RESEARCH PAPER

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TTSS2-deficient *hha* mutant of *Salmonella* Typhimurium exhibits significant systemic attenuation in immunocompromised hosts

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Abbreviations: p.i., post-infection; p.c., post-challenge; p.v., post-vaccination; PBS, phosphate-buffered saline; CFU, colony forming units; TTSS, type-III secretion system; SPI, *Salmonella* pathogenicity island

Non-typhoidal *Salmonella* (NTS) infections are emerging as leading problem worldwide and the variations in host immune status append to the concern of NTS. *Salmonella enterica* serovar Typhimurium is one of the causative agents of NTS infections and has been extensively studied. The inactivation of *Salmonella* pathogenicity island 2 (SPI2) encoded type-III secretion system 2 (TTSS2) has been reported rendering the strain incapable for systemic dissemination to host sites and has also been proposed as live-attenuated vaccine. However, infections from TTSS2-deficient *Salmonella* have also been reported. In this study, mutant strain MT15 was developed by inactivation of the hemolysin expression modulating protein (*hha*) in TTSS2-deficient *S.* Typhimurium background. The MT15 strain showed significant level of attenuation in immune-deprived murine colitis model when tested in *iNos*$^{-/-}$, *IL10*$^{-/-}$, and *CD40L*$^{-/-}$ mice groups in C57BL/6 background. Further, the mutation in *hha* does not implicate any defect in bacterial colonization to the host gut. The long-term infection of developed mutant strain conferred protective immune responses to suitably immunized streptomycin pre-treated C57BL/6 mice. The immunization enhanced the CD4^{+} and CD8^{+} cell types involved in bacterial clearance. The serum IgG and luminal secretory IgA (sIgA) was also found to be elevated after the due course of infection. Additionally, the immunized C57BL/6 mice were protected from the subsequent lethal infection of *Salmonella* Typhimurium. Collectively, these findings implicate the involvement of hemolysin expression modulating protein (Hha) in establishment of bacterial infection. In light of the observed attenuation of the developed mutant strain, this study proposes the possible significance of SPI2-deficient *hha* mutant as an alternative live-attenuated vaccine strain for use against lethal *Salmonella* infections.

Introduction

Salmonella is a bacteria belonging to Enterobacteriaceae family. The serovars of *Salmonella enterica* are facultative intracellular pathogens with efficient colonizing capacity and competence of causing disease in a wide host range. Food products, including poultry, egg, milk, and pork constitutes an important source of *Salmonella* infection in humans.[1] *Salmonella enterica* serovar Typhimurium (hereafter referred to as *S.* Typhimurium) is a broad host range serovar that infects humans, cattle, mice, and chickens, and is one of the major causes of foodborne human salmonellosis.[2,3] In humans, *S. enterica* causes diseases ranging from localized gastroenteritis to disseminated systemic fever.

The pathogenesis of *Salmonella* has been extensively studied in the mouse host.[4-7] In susceptible mice, *Salmonella* causes an acute systemic disease with limited intestinal manifestations.[8] The experimental model to study the *Salmonella* enterocolitis has been developed in streptomycin-pretreated C57BL/6 mice and used with broad scientific acceptance.[9] The *Salmonella* pathogenicity islands (SPIs) 1 and 2 are the two major virulence determinants of *S. enterica*. They encode type III secretion systems (T3SS) that form syringe-like structure on the surface of *Salmonella* and enable the injection of effector proteins directly into the cytosol of eukaryotic cells to establish the infection.[10,11] These effectors ultimately manipulate the cellular functions of the infected host and facilitate the progression of the infection.

A critical step in initiation of salmonellosis is the ability to invade the intestinal cells of the host. The entry process occurs by rearrangement of the cellular membrane in the form of actin-ruffles engulfing the bacteria.[11] The principle behind successful infection by *Salmonella* includes bacterial invasion and survival within nonphagocytic cells, as well as replication within macrophages.[12,13] Host cell invasion depends on the production of SPI1 type III secretion system (TTSS1) that injects effector proteins. The effector proteins trigger rearrangements of the actin cytoskeleton that lead to transient membrane ruffling and bacterial

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uptake.[14,15] Most of the genes that are required for invasion are located at SPI1 genome patch of *Salmonella*;[16,17] however, others proteins important for bacterial survival within the macrophages are located primarily in the SPI2 island. The expression of invasion associated effector proteins is a collective result due to coordinated functioning of SPI1 associated genes. The regulation of SPI1 and SPI2 gene expression involves numerous transcriptional regulators located both inside and outside these pathogenicity islands. Many of the regulatory proteins were reported influencing the expression for invasion associated proteins. Few such proteins include activators HilA, InvF, HilD, HilC, and RtsA and nucleoid-associated proteins H-NS and Hha.[5,18] The activators act in a cascade starting with homologous regulators HilD/HilC/RtsA (Hil activators), followed by the key regulator HilA. It has been shown that two HilD/HilC sites at the *hilA* promoter overlap with upstream binding sites for repressors Hha/H-NS. Hha is a small nucleoid-associated protein (8 kDa) involved in the negative modulation of virulence genes rather than of housekeeping genes in gram-negative bacteria. Hha was originally shown to increase the cytoplasmic expression of hemolysin in *Escherichia coli*[19] and has also been shown to negatively regulate invasion associated genes in *Salmonella*.[20]

Virulence gene regulation in bacterial pathogens is a highly coordinated process involving extracellular sensors and transcription factors that are activated in response to specific environmental cues. In *S.* Typhimurium, the virulence factors required for intracellular growth are encoded on a large pathogenicity island SPI2, which encodes a type III secretion system (TTSS) 2 and a two-component regulatory system called SsrA–SsrB that activates type III system in the intracellular environment.[1] The specific environmental context required for SPI2 activation implied the existence of a repressing system to silence intracellular virulence genes in SPI2 in the absence of an activating environmental signal. The *ssaV* gene is an important structural component of TTSS2 encoded by SPI2. *SsaV* is a structural gene encoding part of the secretion apparatus of TTSS2 complex. SPI2 mutations are attenuating in mice, and there is evidence that SPI2 is required for survival and growth within macrophages, which normally mediate the systemic spread of the organisms. Growing evidence suggests that the roles of SPI1 and SPI2 are not as segregated as previously thought, including the finding that SPI2 is also activated inside the intestinal lumen. However, some of the reports also claim that SPI2-deficient *Salmonella* can also disseminate, though with slow rate, to systemic sites. Also, it has been shown in our previous finding that such *Salmonella* can cause significant lethal infections in various immunocompromised host groups.[21,22] Systemic spread of *S.* Typhimurium *ssaV* mutant, in host system, implicates the involvement of additional proteins favoring the survival of the bacterium.

In this report, we investigated the impact of *hha* mutation in TTSS2-deficient *S.* Typhimurium with respect to systemic colonization and reactivity to immunocompromised hosts. Our results show that TTSS2-deficient *S.* Typhimurium colonizes in the systemic sites of murine colitis models of immunocompromised hosts; however, the access to systemic sites can further be restricted when deletion of hemolysin expression modulating protein Hha is accompanied. Also, we have tried to demonstrate the preliminary observations to establish TTSS2-deficient *hha* mutant of *S.* Typhimurium as live-attenuated vaccine strain safe to be used in immunocompromised mice hosts.

Results

TTSS2-deficient *S.* Typhimurium *hha* mutant (MT15) showed reduced systemic loads in immune-deprived hosts

Salmonella Typhimurium with inactive TTSS2 has been proved to be incompetent in disseminating to host systemic sites to cause lethal infection[23] and thus established as vaccine candidate while being able to elicit protective immune responses in C57BL/6 mice. In our study, we evaluated the pathogenicity of SPI2-deficient *S.* Typhimurium in various isogenic immunocompromised mice of C57BL/6 genetic background; we observed that it causes lethal infections in immune-deprived mice groups. We hypothesized that such dissemination to systemic sites by previously proposed vaccine strain can further be reduced by incorporating additional mutation in the genes possibly involved either in bacterial colonization or systemic replication. Thus, we targeted the *hha* gene of *S.* Typhimurium because of its above discussed properties (see Introduction) and contribution in controlling the TTSS1 associated proteins. We designed a double mutant (MT15) deficient in SsaV structural protein of TTSS2 and Hha hemolysis expression modulating protein of *S.* Typhimurium.

The attenuation characteristics of developed *S.* Typhimurium strains were assessed in C57BL/6 and its immune-deficient mice lines devoid of *iNos*$^{-/-}$, *IL10*$^{-/-}$, and *CD40L*$^{-/-}$. Group of mice (n = 6 each group) were administered with wild-type strain of *S.* Typhimurium (SB300), MT12 (Δ*ssaV*; SPI2-deficient strain), and MT15 (*hha* and Δ*ssaV;* SPI2-deficient *hha* mutant strain). The systemic bacterial dissemination and intestinal colonization was analyzed at day 4 post infection (p.i.). All the strains showed efficient colonization in the gut of various mice lines used in the study (**Fig. 1A**) and we observed no significant differences between the cecal colonization efficacies of subjected mutants among the various mice groups. The bacterial densities in mesenteric lymph node (mLN) of C57BL/6 mice were comparable within the group; however, the bacterial load of MT12 (Δ*ssaV*; SPI2-deficient strain) and MT15 (*hha* and Δ*ssaV;* SPI2-deficient *hha* mutant strain) were significantly different when compared with SB300 (wild-type) load in mLN of all immune deprived mice groups (**Fig. 1B**). It was observed that the strain devoid of SPI2 (MT12) failed to colonize the systemic sites (liver and spleen) of the C57BL/6 mice group; however showed significant colonization to such systemic organs when tested in immunocompromised mice (**Fig. 1C and D**). In comparison to this, the MT15 (SPI2-deficient *hha* mutant) was stringently attenuated in parental C57BL/6 mice and other immune-deprived mice tested in the study (**Fig. 1C and 1D**). Collectively, data suggested that additional inactivation of *hha* in SPI2-deficient *S.* Typhimurium would render the strain incapable of colonizing systemic sites in wild-type mice as well as various immune-deprived host mice tested.

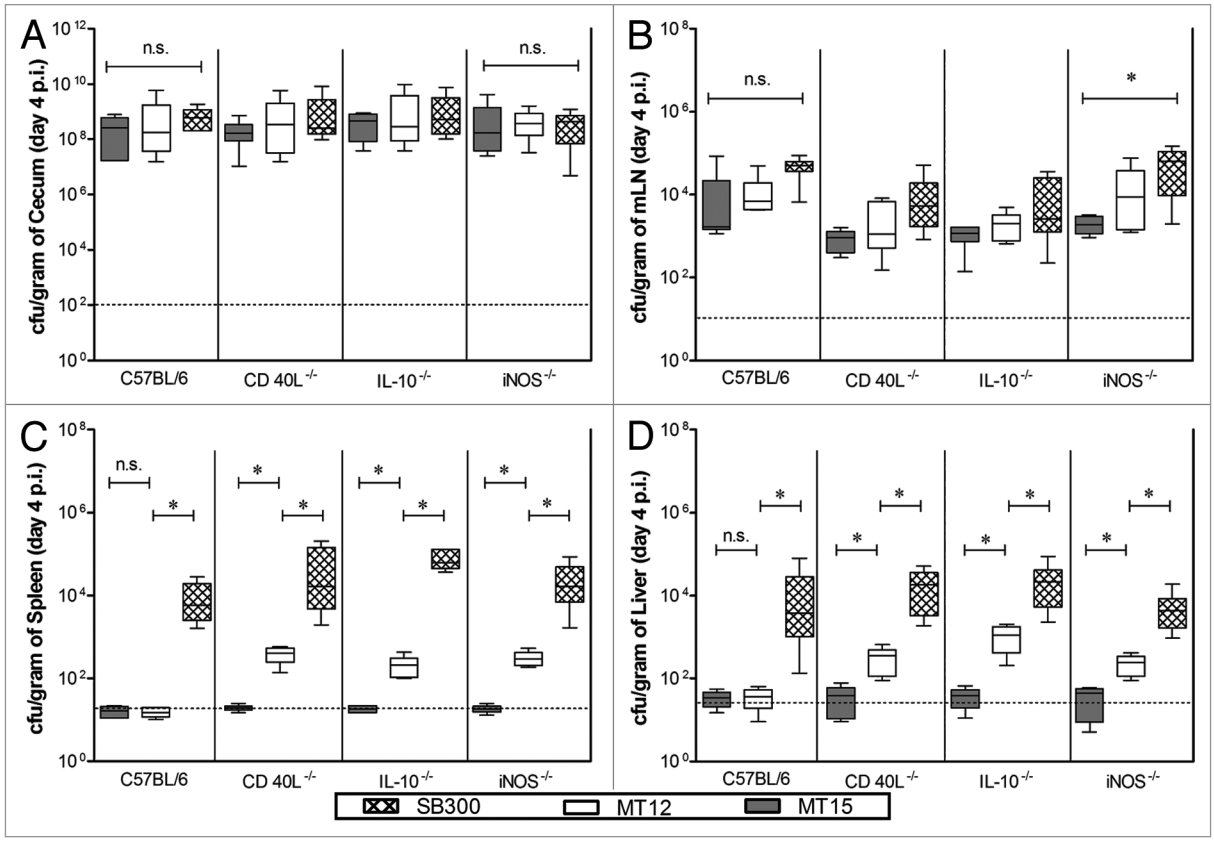

Figure 1. Comparison of M15 fitness in immunocompromised and wild-type C57BL/6 mice. Groups of various immunocompromised mice (*iNos*$^{-/-}$, *IL10*$^{-/-}$, and *CD40L*$^{-/-}$ in C57BL/6 background along with wild-type mice as control group) were infected with 5 × 10^7 cfu of *Salmonella* Typhimurium (wild-type, SB300; *ssaV* mutant, MT12; *ssaV* and *hha* double mutant, MT15) by oral gavage. Mice were sacrificed at 4 d p.i. and bacterial colonization was assessed. Graph represents colonization of *S.* Typhimurium strains at different host sites: (**A**) cecum, (**B**) mesenteric lymph nodes, (**C**) spleen, and (**D**) liver. Broken lines in the graphs shows minimum detection limit. n.s., not significant; *statistically significant ($P < 0.05$, *t* test).

S. Typhimurium MT15 mutant colonizes efficiently to cecal tissues

Intestinal colonization and elicitation of mucosal immune response is an important feature of potent *Salmonella* mucosal vaccine claimed elsewhere. Since the TTSS2-deficient strain was also claimed to be capacitive of efficient cecal colonization and effective luminal immune response elicitation,[24] it was important to check the effect of *hha* mutation on these attributes, especially the intestinal colonization and conferring immune response. Keeping in consideration, wild-type C57BL/6 mice groups (n = 10 mice in each group) were infected/vaccinated with *S.* Typhimurium MT12 (Δ*ssaV*; TTSS2-deficient strain) and MT15 (*hha* and TTSS2-deficient strain). The PBS-treated mice served as control group. We assessed the CFU counts of administered *Salmonella* strains in fecal shedding of infected mice at different time points through the course of infection. Subjected strains reached the cecal densities of 10^8 to 10^9 cfu/g within 24 h of infection. These strains maintained their cecal densities through the course of 30 d infection with a slight decrease during the last 10 d of infection period. However, the fecal shedding of strain MT12 and MT15 was comparable until day 21 p.v. which was suggestive of their efficient gut-colonization efficiency. The bacterial densities of MT15 strain in the feces were slightly reduced at day 30 p.v. ($P < 0.05$) when compared with SPI2-deficient MT12 strain (**Fig. 2A**). To substantiate the observations, the bacterial densities in cecal tissues were also assessed from infected mice groups by euthanizing some of the mice (n = 5) from each group. Equivalent cecal load of MT15 and MT12 strains were reported in cecum at day 30 p.v. These strains disseminated at comparable rate to mLN of infected wild-type C57BL/6 hosts (**Fig. 2B**); however, they failed to infiltrate the systemic organs like spleen and liver. During the infection, the PBS-treated control mice group had no signs of *Salmonella* infiltrations. To facilitate appropriate statistical analysis, the bacterial counts for PBS-treated control group were kept at minimum detection limit. Collectively, competent colonization in the draining lymph node and gut associated mucosa by TTSS2-deficient *hha* mutant (MT15) was suggestive of efficient cecal colonization and possibility of exerting effective immunogenic response in C57BL/6 hosts by the developed MT15 strain.

MT15 strain confers protective immune response

Since the parental TTSS2-deficient *S.* Typhimurium strain (MT12) was previously anticipated as an effective vaccine candidate,[24] we were interested to study the vaccine prospective of developed MT15 strain and to compare the same with its parental strain MT12. To evaluate this, the vaccinated mice groups were

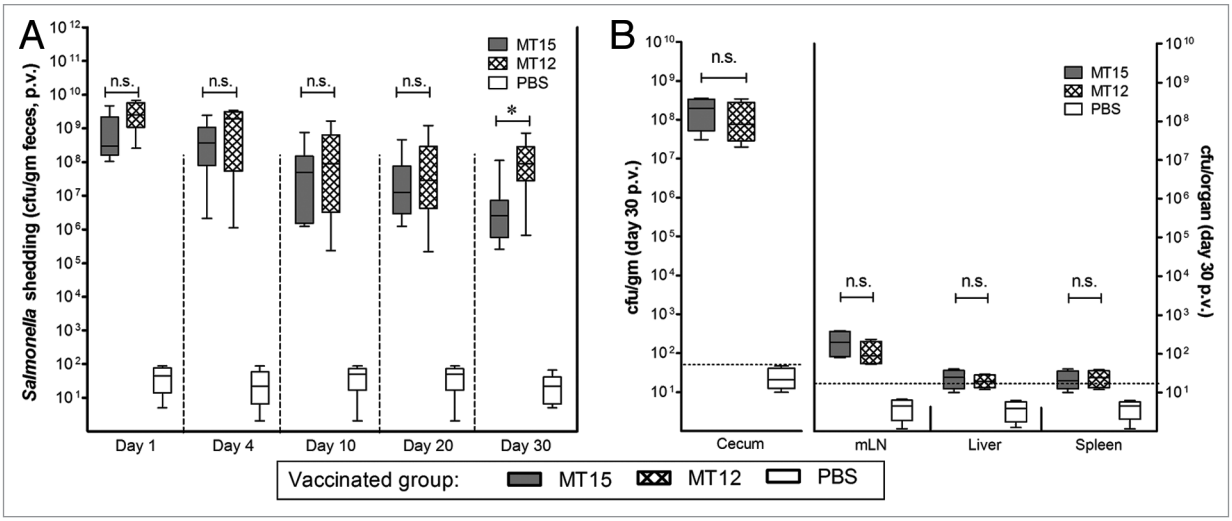

Figure 2. Deletion of *hha* does not impair long-term cecal colonization of MT15. Groups of C57BL/6 mice were vaccinated with 5×10^7 cfu of *Salmonella* Typhimurium MT12 (*ssaV* mutant; $n = 10$) and MT15 (*ssaV* and *hha* double mutant; $n = 10$) by oral gavage. Control mice group was treated with PBS ($n = 10$). (**A**) Fecal shedding of different *Salmonella* strains as analyzed by plating at various time-points. (**B**) Mice from each vaccinated group were sacrificed ($n = 5$) at day 30 post-vaccination (p.v.) to assess the bacterial densities at different host sites. Graph shows the enumeration of bacterial loads at various host sites. Broken lines in the graphs shows minimum detection limit. n.s., not significant; *statistically significant ($P < 0.05$, *t* test).

administered orally with 20 mg of ampicillin in order to clear any colonized test bacterial strains and regrown gut flora from the gut of the remaining mice ($n = 5$) of each vaccinated group (discussed in previous section). After 24 h of ampicillin treatment, these mice groups were challenged with *S.* Typhimurium strain (wild-type; SB300) through oral gavage. These mice were kept under observation and euthanized at day 4 post-challenge (p.c.). Various organ sites were assessed for the bacterial densities and the cecal pathology was gauged. We observed an efficient colonization of the wild-type SB300 challenge bacterial strain to mice gut. These bacterial densities were equivalent with that of the bacterial densities from the control mice group (PBS; $P > 0.05$). The challenged wild-type *Salmonella* SB300 had better access to systemic sites of the non-vaccinated PBS control mice group in comparison to the mice groups those were previously vaccinated with MT15 and MT12 (**Fig. 3A**). Effective infiltration of wild-type *Salmonella* to systemic sites of the PBS-treated mice group was suggestive for absence of any *Salmonella*-specific immune response to counter lethal infections of *Salmonella*. On the other hand, the challenged wild-type SB300 strain failed to colonize to various systemic sites in the mice groups vaccinated with MT12 and MT15; the observation was suggestive of development of successful immunity in vaccinated mice groups (**Fig. 3A**). Assessed cecal pathoscores and representative HE-stained cecal sections of MT15- and MT12-vaccinated mice groups supported in favor of above observations (**Fig. 3B and C**). Collectively, presented observations were supportive of the fact that additional deletion of *hha* in TTSS2-deficient *Salmonella* Typhimurium does not impair its ability to confer protective immune responses in vaccinated C57BL/6 mice.

Vaccination by MT15 enhances T-cell activation

Induction of immune responses depends on the ability of mucosal immunogen to access the gut-associated lymphoid tissues (GALT) of the host to activate the $CD4^+$ and $CD8^+$ cell populations. To analyzing this feature, the impact of *S.* Typhimurium strain MT12 (SPI2-deficient) and MT15 (SPI2-deficient *hha* double mutant) on different cell populations was evaluated and the changes in the $CD4^+$ and the $CD8^+$ cells counts in the mLN was analyzed. The cell populations were analyzed by flowcytometry and were found to be almost equally populated in the MT12 and MT15 vaccinated mice groups but significantly enhanced in comparison to the PBS-treated mice cell counts (compare Q2 of FACS plots in **Fig. 4A**). This clearly indicates that MT15 strain has an ability to induce cell mediated immune response in the vaccinated mice.

Immunization with MT15 shows raised serum IgG and luminal sIgA titers

Immunity toward *Salmonella* infection by *Salmonella* (SPI2-deficient) strain develops basically through luminal secretory IgA (sIgA). The enhanced immune response can also be reflected in titer of IgG in serum.[24] The potential of SPI-2 deficient *hha* mutant (MT15) to mount immune response was validated by analyzing antibody response of subjected mice groups by evaluating serum-IgG and luminal-IgA titers. Thoroughly washed bacterial cells of *S.* Typhimurium (SB300) were treated with suitably diluted serum (1:20, 1:60, 1:120) and gut-wash (undiluted, 1:3, 1:9) samples. Treated cells were incubated with appropriately labeled secondary antibodies (see Materials and Methods section). Washed bacterial conjugates were FACS analyzed and the mean fluorescent intensities were derived with the use of Flow Jo software (v 10.0.5). The serum IgG and intestinal IgA titers observed from the diluted samples of MT12 and MT15 vaccinated mice groups were found comparable with no statistical significance (**Fig. 4B**). However, these antibody titers were found significantly different with observed antibody titers of PBS-treated mice groups. These observations were suggestive of

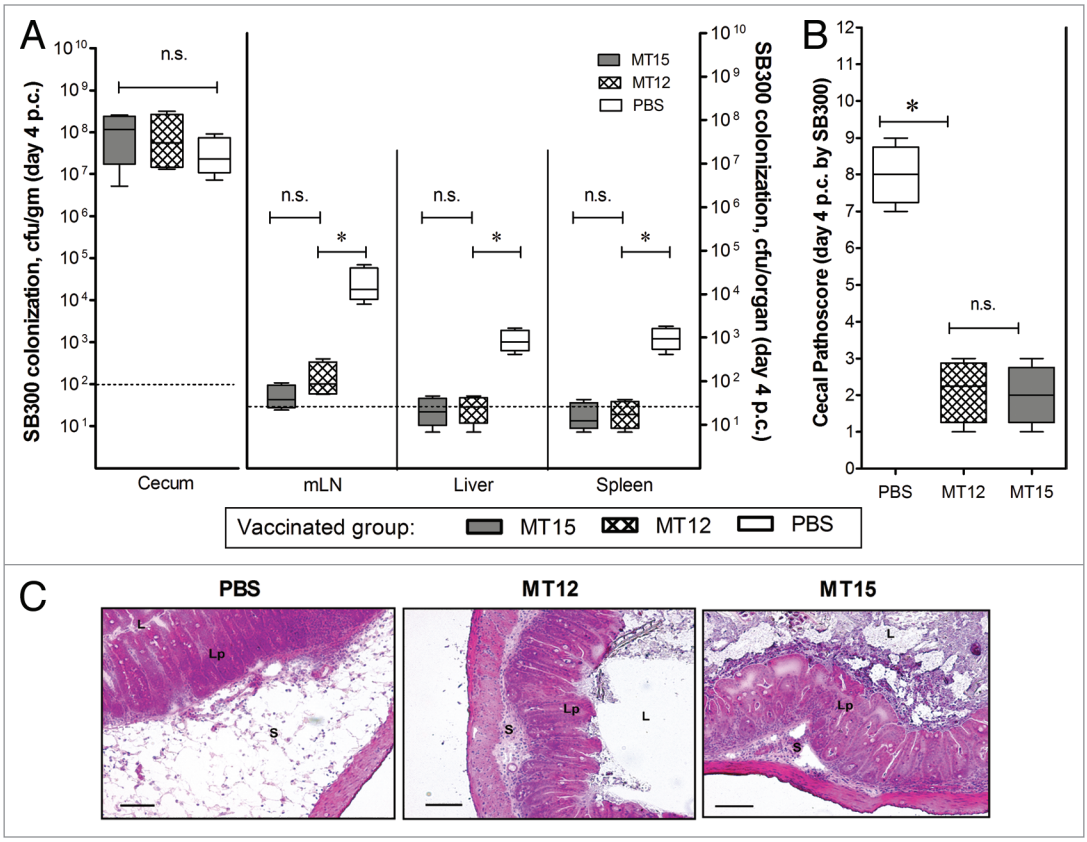

Figure 3. Analysis of vaccination efficacy of M15 *S.* Typhimurium strain. Vaccinated C57BL/6 mice groups (PBS, $n = 5$; MT12, $n = 5$; MT15, $n = 5$) were ampicillin-treated (25 mg by gavage), challenged with wild-type SB300 (amp^r, sm^r) after 24 h of treatment, and euthanized at day 4 post-challenge (p.c.). (**A**) Colonization of SB300 at various host sites after challenge. (**B**) Enumeration of cecal pathology in terms numerical pathoscore was determined as described in material and methods section. Broken lines in the graphs shows minimum detection limit. n.s., not significant; *statistically significant ($P < 0.05$, *t* test). (**C**) Representative HE-stained cecal sections obtained from SB300-challenged mice groups pre-immunized with MT12, MT15, and PBS (control). Bar, 200 μm. S, submucosal edema; Lp, lamina propria; L, lumen.

presentation of unhampered immune response by the MT15 vaccinated mice groups in comparison to the host groups immunized with TTSS2-deficient pre-established MT12 *S.* Typhimurium strain.

Discussion

Non-typhoidal *Salmonella* (NTS) infections are emerging as serious problem for vital appeal worldwide. To date, no permanent solutions are advised for NTS infections. However, the development of live-attenuated vaccines (LAV) targeting the leading causative agents of NTS is of high importance. Young children (<3 y) and HIV-infected individuals were reported prone to develop fatal outcomes upon NTS infection.[25] The Ty21a and the Typhim Vi are the two possible vaccines currently available for the typhoid prevention.[26] Experiments on numerous attenuated *Salmonella* vaccine strains or LAV with protective potential against *Salmonella* infection have employed *S.* Typhimurium murine infection models.[7] Such LAV strains induce relatively efficient immunogenicity than the killed organisms.[27,28] Unfortunately, most of the promising vaccine candidates provide only short-term and incomplete protection and show often an insufficient efficiency in young children.[29-31] The mutant of structural protein of TTSS2 (*ssaV* mutant) has been reported to confer protection against *S.* Typhimurium infection.[24] However, in a recent study, the TTSS2-deficient Typhimurium strain was reported with increased replication within the infected phagocytes and macrophages.[32] The biggest challenge in developing live vaccine candidates is the generation of a *Salmonella* strain that provides both immunogenicity and safety (virulence attenuated) even when used in immune deprived hosts.

In our study, we focused on the bacterial attenuation by inactivating *hha* in SPI2-deficient background. Though it has been discussed and proved that *Salmonella* disseminate to the systemic sites even after TTSS2 inactivation, we showed that complete attenuation can be achieved upon additional inactivation of *hha* that has been reported earlier to be involved in environmental regulation of virulence factors of the bacterium. This can be explained by considering the interaction of *hha* with other genes as seen (**Fig. S1**) from STRING, a web-based protein interaction prediction tool.[33] Hha is a hemolysin expression modulating protein of *E. coli*[19] and its homolog is present in the genome of *S.* Typhimurium (*STM0473*). The direct interaction of Hha and H-NS has been reported to alter the expression if invasion

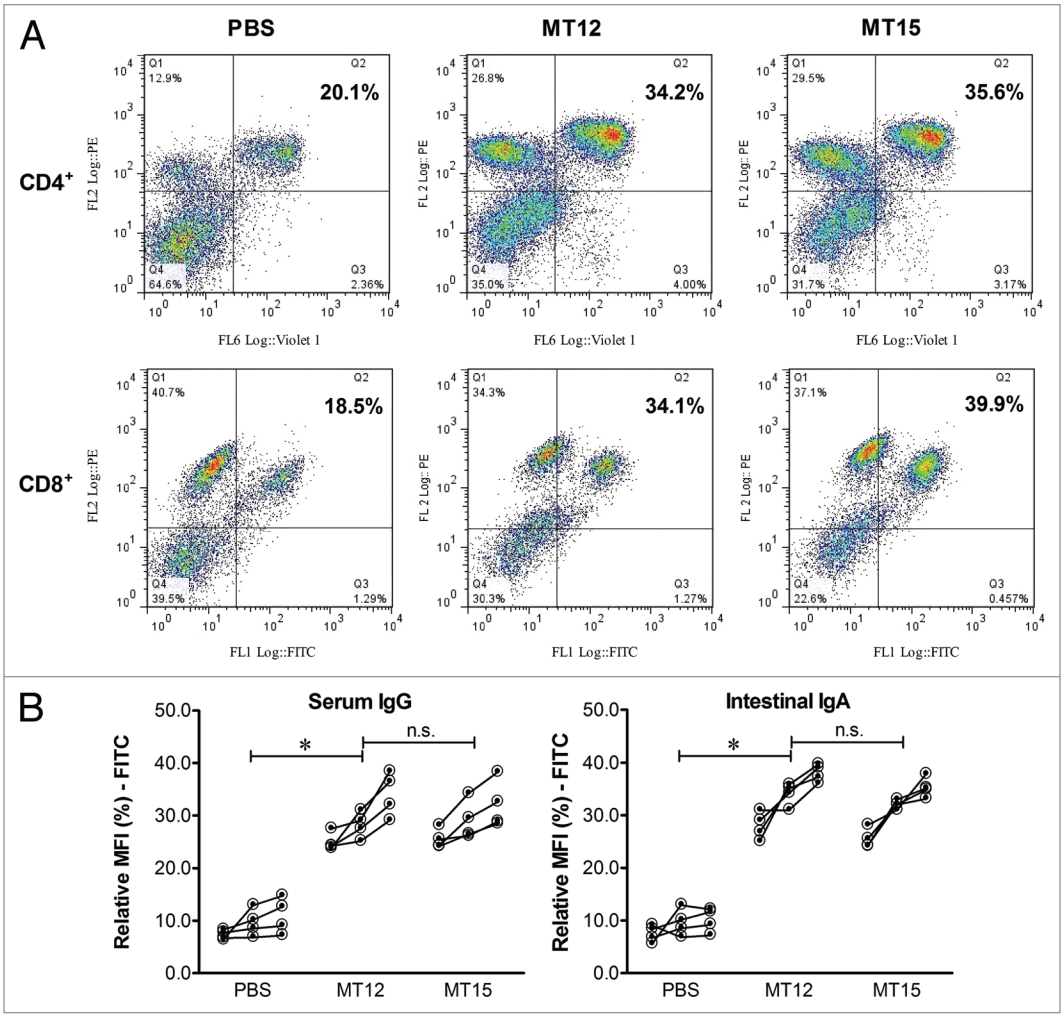

Figure 4. Analysis of host immune response together with assessment of CD4/8$^+$ cell population in immunized mice. The mesenteric lymph nodes (mLN) from remaining mice vaccinated with MT12, MT15, and PBS were collected in 500 μl of RPMI medium (with 10% FCS) at day 30 p.v. The homogenates were prepared and cellular fractions were collected. After processing, cells were reacted with CD4- and CD8-specific antibodies and FACS analyzed for cell densities. (**A**) Representative FACS graphs depicting CD4$^+$ (pacific blue-labeled) and CD8$^+$ (FITC-labeled) cell populations (Q2 in each graph) in various immunized mice groups. (**B**) Assessment of antibody response (serum IgG and intestinal IgA) by bacterial FACS. Different dilutions of serum and gut-wash from vaccinated mice were reacted with prewashed overnight statically-grown wild-type *S.* Typhimurium followed by incubation with FITC-conjugated respective antibodies to mouse IgG or IgA. Cellular fraction was collected, washed, and resuspended for FACS analysis. Figure shows antibody titer for serum IgG and luminal secretory-IgA (sIgA) from collected sample with all tested dilutions.

associated proteins of *Salmonella*.[34] Further, the STRING tool shows Hha interaction with Rck, the outer membrane protein of *Salmonella* that helps in bacterial invasion through PI 3-kinase-Akt signaling-dependent Rac1 activation.[35,36] Such proven and other predicted interactions indicate for the possible involvement of Hha in bacterial fitness during the course of infection.

While considering immunity for bacterial infections, the fine-tuning of activation and restraining of host immune system becomes crucial to minimize host injury. The host T cells plays an important role in establishing equilibrium between bacterial clearance and propagation[37] and many of the reports advocate for the enhanced T-cell response in infections with *Salmonella* strains.[38,39] The T_H cells (CD4$^+$) governs the bacterial clearance from the host tissues possibly by activation of the *Salmonella*-containing mononuclear cells. However, the T_C cells (CD8$^+$) support protection against bacterial infection by clearing intracellular *S.* Typhimurium from infected macrophages.[40] In this study, we observed the induction of CD4/8$^+$ cells in host mice vaccinated with *S.* Typhimurium for 30 d. Such increase in T-cell counts in suitably immunized mice hosts might be considered to grant efficient defense against subsequent infection with *Salmonella*.

As predominant antibody class, sIgA in the external secretions plays an important role in immune protection. The sIgA has been reported in impairing pathogens attachments to epithelial cell receptors. The cholera toxin,[41] reovirus type 1 Lang,[42] and few more exhibit same mechanism. Furthermore, immune exclusion is a suggested mechanism by which sIgA prevents pathogens from attaching, colonizing, and invading mucosal epithelial cells.[43] As per our findings, in *Salmonella*-immunized mice, invasion of *S.* Typhimurium into the epithelium was significantly impaired.

We observed an excellent sIgA response from the immunized mice groups which resulted in protection against subsequent *Salmonella* infection. This cannot be explained by receptor blocking or immune exclusion, rather the sIgA directly affect bacterial virulence factors to impair epithelial invasion by *S.* Typhimurium.[44] A thorough considerate of the sIgA functions in intestinal immunity would be appreciable with respect to designing of competent mucosal vaccines.

As conclusion, the developed double mutant of *Salmonella* Typhimurium strain MT15 confers protection by inducing proficient mucosal and serum immune responses against *Salmonella* Typhimurium infections. This strain (MT15) was found attenuated in various immunocompromised host mice tested in this study. Based on the available reports, it can be speculated that deletion of *hha* in strain MT15 possibly alters the *Salmonella* proteins responsible for its entry to the host cells and thus suppresses the MT15 infection profile. Overall, the study addresses involvement of *hha* in bacterial infection and dissemination and it could be a potential target in developing live-attenuated vaccine particularly benefiting immune-deprived hosts. Further, to have a relative investigation of the niche supporting the attenuation properties of MT12 and MT15 in experimental hosts with respect to their genetic background would be interesting. Additionally, detailed study on involvement of secretory IgA toward the protection against *Salmonella* challenges through MT15 vaccination could possibly reveal unexplored immunological links in conferring protective immunity by such genetically modified strain.

Material and Methods

Bacterial strains used in the study

Bacterial strains (**Table 1**) were cultured in Luria–Bertani medium (LB) supplemented with 0.3 M NaCl for 12 h at 37 °C. For infection experiments, all the bacterial strains were diluted 1:20 in fresh LB medium and sub-cultured for another 4 h under mild aeration until an optical density of 0.6 was achieved. Harvested bacterial cells were washed in ice-cold phosphate buffered saline (PBS) and about 5×10^7 CFU were suspended in 50 μl cold PBS for in vivo experiments. All strains were tested for growth attenuation.

Development of genetic mutant strains

Deletion mutants of *Salmonella* Typhimurium genes *hha* (MT15; *hha::aphT*) and *ssaV* (MT12; Δ*ssaV*) were developed using red-recombinase plasmid pKD46, template plasmid pKD4, and pCP20 flip-recombinase plasmid as per the standard lambda-red recombinase system protocol[45,46] using the primers listed in **Table 1**. The stable double mutant MT15 (*hha::aphT*, Δ*ssaV*) was generated by transducing the *hha::aphT* mutation into the recipient MT12 (Δ*ssaV*) strain. All the mutations were confirmed by PCR using the *hha*- and *ssaV*-specific confirmatory primers listed in **Table 1**.

Ethical statement

All the animal experiments were performed in strict accordance with guidelines laid by the Institutional Animal Ethics Committee (IAEC) of National Center for Cell Sciences (NCCS) Pune, India. This study was approved by the IAEC of NCCS; Permit Number: 7/1999/CPCSEA-09/03/1999. All efforts were made to minimize suffering of animals during experimentation.

In vivo animal experimentation for evaluation of bacterial dissemination

For in vivo animal experimentations, individually ventilated cages were used to house the experimental animals as described previously.[9] Specific pathogen-free (SPF) mice were used for all in vivo experimentations. C57BL/6, *iNos*$^{-/-}$ (B6.129P2-*Nos2tm1Lau*/J), *CD40L*$^{-/-}$ (B6.129S2-Cd40lg^{tm1Imx}/J), and *IL10*$^{-/-}$ (B6.129P2-IL10^{tm1cgn}/J) mice from Jackson Laboratories were bred in the C57BL/6 background at the animal facility of NCCS, Pune. Mice (n = 6 each group) were pretreated with 50 mg of streptomycin, intragastrically, before infecting with any of the mentioned *Salmonella* strain. After 24 h of antibiotic treatment, mice were infected with 5×10^7 CFU of the corresponding bacterial strain (i.e., MT12, MT15, and SB300) with the help of oral gavage. During the course of infection the bacterial loads in the cecum, mesenteric lymph nodes (MLNs), and other systemic sites were enumerated by plating the tissue homogenates on MacConkey agar plates supplemented with appropriate antibiotics (streptomycin, 50 μg/ml; kanamycin, 50 μg/ml; ampicillin, 20 μg/ml; chloramphenicol, 20 μg/ml).

Evaluation of protective efficacy of mutant strains

C57BL/6 mice pretreated with streptomycin were used for vaccination by different *Salmonella* strains as established previously.[9] Concisely, three mice groups (n = 10) were vaccinated (immunized) with mutant MT12 and MT15 *S.* Typhimurium strains; the PBS-treated mice group served as the control. For accounting fecal shedding of bacterial strains, the fecal sample from all the mice of each group was collected at various time points and its suitably diluted homogenates were grown on MacConkey agar supplemented with antibiotics. At day 30 post-vaccination (p.v.), the bacterial load in various host sites along with cecum contents were determined by sacrificing some of the mice (n = 5) from each group. For statistical analysis, samples with no bacterial count were adjusted to the minimal detectable levels. Remaining mice (n = 5) from each vaccinated group were treated orally with ampicillin (25 mg) to clear the residual *Salmonella* or other microbial flora from mice gut. Mice groups were further challenged (infected to see the efficacy of vaccination) after 24 h of ampicillin treatment by introducing 200 cfu of wild-type *S.* Typhimurium SB300 strain harboring pM973 (conferring ampicillin resistance), through oral gavage.[47] The colonization efficiency of challenge strain SB300 at various host sites was assessed at day 4 post-challenge (p.c.). For cryosectioning, mice tissues were collected in an Optimum Cutting Temperature compound (OCT, Sakura Finetek Inc.), snap frozen in liquid nitrogen and stored at −80 °C. Cecal inflammation of the infected tissue was scored as described in the next section.

Evaluation of cecal inflammation

Cryopreserved O.C.T. (Sakura Finetek Inc.) embedded segments of the ileum, cecum, and colon were sectioned at −30 °C to the thickness of 4 μm and collected on glass slides and air-dried. The tissue sections were stained with hematoxylin and eosin (H&E) stains and the pathology was evaluated on the

Table 1. Bacterial strains, plasmids, and primers used in study

Strains	Genetic information	Background	References
SB300	*Salmonella* Typhimurium; Sm^r	Wild type	24
M1525	*Salmonella* Enteritidis P125109 wild type; Sm^r	Wild type	48 and 49
MT12	*S.* Typhimurium Δ*ssaV*; Sm^r	SB300	This study
MT14	*S.* Typhimurium *hha::aphT*; Sm^r, Km^r	SB300	This study
MT15	*S.* Typhimurium *hha::aphT*, Δ*ssaV*; Sm^r, Km^r	MT12	This study
Plasmids	**Relevant genotype (S) and/or phenotype (S)**	**Resistance**	**References**
pKD4	Template plasmid; FRT-*aphT*-FRT	Km^r	45
pKD46	Red recombinase expression plasmid; P_{araB}; oriR101	Amp^r	45
pCP20	FLP recombinase expression plasmid	Cm^r, Amp^r	45
pM973	*bla* P*ssaH* gfpmut2 plasmid with *ori*pMB1	Amp^r	22
Primers	**Sequence (5′ to 3′)**		
Fw-hha	AGGCAGATAA CACCTGCGTG TTCTCTAAAA AGTAATGTAG CGTGATGTGT AGGCTGGAGC TGCTT		
Rw-hha	GTTAGTTTGT CTTGTTAAAA ATTATTACAA TCATAGGTAG AATTTATATG AATATCCTCC TTAGT		
Conf-hha	GTGATCTATA TCACTGTTCT ATAATAGCC		
Fw-ssaV	TCATCGACAA ATAAAATTTC TGGAGTCGCA ATGCGTTCAT GGTTATGTGT AGGCTGGAGC TGCTT		
Rw-ssaV	ATTTCAGCCT CAGACGTTGC ATCAATTCAT TCTTCATTGT CCGCCATATG AATATCCTCC TTAGT		
Conf-ssaV	GCAATGAGTT GTTCTCCACC		

basis of previously described scoring system for the quantitative analysis of cecal inflammation.[9,47,48] All the cecal sections were scored independently on the basis of pathological changes that include epithelial ulceration (0–3), sub-mucosal edema (0–3), loss of goblet cells (0–3), and polymorphonuclear leukocyte infiltration (0–4) with a summation score ranging between 0–13. The individual total score of any cecal tissue reflected the degree of inflammation. The independent pathoscores of each tissue sample were averaged. The combined pathological scores represents the inflammation levels that included intact intestine without any sign of inflammation (pathoscore 0); minimal sign of inflammation (pathoscore 1–2), which is commonly found in the ceca of specific pathogen-free mice and generally not considered as a pathological feature; slight inflammation as a minimal sign of tissue pathology (pathoscore 3–4); moderate inflammation (pathoscore 5–8); and significant inflammation (pathoscore 9–13).[49]

Assessment of CD4/8$^+$ cellular counts by flow cytometry

For evaluation of cellular response the mesenteric lymph node (mLN) from the vaccinated mice groups was collected at day 30 post-vaccination. The mLN were collected in 500 µl of RPMI medium (Lonza) supplemented with 10% fetal calf serum (FCS; Lonza). Homogenates of mLNs were centrifuged at 140× g for 8 min at 4 °C. The cellular fractions were resuspended in 1 ml RPMI-FCS medium. Around 10^6 cells were resuspended in 50 µl of FACS buffer and incubated with different CD4/8$^+$ cell specific antibodies (PacificBlue anti-mouse αCD4, BD PharMingen; FITC anti-mouse αCD8, BD PharMingen) for 90 min at room temperature. Finally, the cells were washed and assessed by FACS Canto (BD Biosciences) and analyzed using FlowJo software (v10.0.5).

Evaluation of serum and gut antibody response

The serum IgG and luminal secretory IgA levels were measured by FACS for assessment of the mucosal immune responses as described previously.[23] Serum and gut washes from vaccinated mice groups were collected at the termination of the vaccination period, i.e., day 30 p.v. For FACS analysis of *Salmonella*-specific IgG and IgA response, the wild-type *S.* Typhimurium (SB300) was grown overnight in static LB broth at 37 °C. The bacterial cells were harvested and washed twice with 1% BSA and 0.05% sodium azide prepared in sterile PBS. Finally, the cells were suspended in PBS to have bacterial density of 10^7 cfu/ml. For assessing serum IgG response, the inactivated mice serum was diluted to 1:20, 1:60, and 1:120. Similarly, for evaluation of *Salmonella*-specific secreted luminal IgA, the clear supernatants of luminal contents from vaccinated mice groups were treated as undiluted, 1:3, and 1:9 dilutions in PBS. Further, a 25 µl of diluted serum samples and the gut washes were mixed separately with 25 µl bacterial suspension of *Salmonella* Typhimurium (SB300) in PBS and incubated for 1 h at 4 °C followed by washing with PBS. The washed bacterial cells were reacted with FITC-conjugated monoclonal anti-mouse IgG and IgA antibodies (Abcam) for serum and gut-wash samples respectively, and incubated for 1 h at 4 °C. Finally, cells were washed twice (in PBS with 1% BSA, 0.05% sodium azide) and resuspended in PBS (with 2% PFA). Samples were processed in FACS Canto analyzer (BD Biosciences).

Statistical analysis

Statistical analyses were performed using *t* test and other suitable statistical analysis parameters (Prism 5; GraphPad Software) when needed. Probability with $P < 0.05$ was considered statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/virulence/article/27605/

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