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TITLE:

The virulence of a non-coding small RNA MicC in outer membrane proteins of *Salmonella* Enteritidis

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Keywords:

Salmonella Enteritidis, MicC, regulation, virulence, outer membrane proteins

Summary:

An λ-Red-mediated recombination system was used to create a deletion mutant of a small non-coding RNA *micC*.

Abstract:

A non-coding small RNA (sRNA) is a new factor to regulate gene expression at the post-transcriptional level. A kind of sRNA MicC, known in *Escherichia coli* and *Salmonella* Typhimurium, could repress the expression of outer membrane proteins. To further investigate the regulation function of *micC* in *Salmonella* Enteritidis, we cloned the *micC* gene in the *Salmonella* Enteritidis strain 50336, and then constructed the mutant 50336Δ*micC* by the λ Red-based recombination system and the complemented mutant 50336Δ*micC*/p*micC* carrying recombinant plasmid pBR322 expressing *micC*. qRT-PCR results demonstrated that transcription of *ompD* in 50336Δ*micC* was 1.3-fold higher than that in the wild type strain, while the

transcription of *ompA* and *ompC* in 50336Δ*micC* were 2.2-fold and 3-fold higher than those in the wild type strain. These indicated that *micC* represses the expression of *ompA* and *ompC*. In the following study, the pathogenicity of 50336ΔmicC was detected by both infecting 6-week-old Balb/c mice and 1-day-old chickens. The result showed that the LD_{50} of the wild type strain 50336, the mutants 50336ΔmicC and 50336ΔmicC/p*micC* for 6-week-old Balb/c mice were 12.59 CFU, 5.01 CFU, and 19.95 CFU, respectively. The LD_{50} of the strains for 1-day-old chickens were 1.13×10^9 CFU, 1.55×10^8 CFU, and 2.54×10^8 CFU, respectively. It indicated that deletion of *micC* enhanced virulence of *S.* Enteritidis in mice and chickens by regulating expression of outer membrane proteins.

Introduction

Non-coding small RNAs (sRNAs) are 40-400 nucleotides in length, which generally do not encode proteins but could be transcribed independently in bacterial chromosomes[1,2,3]. Most sRNAs are encoded in the intergenic regions (IGRs) between gene-coding regions and interact with target mRNAs through base-pairing actions, and regulate target genes expression at the post-transcriptional level[4,5]. They play important regulation roles in substance metabolism, outer membrane protein synthesis, quorum sensing and virulence gene expression[5].

MicC is a 109-nucleotide small RNA transcript present in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium, which could regulate multiple outer membrane protein expression such as OmpC, OmpD, OmpN, Omp35 and Omp36[6-9]. MicC regulates the expression of OmpC by inhibiting ribosome binding to the *ompC* mRNA leader in vitro and it requires the Hfq RNA chaperone for its function in *Escherichia coli*[6]. In *Salmonella* Typhimurium, MicC silences *ompD* mRNA via a ≤12-bp RNA duplex within the coding sequence (codons 23-26) and then destabilizes endonucleolytic mRNA[7]. This regulation process is assisted by chaperone protein Hfq[10]. The OmpC is an abundant outer membrane protein that was thought to be important in environments where nutrient and toxin concentrations are high, such as in the intestine[6]. The OmpD porin is the most abundant outer membrane protein in *Salmonella* Typhimurium and represents about 1% of total cell protein[11]. OmpD is involved in adherence to human macrophages and intestinal epithelial cells[12]. MicC also represses the expression of both OmpC and OmpD porins. It is thought that MicC may regulate virulence. To explore new target genes regulated by MicC and study the virulence regulation function of *micC*, we cloned the *micC* gene in the *Salmonella* Enteritidis (SE) strain 50336, and then constructed the mutant 50336ΔmicC and the complemented mutant 50336ΔmicC/p*micC*. Novel target genes were screened by qRT-PCR. The virulence of 50336ΔmicC was detected by mice and chicken infections.

Protocol

All the experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the National Research Council. The animal care and use committee of Yangzhou University approved all experiments and procedures applied on the animals (SYXK2016-0020).

1. Bacterial strains, plasmids, and culture conditions

1.1. Use the bacteria and plasmids listed in **Table 1**.

1.2. Culture bacteria in LB broth or on LB agar plates at 37 ˚C, in the presence of 50 μg/mL ampicillin (Amp) when appropriate.

1.3. Culture strains containing temperature sensitive plasmids are used for deletion mutant construction at 30 ˚C.

2. Clone *micC* gene of *S.* Enteritidis strain 50336

2.1. Based on the upstream and downstream sequence of *micC* gene of *S.* Typhimurium strain SL1344, design primers vmicC-F and vmicC-R to amplify a fragment containing *micC* gene by PCR using SE50336 genomic DNA as template.

2.2. Mix 5 μL of 10x PCR reaction buffer, 2 μL of dNTP mixture (2.5 mM), 1 μL of vmicC-F and vmicC-R primers, respectively, 5 μL of template, 1 μL of Taq DNA polymerase and 35 μL of ddH_2O together for PCR.

2.3. Use the following PCR reaction conditions: pre-denaturation at 94 ˚C for 4 min; 94 ˚C for 30 s, 53 ˚C for 1 min, 72 ˚C for 1 min for 25 cycles, and extension at 72 ˚C for 10 min.

2.4. Sequence the PCR product to obtain the *micC* gene sequence.

3. Construction of the *micC* deletion mutant

NOTE: The *micC*-negative mutant of *Salmonella* Enteritidis strain 50336 was constructed using λ-Red-mediated recombination as described previously[13,14]. The primers used are listed in **Table 2**.

3.1. Amplify chloramphenicol cassette containing homology fragments of *micC* gene.

3.1.1. Design micC-F and micC-R primers to amplify the chloramphenicol (Cm) cassette from plasmid pKD3, including 50 bp homology extensions from the 5' and 3' of the *micC* gene.

3.1.2. Extract the pKD3 plasmid as the PCR template.

3.1.3. Mix 5 μL of 10x PCR reaction buffer, 2 μL of dNTP Mixture (2.5 mM), 1 μL of micC-F and micC-R primers, respectively, 5 μL of template, 1 μL of Taq DNA polymerase and 35 μL of ddH_2O together as PCR reaction mixture

3.1.4. Amplify the Cm cassette with the following PCR reaction conditions: pre-denaturation at 94 ˚C for 4 min; 94 ˚C for 1 min, 52 ˚C for 1 min, 72 ˚C for 1 min for 10 cycles; 94 ˚C for 1 min, 63 ˚C for 1 min, 72 ˚C for 1 min for 25 cycles, and extension at 72 ˚C for 10 min.

3.1.5. Detect the size of PCR product by agarose gel electrophoresis. Purify and recover PCR product with DNA gel recovery kit, and determine the concentration of DNA by spectrophotometer.

CAUTION: PCR must be carried out twice. The first PCR product was diluted at a ratio of 1:200 and used as a template for the secondary PCR, to eliminate the interference of further recombination by pKD3 plasmid.

3.2. Construct 1st recombinant strain 50336Δ*micC::cat*

3.2.1. Mix 100 μL of SE50336 competent cells with 5 μL of pKD46 plasmid uniformly and incubate on ice for 30 min. Heat shock the above mixture at 42 ˚C for 90 s, and rapidly transfer the mixture to ice for 2 min to transform the pKD46 plasmid to SE50336. Screen positive colonies by culturing overnight at 30 ˚C on an Amp (50 μg/mL) resistant plate.

3.2.2. Add 30 mM L-arabinose to SE50336/pKD46 liquid culture, and induce recombinase expression by a 30 °C shaking culture for 1 h. Then prepare competent cells.

3.2.3. Mix 100 ng of purified PCR product (step 3.1) and 40 μL of SE50336/pKD46 competent cells into an electric shock cup (e.g., Bio-Rad). Carry out electric shock transformation with the parameters of voltage 1.8 kV, pulse 25 μF and resistance 200 Ω.

3.2.4. After electrotransformation, transfer the mixture to 1 mL of SOC medium and a shaking culture at 150 rpm and 30 ˚C for 1 h. Then smear the mixture on a Cm

(34 μg/mL) resistant LB plate and culture at 37 ˚C overnight to screen positive colony.

3.2.5. Culture the above positive colony at 42 ˚C for 2 h. Screen the colony that is sensitive to Amp (50 μg/mL) but resistant to Cm (34 μg/mL) at 37 ˚C overnight to obtain the 1st recombinant strain without pKD46.

3.3. Identify the 1st recombinant strain 50336Δ*micC*::Cat.

3.3.1. Extract 50336Δ*micC*::Cat genomic DNA as the PCR template. Use the same PCR reaction components as in step 2.1. Carry out the PCR reaction with the same conditions as in step 2.1.

3.3.2. Detect the size of PCR product by agarose gel electrophoresis and sequence the PCR product.

3.4. Construct deletion mutant 50336Δ*micC.*

3.4.1. Electroporate 100 ng of plasmid pCP20 into 40 μL of 50336Δ*micC*::Cat competent cells with the parameters of voltage 1.8 kV, pulse 25 μF and resistance 200 Ω, screen positive transformants on both Amp (50 μg/mL) and Cm (34 μg/mL) resistant plate at 30 ˚C.

3.4.2. Transfer above positive transformants into non-resistant LB and culture them overnight at 42 ˚C, and then isolate single colonies on an LB plate at 37 ˚C. Select the colony that is sensitive to both Amp and Cm. This mutant is the *micC* deletion mutant SE50336Δ*micC*.

3.4.3. Verify 50336Δ*micC* by PCR.

3.4.3.1. Extract 50336Δ*micC* genomic DNA as PCR template. Mix 5 μL of 10x PCR reaction buffer, 2 μL of dNTP Mixture (2.5 mM), 1 μL of primer vmicC-F, 1 μL of primer vmicC-R, 5 μL of template, 1 μL of Taq DNA polymerase and 35 μL of ddH_2O together for PCR.

3.4.3.2. Use the following PCR reaction conditions: pre-denaturation at 94 ˚C for 4 min; 94 ˚C for 30 s, 53 ˚C for 1 min, 72 ˚C for 1 min for 25 cycles, and extension at 72 ˚C for 10min.

4. Construction of the *micC* complemented strain

4.1. Design primers pBR-micC-F and pBR-micC-R with NheI and SalI restriction sites.

4.1.1. Amplify full-length *micC* gene with flank sequences using PCR reaction mixture that contains 5 μL of SE50336 genomic DNA as template, primers 1 μL of pBR-micC-F and 1 μL of pBR-micC-R as primers, 5 μL of 10x PCR reaction buffer, 2 μL of dNTP Mixture (2.5 mM), 2 μL of dNTP Mixture (2.5 mM) and 35 μL of ddH_2O.

4.1.2. Use the following PCR reaction conditions: pre-denaturation at 94 ℃ for 4 min; 94 ˚C for 30 s, 52 ˚C for 50 s, 72 ˚C for 1 min for 25 cycles, and extension at 72 ˚C for 10 min. Purify and recover PCR product.

4.2. Digest PCR product and plasmid pBR322 respectively using restriction enzyme *NheI* and *SalI*, and ligate them using T4 ligase at 16 ˚C overnight to obtain the plasmid pBR322-micC.

4.3. Transform pBR322-micC into the SE50336ΔmicC competent cells, and screen positive transformant to obtain the complemented strain SE50336ΔmicC/p*micC*. Extract plasmid pBR322-micC from complemented strain and verify it by restriction enzyme digestion and sequencing.

5. RNA isolation and quantitative real-time PCR

5.1. Culture SE50336, 50336ΔmicC, and 50336ΔmicC/p*micC* in LB medium overnight at 24 ˚C with 180 rpm shake cultivation to an OD_{600} of 2.0. Collect bacterial culture by centrifugation at 13000 rpm for 2 min.

5.2. Extract total RNA using TRIzol reagent. Incubate 50 μL of isolated RNA with 2 μL of DNaseI and 6 μL of 10x buffer at 37 ˚C for 30 min to remove DNA. Determine RNA quantity by pipetting 1 μL of RNA sample to a micro-spectrophotometer.

5.3. Synthesis of cDNA

5.3.1. Use 1 μg of total RNA for cDNA synthesis in 20 μL of reverse transcription reaction system (4 μL of 5x buffer, 1 μL of RT Enzyme mix, 1 μL of RT primer mix, 10 μL of total RNA, and 4 μL of ddH_2O). Incubate above reaction system at 37 ˚C for 15 min and then at 85 ˚C for 5 s.

5.4. Design primers based on the sequence of target genes *ompA*, *ompC* and *ompD*. Perform reverse transcription-PCR using a RT reagent kit. The PCR reaction components contain 2.5 μL of 10x PCR reaction buffer, 1 μL of dNTP mixture (2.5 mM), 1 μL of target gene (*ompA*, *ompC* or *ompD*) primers, 2.5 μL of template, 0.5 μL of Taq DNA polymerase and 17.5 μL of ddH_2O.

5.4.1. Use the following PCR reaction conditions: pre-denaturation at 94 ˚C for 4 min; 94 ˚C for 30 s, 60 ˚C for 1 min, 72 ˚C for 1 min for 25 cycles, and extension at 72 ˚C for 10min.

5.5. Carry out real-time PCR using SYBR green RT-PCR kit in a RT-PCT instrument in triplicates.

5.5.1. Use the following PCR reaction components: 10 μL of 2x SYBR buffer, 0.4 μL of forward primer and reverse primer respectively, 0.4 μL of RoxDye II, 2 μL of cDNA and 6.8 μL of RNase free H_2O.

5.5.2. Use the following PCR reaction conditions: pre-denaturation at 95 ˚C for 1 min for one cycle; 95 ˚C for 5 s, 60 ˚C for 34 s for 40 cycles.

5.5.3. Normalize all data to the endogenous reference gene *gyrA*. Use $2^{-\Delta\Delta CT}$ method for data quantification[15].

6. Virulence assays

6.1. Culture SE50336, 50336ΔmicC and 50336ΔmicC/p*micC* in LB medium to early stationary phase (OD_{600} of 2-3) at 24 ˚C, harvest by centrifugation, and dilute to appropriate CFU mL^{-1} in sterile PBS.

6.2. For mice infections, dilute the cultured strains to 10 CFU/200 μL, 10^2 CFU/200 μL and 10^3 CFU/200 μL gradient resuspensions. Infect groups of five 6-8 week old Balb/c mice per strain by subcutaneous injection. Inject the control group with 200 μL of physiological saline.

6.3. For chicken infections, dilute above three strains to 10^7 CFU/200 μL, 10^8 CFU/200 μL and 10^9 CFU/200 μL gradient resuspensions. Infect groups of twenty 1-day-old chickens per strain by subcutaneous injection.

6.4. Monitor signs of illness and deaths of experimental animals daily. Calculate the LD_{50} (median lethal dose) 14 d post-infection as described previously[16]. Process the data using data analysis software.

6.5. In infection groups, collect the heart, liver, spleen, lung, and kidney of freshly dead chicks.

6.5.1. Weigh 0.5 g of the above tissues separately and grind them with sterile operation. Dilute grinding samples gradually, spread them on LB plate and culture

for 8-10 h at 37 ˚C. Record the amount of *Salmonella* strains colonized in chick tissues.

REPRESENTATIVE RESULTS

Construction of the mutant 50336Δ*micC* and complemented strain 50336Δ*micC* /p*micC*

The *micC* gene clone result indicated that this gene was composed of 109 bp showing 100% identity with that of *S.* Typhimurium. Based on the sequence data, the deletion mutant 50336ΔmicC and the complemented mutant 50336ΔmicC/p*micC* were constructed successfully. In detail, sequencing results showed that a 1.1 kb Cm resistance cassette was amplified and used for constructing the 1st recombinant. The 1st recombinant 50336ΔmicC*::cat* was validated by PCR using primers vmicC-F and vmicC-R with an expected band size of about 1200 bp of PCR products with Cm insertion compared to 279 bp of PCR products in wild type strain (**Figure 1**). In the second recombination, Cm cassette was eliminated by pCP20. The PCR results combined with sequencing confirmed that the isogenic *micC* mutant was constructed successfully and named as 50336ΔmicC (**Figure 1**).

MicC regulates *ompA*, *ompC*, and *ompD* gene expression

To determine the targets of MicC, the expression of *ompA*, *ompC* and *ompD* genes in SE strains 50336, 50336ΔmicC and 50336ΔmicC/p*micC* were analyzed by real-time quantitative PCR using *gyrA* as the normalizing internal standard. The results showed that transcription of *ompA* and *ompC* in 50336ΔmicC increased about 2.2-fold and 3-fold than those in the wild type strain, while *ompD* in 50336ΔmicC was increased slightly (1.3-fold) than that in wild type strain (**Figure 2**). It indicated that *micC* could repress the expression of *ompA*, *ompC* and *ompD*. *OmpA* was probably a potential novel target gene regulated by *micC* directly.

Deleting *micC* enhances *S.* Enteritidis virulence in mice and chickens

We performed LD_{50} assays to quantify the impact of deleting *micC* on *S.* Enteritidis virulence in mice and chickens. After infecting 6-8 week old Balb/c mice with 10^3 CFU of each of the three strains, we observed that the most mice infected by 50336ΔmicC displayed lassitude, inappetence or diarrhea 48 h post infection, and appeared to die in succession 96 h post infection. While the mice infected by WT strain and 50336ΔmicC/p*micC* displayed the above symptoms 72 h post infection, and were dead 120 h post infection. The LD_{50}s were calculated 7 d post-infection. The results showed that the LD_{50} of the WT strain 50336, 50336ΔmicC and 50336ΔmicC/p*micC* for mice were 12.59, 5.01 and 19.95 CFU, respectively. It indicated that the virulence of the mutant 50336ΔmicC enhanced 2.5-fold as compared with WT in mice (**Table 3**). After infecting 1-day-old chickens with 10^9 CFU of each of the three strains, most chickens displayed intestinal hyperemia and diarrhea 10 h post infection. When infected with 10^8 CFU, the chickens infected with

50336ΔmicC showed higher mortality, as compared with WT strain and 50336ΔmicC/p*micC*. The LD_{50}s were calculated for 14 d post-infection. The results showed that the LD_{50} of the WT strain 50336, 50336ΔmicC, and 50336ΔmicC/p*micC* for chickens were 1.13×10^9, 1.55×10^8 and 2.54×10^8 CFU, respectively. It indicated that deletion of *micC* also enhanced virulence of *S.* Enteritidis in chickens. All three strains of *S.* Enteritidis were recovered from the liver, spleen, and caecum of the infected chickens.

Figure 1: PCR verification of the 50336ΔmicC mutants with primers vmicC-F and vmicC-R. A 280 bp PCR product was obtained when the wild-type 50336 genome as template (lane 1). When the Cm cassette gene was inserted to genome of *S.* Enteritidis, the 1st recombinant 50336Δ*mic::cat* was verified by PCR and a 1100 bp PCR product was obtained (lane 2). The Cm cassette gene of 50336Δ*mic::cat* was excised by introducing the FLP recombinase-expressing vector pCP20 and the 2nd recombinant 50336Δ*mic* was obtained and verified by PCR (lane 3). M: molecular mass marker.

Figure 2: Fold changes of *ompA*, *ompC* and *ompD* genes mRNA level were determined in the mutant 50336Δ*mic* and complemented strain 50336Δ*mic*/p*mic* by quantitative RT-PCR compared to the wild type strain. Assays were performed in triplicate. The $2^{-\Delta\Delta CT}$ method was used for data quantification. *Indicates statistically significant difference compared with the wild type strain (p<0.05)

Table 1. Bacterial strains and plasmids used in this study.

Table 2. Primers used in this study

Table 3. Virulence properties of *S.* Enteritidis 50336 strains in mice and chickens

Discussion

S. Enteritidis is an important facultative intracellular pathogen that can infect young chickens and produces symptoms from enteritis to systemic infection and death[17,18]. In addition, *S.* Enteritidis causes latent infections in adult chickens and chronic carriers contaminate poultry products, resulting in food-borne infections in humans[19]. The pathogenic mechanism of *S.* Enteritidis remains to be further probed. To date, some sRNAs such as IsrJ, SroA and IsrM have been found to affect *Salmonella* virulence[20-23]. The non-coding small RNA *micC* gene was identified in many Enterobacteria such as *Escherichia coli*, *Salmonella* Typhimurium, *Salmonella* Bongori and *Shigella flexneri*[6,7,24]. Here, we found that the sequence of *micC* in *S.* Enteritidis 50336 was the same as that in *S.* Typhimurium. It indicates that MicC is a conservative sRNA in Enterobacteria.

To investigate whether MicC mediates virulence in *S.* Enteritidis for animals and identify MicC targets, we constructed the deletion mutant 50336ΔmicC and the complemented mutant 50336ΔmicC/p*micC* expressing *micC* successfully. The results of qRT-PCR indicated that *micC* could repress the expression of *ompA* and *ompC*. OmpA is probably a potential novel target of MicC. The sRNA RybB could repress the synthesis of OmpA by base-pairing with the 5' untranslated regions (5' UTRs) of target *ompA* mRNA[25]. The MicA sRNA also facilitates rapid decay of the *ompA* mRNA by antisense pairing similarly to RybB[25, 26]. Whether MicC uses the similar regulation mechanism to regulate *ompA* is not known and remains to be studied in the near future. In *E. coli*, the deletion of MicC increased the expression of *ompC* 1.5- to 2-fold. Further study showed that MicC was shown to inhibit ribosome binding to the *ompC* mRNA 5' leader[6]. In addition, Pfeiffer found that OmpC was the main targets of MicC[7]. It is supposed that MicC regulates *ompC* in a similar mechanism in *S.* Enteritidis with that in *E. coli* and *S.* Typhimurium. Besides OmpA and OmpC, MicC could also repress the expression of OmpD. The result showed that the transcription of *ompD* in 50336Δ*micC* was increased slightly (1.3-fold) than that in wild type strain. Based on the above results, it demonstrated that MicC could repress the transcription of multiple target mRNAs (*ompA*, *ompC* and *ompD*) in *S.* Enteritidis. MicC is not the only one sRNA that can regulate multiple targets. Some sRNAs such as RybB, DsrA, GcvB, RNAIII and RyhB also act upon multiple targets[25,27-31]. Because sRNAs regulate targets by base-pairing mechanism to accomplish sRNA-target interactions[32], it is possible that conserved sub-regions or domains of sRNAs can bind to different targets.

The outer membrane of Gram-negative bacteria is a key interface in host-pathogen interactions. OmpA, OmpC and OmpD are all important and abundant outer membrane proteins. OmpC plays an important role in abominable environment such as in the intestine[6]. OmpD is involved in adherence to human macrophages and intestinal epithelial cells[12]. It was thought that the change of OMPs expression caused by MicC deletion could influence the virulence of *S.* Enteritidis, and MicC accumulated in stationary-phase cells and especially under growth conditions induced the Salmonella SPI-1 and SPI-2 virulence genes[7]. It is thought that MicC is related to virulence in *Salmonella*, while animal infections experiments were performed to detect virulence of MicC. The results showed that the LD_{50} of the mutants 50336ΔmicC for 1-day-old chickens and 6-week-old Balb/c mice were both declined obviously compared with the wild type strain. It indicated that the deletion of *micC* enhanced virulence of *S.* Enteritidis in mice and chickens. It is supposed that the increase of OmpA, OmpC and OmpD expression, which is caused by MicC deletion lead to virulence enhancement in *S.* Enteritidis.

MicC negatively regulates *S*. Enteritidis virulence in mice and chickens probably by downregulating expression of the major outer membrane proteins OmpA and OmpC.

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Disclosures:

The authors have nothing to disclose.

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Figure 1

Click here to access/download;Figure;fig1.jpg

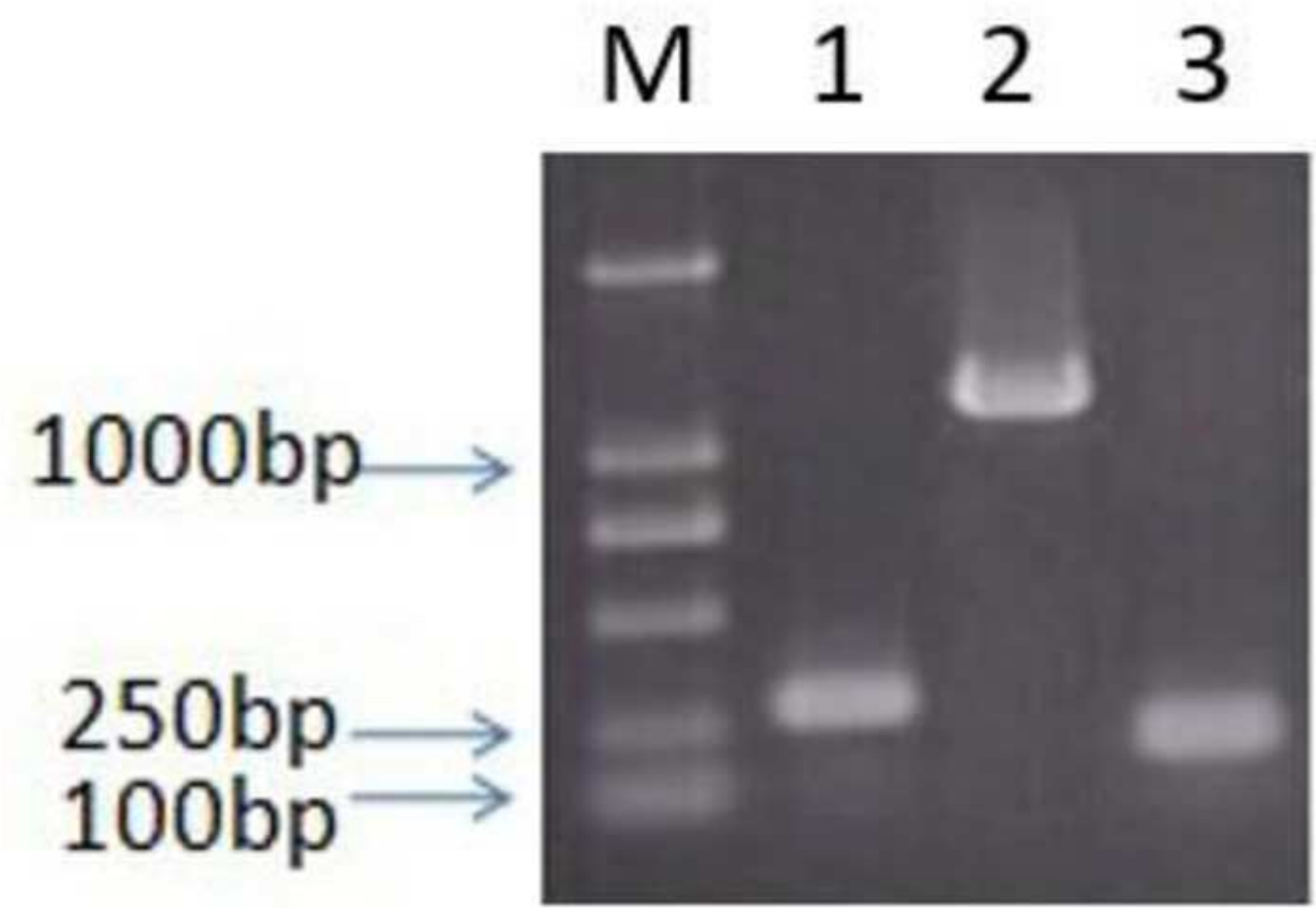
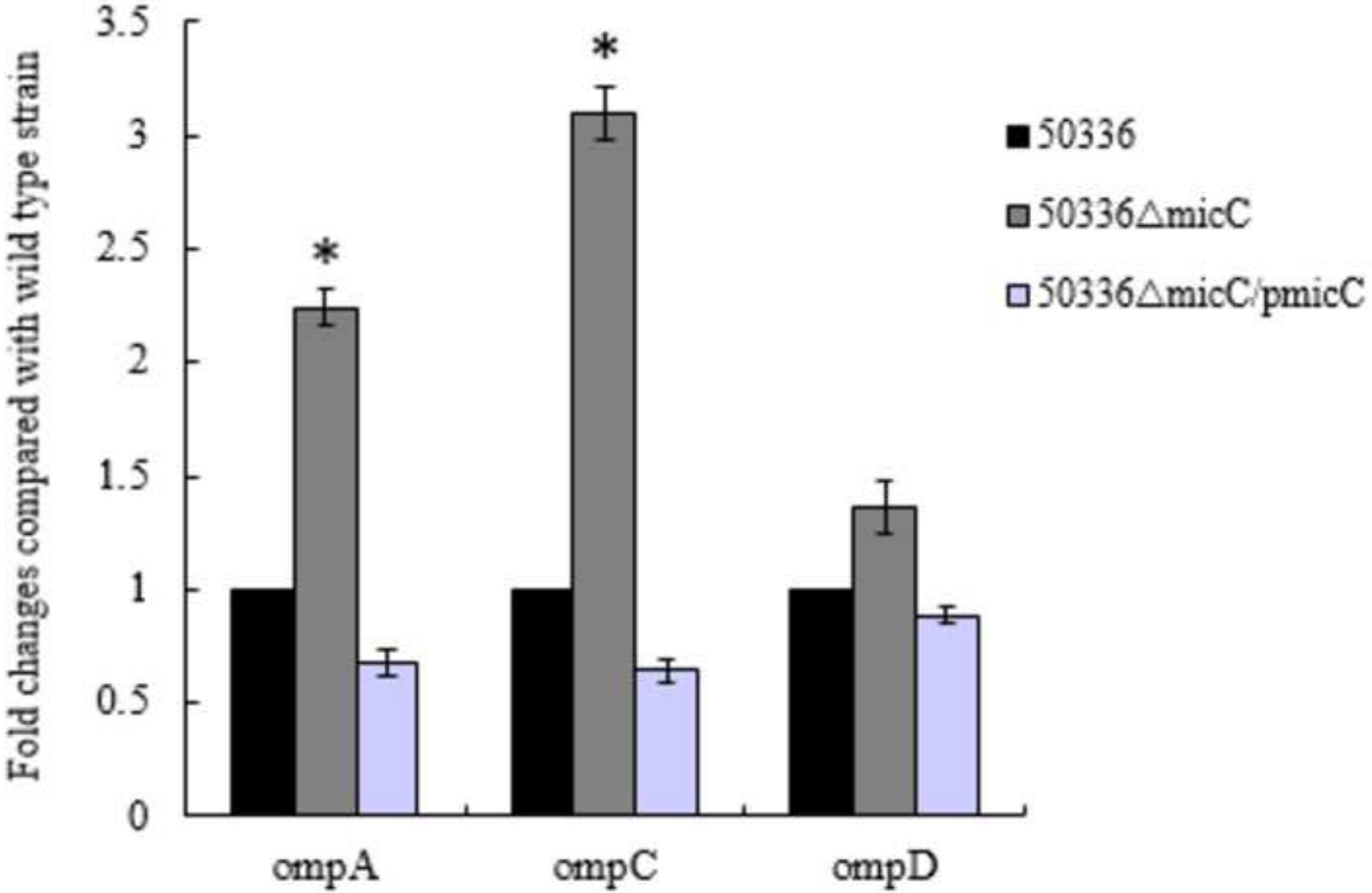


Figure 2



Strains/plasmids	Characteristics
Strains	
CMCC(B)50336	*Salmonella enterica* serovar Enteritidis wild-type
50336△*micC*	*micC* deficient mutant
50336Δ *micC* /*pmicC*	50336Δ*micC* carrying pBR- *micC* (Amp^r)
Plasmids:	
pKD3	Cm^r; Cm cassette teplate
pKD46	Amp^r, λRed recombinase expression
pCP20	Amp^r, Cm^r; Flp recombinase expression
pBR-*micC*	pBR322 carrying the full *micC* gene (Amp^r)
pGEM-T Easy	cloning vector, Amp^r
pMD19 T-simple	cloning vector, Amp^r

References	
NICPBP, China	
This study	
This study	
[13]	
[13]	
[13]	
This study	
Takara	
Takara	

Primer	Sequence (5'-3')
micC-F	TGTCAGGAAAGACCTAAAAAGAGATGTTACCGTTTAATTCAATAATTAATTGTGTAGGCTGGAGCTGCTTCG
micC -R	TGGAAATAAAAAAAGCCCGAACATCCGTTCGGGCTTGTCAATTTATACCATATGAATATCCTCCTTAG
vmicC -F	AGCGAGTTGACGTTAAAACGTTAT
vmicC -R	TTCGTTCGGGCTTGTCAATTTATA
pBR-micC-F	CAGGCTAGCCACTTTATGTACAATGACATACGTCAC
pBR-micC-R	CAGGTCGACAAATATTCTAAGGATTAACCTGGAAAC
ompA-F	actgaacgccctgagcttta
ompA-R	acaccggcttcattcacaat
ompC-F	aaagttctgcgctttgttgg
ompC-R	cgctgacgaacacctgtatg
ompD-F	acggtcagacttcgcatagg
ompD-R	tgttgccacctaccgtaaca
gyrAF	GCATGACTTCGTCAGAACCA
gyrAR	GGTCTATCAGTTGCCGGAAG

Product size (bp)
1114
279/140
434
177
187
184
278

Strains	LD_{50} for mice (CFU)	Fold enhancement	LD_{50} for chickens (CFU)
S. enteritidis 50336	12.59	1	1.13×10^{9}
50336△*micC*	5.01	2.51	1.55×10^{8}
50336△*micC*/p*micC*	19.95	0.63	2.54×10^{8}
Negative control	0	/	0

Fold enhancement
1
7.29
4.45
/

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
dextrose	Sangon Biotech	A610219	for broth preparation
DNA purification kit	TIANGEN	DP214	for DNA purification
Ex Taq	TaKaRa	RR01A	PCR
KH_2PO_4	Sinopharm Chemical Reagent	10017608	for broth preparation
K_2HPO_4	Sinopharm Chemical Reagent	20032116	for broth preparation
L-Arabinose	Sangon Biotech	A610071	λ-Red recombination
Mini Plasmid Kit	TIANGEN	DP106	plasmid extraction
NaCl	Sinopharm Chemical Reagent	10019308	for broth preparation
$(NH_4)_2SO_4$	Sinopharm Chemical Reagent	10002917	for broth preparation
PrimeScript^{R}RT reagent Kit with gI	TaKaRa	RR047	qRT-PCR
SYBR^R Premix Ex Taq II	TaKaRa	RR820	qRT-PCR
T4 DNA Ligase	NEB	M0202	Ligation
TRIzol	Invitrogen	15596018	RNA isolation
Tryptone	Oxoid	LP0042	for broth preparation
Yeast extract	Oxoid	LP0021	for broth preparation
centrifuge	Eppendorf	5418	centrifugation
Electrophoresis apparatus	Bio-Rad	164-5050	Electrophoresis
Electroporation System	Bio-Rad	165-2100	for bacterial transformation
Spectrophotometer	BioTek	Epoch	Absorbance detection
Real-Time PCR system	Applied Biosystems	7500 system	qRT-PCR

Rebuttal letter

Dear editor,

Thank you very much for your nice comments concerning our manuscript. We truly appreciate the constructive comments from the editor and reviewers, and please let us know if you have any questions or requests. I have revised the manuscript according to editorial and reviewers' comments. The point-by-point responses are as follows:

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response: Thanks for your helpful suggestion. We have revised spelling and grammar mistakes

2. Please remove the brackets around the superscripted references.

Response: Thank you. We have revised them.

3. Please specify the PCR reaction recipe throughout. How much of what is used?

Response: Thanks for your helpful suggestion. We have specified the PCR reaction recipe throughout.

4. Please specify all volumes and concentrations used throughout.

Response: Thank you. We have revised them.

5. Much of the protocol is written with very general instructions. Please note that we need explicit stepwise instructions in order to film. The usage of commercial kits is okay but if the step involving the kit is filmed, we need to know what is explicitly done. How much of what is added for how long and into what? etc. Steps 3.2-3.2.4 are examples of sufficient details.

Response: Thank you. We have detailed protocol based on your suggestion.

6. Please highlight up to 3 pages of protocol text for inclusion in the protocol section of the video. Please ensure that these highlighted steps tell a cohesive narrative and all the details to perform the experimental step is included.

Response: We highlighted protocol in part3 "construction of the micC deletion mutant".

7. Please spell out journal titles in the references.

Response: Thank you. We have revised references based on your suggestion.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The authors have addressed the reviewers comments properly and the revised manuscript has improved significantly.

Major Concerns:

However, the English language still require polishing. In addition, there are places where the sentences should be rewritten.

Response: Thanks for your suggestions. We have polished the English language again.

Minor Concerns:

1. Lines 35-37: "was increased"and "increased", not symmetrical. It can be "1.3- fold higher than that in…" and 2.2-fold and 3-fold higher than….

Response: Thanks for your suggestion. We have revised them.

2. Lines 38-39: It does not make sense to say here "OmpA, probably a novel target genes regulated by micC, remains to he known"because you are going to study OmpC, but not OmpA.

Response: Thanks for your suggestion. We have deleted this sentence.

3. Line 37 and 44: They used "it indicated"here, Should be "it"or "these"? Also you should use different expressions as well.

Response: Thank you. We have revised “it” to “these”.

4. Lines 291-296 should be moved as the first paragraph.

Response: We have moved these sentences to the first paragraph.

5. Line 296: 'to'should be "To".

Response: Thank you. We have revised it.

6. The conclusion is the repetition of the major results, not appropriate here. It should be rephrased as "micC negatively regulates S. Enteritidis virulence probably by downregulating expression of the major outer membrane proteins OmpA and OmpC.

Response: Thanks for your suggestion. The conclusion has been revised.

7. Figure 1 should be cropped on the top and bottom parts, simply keeping the

"effective"region to save space.

Response: Thanks for your suggestion. We have revised Figure 1.

8. Figure 2 Y-axis: should be 'Fold changes compared…'

Response: Thank you. We have revised it.

Reviewer #2:

Manuscript Summary:

A non-coding small RNA MicC contributes to virulence involved in outer membrane proteins in Salmonella Enteritidis

Major Concerns:

there is no Major concerns

Minor Concerns:

There are no Minor concerns. The manuscript can be published.

Response: Thank you very much!