FEMS Microbiology Letters, 368, 2021, fnab124

https://doi.org/10.1093/femsle/fnab124
Advance Access Publication Date: 20 September 2021
Research Letter

RESEARCH LETTER – Pathogens & Pathogenicity

sRNA *STnc150* is involved in virulence regulation of *Salmonella Typhimurium* by targeting *fimA* mRNA

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One sentence summary: STnc150 inhibits fimA translation by binding to the translation initiation sequence of fimA mRNA, thereby significantly affecting the adhesion, intracellular survival and virulence of STM to the host cells.

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Editor: Olga Ozoline

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ABSTRACT

Small RNAs (sRNAs) are essential virulent regulators in *Salmonella typhimurium* (STM). To explore the role of sRNA *STnc150* in regulating STM virulence, we constructed a *STnc150* deletion strain (Δ*STnc150*) and its complementary strain (Δ*STnc150*/C). Then, we compared their characteristics to their original parent strain experimentally, identified the target genes of *STnc150* and determined the expression levels of target genes. The results showed that the Δ*STnc150* strain exhibited delayed biofilm formation, enhanced adhesion to macrophages, significantly reduced LD_{50}, increased liver and spleen viral loads and more vital pathological damaging ability than its parent and complementary strains. Further, bioinformatics combined with the bacterial dual plasmid reporter system confirmed that the bases 72–88 of *STnc150* locating at the secondary stem-loop structure of the *STnc150* are complementary with the bases 1–19 in the 5′-terminal of *fimA* mRNA of the type 1 fimbriae subunit. Western blot analysis showed that *fimA* protein level was increased in *STnc150* strain compared with its parent and complementary strains. Together, this study suggested that *STnc150* can down-regulate STM *fimA* expression at the translation level, which provided insights into the regulatory mechanisms of sRNAs in virulence of STM.

Keywords: *STnc150*; virulence regulation; *Salmonella typhimurium*; *fimA* mRNA

INTRODUCTION

Salmonella typhimurium (STM) belongs to the group B serotype non-typhoidal *Salmonella* (NTS; Havelaar *et al.* 2015). It is a Gram-negative facultative intracellular parasite widely distributed in the natural environment (Fierer and Guiney 2001). As an important zoonotic foodborne pathogen, STM can infect humans and animals through animal-derived food, causing acute gastroenteritis or sepsis and posing severe threats to public health and food safety worldwide (Sewell and Farber 2001).

Received: 14 April 2021; **Accepted:** 15 September 2021

Table 1. Primers used in this study.

Primers	Sequences (5′–3′)	Product size(bp)	Target gene
F1	ATTGCCGCGAAGACCGTGAC	366/1223/243	*STnc150*/*STnc150*::*cat*
R1	TTCTCCTGATAATAACAATAAAT		
F2	ATTATCGCGAATATGTAATAACGATAATTGTTAAAAACAAAAACGGGGACGT GTAGGCTGGAGCTGCTTC	1114	*Chloramphenicol*
R2	GGTAAGGCACTTTCAAAAAATAGCCAAATCACACATTATTAAGAAAACCA TATGAATATCCTCCTTAGTT		
F3	AAGCTTATTGCCGCGAAGACCGTGAC	378	*STnc150*
R3	GGATCCTTCTCCTGATAATAACAATAAAT		
F4	CGGGATCCATTGCCGCGAAGACCGTGAC	366	*STnc150*
R4	GGGGTACCTTCTCCTGATAATAACAATAAAT		
F5	CCCAAGCTTAAGAGTTTGCGGCTATTTTT	343	*fimA*
R5	CGGGGTACCATGTACCGCCACTCACGCTCACC		
F6	ATGCCGCGTGTATGAAGAAGG	275	*16S rRNA*
R6	CTAACTCCGTGCCAGCAGCCGC		
F7	CGCTGCCGTGGCTTTCTCTGG	262	*fimA*
R7	AAAGGTGGCGTCGGCATTAGC		
F8	GGATCCATGGAAAGCAAAGTAGTTGT	1263	*icdA*
R8	AAGCTTTTACATATTCGCGATAATCG		
F9	GGATCCTCCATGAAACATAAATTAATG	591	*fimA*
R9	AAGCTTTGGCGTTCCCTGACGGGATTA		

Note: Underlined sequences in the primer indicate the restriction site recognized by *Hind* III, *BamH* I and *Kpn* I, respectively.

When STM enters the host body through the digestive tract, it completes its invasion, intracellular survival and colonization by regulating the expression of various virulence factors to adapt to the complex living environment in the host body (Ilyas, Tsai and Coombes 2017). Existing studies have proved that small RNAs (sRNAs) are one of the important factors involved in the virulence regulation, which can regulate STM biofilm formation, metabolism, intracellular parasitism and virulence, exerting important regulatory roles in the process of STM infection, colonization and pathogenicity (Gong *et al.* 2011). Currently, it is generally believed that sRNAs regulate the target genes' stability at the post-transcriptional level through complementary base pairing with mRNA of the target gene to inhibit or promote the target genes' translation level and achieve their regulatory function (Dutta and Srivastava 2018). At present, more than 280 sRNAs have been detected in the STM SL1344 strain, and most of which are mainly located in the intergenic region (IGR) of the STM genome (Kröger *et al.* 2012). However, the biological functions of most sRNAs found in STM are still unclear.

Srikumar *et al.* (2015) found that the expression of sRNA *STnc150* was significantly upregulated in STM-infected macrophages, suggesting that it may be related to STM infection and intracellular survival. However, the regulatory effects and mechanisms of sRNA *STnc150* on STM infection, intracellular survival and virulence are still unclear. The purpose of this study is to clarify the roles of sRNA *STnc150* in STM biofilm formation, cell invasion ability, intracellular survival and proliferation and mouse virulence regulation using bioinformatics prediction combined with bacterial double plasmid reporter system, qRT-PCR and western blot and verify sRNA *STnc150* target genes at the transcription and translation level, with the hope to reveal the regulatory mechanisms of sRNA *STnc150* on STM virulence.

MATERIALS AND METHODS

Primers design

The primers were designed using the Primer 5.0 software (Premier Inc., Canada) and synthesized by Beijing Huada Bioengineering Company (Table 1). Primers F2/R2 was designed based on pKD3 sequence deposited in GenBank (Accession number: AY048742.1), while primer pairs F4/R4-F10/R10 were designed according to the sequence of STM SL1344 (Accession number: FQ312003.1).

Generation of STM *STnc150* gene deletion and complementation strains

Salmonella enterica serovar Typhimurium SL1344 (Accession number FQ312003.1) was employed for the construction of *STnc150* gene deletion and complementation strains. The strains and plasmids used in this study were listed in Table S1 (Supporting Information). Briefly, the *STnc150* gene and the fragment containing the chloramphenicol gene (*cat*) were amplified using F1/R1 and F2/R2 primers, respectively. Then the *STnc150* gene deletion strain was constructed using the Lambda-Red recombination technique described by Datsenko and Wanner (Datsenko and Wanner 2000). For the construction of complementary strain, the sequence of *STnc150* was amplified with F3/R3 primers containing *Hind* III and *BamH* I (TaKaRa, Japan) recognized sequence, and cloned into pBR322 vector to generate the complementation strain Δ*STnc150*/C. The SL1344, Δ*STnc150* and Δ*STnc150*/C strains were cultured *in vitro* at 37°C, respectively, and their growth curves were plotted to examine the effects of *STnc150* gene deletion on STM growth.

Detection of STM biofilm formation

The bacterial cultures were inoculated into a 96-well microtiter plate (Qiagen, Germany), and the STM biofilm was detected, as reported previously (Kint *et al.* 2010). The formed biofilm was observed under a microscope (Olympus, Japan). After that, the biofilm was dissolved in 90% ethanol, and the OD_{600nm} of each well was measured. The experiments were performed independently three times with eight replicates. The differences in the biofilm formation ability among the SL1344, Δ*STnc150* and Δ*STnc150*/C strains were compared.

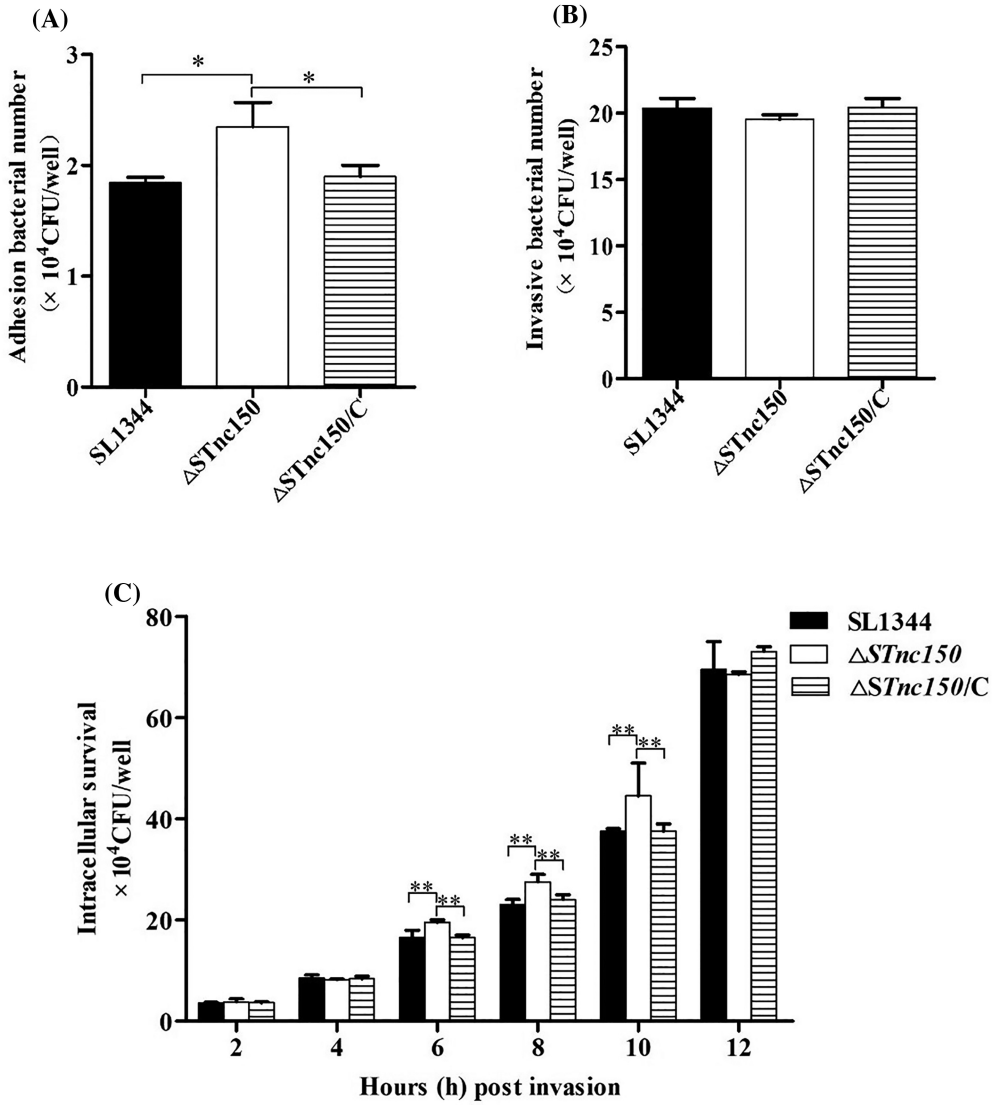


Figure 1. Determination of cellular adhesion, invasion and survival abilities of SL1344, Δ*STnc150* and Δ*STnc150*/C strains in the RAW264.7 cells. **(A)** cellular adhesion of SL1344, Δ*STnc150* and Δ*STnc150*/C strains; **(B)** cellular invasion of SL1344, Δ*STnc150* and Δ*STnc150*/C strains and **(C)** cellular survival abilities of SL1344, Δ*STnc150* and Δ*STnc150*/C strains; Values represent the mean ± SEM (*$P < 0.05$, **$P < 0.01$). CFU = colony-forming unit.

Assay of STM abilities of cell adhesion, invasion, intracellular survival and proliferation

Mouse macrophage Raw264.7 cells were cultured overnight in DMEM medium (Gibco, USA) containing 10% fetal bovine serum (Biological Industries, Israel) and incubated at 37°C in a humidified incubator supplemented with 5% CO_2. When the cells formed a monolayer with about 2×10^5 cells per well, 1 mL of SL1344, Δ*STnc150* or Δ*STnc150*/C DMEM resuspension at 2×10^6 CFU/mL was added, and the ability of STM adhesion cell, invasion, intracellular survival and proliferation were examined as reported previously (Peng *et al.* 2016).

Determination of STM virulence

BALB/c mice aged 6 weeks were randomly divided into one control group and 6 infection groups. Mice in each group were infected by intraperitoneally injecting 0.5 mL of the serially diluted STM SL1344, Δ*STnc150* and Δ*STnc150*/C strains and observed continuously for 10 days. The control group was treated with the same volume as PBS. The LD_{50} of each strain to mice was calculated by the modified Krebs' method (Owei and Isirima 2014). Mice were infected with 2×10^3 CFU/mL SL1344, Δ*STnc150* and Δ*STnc150*/C strains, respectively. The bacterial load in the organs of each mouse was determined as described previously (Kumawat *et al.* 2016). Histopathological changes in the liver, spleen and small intestine collected 5 days after infection were observed after being prepared as pathological sections.

Prediction of *STnc150* secondary structure and its regulatory target genes

The secondary structure of *STnc150* was predicted using Mfold (http://unafold.rna.albany.edu/?q=mfold; Chien *et al.* 2019). *STnc150* putative mRNA targets were predicted using TargetRNA2 (http://cs.wellesley.edu/~btjaden/TargetRNA2/usergui de.html) after inputting sRNA *STnc150* RNA sequence as the reference and screening STM SL1344 genome as the object (Kery *et al.* 2014).

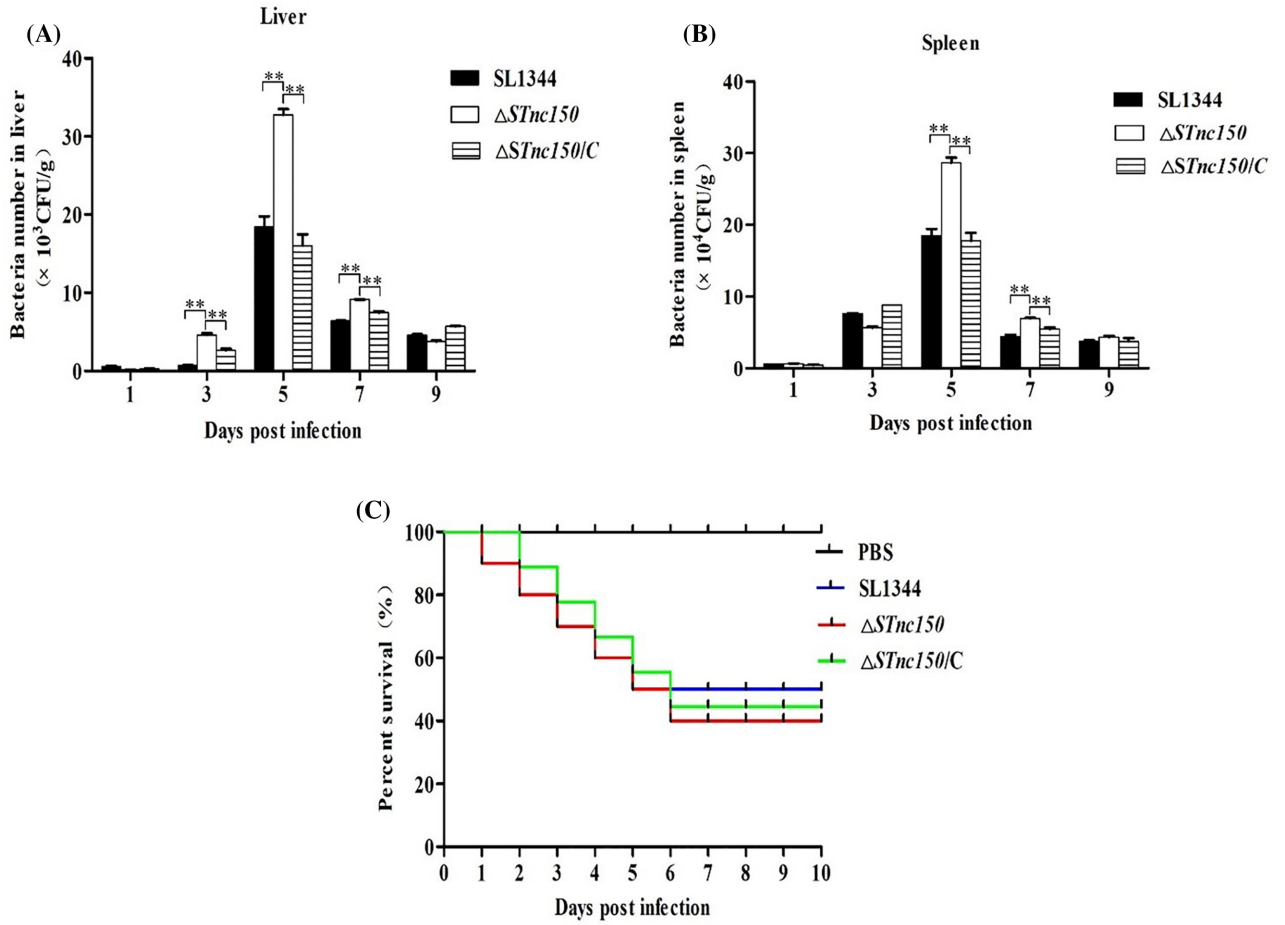


Figure 2. Bacterial loads and survival curves in mice infected by SL1344, Δ*STnc150* and Δ*STnc150*/C strain, respectively. **(A)** Bacterial loads of liver; **(B)** Bacterial loads of spleen and **(C)** Survival curves; Values represent the mean ± SEM (**$P < 0.01$). CFU = colony-forming unit.

Analysis of the interaction between *STnc150* and its target genes

The interaction between *STnc150* and the mRNA of target gene was analyzed using the bacterial dual plasmid co-expression system. Briefly, pUT18C-*STnc150* and pMR-LacZ-*fimA* plasmids were constructed by inserting *STnc150 and fimA gene* into empty vector pUT18C and pMR-LacZ, respectively. Then, the recombinant plasmids were simultaneously transformed into *Escherichia coli* BTH101 competent cells (TaKaRa, Japan), and cultured on agar plates containing X-gal, IPTG, Kan (100 μg/mL) and Amp (50 μg/mL; TaKaRa, Japan) at 37°C, overnight. The lawn on the agar was rinsed off with sterilized saline, and the OD_{420nm} of the bacterial solution was determined using spectrophotometer.

Quantitative qRT-PCR assays

After statically cultured at 30°C for 23 h, bacteria pellets were collected by centrifugation. Total RNA was extracted with Trizol reagent (Invitrogen, USA). cDNA synthesis was performed using the PrimeScript RT reagent Kit (TaKaRa, Japan). The transcription level of *fimA* and isocitrate dehydrogenase (*icdA*) gene was measured by qRT-PCR using SYBR Green Mix (Invitrogen, USA) and primers F6/R6, F7/R7 and F8/R8 on a Roche LightCycler 480 instrument (Roche, Swiss). The *fimA* transcription level was calculated by the $2^{-\Delta\Delta CT}$ method with *16S rRNA* gene as the reference (Livak and Schmittgen 2001). The experiments were performed independently three times with three replicates.

Determination of the translational levels of target genes

Protein levels were examined by western blot as reported previously with slight modification (Chen *et al.* 2020). Briefly, SL1344, Δ*STnc150* and Δ*STnc150*/C strains were statically cultured in Luria–Bertani (LB) medium (Hopebio, China) at 30°C for 23 h, respectively. The cells were collected by centrifugation, resuspended in lysis buffer and then sonicated to extract proteins. Proteins were separated by SDS-PAGE and transferred onto nitrocellulose filter membranes (Biosharp, China). The expression level of *fimA* protein was analyzed by western blot using mouse antibody against recombinant *fimA* protein and HRP-labeled rabbit anti-mouse IgG (Sigma, USA). The signals were visualized using DAB color developing solution (Sigma, USA). The effects of *STnc150* gene deletion on the translation level of target proteins was analyzed using *icdA* as the reference. The experiments were repeated three times independently.

Statistical analysis of data

All data were statistically analyzed by GraphPad Prism 5.0 software (https://www.graphpad.com/). The analysis of variance (ANOVA) was employed to compare continuous variables, while

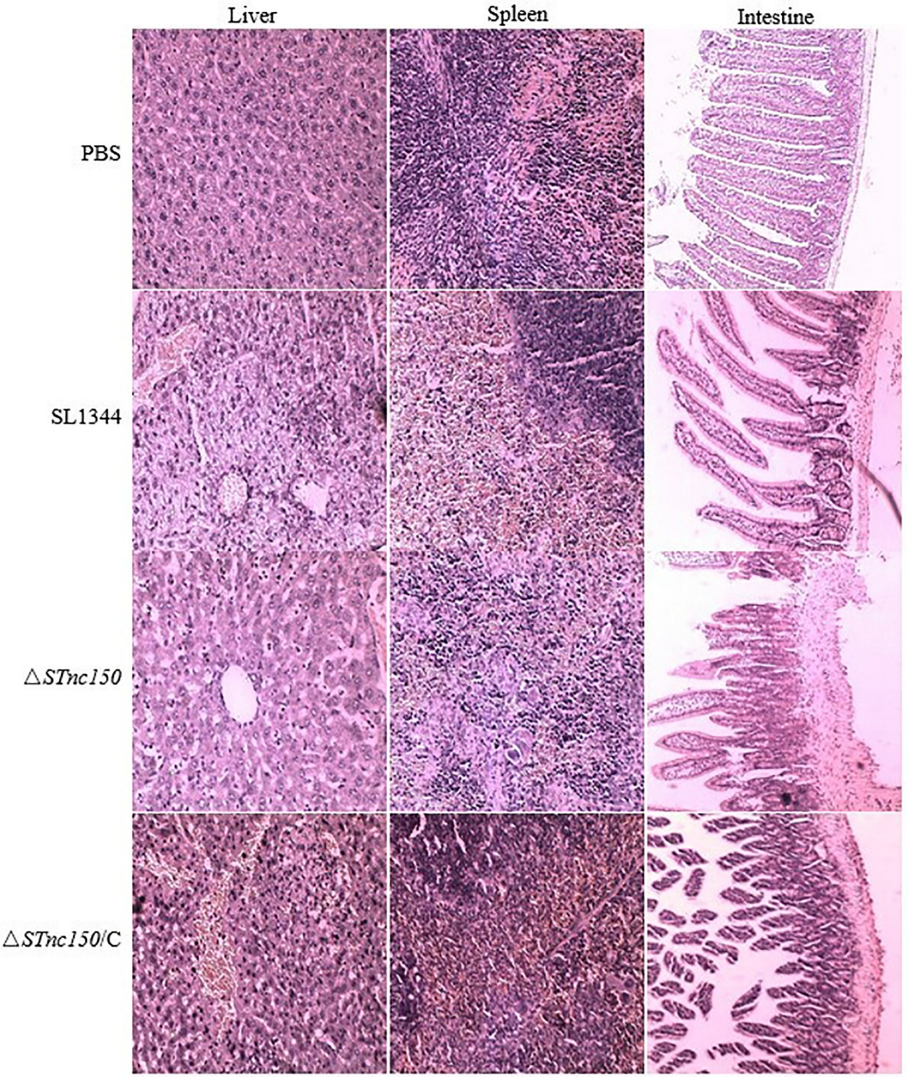


Figure 3. Pathological changes in liver, spleen and intestine of mice infected by SL1344, Δ*STnc150* and Δ*STnc150*/C, respectively. (Liver and spleen, ×200; intestine, ×100; HE staining)

chi-square test was used for the analyses of categorical variables. $P < 0.05$ was considered significant, while $P < 0.01$ was considered extremely significant.

RESULTS

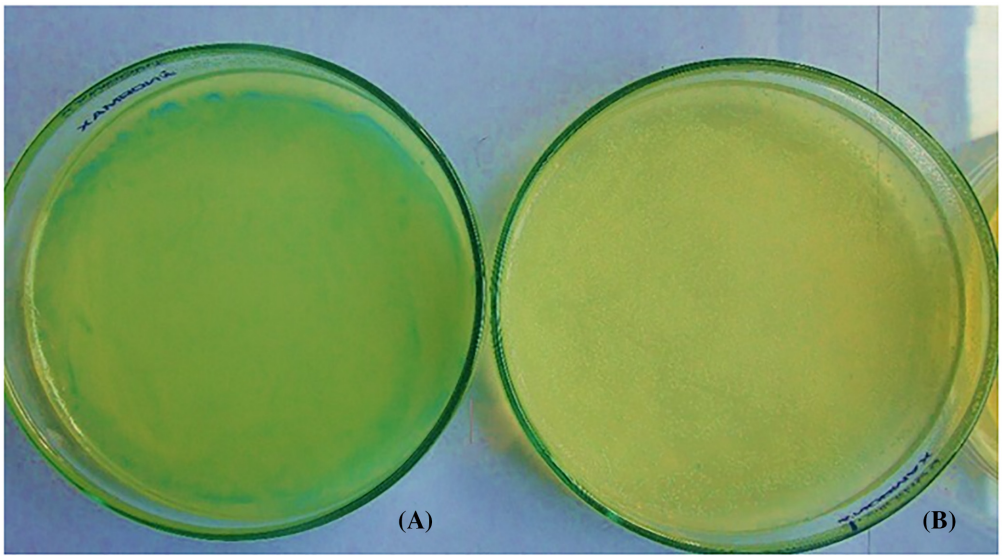
PCR and sequencing verified the successful generations of the deletion strain Δ*STnc150* and the complementary strain Δ*STnc150*/C. There was no significant differences in the growth among SL1344, Δ*STnc150* and Δ*STnc150*/C at 37°C ($P > 0.05$; Figure S1, Supporting Information), indicating that *STnc150* gene deletion did not affect the growth characteristics of SL1344.

After culturing for 22, 23 and 24 h, the SL1344, Δ*STnc150* and Δ*STnc150*/C strains could all form biofilm, but the biofilm formation ability of the Δ*STnc150* strain significantly decreased at 22 h and 23 h ($P < 0.05$; Figure S2a and b, Supporting Information), indicating that *STnc150* gene deletion delayed biofilm formation of STM.

To investigate the effects of the deficiency of *STnc150* gene on the infection and survival, we determined the adhesion, invasion rates and intracellular survival in RAW264.7 cells. Compared with SL1344 and Δ*STnc150*/C strains, the adhesiveness but not the invasiveness of Δ*STnc150* strain to RAW264.7 cells was significantly enhanced ($P < 0.05$; Fig. 1A and B). At 6, 8 and 10 h after infection, the colony number of Δ*STnc150* strain was significantly higher than that of SL1344 and Δ*STnc150*/C strains ($P < 0.01$; Fig. 1C).

The LD_{50} of SL1344, Δ*STnc150* and Δ*STnc150*/C strains was 3.6×10^4 CFU/mL, 1.6×10^4 CFU/mL and 8.5×10^4 CFU/mL, respectively (Table S2, Supporting Information). The survival curve showed that the virulence of Δ *STnc150* was significantly enhanced (Fig. 2C). The liver bacterial load of mice infected with Δ*STnc150* increased significantly on the 3rd, 5th and 7th day of post-infection ($P < 0.01$; Fig. 2A), and the spleen bacterial load increased significantly on the 5th and 7th day of post-infection ($P < 0.01$; Fig. 2B).

On the 5th day of post-infection, mice infected with Δ*STnc150* showed hepatic enlargement, hyperemia and discoloration, and obviously enlarged spleen, compared with mice infected with SL1344 and Δ*STnc150*/C (Figure S3, Supporting Information). Histopathological examination showed deteriorated



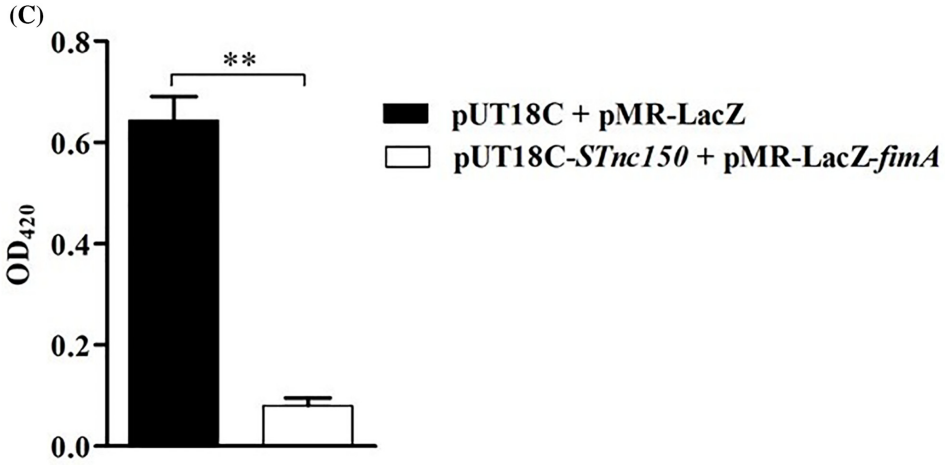


Figure 4. Verification of interaction between *STnc150* and target gene *fimA* mRNA using two plasmids co-expression system. **(A)** Bacterial lawn of BTH101 strain co-transformed by pUT18C and pMR-LacZ plasmid; **(B)** Bacterial lawn of BTH101 co-transformed by pUT18C-*STnc150* and pMR-LacZ-*fimA* plasmid and **(C)** Determination of OD_{420nm} of plates containing X-gal. Values represent the mean ± SEM ($^{**}P < 0.01$).

liver, spleen and small intestine tissues in mice infected with Δ*STnc150* (Fig. 3), indicating that *STnc150* deletion enhanced the virulence of STM in mice.

Prediction of *STnc150* secondary structure using Mfold together with the prediction of *STnc150* target gene using TargetRNA2 showed that bases 72–88 of *STnc150* locating the secondary stem-loop structure (Figure S4a, Supporting Information) of the sRNA is complementary to the bases 1–19 in the 5′-terinmal of *fimA* mRNA sequence (Figure S4b, Supporting Information).

Compared with *E. coli* co-transformed with pUT18C and pMR-LacZ control plasmids, *E.coli* co-transformed with pUT18C-*STnc150* and pMR-LacZ-*fimA* displayed a lighter blue on the plate containing X-gal (Fig. 4A and B), and the OD_{420nm} value of bacterial solution was significant decreased ($P < 0.01$; Fig. 4C), suggesting that *STnc150* may interact with *fimA* mRNA sequence, thereby down-regulating the expression of *fimA* gene at the post-transcriptional level.

The mRNA levels of *fimA* and *icdA* genes were determined by qRT-PCR, respectively. After culturing at 30°C for 23 h, *fimA* and *icdA* genes transcription levels were not significantly different among the three strains ($P > 0.05$; Fig. 5A and B). Moreover, western blot results showed that the expression level of *fimA* protein increased in Δ*STnc150* strain than in SL1344 and Δ*STnc150*/C strains (Fig. 5C), indicating that *STnc150* played a role in regulating *fimA* expression and implying that *STnc150* inhibits *fimA* mRNA translation via a negative regulatory mechanism.

DISCUSSIONS

STM is an important zoonotic intracellular parasitic bacterium infecting various non-phagocytes and phagocytes, propagating and surviving (Fàbrega and Vila 2013). With the help of a variety of virulent and regulatory factors, STM initially completes cell adhesion and internalization process, then forms Salmonella-containing vacuoles (SCV) in the cells for survival and propagation, and migrates between cells (Coburn, Grassl and Finlay 2007; Fresno and Olsen 2018). Existing studies have found that besides proteins, sRNAs are another important type of factors for virulent gene expression in STM (Kröger *et al.* 2013), which can affect the expression, degradation and stability of target genes

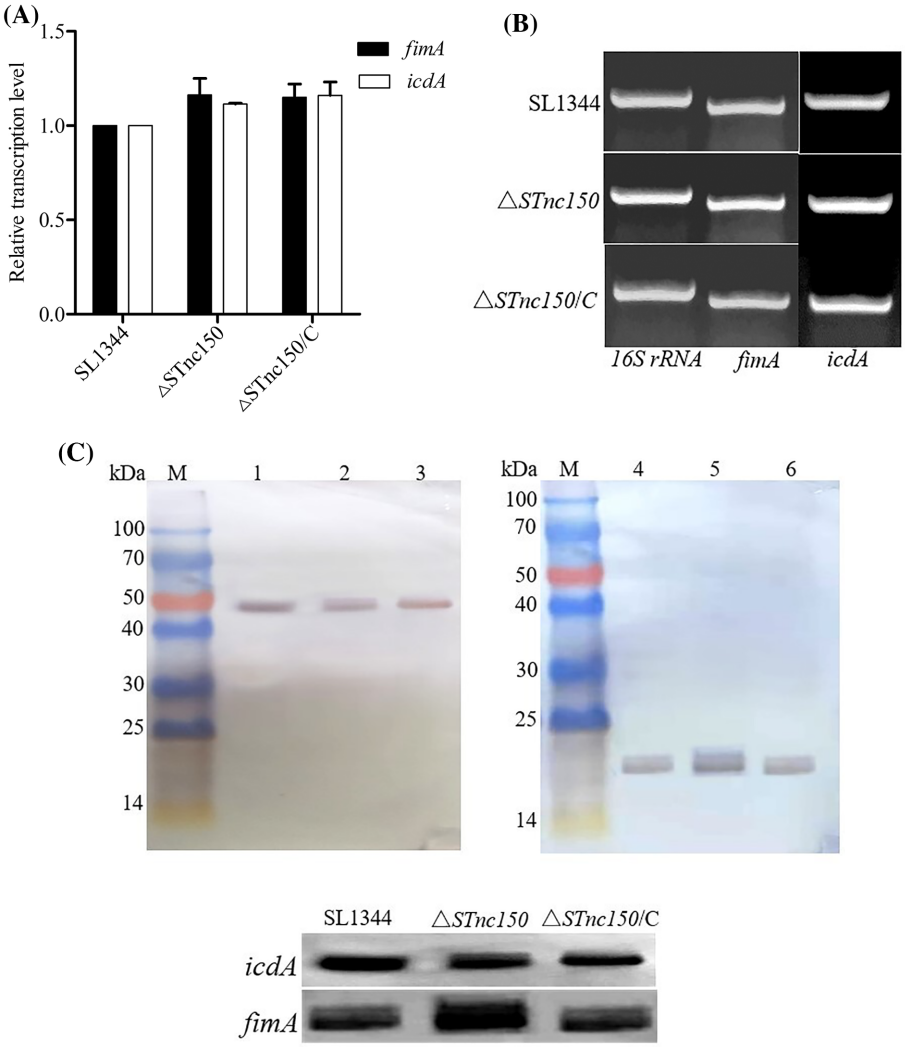


Figure 5. Analysis of the transcriptional and expression levels of *fimA* and *icdA*. **(A)** Transcriptional levels of *fimA* and *icdA* gene in SL1344, Δ*STnc150* and Δ*STnc150*/C; **(B)** Gel electrophoresis analysis of qRT-PCR products and **(C)** Expression levels of *fimA* and *icdA* proteins by western blot; M: pre-stained protein marker (100, 70, 50, 40, 30, 25 and 14 kDa); (1) SL1344-*icdA*; (2) Δ*STnc150*-*icdA*; (3) Δ*STnc150*/C-*icdA*; (4) SL1344-*fimA*; (5) Δ*STnc150*-*fimA* and (6) Δ*STnc150*/C-*fimA*. *icdA* was used as the control.

through complementary pairing with target genes at the post-transcriptional levels, thus playing vital roles in the host cells infecting process (Wagner and Romby 2015).

It has been found that *STnc150* was a 157 nt sRNA up-regulated in STM-infected macrophages (Kröger *et al.* 2012). However, so far, *STnc150*'s target genes and roles in regulating STM virulence have not been explored. To understand the effect of *STnc150* on the virulence of STM, here, we compared the biofilm-forming ability, cell infection and virulence of the *STnc150* deletion strain with its parental and complement strains. The results confirmed that Δ*STnc150* deletion significantly enhanced the adhesion ability, intracellular survival and proliferation of the strain in mouse macrophages, significantly enhanced the liver and spleen bacterial loads and strengthened the pathological damages to the liver, spleen and small intestine. These findings suggested that *STnc150* has a significant regulatory effect on STM virulence.

In this study, the candidate target gene *fimA* of *STnc150* was initially screened using the bioinformatics prediction technology of TargetRNA2 software. To investigate the regulatory effects of *STnc150* on *fimA* gene, a dual plasmid reporter system was established and used to verify the interaction between *STnc150* and *fimA* mRNA. The results confirmed that *STnc150* interacted with *fimA* mRNA. Western blot showed that *STnc150* negatively regulated *fimA* gene expression. Complementarily pairing of *STnc150* with the translation initiation sequence of *fimA* mRNA can inhibit the translation process of *fimA* mRNA (Fig. 6). This regulation mechanism is similar to the negative regulation mechanism of sRNA *RybB* on *ompN* reported by Bouvier *et al.* (2008).

Notably, *fimA* is the main protein of type 1 fimbriae subunits and necessary for type 1 fimbriae biosynthesis (Velden *et al.* 1998; Zeiner, Dwyer and Clegg 2012). Accordingly, the increase of *fimA* expression may enhance the biosynthesis of type 1 fimbriae. In this study, the cell infection test confirmed that *STnc150* deletion significantly enhanced STM adhesion to RAW264.7 cells, and the animal infection test confirmed that *STnc150* deletion enhanced the virulence of STM to mice. Therefore, we speculate that *STnc150* enhances the biosynthesis of type 1 fimbriae by regulating *fimA* expression, which helps STM attach to the cell surface, thereby enhancing its infectivity and virulence to the host cells.

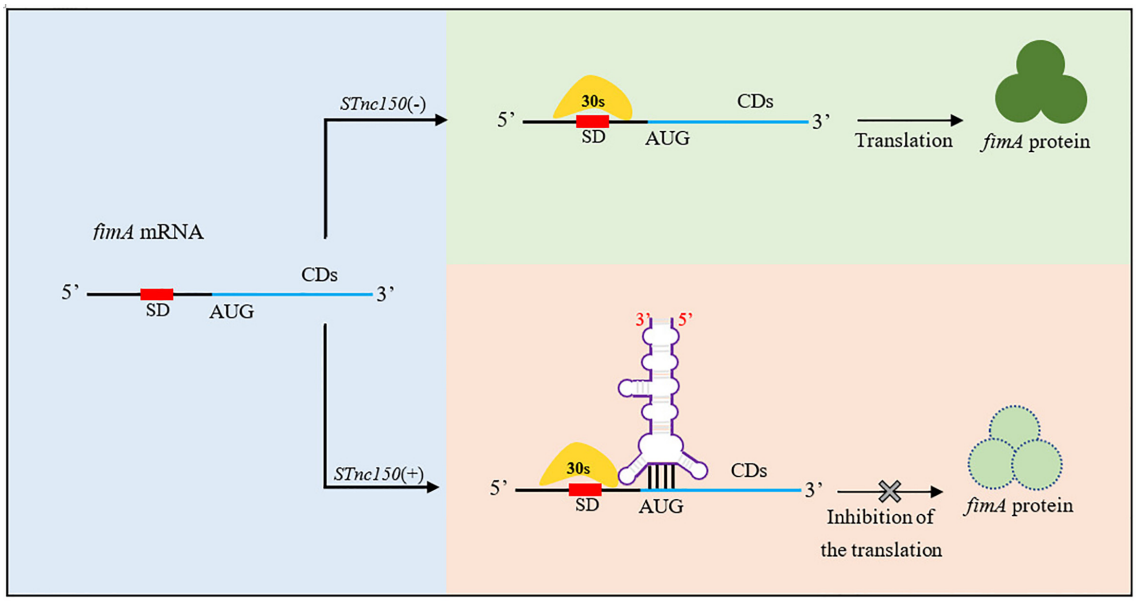


Figure 6. Schematic illustration of the mechanisms of sRNA *STnc150* regulating the expression of *fimA* gene.

Taken together, this study revealed that *STnc150* may inhibit the translation *fimA* protein by binding to the translation initiation sequence of *fimA* mRNA, thereby significantly affecting the adhesion, intracellular survival, and virulence of STM to the host cells. This kind of regulatory mode highlights the diverse roles played by sRNAs, and provides new insights into the regulatory mechanisms of sRNAs in virulence of STM.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSLE online.

ETHICAL APPROVAL

The experiments were carried out in accordance with the guidelines issued by the Ethical Committee of Shihezi University.

ACKNOWLEDGMENTS

The authors thank the field staff for providing the materials for this study.

FUNDING

This work was supported by grant from the National Key Research and Development Program (number 2016YFD0500900), the grant from the Youth Science and Technology Innovation Leader of Xinjiang Production and Construction Corps (number 2016BC001) and the Key Scientific and Technological Project in Agriculture of Xinjiang Production and Construction Corps (number 2019GG026).

Conflicts of interest. None declared.

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