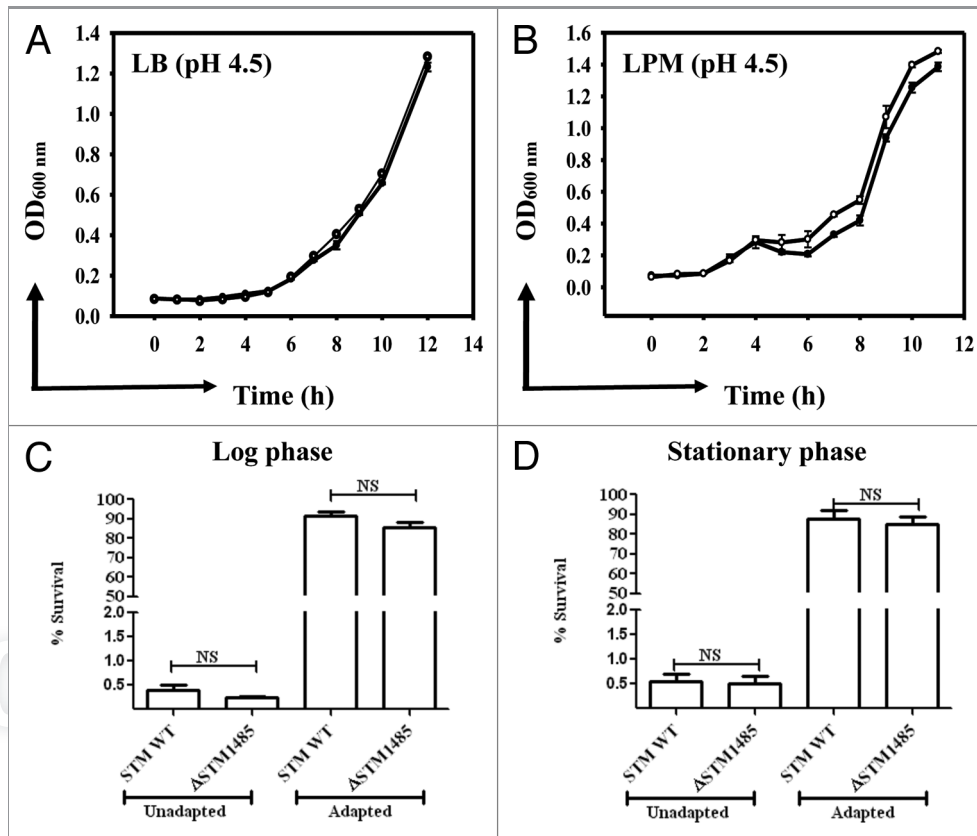
and methods. No difference was observed between growth rates of WT and  $\Delta$ STM1485 in either LB or LPM at pH 4.5 (Fig. 1A and B). However, at pH 4.0 and 3.5 both strains showed very poor growth (data not shown).

Then, we have analyzed the role of STM1485 in the acid tolerance response of *S. Typhimurium* at pH 3.0 after an initial adaptation of both the WT and  $\Delta$ STM1485 at pH 4.5 in LPM medium. Direct acid challenge of the log phase cultures (without adaptation) of both the WT and  $\Delta$ STM1485 from pH 7.0 to 3.0 showed 0.4% and 0.25% survival respectively. In contrast, adapted WT and  $\Delta$ STM1485 exhibited 91% and 87% survival after two hours of incubation at pH 3.0 (Fig. 1C). Same is the case even with the stationary phase acid tolerance response (Fig. 1D). No significant difference was observed in acid tolerance response of the WT and  $\Delta$ STM1485 strains implying that STM1485 is not essential for log and stationary phase acid tolerance response in *S. Typhimurium* in vitro.

**STM1485 expression is upregulated inside the host cells and is dependent on vacuolar acidification.** Before investigating the physiological role of STM1485 gene, we studied the expression of STM1485 in vitro as well as in cell culture. Since STM1485 and its ortholog (STY1582) in *S. Typhi* are upregulated inside the host cells,<sup>17,19,24</sup> semi-quantitative RT-PCR was done to check the expression level of STM1485 in vitro as well as in intracellular bacteria i.e., infected RAW264.7 cells with or without bafilomycin (BAF) pretreatment at 4 h post infection. We used BAF, a specific inhibitor of V-ATPases to block the vacuolar acidification.<sup>25</sup> We first determined the minimal concentration required for inhibiting the acidification of phagosomes. Effect of BAF on acidification of phagosomes was tested by vital staining with acridine orange, a dye that shifts fluorescence emission upon protonation (data not shown).

The results obtained by semi-quantitative RT-PCR showed that STM1485 expression was induced when the bacteria were grown in LB at pH 4.5 and not pH 7.0. More over the expression of STM1485 is not affected in SPI-2 null mutant  $\Delta$ ssaV (Fig. 2A). Pretreatment of RAW264.7 cells with BAF reduced the expression of the gene STM1485 significantly in intracellular WT bacteria compared with the untreated one (Fig. 2B). The SPI-2 gene *spiC* is used as a positive control, whose expression was also reduced with BAF treatment, showing accordance with the previous report by Cirillo et al.<sup>26</sup> Reduced expression of STM1485 in BAF pretreated cells could be attributed to the increase in the vacuolar pH. These results indicate that STM1485 is induced at low pH in vitro as well as inside the cells.

To check the STM1485 expression at protein level, we made a knock-in strain where six histidine amino acids were tagged to the C-terminal of the chromosomal STM1485. As shown in Figure 2C, no band was observed in the case of *Salmonella* grown in LB at pH 7.0. However, a 10 kDa band corresponding to His-tagged STM1485 protein was induced at pH 4.5. The WT strain has been used as control (Fig. 2C). The expression of STM1485 in different epithelial cells (INT-407, Caco-2 and HeLa) was assessed. STM1485 gene was upregulated in all the epithelial cell lines tested both at mRNA as well as protein level, when compared with its expression in bacteria grown in DMEM



**Figure 1.** In vitro characterization of the  $\Delta$ STM1485. (A) and (B) Growth kinetics of the  $\Delta$ STM1485 and its parental strain at acidic pH. Growth kinetics was done in LB (A) and LPM (B) at pH 4.5 buffered with sodium citrate. Log (C) and stationary (D) phase acid tolerance response (ATR) of the  $\Delta$ STM1485 strain. Overnight grown cultures were sub cultured into LPM media at pH 7.0 to an OD<sub>600</sub> nm of 0.3 (log phase) or 1.5 (stationary). For adaptation, bacteria were resuspended in LPM media at pH 4.5 for 2 h, followed by shifting of pH to 3.0 and growth for 2 h. Unadapted cells were directly shifted to pH 3.0. Number of viable bacteria was enumerated by plating on selective LB plates. Shown is the data of two independent experiments. Filled circles, WT; open circles,  $\Delta$ STM1485; NS, not significant.

(Fig. 3A and B). These results suggest the importance of the STM1485 in epithelial cells, which Salmonella encounters during its course of infection.

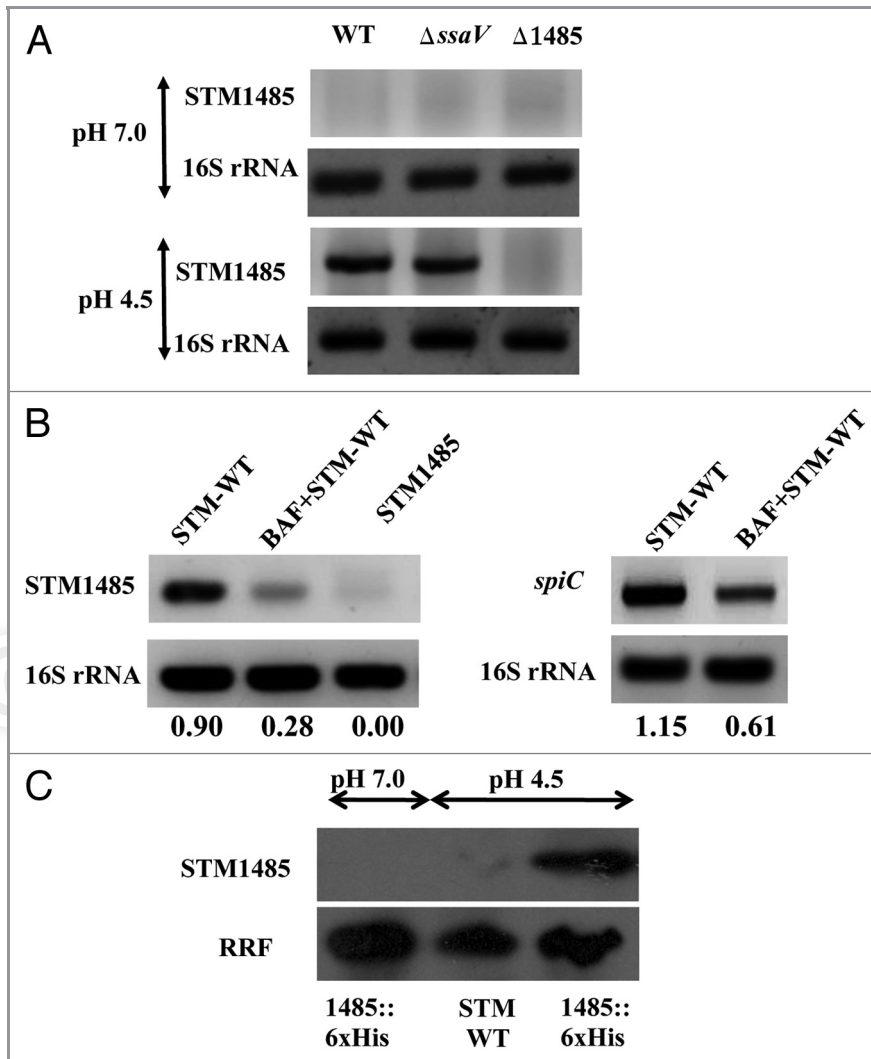
**$\Delta$ STM1485 strain is replication defective in the macrophage and epithelial cells.** Salmonella encounters epithelial and macrophage cells during its course of pathogenesis.<sup>27</sup> Therefore, the intracellular replication capacity of the  $\Delta$ STM1485 in human epithelial and murine macrophage cells was analyzed. The  $\Delta$ STM1485 is highly defective in intracellular replication compared with that of the WT in human epithelial (Fig. 4B) and murine macrophage cells (Fig. 5A) ( $p < 0.005$ ). However, the number of  $\Delta$ STM1485 colonies obtained after 30 min of infection was comparable to that of the WT strain showing that the strain is not invasion defective (Fig. 4A). This decrease in the fold replication was not seen in the  $\Delta$ STM1485 complemented strain when tested in RAW264.7 and INT-407 cells (Fig. 5B and C). Complementation studies also suggested that the STM1485 gene deletion is indeed non polar.

**Effect of vacuolar pH neutralization on survival and replication of the  $\Delta$ STM1485 strain.** Salmonella containing vacuoles (SCVs) acidify soon after their formation and remain acidified for at least 5–6 h post infection. The vacuolar proton ATPases

(V-ATPases) play critical role in vacuolar acidification and this pH drop is independent of phagosome-endosome/lysosomal fusion. The low vacuolar pH is required for the intracellular replication of Salmonella.<sup>7,28</sup> Therefore we sought to determine the effect of neutralization of vacuolar pH on intracellular survival of the WT and the  $\Delta$ STM1485 in RAW264.7 macrophage cells.

Pretreatment of RAW264.7 cells with BAF (50 nM), reduced the intracellular fold replication of the WT from 2 to 16 h. In contrast, the  $\Delta$ STM1485 did not show any further reduction in intracellular fold replication (Fig. 6A) ( $p < 0.005$ ). In addition to BAF, we observed the same phenomenon with folimycin (Concanamycin A) and ammonium chloride pretreatment which inhibit the vacuolar pH through different mechanisms (Fig. 6B and C). These results suggest that Salmonella needs the acidification for induction of some of their genes protecting it from bactericidal effects different from the acidification. These results also hint that STM1485 might confer survival advantage to Salmonella inside the acidic phagosomes.

**$\Delta$ STM1485 exhibited reduced surface translocation of SPI-2 encoded translocon proteins.** Low pH (between 4 and 5) is an environmental cue for the secretion of translocon proteins and formation of translocon encoded by SPI-2 type three



**Figure 2.** STM1485 gene expression in in vitro grown bacteria and intracellular bacteria. (A) and (B) cDNA was synthesized from the RNA isolated from in vitro grown bacteria (in LB pH 7.0 and 4.5 for 4 h) and from infected RAW264.7 cells (4 h post-infection) with and without BAF treatment. STM1485 and *spiC* (representative from SPI-2 island) were amplified by PCR. 16S rRNA served as internal control. (C) Expression of the His tagged STM1485 protein from STM1485::6xHis knock in strain grown in LB at pH (4.5 and 7.0) by immunoblot. Ribosome Recycling Factor (RRF) probing was done to equalize the amount of bacterial protein loaded for the SDS PAGE. Numbers below the gels indicate the values of densitometric image analysis using Multi Guage software. Images are representative of two independent experiments.

secretion system of *S. Typhimurium* inside the macrophages.<sup>14-16</sup> Furthermore, a recent study by Yoon et al. identified STM1485 as one of the genes which was co-regulated with SPI-2 genes.<sup>29</sup> Therefore we hypothesized that STM1485 might be playing an important role in the formation of SPI-2 translocon at low pH. SseB is the first SPI-2 T3SS translocon protein to be identified whose surface secretion is controlled by pH of the medium.<sup>15,30</sup> The surface translocation of SseB in vitro at low pH in ISM medium was studied. Proteins isolated from three fractions (pellet, surface and supernatant) were analyzed for the presence of SseB by immunoblotting. A band size corresponding to SseB (-23 kDa) was detected in the surface fraction and cell pellet fraction but not in the supernatant fraction. Compared with the WT, SseB

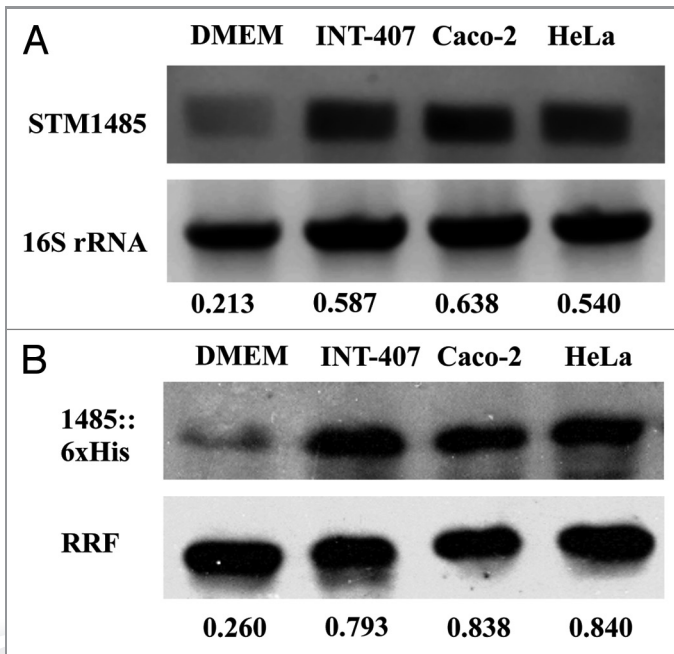
translocation on to the cell surface was visibly reduced in the  $\Delta$ STM1485, when the bacteria were cultured at pH 5.0. In contrast, the level of SseB in the pellet fraction of the  $\Delta$ STM1485 strain was higher than the pellet fraction of the WT (Fig. 7A).

These results were confirmed by analyzing the localization of SseD on the Salmonella grown in in vitro and in vivo conditions using confocal microscopy. Antiserum raised against SseB was used for detecting surface appendages formed during the growth of the bacteria. A punctuate appearance of SseD on one pole of the bacterial cell was observed in WT but not in  $\Delta$ STM1485 both under in vitro growth conditions (Fig. 7C) and in infected cells (Fig. 7D). These results indicate that SPI-2 translocon formation is affected in the absence of STM1485.

Further experiments were performed to establish that STM1485 is required for the SPI-2 translocon formation. Secretion of SPI-2 effector SseJ under in vitro growth conditions was studied by immunoblotting. STM-WT,  $\Delta$ STM1485 and  $\Delta$ ssaV mutant strains carrying the His-tagged SseJ construct were grown under SPI-2 inducing conditions (F media, pH 5.0) for overnight and secreted proteins were precipitated from the culture supernatant. We evaluated the cell pellet and supernatant fractions for intracellular and secreted levels of the SseJ-His by immunoblotting. A band size corresponding to SseJ (-50 kDa) was detected in the supernatant as well as in the cell pellet fraction. We found that SseJ secretion was noticeably reduced in the supernatant of  $\Delta$ STM1485 compared with WT. RRF was not observed in any of the secreted fractions suggesting that bacterial cell integrity was maintained throughout the experiment. No secretion of SseJ-His was observed in the supernatant of  $\Delta$ ssaV mutant (Fig. 7B). Taken together these

observations suggest that STM1485 is important for SPI-2 translocon formation.

**$\Delta$ STM1485 containing phagosome progresses along the degradative pathway and fuses with lysosomes.** Salmonella is an intracellular pathogen that requires a set of virulence proteins encoded in the SPI-2 for its intracellular survival. The effector proteins encoded by SPI-2 allows Salmonella to establish SCV by preventing the fusion of SCV with lysosomes and interferes with the trafficking of NADPH oxidase and iNOS containing vesicles to the SCV inside the host cell.<sup>11,31-33</sup> The reduction in the surface translocation of SPI-2 needle proteins in  $\Delta$ STM1485 might have an effect on the SPI-2 function and hence the intracellular fate of the bacteria. To test this, we investigated the co-localization of



**Figure 3.** Evaluation of STM1485 expression in epithelial cells (INT-407, Caco-2 and HeLa). STM1485 expression was studied using (A) RT-PCR assay. (B) Immunoblot method using the STM1485::6xHis tagged strain. Bacteria were isolated from the infected epithelial cells at 6th h post infection. Bacteria grown in DMEM at 37°C under 5% CO<sub>2</sub> were served as control to determine expression outside host cells. Numbers below the gels indicate the values of densitometric image analysis using Multi Guage software. Results are representative of two independent experiments.

GFP bacteria with the endocytic probe texas red conjugated ovalbumin (TROv).<sup>34</sup> TROv gets distributed throughout the endocytic network after pinocytosis and can be chased into lysosomes to serve as lysosomal marker. Approximately 20% of the WT bacteria were co-localized with TROv at each time point; in contrast,  $\Delta$ STM1485 showed ~19%, ~34% and ~44% of co-localization with TROv at 2, 8 and 16 h respectively. Co-localization of the  $\Delta$ STM1485 was significantly higher than the WT at 8 and 16 h post infection ( $p = < 0.05$ ) (Fig. 8A and B). These results indicate that the lack of STM1485 results in the maturation of SCV along the degradative pathway.

**$\Delta$ STM1485 strain failed to compete with the WT in mouse model.** In order to evaluate the contribution of STM1485 gene in virulence of Salmonella in vivo, we tested the ability of the  $\Delta$ STM1485 strain to compete with the WT strain in mixed infections in murine typhoid model (Table 2). Competitive index is an alternative measure to LD<sub>50</sub> to determine the degree of virulence attenuation. The WT and mutant strains were recovered 4 d after intra-gastric inoculation of 10<sup>7</sup> CFU/mouse at a ratio of 1:1. As shown in the Table 2, the  $\Delta$ STM1485 strain showed significantly reduced competitive indices (CI) in spleen, liver and mesenteric lymph nodes (MLN), suggesting that STM1485 gene is essential for the in vivo colonization of *S. Typhimurium*. The pQE601485 complemented strain was found to compete equally against the WT strain.

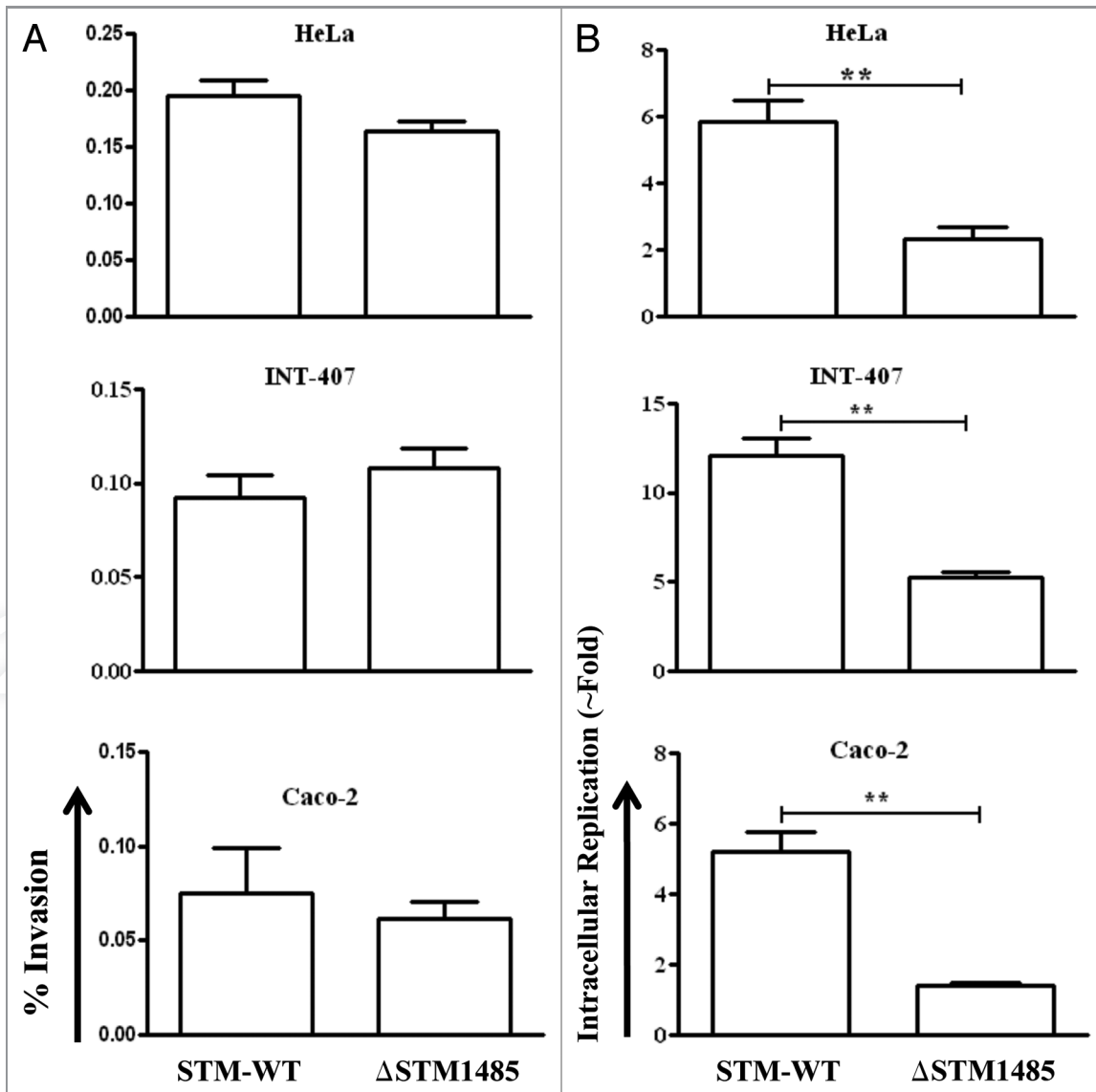
## Discussion

Adaptations to environmental changes allow pathogens to survive and perpetuate during their encounter with nature's extremes. One such natural stress situation confronted by Salmonella in host and non-host environments is acid stress. *S. Typhimurium* can survive lethal acidic conditions (pH between 2 and 3) if it is first adapted to mild acidic conditions and the phenomenon involves production of acid shock proteins.<sup>35</sup> These ASPs belong to different groups including those of membrane bound transporters, periplasmic proteins, cytoplasmic enzymes and extracellular components.<sup>36</sup> The physiological function of one such gene, STM1485, which is induced under acidic pH, in Salmonella pathogenesis is not known. Moreover, in spite of the acidic pH of the phagosome inside the host cells, Salmonella can multiply within the phagosomes of macrophages and use acidic pH as a cue for regulation of virulence genes or assembly of SPI-2 T3SS. We speculated if STM1485 induced at low pH has any role in counteracting the acidic pH inside the phagosomes.

STM1485 gene is induced when bacteria are grown in acidic pH (pH 4.5) in LB media but not at neutral pH. The expression of STM1485 in infected macrophages was reduced with BAF pretreatment indicating that the STM1485 gene might be required for survival at acidic pH inside the phagosomes of the host. The fact that the deletion of STM1485 did not affect the growth of *S. Typhimurium* at low pH in vitro and its acid tolerance response (log and stationary phase) suggests that STM1485 gene is dispensable for Salmonella survival at low pH or for acid tolerance response under in vitro conditions. The  $\Delta$ STM1485 was attenuated in all the cell lines tested and could not compete with the WT in mixed infections in murine typhoid model. Indispensability of STM1485 gene for survival and replication of *S. Typhimurium* is confirmed by complementation of STM1485 gene which fully restored the phenotype back to wild-type. These results clearly state that the STM1485 gene is essential for intracellular replication and Salmonella pathogenesis. Our data suggest that though STM1485 is induced at low pH conditions, it is not involved in counteracting acidic conditions in vitro but indirectly helps in counteracting the other anti-bactericidal mechanisms inside the host.

Intracellular replication of WT and not  $\Delta$ STM1485 was compromised by vacuolar pH neutralizing agents (BAF, folimycin and ammonium chloride). Loss of WT intracellular replication with phagosomal pH neutralization may be due to the absence of expression of virulence genes or lack of SPI-2 encoded needle which are required for survival and growth within the macrophages.<sup>14,15</sup> Our RT-PCR studies of intracellular bacteria with and without BAF treatment shows that STM1485 gene expression is reduced upon BAF treatment compared with the untreated ones. So we reason that reduction in the STM1485 gene expression in WT with BAF has an equivalent effect as that of  $\Delta$ STM1485.

Infection of macrophages by Salmonella leads to expression of SPI-2, which allows Salmonella to establish replicative vacuoles, called SCV. The type three secretion system encoded by SPI-2 is used to translocate cohorts of bacterial effector proteins directly into the host cell. There have been several studies to identify

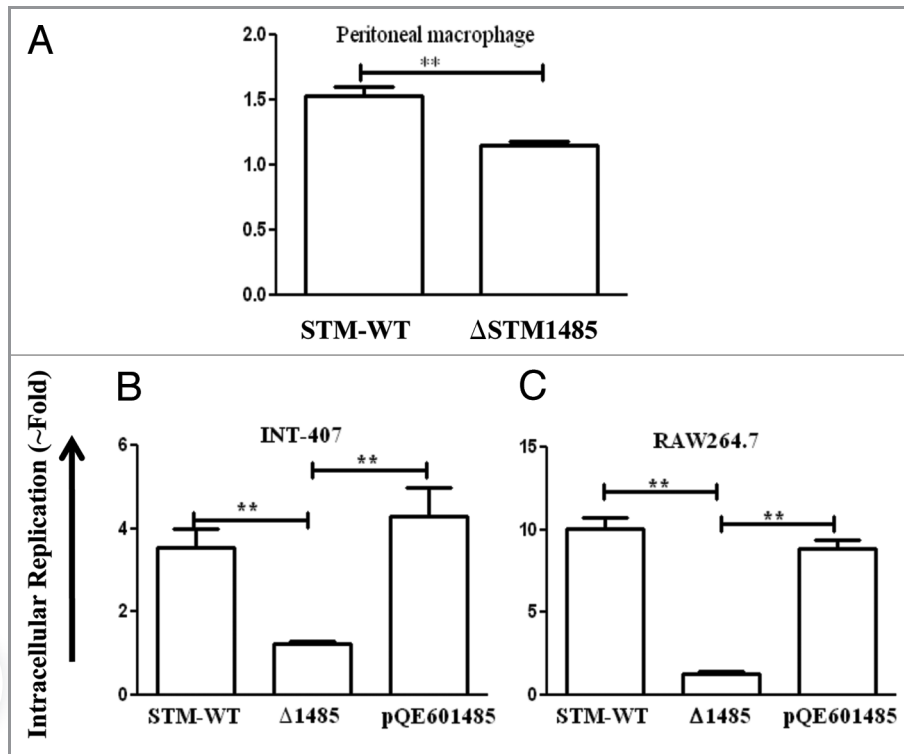


**Figure 4.** Invasion and intracellular fold replication of the  $\Delta$ STM1485 strain inside epithelial cells (INT-407, Caco-2 and HeLa). (A) Invasion of  $\Delta$ STM1485 was determined by lysing the infected cells 30 min post-infection. (B) Fold replication was determined by lysing the cells infected with the WT and the  $\Delta$ STM1485 at 2 h and 16 h post infection. Graphs are representative of three independent experiments, each with triplicate samples. Standard error bars are shown. (\*\* $p < 0.005$ ) (Student's t-test).

environmental cues that regulate either expression or assembly of SPI-2 T3SS. Acidic pH (pH 4.5–5.0) of SCV is an important cue, in part required for the assembly of T3SS.<sup>14-16</sup> SPI-2 T3SS secretes translocon proteins (SseB, SseC and SseD) that form translocon or needle through which SPI-2 T3SS effector proteins (SpiC, SseF, SseG, SifA, SseI and SseJ) are delivered into the host cytosol. Some of the SPI-2 proteins forming needle components are present in the periplasmic region of bacteria, e.g., SsaV, SsaJ and SsaC.<sup>30</sup>

When the Salmonella are exposed to the harsh environmental conditions like low pH inside the phagosome, proteins present in the periplasm are more vulnerable to such conditions due to the porous nature of the periplasm, thus requiring greater protection than the cytoplasmic proteins. Periplasmic proteins or chaperons

play a crucial role in the biogenesis of outer membrane proteins or formation of functional type three secretion systems, when Salmonella encounters harsh environmental conditions inside the host cells.<sup>37,38</sup> We speculate that STM1485 encoded protein might be located in the periplasm based on the conservation of signal sequence of STM1485 with that of *E. coli asr* signal sequence.<sup>22</sup> STM1485 encoded protein may play an important role in outer membrane protein biogenesis and formation of functional T3SS, which are important for the survival of Salmonella inside the host cells. Interestingly, in our study we have observed that the surface secretion of SseB and SseD and secretion of SseJ effector protein are affected in  $\Delta$ STM1485 compared with WT, which implies that STM1485 encoded



**Figure 5.** Intracellular survival assay of  $\Delta$ STM1485 in mouse macrophage cells (A) and complementation studies in INT-407 (B) and RAW264.7 (C). Cells were infected with WT or  $\Delta$ STM1485 or  $\Delta$ STM1485pQE601485 strains were lysed at 2 and 16 h post infection. Bacterial load was shown as fold increase in CFU from 2 h to 16 h. Graphs are representative of two independent experiments with similar results. Statistical significance was defined as follows (\*\* $p < 0.005$ ) (Student's t-test).

protein is playing a crucial role in the secretion of translocon proteins. The possible reasons for this decreased surface secretion are either defect in needle formation or loss of membrane integrity in the  $\Delta$ STM1485.

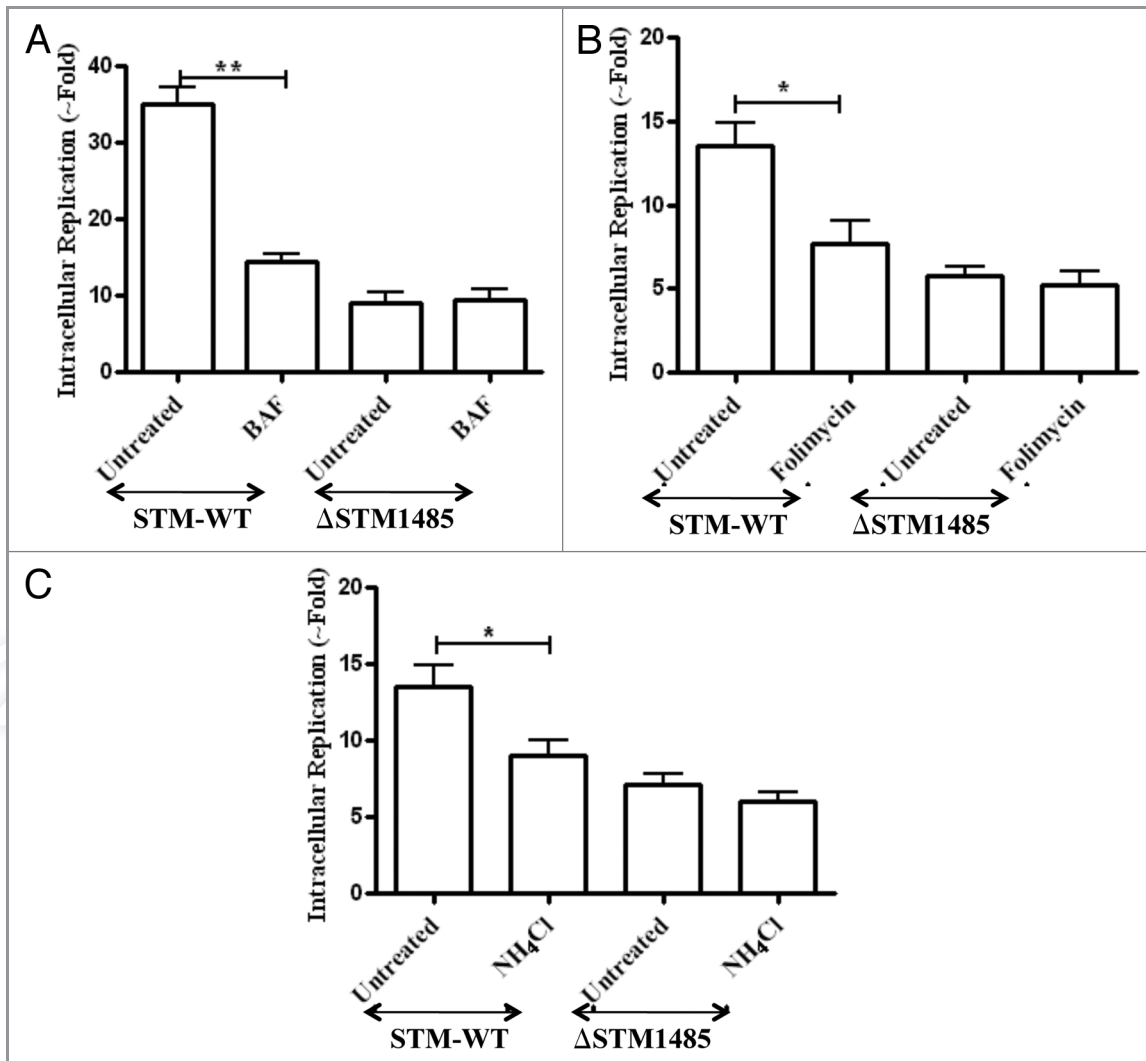
Co-localization studies of GFP bacteria with the endocytic fluid phase marker texas red conjugated ovalbumin (TROv) showed increased co-localization of  $\Delta$ STM1485 with the TROv (lysosomes) compared with the WT. This can be explained by the reduced SPI-2 translocon formation as reported earlier that the reduction in the SPI-2 expression leads to efficient interaction of SCV with the components of the late endosomal or lysosomal system.<sup>31,32</sup> Therefore, the inability of  $\Delta$ STM1485 in assisting the formation of functional SPI-2 T3SS translocon explains its high level of attenuation inside the cell line and its incapability to compete with the WT in murine model.

Based on our results, we conclude that perhaps Salmonella senses low pH using unknown sensors or two component regulatory systems like PhoPQ and synthesize the STM1485 encoded protein. STM1485 encoded protein in turn regulates the assembly of SPI-2 encoded T3SS at low pH inside the host cell. Absence of STM1485 results in the decreased SPI-2 T3SS needle formation thereby increasing the fusion of SCV with the degradative compartments. Hence, STM1485 is one of the key players in regulating the SPI-2 translocon thereby contributing to Salmonella pathogenesis.

## Materials and Methods

**Bacterial strains and growth conditions.** The bacterial strains and plasmids used in this study are listed in the Table 1. Antibiotics namely kanamycin, carbenicillin, nalidixic acid (each at 50  $\mu$ g/ml) and chloramphenicol (20  $\mu$ g/ml) were added when needed for the selection of strains. Except where indicated, bacteria were grown at 37°C with aeration. Luria broth (LB) at different pH values (4.5, 4.0 and 3.5) buffered by sodium citrate (0.05 M); F-media with 50 mM KCl, 75 mM  $(\text{NH}_4)_2\text{SO}_4$ , 5 mM  $\text{K}_2\text{SO}_4$ , 10 mM  $\text{K}_2\text{HPO}_4$ , 1 mM Bis-tris, 30  $\mu$ M  $\text{MgSO}_4$ , 38 mM glycerol and 0.1% casamino acids;<sup>15</sup> Low phosphate salt glucose media (LPM) with 50 mM Tris-base, 20 mM KCl, 75 mM  $(\text{NH}_4)_2\text{SO}_4$ , 240  $\mu$ M  $\text{MgCl}_2$ , 18.5  $\mu$ M  $\text{CaCl}_2$ , 6.5 mM  $\text{FeCl}_3$ , and 0.2% Glucose Bactopectone-0.6%);<sup>22</sup> ISM media (170 mM  $\text{K}_2\text{PO}_4/\text{KH}_2\text{PO}_4$ , 0.5 mM  $\text{MgSO}_4$ , 1 mM  $\text{CaCl}_2$ , 6 mM  $\text{K}_2\text{SO}_4$ , 5 mM  $\text{NH}_4\text{Cl}$ , 5 mM NaCl, 0.4% glucose, 2 mg/mL nicotinic acid at pH 5 or 7.5).<sup>16</sup> Heat killed bacteria were prepared by heating the 0.3 OD adjusted cultures at 65°C for 20 min.

Growth study of  $\Delta$ STM1485 strain was done in different media such as LB (pH 7.5), minimal media (pH 7.5), F-media (pH 5.0) and ISM media (pH 5.0). For growth kinetics at low pH, cultures were grown in LB or LPM buffered at different pH values (pH 4.5, 4.0 and 3.5) by sodium citrate (0.05 M). Overnight grown cultures were sub-cultured (equal number of



**Figure 6.** Effect of phagosomal pH neutralization on intracellular replication of  $\Delta$ STM1485 strain. RAW264.7 cells were pretreated with (A) bafilomycin A1 (50 nM), (B) Folic acid (100 nM) and (C)  $\text{NH}_4\text{Cl}$  (100 mM) followed by infection as described in materials and methods. Infected cells were lysed at 2 h and 16 h post infection and bacterial load was shown as fold increase in CFU from 2 h to 16 h. Graphs are representative of three independent experiments with similar results. Statistical significance was defined as follows (\* $p < 0.05$ , \*\* $p < 0.005$ ) (Student's t-test).

bacteria) into the respective media and growth was monitored by measurement of optical density at 600 nm every 1 h. Acid tolerance response of *Salmonella* was done as described previously with some modifications.<sup>22</sup>

For ATR analysis, cultures were grown at 37°C in LPM (pH 7.0) to an  $\text{OD}_{600}$  of 1.5 for the stationary phase ATR assay and to an  $\text{OD}_{600}$  of 1.5 for log-phase assay. For acid adaptation, cells were resuspended in fresh LPM at pH 4.5 and incubated for 2 h. Unadapted and adapted cultures were inoculated into fresh LPM (pH 3.0) at approximately  $1 \times 10^8$  cells/ml. Cultures were incubated for 2 h at pH 3.0 and viable bacteria were calculated by plating aliquots of serially diluted cultures onto the LB (pH 7.5) plates at 0 h and 2 h. The number of viable bacteria was expressed as % survival after 2 h incubation at pH 3.0.

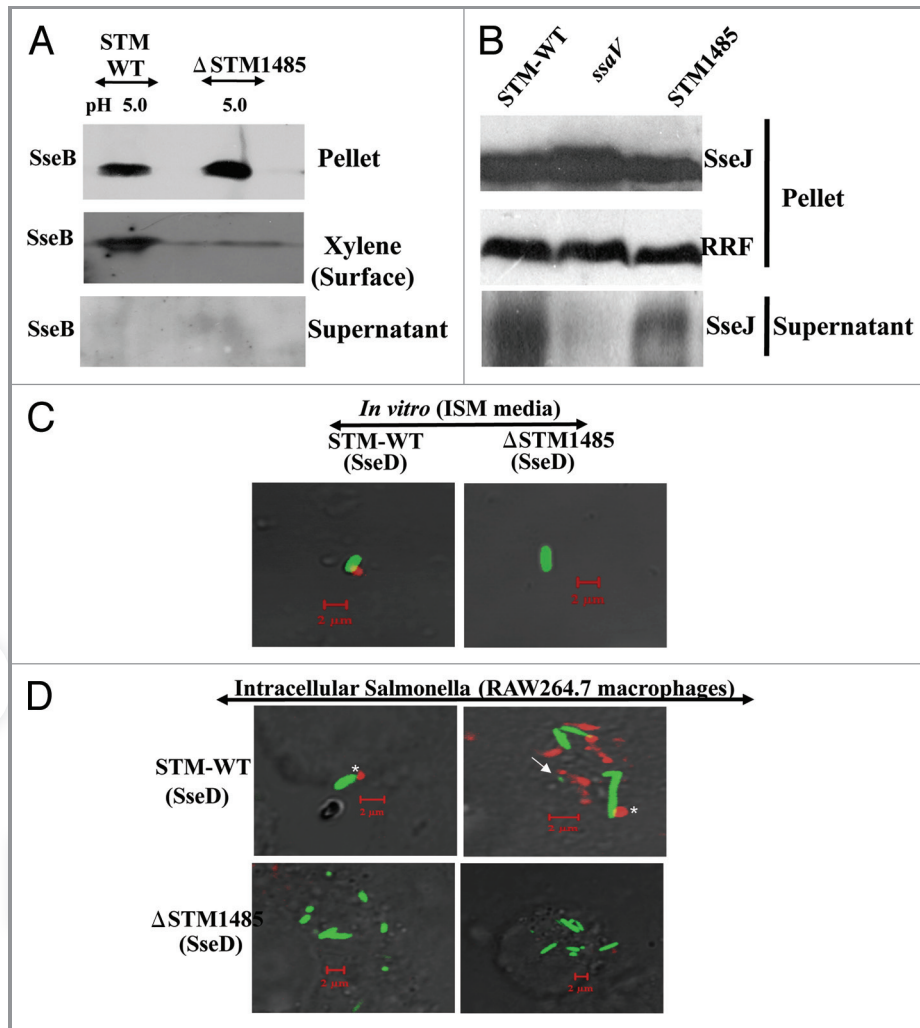
**Eukaryotic cell lines and growth conditions.** RAW264.7 cells were a kind gift of Prof. Anjali Karande (Department of Biochemistry, Indian Institute of Science, India). INT-407, HeLa

and CaCo-2 cells were obtained from National Center for Cell Science (NCCS), Pune, India. The cells were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma) or RPMI 1640, both supplemented with 10% fetal calf serum (Sigma). Elicited mouse macrophages were harvested from BALB/c mice by peritoneal lavage with RPMI 1640 medium (Sigma) 5 d after intraperitoneal injection of 5 ml of brewer's thioglycolate medium (Hi-media). All cells were maintained at 37°C and 5%  $\text{CO}_2$ . For Caco-2 cells, 1% non-essential amino acids was added to the medium.

**Construction of the STM1485 mutant in *Salmonella Typhimurium*.**  $\Delta$ STM1485 strain was constructed by one-step gene deletion strategy.<sup>23</sup> The knockout was selected for chloramphenicol resistance and confirmed by colony PCR using specific confirmatory primers (Table S1).

**Complementation of the  $\Delta$ STM1485 strain.** First, STM1485 gene was amplified by polymerase chain reaction (PCR) using





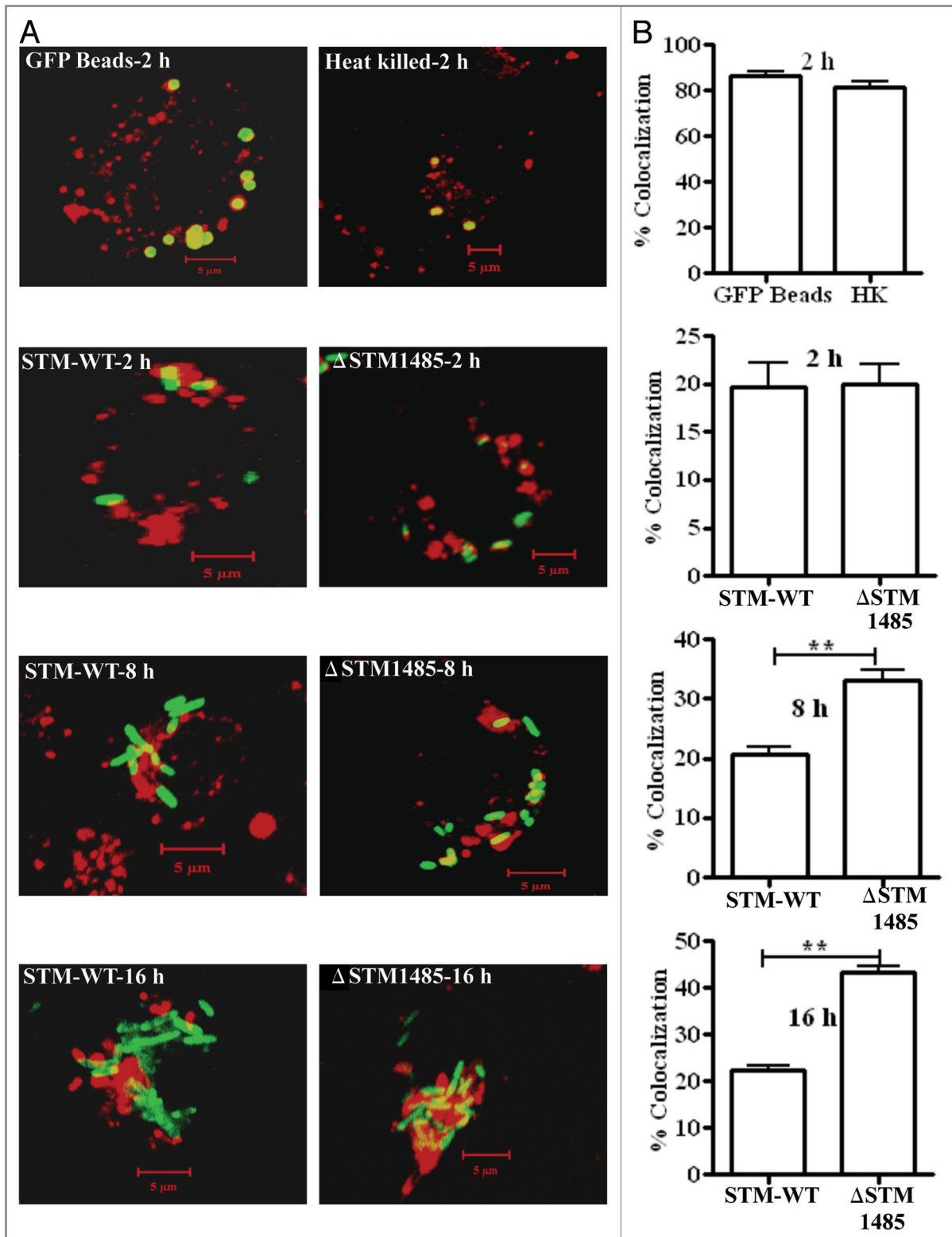
**Figure 7.** STM1485 is required for the SPI-2 translocon formation. Surface translocation of SPI-2 encoded translocon proteins (SseB and SseD) in the  $\Delta$ STM1485 (A). WT and  $\Delta$ STM1485 were grown in ISM media at pH 5.0 and then cell pellet, surface and supernatant fractions were isolated as described in Materials and Methods. By immunoblotting, the presence of SseB protein in all three fractions was analyzed. Secretion of SPI-2 effector protein (B). STM-WT,  $\Delta$ STM1485 and  $\Delta$ ssaV mutant strains carrying the His-tagged SseJ construct were grown under SPI-2 inducing conditions (F-media, pH 5.0) for overnight and secreted proteins were precipitated from the culture supernatant. We evaluated the cell pellet and supernatant fractions for intracellular and secreted levels of the SseJ-His by immunoblotting. Detection of SseD in vitro and in intracellular bacteria (C and D). WT-GFP and  $\Delta$ STM1485-GFP were grown in ISM media at pH 5.0 for overnight. Bacteria were harvested and preceeded for immunostaining (C). RAW264.7 cells were infected with WT-GFP and  $\Delta$ STM1485-GFP. Twelve hours post-infection cells were fixed and immunostained (D). Samples were analyzed using a confocal laser scanning microscope (LSM Meta, Zeiss). Note the appearance of SseD (red) on the surface of intracellular bacteria (green) (\*) and also distant to the bacteria (arrow). Scale bar represent the 2  $\mu$ m.

primers listed in Table S1 and cloned into pQE60 vector. Plasmid containing STM1485 gene was confirmed by restriction digestion and using vector specific primers (Table S1). The resulting plasmid DNA was electroporated into the  $\Delta$ STM1485 strain to get pQE601485 strain.

**Construction of His-tagged SseJ.** To generate His-tagged fusion protein, the *sseJ* ORF along with its promoter was amplified using primers listed in Table S1. Plasmid pHG86 and the PCR product were digested with EcoRI and BamHI and ligated together, yielding His-tagged construct. In this strategy, reverse primer was modified to carry a sequence coding for 6X histidine residues at 5' end followed by a stop codon so as to obtain only a SseJ tagged with 6X His at C-terminal end. The

His-tagged SseJ construct was introduced into STM-WT strain,  $\Delta$ STM1485 strain and  $\Delta$ ssaV mutant strain by electroporation.

**Construction of the Salmonella strain expressing the chromosomal STM1485::6xHis fusion protein.** The Salmonella strain expressing the STM1485::6xHis fusion protein was engineered in serovar Typhimurium by a modified method from the one-step deletion strategy as described previously.<sup>39</sup> Briefly, electrocompetent cells from serovar Typhimurium transformants, carrying a red helper plasmid (pKD46), were prepared. In this strategy, the forward knockout primer was modified to carry a sequence coding for six histidine residues at 5' end. This histidine tag containing primer was targeted against the region immediately downstream of the STM1485 gene (including the STM1485 stop



**Figure 8.** For figure legend, see page 132.

codon). The knockout primer set effectively knocked out the 270 bp of intergenic region downstream of STM1485 while concomitantly tagging STM1485 on its C-terminal with the

sequence coding for 6 histidine residues. This was followed by another stop codon so as to obtain only a STM1485::6xHis fusion protein. PCR product containing the kanamycin resistance gene

**Figure 8 (See previous page).** Co-localization of GFP expressing bacteria, latex beads and heat killed bacteria with TROv in RAW264.7 cells (A). Prior to infection, RAW264.7 cells were pulse-chased with 50 µg/ml of Texas Red-Ovalbumin (TROv) for 30 min, washed and then incubated for another 30 min. Then cells were infected with GFP expressing WT and ΔSTM1485 and fixed at different time points. Cells were also incubated with latex GFP beads and heat killed bacteria. Heat killed bacteria were detected with antibody specific to Salmonella LPS followed by FITC-conjugated secondary antibody. Samples were analyzed using a confocal laser scanning microscope (LSM Meta, Zeiss). At least 50 cells were counted in each case in each experiment. (B) Results of three experiments in which SCVs from cells infected with each strain or phagosomes containing latex beads and heat killed bacteria were scored for TROv co-localization at 2, 8 and 16 h post infection. Shown are the representative images of a single XY section taken from Z-stack. The values are given as mean ± standard error in percentages. Statistical significance was defined as follows (\*\*p < 0.005) (Student's t-test).

**Table 1.** Strains and plasmids used in this study

Strain	Description	Reference
STM-WT 12023	Wild-type 12023, parental strain for all mutants, resistant to nalidixic acid	15
ΔSTM1485	Δ1485::CAT <i>S. Typhimurium</i>	This study
WT-GFP	WT 12023 with pFPV25.1, resistant to carbenicillin (used in confocal and competition experiments)	This study
ΔSTM1485 GFP	ΔSTM1485with pFPV25.1 (used in confocal studies)	This study
STM1485::6xHis	<i>S. Typhimurium</i> expressing the 1485::6xHis fusion protein	This study
STMΔssaV	SPI-2 null mutant strain	15
Plasmid	Description	
pQE601485	<i>S. Typhimurium</i> 1485 complementing vector (carb')	This study
pKD46	Plasmid expressing λ red recombinase (carb')	23
pKD3	Plasmid with FRT-flanked chloramphenicol resistance gene	23
pKD4	Plasmid with FRT-flanked kanamycin resistance gene	23
pFPV25.1	Plasmid expressing GFP	45
pHG86	Promoterless reporter plasmid (carb')	46
pHG86sseJ-His	Plasmid expressing 6X His tagged SseJ	This study

(from pKD4 plasmid) flanked by sequences upstream and downstream of the STM1485 gene was prepared. This DNA was then electroporated in *Salmonella Typhimurium* carrying pKD46. The mutants were selected by kanamycin resistance and confirmed by PCR using the kanamycin internal forward and STM1485 cloning forward primers. In the knock-in strain, a band corresponding to 850 bp was amplified whereas in the WT strain, no such product could be detected. We also confirmed the chromosomal knock-in strain in the bacterial lysate by immune-blot using anti-His antibody staining. A 10 kDa single band corresponding to the STM1485:: 6xHis fusion protein was observed.

**Intracellular proliferation assay.** Different cell lines were seeded in 24-well plate at a concentration of  $1 \times 10^5$  to  $2 \times 10^5$  per well 24 h prior to infection. For the infection of RAW264.7 cells, the *S. Typhimurium* strains were grown to stationary phase in LB with the appropriate antibiotic. For infection of epithelial cells, stationary-phase cultures were diluted 1:33 in LB and grown for 3 h

(late-exponential phase) prior to infection to induce *Salmonella* pathogenicity island 1 (SPI-1) encoded genes required for invasion of non-phagocytic cells.<sup>40</sup> The gentamicin protection assay was done as described previously.<sup>41</sup> The fold intracellular replication was calculated by dividing the intracellular bacterial load at 16 h by the bacterial load at 2 h. Alternatively, GFP latex beads and heat killed bacteria (at a ratio of 10 to 1) were added to the cells for 20 min and followed further as in the case of infection. To inhibit the vacuolar acidification, inhibitors like bafilomycin A1 (BAF) (10–100 nM), folimycin (100 nM) and NH<sub>4</sub>Cl (100 mM) (purchased from Calbiochem) were used. Cells were pretreated with these inhibitors for 30 min followed by the infection and gentamicin protection assay.

**Bacterial RNA extraction from infected cells and in vitro grown bacteria.** This was done as described previously.<sup>17</sup> After 6 h of infection, cells were lysed on ice for 30 min in 1 ml of 0.1% SDS, 1% acidic phenol, and 19% ethanol in water. *Salmonella* were

**Table 2.** Competitive Indices of mixed infections using the WT, ΔSTM1485 and the pQE601485 complemented strains

	Strain-1	Strain-2	Competitive index of strain 2 with respect to strain 1		
			Spleen	Liver	MLN <sup>†</sup>
1	WT <sup>#</sup>	WT <sup>§</sup>	1.121 ± 0.12	0.975 ± 0.17	1.124 ± 0.09
2	WT <sup>#</sup>	ΔSTM1485	0.413 ± 0.05**	0.341 ± 0.07**	0.206 ± 0.13**
3	WT <sup>#</sup>	pQE601485	1.035 ± 0.14	1.367 ± 0.09	1.107 ± 0.12

<sup>#</sup>Resistant to nalidixic acid and the parent strain for knock out. <sup>§</sup>Resistant to Carbencillin. Both WT strains were isogenic. <sup>†</sup>Mesenteric lymph nodes. \*\*p < 0.005 (Student's t-test); statistical difference between 1 and 2.

isolated from the lysate by centrifugation and RNA was isolated using TRI reagent (Sigma) according to manufacturer's protocol. RNA from different in vitro-grown bacterial strains (WT and  $\Delta$ STM1485) was obtained by growing bacteria in LB at neutral pH values (pH 7.0 and 4.5) at 37°C to mid-log phase using TRI reagent (Sigma). Bacteria grown under static condition at 37°C to mid-log phase in DMEM/5% CO<sub>2</sub> were used as control. These conditions mimicked those used for the cell-infection experiments.

**RT-PCR.** Bacterial RNA (extracted either from infected cells, bacteria grown in DMEM medium or from bacteria grown in LB medium) was reverse transcribed using reverse transcription system (Fermentas) for STM1485 gene and control 16S rRNA gene with primers mentioned in (Table S1). Samples were pretreated with DNase I to remove the genomic DNA. To check the expression level of specific gene, cDNA was amplified for 35 cycles using gene specific primers (Table S1). Control reactions were done without reverse transcription to verify the absence of DNA in RNA preparations.

**Antibodies and confocal laser scanning microscopy (CLSM).** Antisera raised in rabbits against recombinant SseB, and SseD have been described before by Nikolaus et al.<sup>18</sup> RAW264.7 cells were seeded on a coverslip at  $1 \times 10^5$  cells/well in 24-well plates and infection was done as described above. Infected cells were fixed with 3.5% paraformaldehyde in PBS (pH 7.4) (Sigma) for 15 min at indicated time points and then cells were washed thrice with PBS. Cells were incubated with specific antibody [LPS (HyTest Ltd.); SseD (kind gift from Michael Hensel)] diluted in blocking buffer [0.1% saponin, 2% BSA and 2% goat serum (all from Sigma) in PBS] for 1 h. Cells were then washed twice with PBS and incubated with appropriate fluorescent secondary antibodies (DyNova or Jackson Laboratory) diluted in the blocking buffer (SseB: Cy3-conjugated anti-rabbit IgG). In some experiments, Salmonella and heat killed bacteria were stained for LPS using the monoclonal anti-Salmonella Typhimurium LPS antibody (HyTest) raised in mouse, as the primary antibody and FITC-conjugated goat anti-mouse IgG antibody (Dianova) as the secondary antibody. The cells were washed thrice with PBS and coverslips were mounted on a glass slide. After staining images were taken using a confocal laser scanning microscope (LSM Meta, Zeiss).

For detection of SseD in vitro, bacteria expressing GFP were grown in ISM media (pH 5.0) overnight at 37°C. Bacteria were recovered by centrifugation at 5,000 g for 10 min and fixed for 20 min in 3.5% PFA in PBS. Subsequently bacteria were washed twice with PBS very gently. Antibodies were diluted 1:100 in PBS containing 2% BSA and 2% goat serum and added to the fixed bacteria. Antibody staining was performed under shaking conditions for 2 h followed by Cy3-conjugated anti-rabbit IgG for 1 h. After 3–4 washes with PBS, the final pellet was resuspended in 50% glycerol in PBS. Aliquot of stained bacteria were mounted on glass slides, covered and sealed and analyzed for CLSM. Adobe Photoshop 7 was used to adjust the contrast and brightness of the images.

**Texas Red-Ovalbumin (TROv) pulse chasing to load the lysosomes.** RAW264.7 cells were seeded on glass coverslips and were labeled by pulsing with 50  $\mu$ g/ml of TROv (Molecular

Probes) for 30 min at 37°C in 5% CO<sub>2</sub>.<sup>34</sup> The labeling media was replaced with fresh DMEM medium after three washes and incubated further for 30 min. After 30 min of incubation, cells were infected as described above. Infected cells were fixed at respective time points and analyzed. In some experiments, cells were incubated with fluorescent yellow-green latex beads of 1  $\mu$ m size (L4655, Sigma-Aldrich). Images were obtained as projections of Z-stacks and co-localization analysis was done using FCS-CLSM META software.

**Preparation of cell fraction and protein gel blot analysis.** Bacterial strains WT and  $\Delta$ STM1485 were grown overnight in ISM media at pH 5.0. Bacterial lysate, surface and secretory proteins were isolated from the culture as described by Beuzon et al. with slight modifications.<sup>30</sup>

Aliquots containing equal amount of protein were loaded onto 12% SDS-PAGE gel and transferred to nitrocellulose membrane (Millipore) using semi dry gel transfer apparatus. The membranes were treated with 5% skimmed milk for 1 h to block non-specific binding and incubated with primary antibody raised in rabbit against SseB (kind gift from Michael Hensel) (1:2,000) for 2 h and followed by washes. The blots were further stained with goat anti-rabbit IgG-horseradish peroxidase conjugate (AP-Biotech, 1:2,000) for 2 h. The immune complex on the blots were detected with enhanced chemiluminescence substrate (Perkin Elmer) and exposed to Eastman Kodak XAR X-ray film.

For the expression of STM1485::6xHis fusion protein, both the strains were inoculated in LB broth and epithelial cells were infected separately with each strain. Equal amount of total protein from the whole cell lysate (purified bacteria either from infected cells or from those grown in DMEM or LB medium) was processed for immunoblotting.

**Protein secretion analysis.** STM-WT strain,  $\Delta$ STM1485 strain and  $\Delta$ ssaV mutant carrying the His-tagged SseJ construct were grown in F media, pH 5.0 (SPI-2 inducing conditions). Bacterial lysate and secretory proteins were isolated from the culture as described by Freman et al.<sup>42</sup> with slight modifications.

STM1485::6xHis and SseJ::6xHis fusion proteins were probed using HRP-conjugated anti-His antibody (1:20,000, Sigma). As loading control anti-RRF (ribosome recycling factor) antibody was used (a kind gift of Professor Umesh Varshney, Indian Institute of Science, India. Polyclonal, 1:2,000). The WT Salmonella was used as a control to study the expression of His-tagged protein in Salmonella strain expressing STM1485::6xHis fusion protein.

**Competitive index (CI).** Competitive index assay was performed as described previously.<sup>43</sup> The competitive index (CI) was calculated by dividing the number of mutant bacteria recovered from infected animals by the number of wild-type bacteria recovered.<sup>44</sup> This value was then corrected by the initial ratio of mutant to wild-type bacteria used to infect each animal. Values are mean  $\pm$  SD for ten mice from two experiments.

**Statistical analysis and software.** For analyzing data Student's t-test was employed using commercially available software (GraphPad Prism). Statistical significance was defined as follows: \*\*,  $p < 0.005$ ; \*,  $p < 0.05$ . Densitometric analyses performed using Fuji Multi Gauge V2.3 software.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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## Supplemental Material

Supplemental materials may be found here: [www.landesbioscience.com/journals/virulence/article/19029](http://www.landesbioscience.com/journals/virulence/article/19029)

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