Small non-coding RNA STnc640 regulates expression of fimA fimbrial gene and virulence of Salmonella enterica serovar Enteritidis

Xia Meng 1,2†*, Xianchen Meng 1,2†, Jinqiu Wang 3†, Heng Wang 1,2, Chunhong Zhu 4, Jie Ni 1,2 and Guoqiang Zhu 1,2*

Abstract

Background: Small non-coding RNAs (sRNAs) regulate bacterial gene expression at the post-transcriptional level. STnc640 is a type of sRNA that was identified in Salmonella Typhimurium.

Results: In this study, STnc640 in Salmonella Enteritidis was confirmed to be an Hfq-dependent sRNA. TargetRNA software analysis showed that fimbrial genes fimA and bcfA were likely to be the target genes of STnc640. To investigate the target mRNAs and function of STnc640 in pathogenicity, we constructed the deletion mutant strain 50336 △stnc640 and the complemented strain 50336 △stnc640/pstnc640 in Salmonella Enteritidis 50336. The RT-qPCR results showed that the mRNA level of fimA was decreased, while bcfA was unchanged in 50336 △stnc640 compared with that in the wild type (WT) strain. The adhesion ability of 50336 △stnc640 to Caco-2 cells was increased compared to the 50336 WT strain. The virulence of 50336 △stnc640 was enhanced in a one-day-old chicken model of S. Enteritidis disease as determined by quantifying the 50% lethal dose (LD50) of the bacterial strains.

Conclusions: The results demonstrate that STnc640 contributes to the virulence of Salmonella Enteritidis.

Keywords: Salmonella Enteritidis, STnc640, Regulation, Adhesion, Virulence

Background

Small non-coding RNAs (sRNAs) in bacteria are stable transcripts approximately 50–500 nucleotides in length, often encoded in intergenic regions (IGRs), that play important roles in regulating gene expression at the post-transcriptional level [1–4]. sRNAs regulate many physiological processes, including metabolism, iron homeostasis, outer membrane protein biosynthesis, quorum sensing, and virulence [5–8]. Many of these sRNAs require the RNA-chaperone Hfq [9]. Nearly 100 distinct sRNAs have been identified in Salmonella [10].

Salmonella enterica serovar Enteritidis is an important Gram-negative intracellular pathogen with a broad host range. It can infect young chickens and cause symptoms such as enteritis or systemic infection [11]. Adult chickens infected with Salmonella Enteritidis may have subclinical infections and become chronic carriers, leading to contamination of chicken meat and egg products and the resulting food-borne diarrheal illnesses in humans [12]. Adhesion to intestinal epithelial cells mediated by bacterial fimbriae is a necessary first step for colonization [13–16]. Whole-genome sequencing has identified 13 fimbriae operons in the Salmonella Enteritidis strain P125109 [17]. The fim operon directs the assembly of type I fimbriae, which are involved in reproductive tract infection and in egg contamination [15]. Type I fimbriae and other multiple fimbrial adhesins are also required for the colonization of the intestinal lumen and for the virulence of Salmonella Typhimurium in mice [18].

STnc640 is a novel Hfq-binding sRNA that was identified in Salmonella Typhimurium through deep sequencing and transcriptomic analysis of Hfq-bound sRNAs and mRNAs [19]. Here we constructed a stnc640 deletion...
mutant and characterized the role of this sRNA in bacterial adhesion and virulence.

Results

Hfq plays a positive role on STnc640 stability
To determine whether the stability of STnc640 depends on the sRNA chaperone protein Hfq, the abundance of stnc640 transcripts in S. Enteritidis WT strain 50336, mutant 50336Δhfq and the complemented mutant 50336Δhfq/phfq were determined using RT-qPCR. The abundance of stnc640 was significantly reduced in 50336Δhfq, exhibiting only about 2% of that in the WT strain (P < 0.01) and was restored in the 50336Δhfq/phfq mutant (Fig. 1). This indicated that Hfq played a positive role on STnc640 stability.

Candidate mRNA targets of STnc640
Candidate mRNA targets of STnc640 were predicted using TargetRNA2 [20]. There were nine consecutive hybridization seeds between the AU-rich region of STnc640 (nts 263–277) and bcfA (nts 37–51). There were 11 consecutive hybridization seeds between the coding sequences (codons 8–25) of fimA mRNA and STnc640 (codons 99–125).

Construction and growth characteristics of the mutant 50336Δstnc640 and complemented strain 50336Δstnc640/pstnc640
S. Enteritidis strain 50336 contains an stnc640 gene with 97% identity to the S. Typhimurium strain LT2 stnc640 gene. STnc640 was located in a non-coding region between the genes SEN1810 and icdA in S. Enteritidis. In the construction of the deletion and the complemented strains, a 460 bp DNA fragment of the non-coding region was deleted and complemented. We constructed an stnc640 deletion mutant 50336Δstnc640 and compared its growth to the WT and complemented strains. The growth rate of 50336Δstnc640 was significantly reduced during the log phase from 2 h to 3 h (P < 0.05) (Fig. 2).

STnc640 regulates fimA expression and affects adherence and invasion to Caco-2 cells
To determine whether bcfA and/or fimA expression are regulated by STnc640, we quantified bcfA and fimA expression using RT-qPCR. The fimA but not bcfA transcript abundance was reduced in the Δstnc640 mutant compared with the WT strain (Fig. 3). To investigate whether deleting stnc640 affected bacterial adhesion and invasion by regulating fimA, we performed bacterial adhesion and invasion assays. Δstnc640 was enhanced in adhering and invading to Caco-2 cells compared with the WT strain (Fig. 4).

Deleting stnc640 enhances virulence in chickens
LD50 assays were performed to analyze the effect of stnc640 on S. Enteritidis virulence in chickens. All of the chickens displayed intestinal hyperemia and diarrhea 10 h post infection. Higher mortality appeared when infected by 50336Δstnc640 compared to the WT strain and the complemented strain. The mortality rates for 10^7, 10^8 and 10^9 CFU bacteria treatment were 5, 57 and 95% separately when infected by 50336Δstnc640. The mortality rates for the above three dose treatment were 5, 50 and 85% separately when infected by the WT.
strain, and the rates were 0, 5 and 62% when infected by the complemented strain. The LD50s were calculated 14 days post-infection. The LD50 values of the WT strain 50336, 50336Δstnc640 and 50336Δstnc640/pstnc640 were 2.9 × 10^8, 2.0 × 10^8 and 5.1 × 10^8 CFU, respectively. This indicated that the virulence of 50336Δstnc640 was enhanced approximately 1.5-fold compared with the WT. The virulence of complemented strain 50336Δstnc640/pstnc640 has attenuated compared with the WT strain and the 50336Δstnc640 mutant. Tests of isolation and identification of bacteria showed that all three strains of S. Enteritidis were widely distributed in the liver, spleen, and caecum of the infected chickens.

**Discussion**

sRNAs are a ubiquitous class of molecules that can regulate gene expression at the post-transcriptional level. Most sRNAs can interact with their target mRNAs by base-pairing actions and then modulate translation, degradation, or stability of mRNA [4]. In this study, an sRNA gene stnc640 of S. Enteritidis strain 50336 was cloned and showed 97% identity with stnc640 of S. Typhimurium. This indicated that stnc640 has very high homology within the genus. Identification of the STnc640 target gene is important for the study of sRNA function. To date, the target genes and the function of STnc640 remain unknown. We thus identified likely candidate mRNA targets of STnc640 (fimA and bcfA) by bioinformatics prediction technology using TargetRNA2.

The growth rates of the WT strain, 50336Δstnc640, and 50336Δstnc640/pstnc640 were determined by measuring OD600. The growth rate of 50336Δstnc640 was lower than those of the WT strain and 50336Δstnc640/pstnc640 in the log phase. Many sRNAs can directly sense multiple environmental signals such as fluctuations in temperature, pH, and metabolites [3, 21]. The deletion of STnc640 apparently weakened environmental adaptation, leading to the decline in growth rate at the log phase, but the final concentration of bacteria was not affected.

The STnc640 candidate targets fimA and bcfA were verified by detecting their mRNA levels by RT-qPCR. The expression of fimA was down-regulated in 50336Δstnc640 compared to the WT strain. This suggested that that STnc640 could regulate fimA expression. In other words, fimA was a likely target of STnc640. However, the regulation mechanism needs further study. FimA is a major fimbrial subunit in Salmonella enterica. The Type I fimbriae can alter virulence of S. Typhimurium toward mice [18]. Type I fimbriae are also involved in clearance of S. Enteritidis from the blood and in egg contamination by S. Enteritidis in laying hens [15]. Deletion of STnc640 led to a decrease of fimA expression, but the ability of adhesion to Caco-2 cells of the STnc640 mutant was stronger than that of the wild type strain. This indicated that there is no direct relationship between fimA expression and adhesion ability. Rajashekara found that deletion of the fimA gene in S. Enteritidis did not affect the ability to invade Caco-2 cells and colonize the chicken caecum [22], which is consistent with our result. Multiple fimbrial adhesins are required for Salmonella colonization of the chicken intestine tract. We supposed that up-regulation of other adhesion-related genes expression, but not down-regulation of the fimA gene, caused the adhesion ability enhancement in the STnc640 deletion mutant. Adhesion to and colonization of host cells are important factors for
virulence. In our study, the STnc640 deletion in S. Enteritidis strengthened the ability to adhere to Caco-2 cells and thus increased the virulence in chickens. We inferred that STnc640 could inhibit S. Enteritidis virulence by affecting adhesion. For further confirm of whether STnc640 could inhibit virulence, overexpression of STnc640 in the wild type strain and comparison that with wild type need to be performed in the future.

Conclusions
Small non-coding RNA STnc640 could regulate the expression of fimA fimbrial gene in S. Enteritidis. The deletion of STnc640 in S. Enteritidis strengthened the ability to adhere to and colonize in Caco-2 cells and thus increased the virulence in chickens. It was supposed that STnc640 could inhibit S. Enteritidis virulence by affecting adhesion.

Methods
Bacterial strains, plasmids and cell culture conditions
The bacteria strains and plasmids used in this study are listed in Table 1. 

| Strains/plasmids | Characteristics | References |
|------------------|----------------|-----------|
| Strains          |                |           |
| CMCC(B)50336     | *Salmonella enterica* serovar Enteritidis wild-type | NICPBP, China |
| 50336Δstnc640    | stnc640 deficient mutant | This study |
| 50336Δstnc640/pstnc640 | 50336Δstnc640 carrying pBR-stnc640 (Amp') | This study |
| 50336Δhfq       | hfq deficient mutant | [23] |
| 50336Δhfq/phfq   | 50336Δhfq carrying pBR-hfq (Amp') | [23] |
| Plasmids         |                |           |
| pKD3             | Cm', Cm cassette template | [24] |
| pKD46            | Amp', λRed recombinase expression | [24] |
| pCP20            | Amp', Cm', Fip recombinase expression | [24] |
| pBR-stnc640      | pBR322 carrying the full stnc640 gene (Amp') | This study |
| pGEM-T Easy      | cloning vector, Amp' | Takara |
| pMD19 T-simple   | cloning vector, Amp' | Takara |

Stability detection of STnc640 in hfq mutants
*S. Enteritidis* WT strain 50336, the mutant 50336Δhfq, and the complemented mutant 50336Δhfq/phfq were grown to an OD600 of 2.5 and collected by centrifugation. Total RNA was extracted and reverse transcribed to cDNA. The mRNA transcripts of *stnc640* in WT 50336, 50336Δhfq, and 50336Δhfq/phfq were detected by real-time quantitative PCR (RT-qPCR) using primers stnc640-F and stnc640-R.

Prediction of candidate mRNA targets of STnc640
Candidate mRNA targets of STnc640 were predicted using TargetRNA2 [20] (http://old-tempest.wellesley.edu/~btjaden/TargetRNA2/index.html.oldtempest). Using this website, we selected the *Salmonella* Enteritidis strain P125109 genome, input the STnc640 sequence, and then specified 90 nucleotides upstream and 30 nucleotides downstream of the translation start sites of candidate targets. Candidate targets were identified by specifying at least nine consecutive hybridization seeds corresponding to an initial interaction between the sRNA and mRNA with a p-value below 0.01.

Construction of the stnc640 deletion mutant and the complemented strain
The primers used are listed in Table 2. The stnc640 gene was cloned using PCR primers that flank the stnc640 gene in *Salmonella* Typhimurium. The construction of stnc640-negative mutants of S. Enteritidis 50336 was generated by the phage λ-Red-mediated recombination system as described previously [24, 25]. Primers P3 and P4 were used to amplify chloramphenicol resistance-encoding genes to construct the first recombinant strain 50336Δstnc640::cat. The stnc640 complete deletion mutant 50336Δstnc640 was confirmed by PCR using primers (P1, P2) and sequencing.
the PCR product that contained the primers P3 and P4 sequences and lacked of stnc640 sequences using fluorescence-based chain-termination method with a DNA sequencer ABI 3730XL. The complemented strain was generated by cloning the full-length stnc640 gene into plasmid pBR322, which was transferred to the stnc640 mutant.

The mutant 50336 △ hfq and complemented mutant 50336 △ stnc640/pstnc640 were described previously [23].

RNA extraction and real-time quantitative PCR
Bacteria were grown to an OD 600 of 2.5 in LB medium and collected by centrifugation. Total RNA was extracted using TRIzol reagent (Invitrogen, NY, USA). cDNA was synthesized using the PrimeScript RRT reagent kit with gDNA Eraser (Takara Bio, Shiga, Japan). Transcript abundance was quantified using RT-qPCR with SYBR Premix Ex Taq II (Takara) and the primers listed in Table 2 using an ABI7500 instrument (Applied Biosystems, USA). Assays were performed in triplicate, and all data were normalized to the endogenous reference gene gyrA using the 2−ΔΔCT method [26].

Bacterial adherence and invasion assays
Bacterial adherence and invasion assays were performed as described previously [27]. Bacteria were incubated with a monolayer of 1 × 10⁵ Caco-2 cells at a multiplicity of infection (MOI) of 100 at 37 °C in 96-well tissue culture plates (Corning) for 2 h. Infections were carried out in triplicate. Infected cell monolayers were gently washed three times with PBS to remove loosely adherent bacteria. Cells were lysed with 0.5% Triton X-100 for 30 min. The lysates were serially diluted and plated onto LB agar plates for the enumeration of adherent and invaded bacteria.

Animal infections
One-day-old chickens (National Chickens Genetic Resources, Yangzhou, China) were randomly divided into one control group and three infection groups (n = 20, 10 females and 10 males). Salmonella Enteritidis strains 50336, 50336 △ stnc640 and 50336 △ stnc640/pstnc640 were grown to early stationary phase with an OD₆₀₀ of 2.5 in LB medium, harvested by centrifugation, washed, and resuspended to 5 × 10⁻⁷ CFU/mL, 5 × 10⁻⁸ CFU/mL and 5 × 10⁻⁹ CFU/mL gradient suspensions in sterile PBS prior to inoculation into infection group chickens. Three infection groups were separately inoculated with 200 μL 5 × 10⁻⁷ CFU/mL, 5 × 10⁻⁸ CFU/mL or 5 × 10⁻⁹ CFU/mL bacterial suspensions, while the control group received 200 μL PBS by subcutaneous injection. Signs of chickens illness and death were monitored daily. The 50% lethal dose (LD₅₀) was calculated 14 days post-infection using the method described previously [23]. Briefly, the numbers of dead and surviving chickens were recorded daily. The summation of cumulative dead and surviving chickens of each dose was taken. The LD₅₀ was calculated using the data on percent mortality using the arithmetical method of Reed and Muench [28]. All live chickens were euthanized by pentobarbital after the assays. All procedures complied with institutional animal care guidelines and were approved by the Animal Care and Ethics Committee of the Yangzhou University (Approval ID: YZUDWSY2017–0026).

Statistical analysis
Data were analyzed using Student’s t test for independent samples. Differences were considered significant if P ≤ 0.05.

Abbreviations
Amp: Ampicillin; Caco-2: Human colorectal adenocarcinoma epithelial cells; Cm: Chloramphenicol; IGRs: Intergenic regions; LB: Luria-Bertani broth; LD₅₀: 50% lethal dose; OD₆₀₀: Optical density at 600 nm; RT-qPCR: Real-time quantitative PCR; sRNAs: Small non-coding RNAs; WT: Wild type

Acknowledgments
We thank LetPub (www.letpub.com) for its linguistic assistance during the preparation of this manuscript.
Authors’ contributions
XiaM, GZ and JW conceived, designed and drafted the manuscript. XiaM and XianM performed the majority of the experiments. HW helped with experiments and provided valuable discussion and modified the final manuscript. CZ and JN participated in experimental procedures and data analysis. All authors read and approved the final manuscript.

Funding
This study was supported by grants from the National Key R & D Program (2017YFD0500203), Chinese National Science Foundation (Nos. 31101826 and 31672579), A Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD), Jiangsu Higher Education Science Foundation (No.14KJB230002), State Key Laboratory of Veterinary Biotechnology (No.SKLVBF201509) and Nature Science Foundation of Yangzhou of China. The protocol was approved by the Animal Care and Ethics Committee of Yangzhou University (Approval ID: YZUDWS2017–0026).

Availability of data and materials
The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
One-day-old chickens were provided by the National Chickens Genetic Resources in Yangzhou of China. The protocol was approved by the Animal Care and Ethics Committee of Yangzhou University (Approval ID: YZUDWS2017–0026).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Author details
1College of Veterinary Medicine, Yangzhou University, Yangzhou 225009, China. 2Jiangsu Co-Innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou 225009, China. 3Department of Animal Husbandry and Veterinary Medicine, Beijing Vocational College of Agriculture, Beijing 102442, China. 4Jiangsu provincial key lab for genetics and breeding of poultry, Jiangsu Institute of Poultry Science, Yangzhou 225125, China.

Received: 10 February 2019 Accepted: 27 August 2019
Published online: 05 September 2019

References
1. Hebrard M, Kroger C, Srikumar S, Colgan A, Handler K, Hinton JC. sRNAs and the virulence of Salmonella enterica serovar typhimurium. RNA Biol. 2012;9:437–45.
2. Majdalani N, Vanderpool CK, Gottesman S. Bacterial small RNA regulators. Crit Rev Biochem Mol Biol. 2005;40:93–113.
3. Vogel J. A rough guide to the non-coding RNA world of Salmonella. Mol Microbiol. 2009;71:1–11.
4. Waters LS, Storz G. Regulatory RNAs in bacteria. Cell. 2009;136:615–28.
5. Bejerano-Sago M, Xavier KB. The role of small RNAs in quorum sensing. Curr Opin Microbiol. 2007;10:189–98.
6. Gripenland J, Netterling S, Loh E, Tiensu T, Toledo-Arana A, Johansson J. sRNAs: regulators of bacterial virulence. Nat Rev Microbiol. 2010;8:857–64.
7. Masse E, Salviall H, Desnoyers G, Arguin M. Small RNAs controlling iron metabolism. Curr Opin Microbiol. 2007;10:140–5.
8. Papenfort K, Vogel J. Regulatory RNA in bacterial pathogens. Cell Host Microbe. 2010;8:116–27.
9. Chao Y, Vogel J. The role of Hfq in bacterial pathogens. Curr Opin Microbiol. 2010;13:24–33.
10. Sridhar J, Papenfort K, Vogel J. The small RNAs of Salmonella. Salmonella from genome to function. New York: Horizon press; 2011. p. 123–48.
11. Saeed AM, Gast RK, Potter ME, Wall PG. Salmonella enterica serovar Enteritidis in humans and animals: epidemiology, pathogenesis, and control. Ames: Iowa State University Press; 1999.
12. De Buck J, Van Immerseel F, Haeberlauk F, Ducatelle R. Colonization of the chicken reproductive tract and egg contamination by Salmonella. J Appl Microbiol. 2004;97:233–45.
13. Baumerl AJ, Tsolis RM, Heffron F. The lpf fimbrial operon mediates adhesion of Salmonella typhimurium to murine Peyer's patches. Proc Natl Acad Sci U S A. 1996;93:279–82.
14. Baumerl AJ, Tsolis RM, Heffron F. Contribution of fimbrial operons to attachment to and invasion of epithelial cell lines by Salmonella typhimurium. Infect Immun. 1996;64:1862–5.
15. De Buck J, Van Immerseel F, Haeberlauk F, Ducatelle R. Effect of type 1 fimbriae of Salmonella enterica serotype Enteritidis on bacteremia and reproductive tract infection in laying hens. Avian Pathol. 2004;33:314–20.
16. Weening EH, Barker JD, Laarakker MC, Humphries AD, Tsolis RM, Baumerl AJ. The Salmonella enterica serotype typhimurium lpf, bcf, stb, stc, std, and sth fimbrial operons are required for intestinal persistence in mice. Infect Immun. 2003;71:3358–66.
17. Thomson NR, Clayton DJ, Windhorst D, Vemvisikos G, Davidson S, Churicher C, et al. Comparative genome analysis of Salmonella Enteritidis PT4 and Salmonella Gallinarum 287/91 provides insights into evolutionary and host adaptation pathways. Genome Res. 2008;18:1624–37.
18. Van der Velden AW, Baumerl AJ, Tsolis RM, Heffron F. Multiple fimbrial adhesions are required for full virulence of Salmonella typhimurium in mice. Infect Immun. 1998;66:2808–33.
19. Sittka A, Lucchini S, Papenfort K, Sharma CM, Rolle K, Binnewies TT, et al. Deep sequencing analysis of small noncoding RNA and mRNA targets of the global post-transcriptional regulator, Hfq. PLoS Genet. 2008;4:e1000163.
20. Key MB, Feldman M, Livny J, Tjaden B. (2014) TargetRNA2: identifying targets of small regulatory RNAs in bacteria. Nucleic Acids Res. 2014;42:124–9.
21. Klinkert B, Narberhaus F. Microbial thermosensors. Cell Mol Life Sci. 2009;66:2661–76.
22. Rajashekara G, Munir S, Alexeyev MF, Halvorson DA, Wells CL, Nagaraja KV. Pathogenic role of SEF14, SEF17, and SEF21 fimbriae in Salmonella enterica serovar enteritidis infection of chickens. Appl Environ Microbiol. 2006;72:159–63.
23. Meng X, Meng X, Zhu C, Wang H, Wang J, Nie J, et al. The RNA chaperone Hfq regulates expression of fimbrial-related genes and virulence of Salmonella enterica serovar Enteritidis. FEMS Microbiol Lett. 2013;346:90–6.
24. Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc Natl Acad Sci U S A. 2000;97:6640–5.
25. Duan Q, Zhou M, Zhu X, Yang Y, Zhu J, Bao W, et al. Flagella from F18+ Escherichia coli play a role in adhesion to pig epithelial cell lines. Microb Pathog. 2013;55:32–8.
26. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2–(Delta Delta C(T)) method. Methods. 2001;25:402–8.
27. Jouve M, Garcia MI, Courcoux P, Labigne A, Gounon P, Le Bouguenec C. Adhesion to and invasion of HeLa cells by pathogenic Escherichia coli carrying the afa-3 gene cluster are mediated by the AfaE and AfaD proteins, respectively. Infect Immun. 1999;67:482–9.
28. Reed LJ, Muench H. Asimple method of estimating fifty percent end points. Am J Hyg. 1938;27:493–7.