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Original article

Deletion of *invH* gene in *Salmonella enterica* serovar Typhimurium limits the secretion of Sip effector proteins

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Abstract

The type-III secretion system-I (T3SS-I) of *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) is an essential component to mediate active invasion and subsequent inflammation in genetically susceptible C57BL/6 mice. *S.* Typhimurium translocates its effector proteins through Salmonella Pathogenicity Island-I (SPI-I) encoded T3SS-I needle complex. This study focuses on *invH* gene of *S.* Typhimurium, which plays an active role in SPI-I mediated effector protein translocation. The deletion of *invH* gene in *S.* Typhimurium reduced the invasion efficiency of the bacterium to 70–80% as compared to wild-type *S.* Typhimurium (SB300) in vitro. To further investigate the role of *invH* gene exclusively in SPI-1 mediated inflammation, C57BL/6 mice were infected with *S.* Typhimurium double mutant deficient in *invH* and *ssaV*. Results indicated significant difference in the degree of cecal inflammation between wild-type *S.* Typhimurium and double mutant at 12 h and 48 h post infection. However this difference was found to be more prominent at 12 h p.i. In line with our findings, analysis of effector protein secretion in *invH*, *ssaV* double mutant showed reduced secretion of Sip effector proteins (SipA, SipB, SipC and SipD) as compared to the wild-type strain. The inflammation phenotype was restored on complementing *invH* to its respective double mutant strain. Altogether, the current study proposes a possible role of *invH* gene in early cecal inflammation by *Salmonella* Typhimurium in mice colitis model.
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Keywords: *Salmonella* Typhimurium; SPI-I; SPI-II; Type III secretion system; *invH*

1. Introduction

S. Typhimurium is one of the most common food and water borne pathogen causing diseases ranging from severe gastroenteritis to systemic infections in human. Invasion of *S.* Typhimurium into the intestinal epithelium involves the subversion of the host signal transduction pathways that induce cytoskeletal changes and membrane ruffling to help internalization of the bacteria [1–3]. The process by which *S.* Typhimurium invades the epithelial cells depends upon T3SS-I encoded by SPI-I present at the centisome 63 and controlled by two regulators, *invF* and *hilA* [4–6]. This machinery secretes various virulence effector proteins like InvJ, SpaO, SipA, SipB, SipC, SipD, SptP and AvrA and may also translocate a subset of these into the host cell cytosol [7–9].

SPI-I of *S.* Typhimurium encodes a series of genes of the *prg*, *hil*, *sip*, *spa* and *inv* operon [4]. These set of genes along with non-SPI encoded effector proteins are responsible for stimulating inflammatory responses in cultured epithelial cells and developing inflammation in the gut of different hosts including streptomycin pre-treated C57BL/6 mice [10,11]. Genes like *invA*, *invC* and *invG* are known to be a part of the secretion apparatus while *invB* gene being a chaperone restricts the translocation of effector proteins when knocked out from the SPI-I gene complex [12–14]. Mutation in *invA*, *invB*, *invC* and *invG* genes of *inv* operon results in the reduction of virulence and colonization of *Salmonella* by the oral route [12,14,15]. Except for *invH* all genes of *inv* operon

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are well known for their contribution in the virulence of *S.* Typhimurium. InvH is an outer membrane lipoprotein which facilitates the translocation of InvG from the cytoplasm onto the membrane [16]. In an earlier study, mutation in *invH* gene has been shown to reduce enteritis in the calf model [17]. However the role of *invH* in T3SS-I mediated host cell manipulation has remained poorly defined. To examine the effect of *invH* deletion exclusively in SPI-1 induced *S.* Typhimurium virulence, we investigated the inflammation profiles in C57BL/6 mice infected with *invH*, *ssaV* double mutant for 12 h and 48 h p.i. (post infection). In contrast to bovine and chick model, our study showed a delayed inflammation in the streptomycin pretreated mouse model of *Salmonella* induced colitis due to inadequate secretion of Sip effector proteins [18,19].

2. Materials and methods

2.1. Ethics statement

All animals were handled in strict accordance with good animal practice as defined by the relevant national and/or local animal welfare bodies, and all animal work was approved by the appropriate committee (Kantonales Veterinaramt Zurich, Zurich Switzerland, license number ZH201/2007).

2.2. Bacterial strains and plasmids

The naturally streptomycin resistant *S.* Typhimurium strain SB300 was taken as the wild-type strain [20]. The isogenic *invH*$^-$ and *ssaV*$^-$ double mutant (MT1; *invH::aphT*, *ssaV::cat*), T3SS-I$^-$ (MT7; *invC::aphT*), T3SS-II$^-$ (MT2; *ssaV::cat*), T3SS-I$^-$ and T3SS-II$^-$ double mutant (MT3; *invC::aphT*, *ssaV::cat*) of SB300 were generated by P22-transduction. The strains are listed in Table 1. All strains were verified by PCR for mutation at specific locus in the mutants. Plasmid pM973, expressing GFP under the control of a T3SS-II *ssaG* promoter (i.e. after host tissue invasion) was used for the localization study of the bacteria in vivo [21]. In the strain MT1 (*invH::aphT*, *ssaV::cat*), *invH* mutation was complemented by a plasmid pInvH (MT9).

2.3. Bacterial growth condition

Bacteria were grown for 12 h at 37 °C in Luria–Bertani (LB, 0.3 M NaCl) broth, diluted 1:20 in fresh LB medium and sub cultured for 4 h under mild aeration. Bacteria were washed in ice cold phosphate buffered saline (PBS). For mice infections, 5×10^7 colony forming units (CFU) were suspended in 50 µl cold PBS; and for tissue culture infections, various CFU dilutions in PBS were used.

Table 1
Strains used in this study.

Strains	Genetic information	Background	Reference
SB300	Serovar Typhimurium, Sm^R	Wild Type	[14]
MT1	*invH::aphT*, *ssaV::cat*	SB300	This work
MT2	T3SS-II$^-$, *ssaV::cat*	SB300	This work
MT3	T3SS-I&2^{--}(*invC::aphT*, *ssaV::cat*)	SB300	This work
MT7	T3SS-I$^-$(*invC::aphT*)	SB300	This work
MT9	*invH* complemented to MT1	SB300	This work

2.4. Mice infection experiment

The in vivo experiments were executed in streptomycin pretreated specific pathogen free (SPF) C57BL/6 mice because of their susceptibility to many broad and narrow host range *Salmonella* species [22]. Infections of mice were conducted in individual ventilated cages [23]. All the mice were bred at the Rodent Center HCI, ETH Zürich. Five mice were placed in each group. Mice were pretreated intra-gastrically with 50 mg of streptomycin and infected 24 h later with 5×10^7 CFU (by gavage) of the corresponding bacterial strain. Bacterial loads in cecal content, mesenteric Lymph Node (mLN), liver and spleen were determined by plating homogenates on MacConkey agar plates supplemented with appropriate antibiotic/s [24].

2.5. Histopathological evaluation

Cryosections of cecal tissue were stained with hematoxylin and eosin and the pathoscores were determined by an expert pathologist as described earlier [24]:

A. The submucosal edema (se) was scored from the extension of the submucosa and scored by morphometric analysis according to the formula: % se = $(b - a)/c$ where a = area enclosed by the mucosa; b = area enclosed by the borderline between the submucosa and tunica muscularis; c = area enclosed by the outer edge of the tunica muscularis. Submucosal edema: 0 = If no pathological changes; 1 = Slightly detectable edema; 2 = Moderate edema; 3 = Profound edema.
B. PMN (polymorphonuclear neutrophils) infiltration into the lamina propria: PMN in the lamina propria were enumerated in 10 high power fields (hpf) and the average number of PMN/hpf was calculated. The scores were: 0 = If less than 5 PMN/hpf; 1 = If 5–20 PMN/hpf; 2 = If 21–60 PMN/hpf; 3 = If 61–100 PMN/hpf; 4 = If more than 100 PMN/hpf.
C. Goblet cells: The average number of goblet cells per hpf (400X) was calculated from 10 different regions of the tissue section. The scores were 0 = If more than 28 goblet cells per high power field. In the cecum of the normal SPF mice we observed an average of 6.4 crypts per high power field and the average crypt consisted of 35–42 epithelial cells, 25–35% of which were differentiated into goblet cells; 1 = If 11–28 goblet cells per hpf; 2 = If 1–10 goblet cells per high power field; 3 = If less than 1 goblet cell per hpf.
D. Epithelial integrity: The scores were: 0 = If no detectable changes in the pathology; 1 = epithelial desquamation; 2 = erosion in the epithelial surface; 3 = epithelial ulceration (gaps of >10 epithelial cells per lesion).

The individual pathoscore for each tissue sample was determined as the sum of these averaged scores. 0 = tissue

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