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Salmonella spv locus suppresses host innate immune responses to bacterial infection

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ABSTRACT

Salmonella enterica serovar typhimurium (*S.* typhimurium) is globally distributed and causes massive morbidity and mortality in humans and animals. *S.* typhimurium carries *Salmonella* plasmid virulence (*spv*) locus, which is highly conserved and closely related to bacterial pathogenicity, while its exact role in host immune responses during infection remains to be elucidated. To counteract the invaders, the host has evolved numerous strategies, among which the innate immunity and autophagy act as the first defense. Recently, zebrafish has been universally accepted as a valuable and powerful vertebrate model in analyzing bacteria-host interactions. To investigate whether *spv* locus enhances the virulence of *Salmonella* by exerting an effect on the host early defense, zebrafish larvae were employed in this study. LD_{50} of *S.* typhimurium to zebrafish larvae and bacterial dissemination were analyzed. Sudan black B and neutral red staining were performed to detect the responses of neutrophils and macrophages to *Salmonella* infection. Autophagy agonist Torin1 and inhibitor Chloroquine were used to interfere in autophagic flux, and the protein level of Lc3 and p62 were measured by western blotting. Results indicated that *spv* locus could decrease the LD_{50} of *S.* typhimurium to zebrafish larvae, accelerate the reproduction and dissemination of bacteria by inhibiting the function of neutrophils and macrophages. Moreover, *spv* locus restrained the formation of autophagosomes in the earlier stage of autophagy. These findings suggested the virulence of *spv* locus involving in suppressing host innate immune responses for the first time, which shed new light on the role of *spv* operon in *Salmonella* pathogenicity.

1. Introduction

Salmonella enterica serovar typhimurium (*S.* typhimurium) is one of the major food borne pathogen causing worldwide infections, and results in gastroenteritis and systemic infection in a broad range of hosts including a variety of livestock, poultry and humans, and it remains a global health problem [1]. *S.* typhimurium generally carries a virulence plasmid with about 8 kb region of highly conserved gene sequence, called *spv* (*Salmonella* plasmid virulence) locus, which is related to bacterial serum resistance, adhesion and colonization, and can promote bacterial growth and reproduction in host cells and tissues [2,3].

Among the complex process of host immune responses to *S.* typhimurium infection, the innate immune response is the first defense line. Neutrophils and macrophages are phagocytes in host innate immune system, which play crucial roles in the clearance of *Salmonellae*. Neutrophils are very important for limiting dissemination of *S.* typhimurium in humans by shifting to the microbial invader and exerting phagocytosis [4]. As facultative intracellular bacteria, *Salmonellae* reside in both epithelial cells and macrophages. By promoting inflammation and phagocytosis, macrophages play pivotal roles in fighting infectious disease [5]. Furthermore, the invasion of the causative agent could result in rapid induction of autophagy, a significant host immune defense against intracellular microorganisms. Autophagy is a dynamic process, consisting of three sequential stages including the formation of phagophore, then autophagosome and ultimately the autophagosome fuses with the lysosome [6]. Autophagy is an evolutionarily conserved process in which intracellular membrane structures sequester proteins and organelles for lysosomal degradation [7]. Unfortunately, pathogens involve different strategies to escape from the host defense. It is well known that *spv* locus is closely associated with pathogenicity of *S.* typhimurium, while the

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interaction between *spv* locus and the host early defense including the responses of neutrophils, macrophages and autophagic flux remains undefined.

Growing evidence suggests that zebrafish with many advantages could be an efficient animal model for study on immunity and infectious diseases. The advantages of the zebrafish model include small size, simple structure, rapid growth, relatively short life cycle, ease of breeding, and a transparent body in early life stages *etc*. In addition, as a vertebrate model, zebrafish possesses a complete immune system comprising the similar primary components as humans [8]. Taken together, zebrafish is increasingly used as a model to study infections with pathogens. Both zebrafish embryo and larvae model proved especially useful due to their transparency and only with the innate immune system [9–11]. Especially, zebrafish was regarded as a new model for the *in vivo* study of bacteria interaction with phagocytes and autophagy [12]. As an operon structure, the *spv* comprises five open reading frames (ORF), including *spvABCD* genes and the upstream transcriptional activator *spvR* [13]. In a recent study, we employed the zebrafish model and found that *spvB* gene could enhance bacterial virulence by inhibiting autophagy [14]. While host responses to the whole operon as well as the interaction between *spv* locus and host phagocytes upon *Salmonella* infection are still unclear. In the present research, the effect of *spv* locus on host innate immunity including the responses of neutrophils, macrophages and autophagic flux was further explored using zebrafish larva model.

2. Materials and methods

2.1. Bacterial strains and culture

The virulent wide type strain *S.* typhimurium UF009 (STM-WT) and *spv* locus deletion-mutant strain UF110 (STM-*Δspv*) were kindly supplied by Professor Paul A. Gulig [15]. Both strains were grown to mid-logarithmic phase at 37 °C in Luriae Bertani (LB) broth. Strains with red fluorescence protein (RFP-STM-WT and RFP-STM-*Δspv*) were constructed in our laboratory, and were cultured in the media with 100 μg/ml Ampicillin. Bacteria were harvested by centrifugation at 3000×*g* for 10 min and washed with the sterile Holtfreter buffer. They were quantified spectrophotometrically by determining the optical density at 600 nm along with viable plate counts.

2.2. Zebrafish embryo collection and maintenance

The zebrafish wild type AB were maintained in the Center for Circadian Clocks at Soochow University. Zebrafish adults were raised under standard conditions at 28.5 °C, living in the loop filter water. We collected embryos from natural spawning, with the method as previously described [16]. Embryos were observed every 2 h to remove unfertilized eggs promptly. The water was renewed at least once a day to avoid oxygen limitation, keeping zebrafish embryo healthy.

2.3. Assessment of spv locus on median lethal dose (LD_{50}) of S. typhimurium

Both STM-WT and STM-*Δspv* were cultured overnight in LB broth at 37 °C with shaking at 200 rpm and washed. OD_{600nm} of both bacteria suspensions were adjusted to 0.6 (10^9 CFU/ml). Subsequently, they were gradually diluted to 10^8 CFU/ml, 10^7 CFU/ml, 10^6 CFU/ml, 10^5 CFU/ml and 10^4 CFU/ml. Naturally hatched zebrafish at 3 days post fertilization (dpf) were used to be infected with the two strains bacteria respectively. The details were the same as our previous study [14]. The LD_{50} was calculated separately with the Reed-Muench method.

2.4. Analysis of spv locus on Salmonella reproduction and dissemination

The RFP-STM-WT and RFP-STM-*Δspv* were cultured overnight in LB broth with Ampicillin to reach stationary phase, then 500 μl of the bacteria suspension in serial doubling dilutions was inoculated into a 24-well clear-bottomed cell culture plate. The red fluorescence intensity was measured and quantified using *in vivo* imaging system (Maestro EX, USA). At the same time, the diluted bacteria suspension was incubated on LB agar to detect CFU counting data. The linear relationship between CFU and red fluorescence intensity was determined by plotting fluorescence against CFU. The embryos were treated with 0.003% 1-phenyl-2-thiourea (PTU; Sigma, #P7629) to reduce pigmentation at 12 hpf. Then the larvae at 72 hpf were immersed in loop filter water containing *S.* typhimurium at a final concentration of 10^9 CFU/ml. The larvae infected for different time were collected, bacterial reproduction and dissemination were detected using the Auxis Imager, M2 fluorescence microscope.

2.5. Evaluation of spv locus on Salmonella survival in zebrafish

In order to determine the bacterial load, zebrafish larvae were assigned to two groups (n = 10/group) randomly. Each group of zebrafish were immersed in filtering water containing bacteria at the ultimate concentration of 1×10^9 CFU/ml. The protocol for bacterial survival evaluation was according to our previously study [14]. In brief, infected larvae were euthanized with tricaine (MS-222, Sigma, USA) and washed in PBS, then each larva was homogenized in 100 μl PBS, and 50 μl aliquot was diluted and incubated overnight for CFU counting of whole-larvae. The remaining 50 μl was treated with 100 μg/ml AMK for 2 h to remove extracellular bacteria, and incubated for intracellular CFU counting.

2.6. Assessment of spv locus on host phagocytes responses

72 hpf zebrafish larvae were infected with STM-WT and STM-*Δspv*. 10 zebrafish larvae in each group were collected at 2, 4 and 8 h post infection (hpi) and fixed by 4% paraformaldehyde overnight, then washed by PBST (1 × phosphate buffered saline adding 0.1% Tween-20) and specifically stained neutrophils with 0.18% Sudan black B (Solarbio, Serva, USA), the neutrophils recruitment was observed by microscope. To judge macrophages phagocytosis, 10 larvae infected with STM-WT or STM-*Δspv* were cultured in 2.5 μg/ml neutral red (Solarbio, Amresco, USA) dye solution, the phagocytosed neutral red by macrophages was observed under microscope at 2, 4 and 8 hpi after zebrafish anesthesia.

2.7. Analysis of spv locus on autophagy flux during Salmonella infection by western blotting

To investigate the interaction between *spv* locus and autophagic flux of zebrafish, the protein level of Lc3 and p62 were detected, and autophagy agonist Torin1 (Tocris Bioscience, USA) as well as lysosomal inhibitor Chloroquine (CQ) (Sigma, USA) were used. Zebrafish larvae at 72 hpf pretreated by 0.4 μmol/L Torin1 or 50 μmol/L CQ for 24 h were immersed in STM-WT or STM-*Δspv* suspensions at a final concentration of 10^9 CFU/ml for 8 h. For western blotting analysis, equal amounts of protein (100 μg) from each sample were loaded into each lane. Proteins were fractionated on 12% SDS–polyacrylamide gel and blotted onto polyvinylidene difluoride membrane (PVDF). After blocking with 5% non-fat milk in TBST, the membrane was probed with Lc3/p62 antibody (MBL, Japan) for one night at 4 °C and washed 3 times with TBST. Then the