The small RNA *STnc1480* contributes to the regulation of biofilm formation and pathogenicity in *Salmonella Typhimurium*

jing li
Shihezi University
Chengcheng Ning
Shihezi University
Na Li
Shihezi University
Yun Guo
Shihezi University
Chunhui Ji
Shihezi University
Xiaozhen Zhu
Shihezi University
Xingxing Zhang
Xinjiang Academy of Agricultural and Reclamation Science
Qingling Meng
Shihezi University
Xianzhu Xia
Shihezi University
Xuepeng Cai
Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences
Jun Qiao (qj710625@shzu.edu.cn)
Shihezi University

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Abstract

Salmonella Typhimurium (STM) is one of the most important food-borne bacteria that seriously harm livestock and human beings, which is capable of regulating the expression of its own genes in a variety of ways to adapt to a wide variety of adverse environmental stresses. To understand the regulatory roles of sRNA *STnc1480* on the capability of STM, the *STnc1480* gene-deficient strain △STnc1480 and its complement strain △STnc1480/STnc1480 were generated, and the impacts of *STnc1480* gene deficiency on the capability of responding to different environmental stresses, BF formation and pathogenicity were analyzed, respectively. Then, the target genes that were regulated by STnc1480 were also analyzed and explored. Compared with parent and complement strains, the deficiency of the *STnc1480* gene significantly reduced the BF formation. Moreover, the capacities of adhesion and invasiveness of the △STnc1480 strain to macrophages were also significantly reduced, while the LD_{50} in mice was significantly increased. The bacterial loads in liver and spleen were significantly reduced, and the pathological damage was alleviated. It was confirmed that the STnc1480 could be complementary to the 5′-UTR (-52 to -71 bases) region of *lpfA* mRNA. The bacterial dual plasmid reporting system confirmed that STnc1480 was capable of interacting with the mRNA of the *lpfA* gene, suggesting that STnc1480 can regulate the 5′-UTR of the *lpfA* mRNA at post transcription level to reduce the expression of the bacterial fimbria, thus reducing the BF formation and pathogenicity of STM.

Introduction

Salmonella Typhimurium (STM) is a Gram-negative intracellular bacterium with a wide range of host, which is widely distributed in the digestive tracts of livestock, birds and human[1,2]. As one of important food-borne pathogens, STM can infect human through the contaminated meat, milk, eggs and other animal-derived foods, resulting in human acute gastroenteritis, which has posed a serious threat to global food hygiene and safety. In the recent years, food poisoning caused by STM has occurred frequently in many countries, especially in the developing countries, causing serious harm to human health[3].

Small RNA (sRNA) is non-coding RNA with about 50 to 400 nucleotides (nt) in length[4]. They are widely found in the genomes of many bacterial species[5]. The currently available studies have found that sRNA is involved in regulation of the expression of certain genes of bacteria in adverse stress environments, and play an important role in responding the environmental stresses, community effect and the pathogenicity of bacteria[4, 6]. A number of studies have confirmed that bacterial sRNA can perform a variety of biological functions by binding to mRNAs of their target genes to adapt to the environmental changes by regulating them at the post-transcriptional level[7].

Recently, more than 100 non-coding-sRNAs have been found and identified in *Salmonella*[8]. However, the biological functions of most of these sRNAs are not yet known. A few studies have revealed that the expression level of sRNA *STnc1480* with 395 nucleotides in length was significantly increased during the infection process of STM into macrophages[8, 9]. However, the regulatory roles of *STnc1480* on the response of STM to environmental stress, biofilm (BF) formation and pathogenicity of STM remain unclear. Therefore, the main purpose of this study was to understand the regulatory roles of *STnc1480* on environmental stress, BF formation and pathogenicity in STM. Here, we constructed and analyzed the response to environmental stresses, BF formation and pathogenicity of the *STnc1480* gene-deficient strain and complement strain, and identified the target genes regulated by sRNA *STnc1480*, aiming to provide new insights into the molecular mechanism of sRNA regulation in STM.

Materials And Methods

1.1 Design of the primers

According to the STM SL1344 (accession number: FQ312003.1) sequence and pKD3 (AY048742.1) plasmid sequence deposited in the GeneBank, designed the specific primers by using Primer 5.0 software (Premier Inc., Canada), and synthesized by Beijing Genomics Institute (China). All the primers used in this study are listed in Table 1.

Table 1
Primers used in this study

Primer	Sequences (5'–3')	Product size(bp)	Template plasmid/Gene target
F1	TAAGTCGGATGCGTTAGTTTA	760/1394/498	SL1344/ △STnc1480: cat/ △STnc1480
R1	CAGACCTTATGGCGCGAGTATGA		
F2	CGGCATCGATTTAACACTAACGAAAATAAAATAGCCTTTATTATTACGCGGTGTAGGCTGGAGCTGCTTC	1114	pKD3
R2	TATTCCGCGAATTGTTTCAGAAACAGAGCGCCAATCAAAGTGCATAACCATATGAATATCCTCCTTAGTT		
F3	AAGCTTTAAGTCGGATGCGTTAGTTTA	760	SL1344/pBR322-STnc1480
R3	GGATCCCAGACCTTATGGCGCGAGTATGA		
16s-F	ATGCCGCGTGTATGAAGAAGG	132	SL1344
16s-R	GCGGCTGCTGGCACGGAGTTAG		
lpfA-F	GCCATGCGTCGTTTCTACTG	294	SL1344
lpfA-R	GTGGATTTACCGGTGTTCATTTC		
F4	CGGGATCCATGCGTTAGTTTACCATAG	642	SL1344/ pUT18C-STnc1480
R4	GGGGTACCAAACAGAGCGCCAATCAAAGTGC		
F5	CCCAAGCTT AATTATTTTTATATATAC	285	SL1344/ pMR-LacZ-lpfA
R5	CGGGGTACCATTGACCCAGCACAACTTCC		

1.2 Generation of STnc1480-deficient mutant strain and complement strains

The strains and plasmid used are listed in Table 2. STM Wild Type (WT) SL1344 strains was cultured with brain-heart immersion (BHI) broth (Hopebio, China) at 37 º C for 12–16 hours(h). The gene sequence of STM STnc1480 was amplified with F1/R1 primers. The STnc1480- deficient mutant strain was constructed by using λ-Red recombination technology[10]. Briefly, chloramphenicillin-containing gene products were first amplified by PCR with F2/R2 primer pair, which were used to construct recombinant strain △: STnc1480: cat. Then, the STnc1480-deficient mutant strain was identified by PCR amplification with F1/R1 primer pair in combination with sequencing technology. The complement strain △STnc1480/STnc1480 was constructed by using F3/R3 primer pair.

Table 2
Strains and plasmids used in this study

Strains	characteristic	references
Salmonella enterica serovar Typhimurium SL1344	Wild type	8
△STnc1480: cat	STnc1480 deletion mutant carrying cat, Cm^r	this study
△: STnc1480	STnc1480 deletion mutant	this study
△: STnc1480/STnc1480	Complementation of △: STnc1480,Amp^r	this study
BTH101 *Escherichia coli*		this study
Plasmids		
pKD3	Contains FRT -flanked chloramphenicol resistance gene	11
pKD46	Expresses lambda Red recombinase, Amp^r	11
pCP20	Expresses FLP recombinase, Amp^rCm^r	11
pBR322	Cloning vectors, Amp^r	this study
pMD19-T	Cloning vectors, Amp^r	Takara
PUT18C	Amp^r	this study
pMR-LacZ	Kan^r	this study

1.3 Environmental stresses assay

Briefly, the strains of SL1344, △STnc1480 and △STnc1480/STnc1480 were inoculated into 50 mL of BHI liquid medium, and cultured at 37° C overnight. The bacterial optical density at 600 nm (OD_{600nm}) value was adjusted to 0.5, the bacteria solution was inoculated at 1:100 ratio in the BHI liquid medium and cultured continuously at 37° C, their OD_{600} values were measured every hour and their growth curves were plotted. At the same time, the bacterial solutions were inoculated at a ratio of 1:100, respectively, to HCl-adjusted pH-4.5 BHI liquid medium, NaOH-adjusted pH-10 BHI liquid medium and BHI liquid medium containing 2 M NaCl and 2 mM 30% H_2O_2. The OD_{600} values were measured every hour and their growth curves were plotted. The effects of *STnc1480* gene deficiency on the responses of STM to different environmental stress conditions were examined and analyzed.

1.4 Biofilm formation assay

The overnight-cultured active bacterial solution was inoculated in 1:100 ratio into the BHI liquid medium and cultured up to its OD_{600nm} of 0.2. Then 100 μL of bacteria solution was aspired and transferred to 96-well microplates. Each sample was divided into two groups and 8 parallel repetitions were set for each group. BF of STM was measured according to methods reported in the literature[11]. After being cultured for 22 h, 23 h and 24 h, the differences in BF formation capability between SL1344, △STnc1480 and △STnc1480/STnc1480 strains were compared and the effects of *STnc1480* gene deficiency on STM BF formation capacity were analyzed.

1.5 Cell adhesion, invasion and intracellular survival assay

Briefly, mouse macrophages Raw264.7 were placed in culture incubator a 37 ° C and 5% carbon dioxide (CO_2) and cultured overnight to grow into a single layer with about 2×10^5 cells per well. The bacterial adhesion and invasion tests were performed according the methods described in literature with a slight modification[12]. After infection of 1 h, for the cell invasion test, the bacterial cells that did not enter the macrophages were killed with Dulbecco's modified Eagle medium (DMEM) (Gibco, USA) containing gentamicin [(100 mg/mL) (BIOTOPPED, China) at 37 ° C. The remaining steps were the same as those described above. The adhesion rates and invasion rates were calculated. In intracellular survival tests were conducted with reference to literature[13]. At 2 h, 4 h, 6 h, 8 h, 10 h, and 12 h after culture, cultured solution was aspired, cleaned and lysis. The bacterial colonies were counted. The test was repeated 3 times and 3 parallels were set each time.

1.6 Animal infections

One hundred and fourteen 6-week-old BALB/c mice were randomly divided into one control group and six infection groups. STM SL1344, △STnc1480 and △STnc1480/STnc1480 strains were cultured in BHI liquid medium, respectively, and used to infect mice when the logarithmic phase OD_{600} was 0.58. The mice in each subgroup were intraperitoneally injected with 0.5 mL of serially diluted cultures and the control group was treated with the same volume of phosphate buffered saline (PBS) and observed continuously for 10 days, and the LD_{50} of the mice was calculated by the modified Kurt method[14]. Six-week-old mice were infected with 2×10^4 cfu/ mL SL1344,△STnc1480 and △STnc1480/STnc1480 strains, respectively. Mice were sacrificed on days 1, 3, 5, 7 and 9 after infection, and the amounts of bacteria in these organs were simultaneously measured[15]. Meanwhile, the liver

and spleen were collected and pathological tissue sections were prepared 5 days after injection and the histopathological changes were observed by optical microscopy (Olympus, Japan).

1.7 Prediction and screening of target genes regulated by STnc1480

TargetRNA2 online software was used to predict the target genes of *STnc1480*[16]. The STM SL1344 genome was selected and the *STnc1480* RNA sequence was entered. The genes with longer bases being paired with *STnc1480* were selected as the target genes.

1.8 RNA extraction and real-time quantitative PCR

Briefly, the bacteria were cultured to BF formation state, and then collected by centrifugation. Total RNA was extracted with TRIzol reagent (Invitrogen, USA). Then complementary DNA (cDNA) was synthesized using PrimeScript-RRT kit including gDNA Eraser (TaKaRa Bio, Inc., Japan). Briefly, using 16sF/16sR and *lpfA*F/*lpfA*R as primers, the transcription levels of the target genes was determined by real-time quantitative PCR (RT-qPCR) using the ABI7500 instrument (Applied Biosystems, USA). Using the 16sRNA gene as the reference gene, the relative transcription levels of the target genes were calculated by the $2^{-\triangle\triangle CT}$ method[17]. Each test was repeated 3 times.

1.9 The bacterial dual plasmid reporting system experiment

The interaction between *STnc1480* and its target genes was analyzed using the bacterial dual-plasmid reporter system. In brief, F4/R4 and F5/R5 primers were used to amplify the operon DNA sequences of *STnc1480* and *lpfA*. Then, the pUT18C-*STnc1480* and pMR-LacZ-*lpfA* plasmids were constructed; the constructed recombinant plasmids were co-transfacted into BTH101 *E. coli* competent cells *via* electroporation. The positive clones on a Kan^r (100 mg/ mL) Amp^r (100 mg/ 9mL) (BIOTOPPED, China) double-resistant plate coated with X-gal (TaKaRa Bio, Inc., Japan) and Isopropyl-beta-D-thiogalactopyranoside(IPTG) (Solarbio Science & Technology Co., Ltd., China) were screened out and placed them at 37 ° C overnight and the color change of the bacterial lawn was observed.

1.10 Statistical analysis of data

GraphPad Prism 5.0 software (https://www.graphpad.com/, USA) was used for data analysis, and *t* test was used to conduct data significance analysis. The value of $P < 0.05$ was considered significantly different, while $P < 0.01$ was considered extremely significant different.

Results

2.1 STnc1480 affects the growth of STM in alkaline environment

Through PCR and sequencing confirmation, the deletion strain △STnc1480 and the complement strain △STnc1480/STnc1480 were obtained (Supplementary Fig. S1 and Supplementary Fig. S2), respectively. The growth rates of the SL1344, △STnc1480 and △STnc1480/STnc1480 at 37 ° C were not significantly different ($P > 0.05$). Under the condition of pH 10, however, the growth rate of △STnc1480 strain was significantly slower after 4 h *(*$P < 0.01$), indicating that the alkali tolerance ability of △STnc1480 strain was weaker than those of SL1344 and △STnc1480/STnc1480; when being cultured under the condition of 2 M NaCl and 2 mM 30% H_2O_2, the growth of △STnc1480 was not significantly different from those of the complement strain and the parent strain ($P > 0.05$) (Fig. 2 and Supplementary Fig. S4)

2.2 STnc1480 affects biofilm formation of STM

Culture for 22, 23 and 24 h respectively, the SL1344, △STnc1480 and △STnc1480/STnc1480 were all capable of forming BF (Fig. 2A), but the BF forming ability of △STnc1480 strain was significantly weakened ($P < 0.05$) (Fig. 2B), indicating that *STnc1480* has an influence on BF formation of STM.

2.3 STnc1480 affects adherence, invasion and intracellular survival of STM to cells

Compared with those of SL1344 and △STnc1480/STnc1480, the adhesion of △STnc1480 to RAW264.7 cells was extremely significantly weakened ($P < 0.01$) (Fig. 3A), and the invasion ability was also significantly weakened ($P < 0.05$) (Fig. 3B). At 6 h, 8 h, 10 h and 12 h after infection, the quantity of △STnc1480 strains was significantly lower than those of SL1344 and △STnc1480/STnc1480 strains ($P < 0.01$) (Fig. 3C and Supplementary Fig. S5), indicating that *STnc1480* affects the intracellular survival of STM.

2.4 Deleting STnc1480 attenuates virulence of STM in mice

The LD_{50} of SL1344, △STnc1480 and △STnc1480/STnc1480 were 3.6×10^4 cfu/ mL, 4×10^5 cfu/ mL and 8.5×10^4 cfu/ mL, respectively (Table 3). The survival curves indicated that the pathogenicity of △STnc1480 group was significantly reduced (Fig. 4C). On the 5th, 7th and 9th days after infection, as compared with those the SL1344 and △STnc1480/STnc1480 groups, the bacterial loads of △STnc1480 group in the liver and spleen of mice were significantly lower ($P < 0.01$) (Fig. 4A-B). Compared with those of SL1344 and △STnc1480/STnc1480 groups, the histopathological damages in liver, spleen and small intestine of the mice infected with the △STnc1480 was alleviated (Fig. 5).

Table 3
Determination of LD_{50} of SL1344、△STnc1480 and△STnc1480/STnc1480 in 6-weeks-old mice after intraperitoneal injection.

Strains	cfu/ mL	Mice	Mouse death	LD_{50}(cfu/ mL)
SL1344	2×10^2	6	1	3.6×10^4
	2×10^3	6	1	
	2×10^4	6	2	
	2×10^5	6	5	
	2×10^6	6	6	
	2×10^7	6	6	
△STnc1480	2×10^2	6	0	4×10^5
	2×10^3	6	0	
	2×10^4	6	1	
	2×10^5	6	2	
	2×10^6	6	6	
	2×10^7	6	6	
△STnc1480/STnc1480	2×10^2	6	0	8.5×10^4
	2×10^3	6	1	
	2×10^4	6	1	
	2×10^5	6	5	
	2×10^6	6	6	
	2×10^7	6	6	
control	PBS	6	0	-

cfu = colony-forming unit; LD_{50} = 50% lethal dose.

2.5 STnc1480 regulates lpfA expression

TargetRNA2 prediction of the target genes of *STnc1480* led to the finding that the *STnc1480* were complementarily paired with the 5'-UTR (-52 to -71 bases) of *lpfA* mRNA (Fig. 6A-B). In the state of BF formation, RT-qPCR analysis results showed that compared with SL1344 and △STnc1480/STnc1480, the transcription level of *lpfA* gene in △STnc1480 strain was significantly lower ($P < 0.01$) (Fig. 7), suggesting that the expression of *lpfA* gene may be regulated by the *STnc1480*.

2.6 STnc1480 can interact with target gene lpfA

The pMR-LacZ-*lpfA* and pUT18C-STnc1480 plasmids were constructed (Supplementary Fig. S3). Compared with the transformed pUT18C, pMR-LacZ and the co-transformed pUT18C and pMR-LacZ empty plasmids, the *E. coli* moss transformed with pUT18C-*STnc1480* and pMR-LacZ-*lpfA* turned blue on the X-gal plate(Fig. 8), indicating that *STnc1480* plays a positively regulatory role on the expression of *lpfA* gene.

Discussions

Many studies have shown that sRNA may regulate the expression of target genes in a variety of ways at the post-transcriptional levels. A large majority of sRNA regulatory factors block the entry of 30S ribosome by combining mRNA's Shine–Dalgarno (SD) sequences or AUG start codon, thus preventing the start of translation. Alternatively, sRNA can also be paired with the target gene base to protect it from cell RNA degradation, thereby stabilizing the protein expression of the target gene[18, 19]. In addition, sRNA can be paired with the 5'-non-coding (UTR) regions of mRNA to open the secondary structures of the masking SD sequence and promote the binding of the SD sequence to the 30S ribosome, thus activating the expression of the target genes[20].

In the recent years, many investigators have carried out research work on sRNAs of STM. To date, approximately 280 sRNAs expressed under various conditions have been detected in the STM LT2 strain[21]. Shabarinath et al.[9]. found a significant increase in the expression level of 31 sRNAs through transcriptome studies of STM-infected mouse macrophages, while expression levels of some sRNAs were very low during extracellular growth, but were significantly higher in macrophages, suggesting that these sRNAs may be related to STM's pathogenicity and intracellular survival[22].

It has been found that expression level of sRNA *STnc1480* was significantly increased after STM infected cells[9]. However, the regulatory roles of STnc1480 and its target genes have not yet been studied. Here, we confirmed that the cell invasion ability and pathogenicity of the STnc1480-defficient strain were significantly reduced, suggesting that the *STnc1480* was involved in regulation of pathogenicity in STM. Bioinformational predictions revealed that *lpfA* was one of the target genes regulated by *STnc1480*. It has been proved that *lpfA* was the main subunit of *lpf* fimbria, which helps STM form BF in the infected intestinal epithelia and increases ability of STM's intestinal colonization and pathogenicity to mice[23, 24]. RT-qPCR analysis further confirmed that expression level of *lpfA* in the △STnc1480 was lower than that of SL1344, indicating that *STnc1480* contributes to the expression regulation of *lpfA* gene, thus resulting in the reduced capacities of BF formation, cell invasion and pathogenicity of STM.

To confirm the interaction between *STnc1480* and 5′-UTR of *lpfA* mRNA, the bacterial dual-plasmid reporting system was employed in this study. By co-transacting the plasmid expressing sRNA and the plasmid containing their target mRNA binding sites and the reporting gene, the effects of *STnc1480* and *lpfA* on LacZ gene expression using X-gal as color agent were analyzed. The results revealed that *STnc1480* could bind with the 5′UTR region of *lpfA* mRNA. Therefore, we speculated that the molecular mechanism of *STnc1480* is to regulate the expression level of the *lpfA* gene by binding to mRNA of *lpfA* gene, protecting it from RNase degradation, and thus controlling the expression levels of the target genes, which posses similar regulatory mechanisms with the literature[25].

In conclusion, this study revealed the regulatory roles of sRNA *STnc1480* on BF formation and pathogenicity in STM, which provided new insights into the regulatory mechanism of sRNAs in STM.

Declarations

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Conflict of interests

This manuscript has not been simultaneously submitted for publication in another journal and been approved by all co-authors. The authors declare that they do not have any conflict of interest.

Ethical approval

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

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Figures

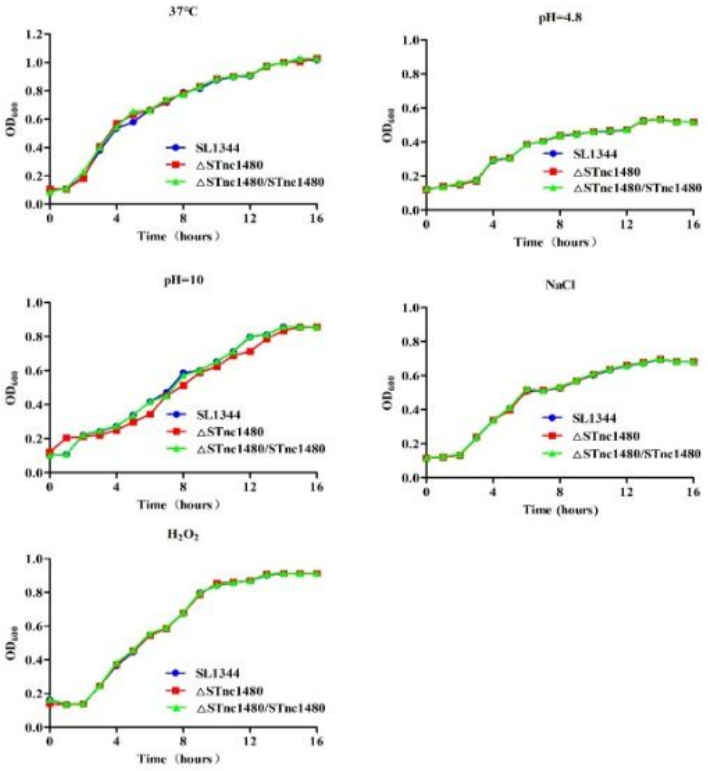

Figure 1

Growth curves of SL1344, △STnc1480 and △STnc1480/STnc1480 strains under different conditions

A-F: Growth curves of SL1344, △STnc1480 and △STnc1480/STnc1480 strains at 37 °C, pH4.8, pH10, 2 M NaCl, 2mM 30% H_2O_2, respectively.

OD_{600}= optical density at 600 nm.

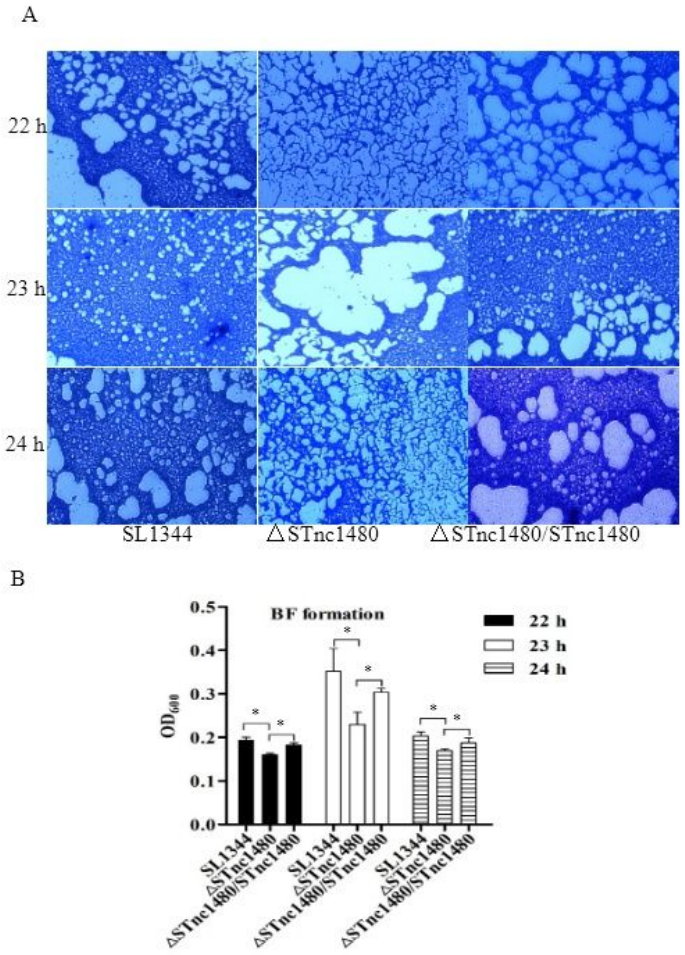

Figure 2

Assay of biofilm formation ability of SL1344, △STnc1480 and △STnc1480/STnc1480 strain, respectively

A: Biofilm observation by microscopic (40×10)；B: Biofilm forming abilities determined by OD600. Values represent the mean ± SEM (*P＜0.05；

OD600= optical density at 600 nm.

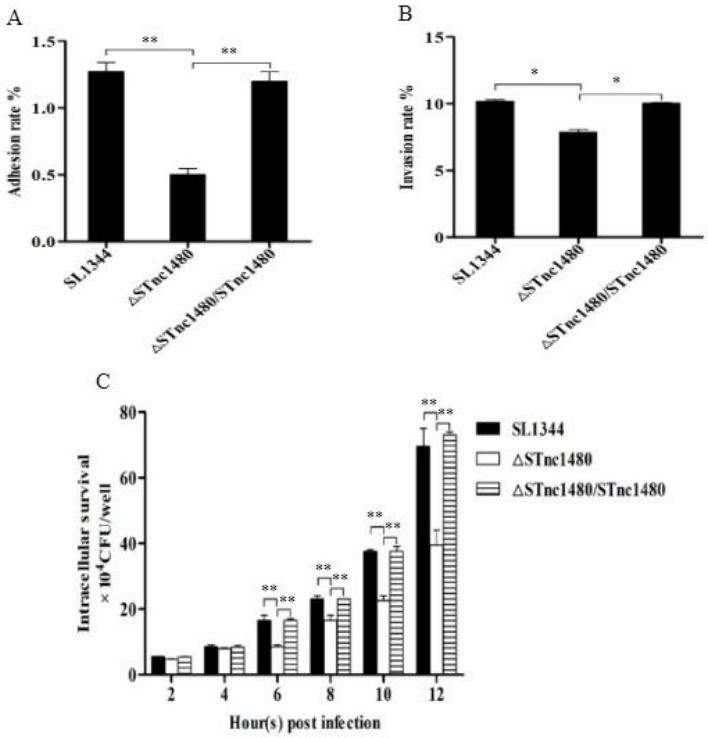

Figure 3

Determination of cellular adhesion, invasion and survival abilities in the RAW264.7 cell line.

A: cellular adhesion rate ; B: cellular invasion rate ; C: Intracellular survival abilities ; Values represent the mean ± SEM (*P<0.05;**P<0.01;

CFU =colony-forming unit.

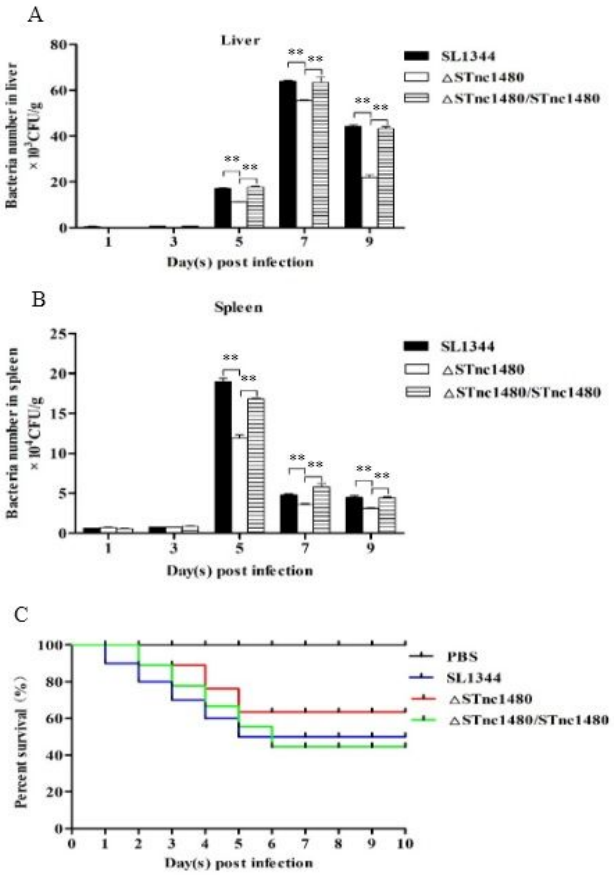

Figure 4

Bacterial loads and survival curves in mice infected by SL1344, △STnc1480 and △STnc1480/STnc1480 strain, respectively

A: Liver; B: Spleen; C: Survival curve.

Values represent the mean ± SEM (** P 0.01).

CFU =colony-forming unit.

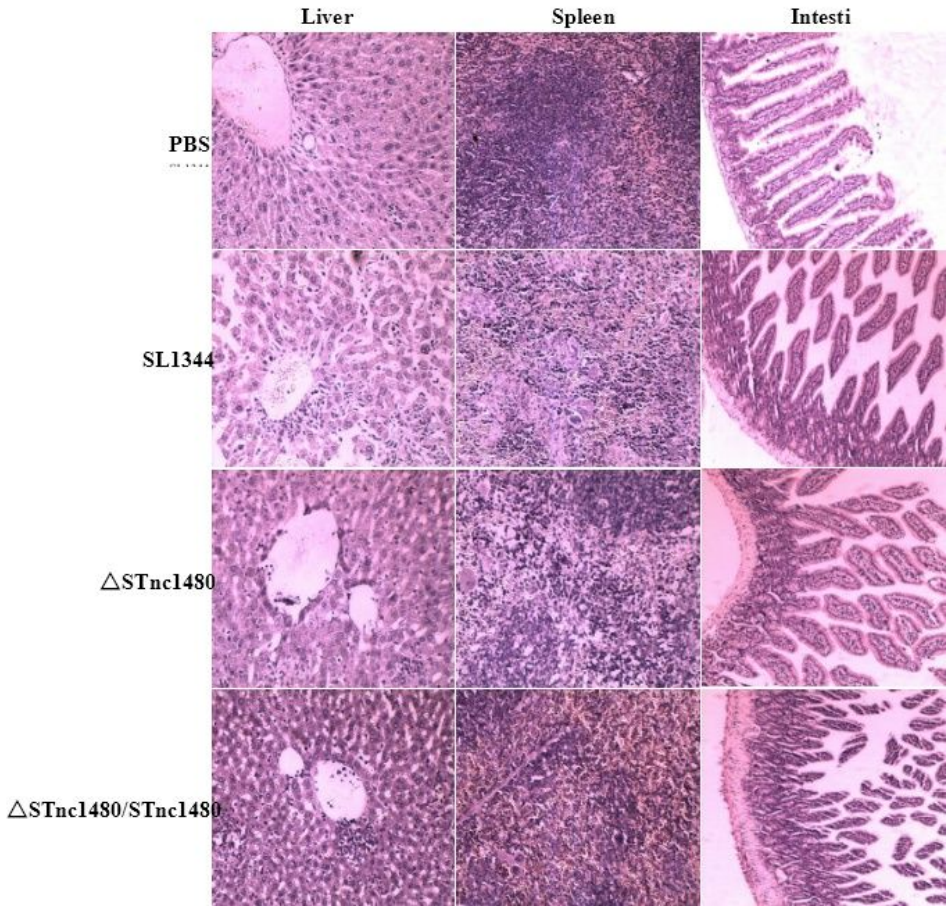

Figure 5

Histopathological changes in liver, spleen and intestine of mice inoculated by PBS, SL1344, △STnc1480 and△STnc1480/STnc1480 strains, respectively.

(magnification, ×200, hematoxylin and eosin

stain(HE) staining)

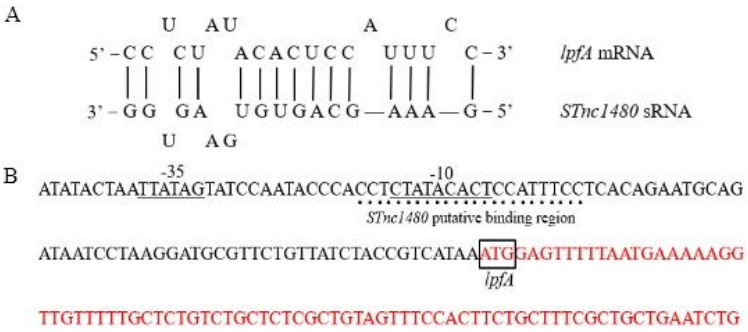

Figure 6

Complementary pairing position of sRNA *STnc1480* with mRNA of *lpfA* gene of STM

A: The schematic represents the pairing region of *STnc1480* sRNA with *lpfA* gene;

B: The -35, -10 region of *lpfA* gene promoter were underlined. The complementary sequences for *STnc1480* targeting region was shown by black spots.

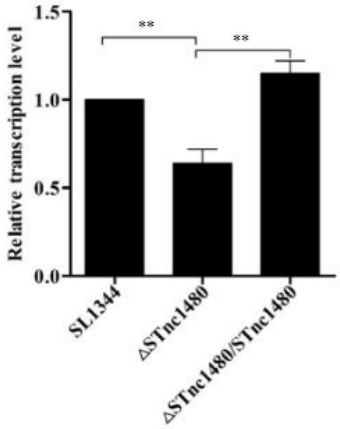

Figure 7

Determination of the transcription levels of *lpfA* gene in △STnc1480, △STnc1480/STnc1480 and SL1344 by qRT-PCR.

Values represent the mean ± SEM (** *P*0.01

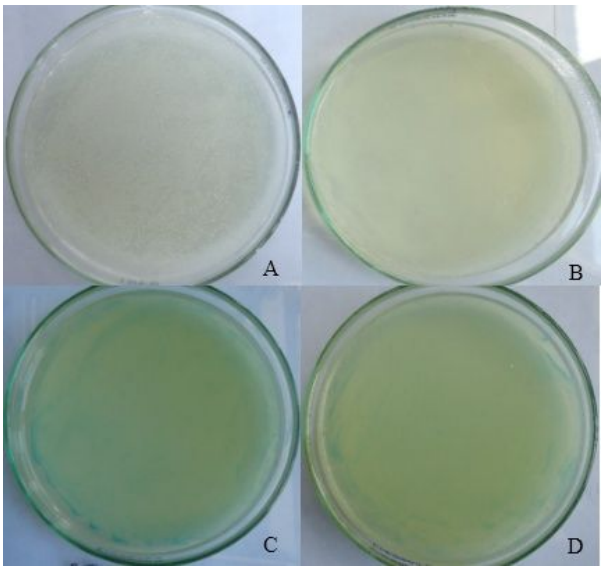

Figure 8

Verification of interaction between *STnc1480* and target gene *lpfA* using two plasmids co-expression system

A: Bacterial lawn of BTH101 transformed by pUT18C;

B: Bacterial lawn of BTH101 transformed by pMR-LacZ;

C: Bacterial lawn of BTH101 co-transformated by pUT18C and pMR-LacZ;

D: Bacterial lawn of BTH101 co-transformated by pUT18C-STnc1480 and pMR-LacZ-*lpfA*.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementaryfigures.docx