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EsR240, a non-coding sRNA, is required for the resistance of *Edwardsiella tarda* to stresses in macrophages and for virulence

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ABSTRACT

Bacterial small non-coding RNAs (sRNAs) are gene expression modulators that respond to environmental changes and pathogenic conditions. In this study, 13 novel sRNAs were identified in the intracellular pathogen, *Edwardsiella tarda* (*E. tarda*) ET13 strain, based on RNA sequencing and bioinformatic analyses. Eight of the 13 putative sRNAs from the ET13 strain were transcribed (as indicated by RT-PCR) following exposure to different stresses. The transcription levels of three sRNAs (EsR128, EsR139 and EsR240) were all highly induced under these stress conditions. Northern blot hybridization was employed to verify that EsR240 was expressed in the ET13 strain under both logarithmic and stationary growth phases, and that it formed a single copy transcript in the chromosomes of the ET13 strain. The precise start and end points of EsR240 were determined using 5'and 3' RACE. The conservation of EsR240 was in agreement with the characteristics of sRNA, as indicated by a BLAST analysis. Furthermore, the survival rates of EsR240 mutant were lower than the rates of the wild type ET13 under stress conditions. When the infection time was extended 4 or 6 h, the CFUs of the wild type bacteria increased more significantly within macrophages compared to the mutant. When the intra-peritoneal (i.p.) route of infection was used in mice, the bacterial loads of the tissues in the mice infected with the wild type bacteria were significantly higher than in the mice infected with the mutants. The virulence of the EsR240 mutant was 6.79-fold lower than the wild type bacterium based on the LD_{50}. In addition, the IntaRNA program was used to predict the target genes of EsR240. Out of the top 10 predicted target genes, 9 genes were regulated by EsR240. These target genes may encode FtsH protease modulator YccA, Na^+ and H^+ antiporters, FtsX-like permease family protein, glycoside hydrolases or various other proteins. Therefore, EsR240 may positively regulate its target genes in *E. tarda* to maintain intracellular survival within host macrophages and to increase its virulence.

Edwardsiella tarda (*E. tarda*), a gram-negative member of the *Enterobacteriaceae*, is a common inhabitant of a broad range of environment niches, as well as the normal gut (Janda et al., 1991). It also is a causative agent of Edwardsiellosis and widely distributed zoonoses. *E. tarda* infects a broad range of fish, reptiles, and amphibians, and had caused severe economic losses in aquaculture (Wilson et al., 1989; Romero et al., 2014). As the only human opportunistic *Edwardsiella* bacterium, *E. tarda* is associated with Salmonella-like gastroenteritis and extraintestinal infections in humans (Janda and Abbott, 1993; Leung et al., 2012; Nelson et al., 2009; Schlenker and Surawicz, 2009).

The virulence of *E. tarda* depends on its ability to survive and replicate within host macrophages (Gao et al., 2016a). The pathogen has to sense and quickly adapt to changes in the environment (Ishibe et al., 2008). This entails a coordinated regulation of its virulence genes in response to various environmental stresses by a complex signaling cascade of regulatory factors. The transcriptional regulation in *E. tarda* has since been studied extensively, and analyses have revealed a complex regulatory network that includes a two-component system (TCS) of signal transduction (Leung et al., 2012), quorum sensing (Romero et al., 2014), Eha regulator (Gao et al., 2001, 2016a), and other signaling molecules. However, little is known about these sRNAs and their potential regulatory actions in *E. tarda*.

The lengths of bacterial sRNAs are generally between 50 and 500 nucleotides (nt). In most cases, these transcripts do not encode proteins, so the term "non-coding RNA (ncRNA)" is often used synonymously (Kavita et al., 2017; Vogel and Wagner, 2007). These molecules are divided into cis-encoded and trans-encoded sRNAs based on how they regulate their targets. The cis-acting sRNAs are generally located in the middle of genes encoding proteins. They are perfectly complementary to an mRNA target expressed on the opposite strand of the DNA. In

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Table 1
Oligonucleotides used in this study.

Prime name	Prime sequence	Purpose
EsR 77-F	TGGCAACAGAGACTGTAGAA	EsR 77 RT-PCR
EsR 77-R	CCTGAAGGAGGGCGAAATGA	
EsR 128-F	GATACGCGTCGCGATCTGGG	EsR 128 RT-PCR
EsR 128-R	ACTACTGGCGGTGCTGCTGT	
EsR 139-F	GTGCGCCAATTACGACAAAA	EsR 139 RT-PCR
EsR 139-R	ATCATCGCCAATACCCAAGC	
EsR 150-F	TAACGATGGATGGGGGTAGT	EsR 150 RT-PCR
EsR 150-R	CATGAATTTGGGGGGAGACG	
EsR 169-F	ACCGATGGAGCAGGGGGATA	EsR 169 RT-PCR
EsR 169-R	AGCTGATTGTGGTTGAGTAA	
EsR 190-F	CATTTACCCATTGAGTTATCCA	EsR 190 RT-PCR
EsR 190-R	CATCGTCTTCTGTTGCGCCTGT	
EsR 213-F	GAAGCCGTAATAGCAAGAAT	EsR 213 RT-PCR
EsR 213-R	GCGGAGATCGAGTTGAAGAT	
EsR 214-F	TTCCAGCCCATCGCCATAAAAAA	EsR 214 RT-PCR
EsR 214-R	TGAAGGAGGTGCGTCTGACTCAC	
EsR 240-F	CGCGGTCATGAGAGATCAGGATC	EsR 240 RT-PCR
EsR 240-R	GTCGGGAAGAATGGCTGTGGGAA	
EsR 255-F	AAACTAAACCAATGTTAAACCA	EsR 255 RT-PCR
EsR 255-R	TACTACTGAGCGATACCGACCG	
EsR 261-F	TTTCTGTGTGAATTCTGTT	EsR 261 RT-PCR
EsR 261-R	TGGATATCGTCTGGGACCA	
EsR 285-F	AACCCATCCCACAGAGAA	EsR 285 RT-PCR
EsR 285-R	TTGAAGCCGTGATAAAGA	
EsR 289-F	TTTTTAAAACGTTAAGCG	EsR 289 RT-PCR

(*continued on next page*)

Table 1 (*continued*)

EsR 289-R	TGTAGTGATCGAAATCCC	
16SrRNA-F	TAGGTCGCTTAGGACATCTC	16SrRNA RT-PCR
16SrRNA-R	AGGGCCGGCTTGGCGACCGT	
Dig-16S –F	ACCCTT ATCCTTTGTTGCCA)	EsR240 Northern blot
Dig-16S –R	CTTTATGAGGTCCGCTTGCT	
Dig-EsR 240-F	CGTCCCGGGGTAGCTTTTTA	EsR240 Northern blot
Dig-EsR240-R	TTGTGCCGCTCCGATC TGTT	
5'RACE - Outer	ACCTCCCGGCAAACTGGGCGATGG	EsR240 RACE
5'RACE - Inner	TGGAGACGCCGGCACTGGAGTATG	
3'RACE – Outer	TGAAGCCGAGTCCATGCAACAGGC	EsR240 RACE
3'RACE – Inner	AGGGCGATGGCGTTACCGTCGTTG	
F1-F2-F	TGTTGTAATGGCGACTCA	EsR240mutant
F1-F2-R	GGTAAGCTCCCTCGGGTT	
EsR240-F	CGCGGTCATGAGAGATCAGGATC	EsR240mutant
EsR240-R	GTCGGGAAGAATGGCTGTGGGAA	
C-EsR240-F	CGGAATTCGGATGGATGATCTTGTCCAC	EsR240Comp
C-EsR240-R	CCCAAGCTTGGGCTCGGCAACGATCAACCCC	
RS06220-F	ATCGTGCCATTGGTGTTTC	qRT-PCR
RS06220-R	CGGTGGAGTTGAGTAGGGTC	
RS06965-F	GCACCAACTACATCGCCTCC	qRT-PCR
RS06965-R	TGCCAGAGCATCATGCCTAACT	
RS01475-F	CCGAGCCGGAATAGCTGAA	qRT-PCR
RS01475-R	ATTGCCTGATTTGCGTGGG	
RS03715-F	CGACCGTTTCCTGCTACACT	qRT-PCR
RS03715-R	CCAGACCCATAGCGATACCC	
RS03265-F	TCACCGCCGTGAATGATAT	qRT-PCR
RS03265-R	CTGGTCTTCTTCTGCTGCTC	

(*continued on next page*)

Table 1 (*continued*)

RS11955-F	GGCAGCAATGAGGGATACGG	qRT-PCR
RS11955-R	CAAAGGCGAAGACGGTGGG	
RS01115-F	CACTGTTCGTAGTAGCGTTGGA	qRT-PCR
RS01115-R	AGCCTCTGGCGTTATCTGC	
RS05140-F	CGACCTGAACCTGTGCCAAGA	qRT-PCR
RS05140-R	CGCCGAGATCACCAGCCTAC	
RS15050-F	GCTCGGCTGACCCAACAAG	qRT-PCR
RS15050-R	AACCGTGAGTCTGACATAAAGTG	
RS09555-F	CGCAGGTAATCCTCGTAGC	qRT-PCR
RS09555-R	ACCTTAGTCGGCGATTTGA	

F：forward primer. R: reverse primer.

contrast, the trans-acting sRNAs are generally located in the intergenic regions between protein-coding sequences. They are only partially complementary to one or more mRNA targets encoded elsewhere in the genome.

The more common post-transcriptional regulation by the trans-encoded sRNAs works through imperfect base-pairing with target mRNAs, which modulates mRNA translation and/or stability. The main functions of the trans-acting sRNAs are as coordinators of the adaptation processes that occur in response to environmental or other changes (Holmqvist and Wagner, 2017; Kacharia et al., 2017). The sRNAs are able to regulate the expression of their target genes to allow the organism to rapidly adapt for stress and changes in the metabolic requirements by base-pairing with their target mRNAs. They are involved in many other biological processes, including regulation of iron homeostasis (Masse et al., 2007, 2005; Vecerek et al., 2007), expression of outer membrane proteins (Guillier and Gottesman, 2006), quorum sensing (Lenz et al., 2005; Tu and Bassler, 2007), and bacterial virulence (Romby et al., 2006; Toledo-Arana et al., 2007) through binding to target mRNAs or proteins.

High-throughput RNA sequencing (RNA-Seq) approaches have recently provided invaluable insights into the transcriptional expression patterns of bacterial genes and sRNAs. We have previously applied RNA-Seq to analyze the transcriptome of *E. tarda* (Gao et al., 2017). In the present study, we describe a novel sRNA EsR240 in *E. tarda* identified by RNA-Seq, bioinformatics and experimental confirmation. Furthermore, we systematically investigated EsR240 and herein provide the first evidence that it modulates *E. tarda* by allowing it to adapt for intracellular survival within macrophages.

1. Materials and methods

1.1. Bacteria and growth conditions

The wild type ET13 strain of *E. tarda* was provided by Dr. Janda (California Department of Health Services Microbial Diseases Laboratory). Bacteria were grown in Luria-Bertani (LB) broth (Sunshine Biotechnology, Nanjing, China). GEM broth was made from $MgSO_4.7H_2O$ (0.2 g/L), citric acid.H_2O (2.0 g/L), K_2HPO_4 (10.0 g/L), $NaNH_4HPO_4.4H_2O$ (3.5 g/L) and glucose (20 g/L) (Zhong et al., 2016).

1.2. Cell culture

Murine RAW264.7 macrophages were obtained from the Research Institute of Beijing Chuanglian North Carolina Biotechnology (Shanghai, China) and were cultivated at 37 °C in a 5% CO_2 incubator. The cell medium was Dulbecco's Minimal Essential Medium (DMEM) (Gibco, Shanghai, China) including 10% fetal calf serum (FCS) (Gibco, Shanghai, China).

1.3. Animals and animal care

BALB/c female mice, 6 weeks of age and weighing between 18 g and 22 g, were obtained from the laboratory animal center of Yangzhou University. Mice were maintained under specific pathogen-free-conditions and allowed unlimited food and water. The animal experiments were carried out following approval by the Animal Ethical and Welfare Committee.

1.4. RNA extraction, RNA sequencing, and Northern blotting

After the ET13 strain was cultured in LB (pH = 7.2) to an OD_{600} of 0.8 at 37 °C, the bacteria were transferred to LB (pH = 6.3) for 2 h. Total RNA was isolated from the bacteria using a RNA extraction kit (TaKaRa, Dalian, China). Samples were sent to Shenzhen Hua Da Gene Company and sequenced using the Illumina sequencing 2000 platform. Data analysis and base calling were accomplished using the Illumina instrument software. The RNA-Seq data have been deposited in the National Center for Biotechnology Information Sequence Read Achieve under accession number SRX1898774.

For the detection of transcripts, digoxigenin (DIG)-labeled RNA probes of 16SrRNA and EsR240 were synthesized according to the manufacturer's instructions (Roche Company, Shanghai). The probes and primers for Dig-16S and Dig-EsR240 are listed in Table 1. Northern blotting was performed as described previously (Beckmann et al., 2010).

1.5. Bioinformatics analysis

New transcripts were mapped with a non-redundant proteins (NR) library (https://ftp. ncbi. nlm.nih. gov/blast/db/ fasta) to remove gene sequences. The sRNAMap, sRNATarBase, and SIPHT programs were searched to annotate sRNAs with the online tools available at http://srnamap.mbc.nctu.edu. tw/, http://ccb1. bmi. ac. cn/ srnatarbase / and http:// bio.cs.wisc.edu/sRNA/, respectively. The Promoter 2.0 (http://www. fruitfly.org/seq_tools/promoter.html) and RNA Motif (http://regrna2. mbc.nctu. edu.tw/) programs were respectively used to predict

the promoters and terminators of candidate sRNAs. The BLAST (http://blast. ncbi.nlm.nih.gov/blast.cgi) was used to search for the conservation of EsR240. IntaRNA (http://rna.informatik.unifreiburg.de/IntaRNA /Input.jsp) was used to predict the target genes of EsR240.

The BPROM software (http://www.softberry.com/berry.phtml?topic = bprom&group = programs&subgroup = gfindb) was used to predict the promoters of EsR240. The promoter prediction was conducted to search the region 200 bp upstream of the start site of EsR240. The RNA Fold program (http://www.softberry.com/berry.phtml?topic = foldrna& group = programs&subgroup = rnastruct) was used to carry out the prediction of the secondary structure based on the lowest folding energy.

1.6. Bacterial growth conditions, RNA isolation, and RT-PCR

After the ET13 strains were cultured in LB (pH = 7.2) to an OD_{600} of 0.8 at 37 °C, the bacteria were treated for 2 h with LB at different pH levels (4.0 or 6.3), LB containing different concentrations of H_2O_2 (50 mM, 100 mM, 200 mM, 300 mM or 400 mM), LB containing different concentrations of NaCl (100 mM, 200 mM, 300 mM or 400 mM), or in GEM medium (Zhong et al., 2016).

Reverse transcription PCR (RT-PCR) was performed with the total RNA obtained from the different growth conditions described above, and the RNA was isolated with a RNA extraction kit (TaKaRa company, Dalian, China). Equal amounts of RNA (1 mg) were used to generate cDNA with random primers. RT-PCR was performed using a reverse transcription kit (Sunshine Biotechnology, Nanjing, China). The expression of 13 potential sRNAs was then examined by RT-PCR using the primers for EsR77, EsR128, EsR139, EsR150, EsR169, EsR190, EsR213, EsR214, EsR240, EsR255, EsR261, EsR285 and EsR289 (Table 1).

1.7. 5' and 3' rapid amplification of cDNA ends (RACE)

5'and 3' RACE were carried out using a Full RACE kit (Takara Biochemicals) as recommended by the manufacturer. Prior to initiating the 3' RACE protocol, total RNA was polyadenylated by treatment with poly (A) polymerase. The PCR products with the primers for 5' RACE and 3'RACE (Table 1) were cloned into the pMD19-TVector (Takara Biochemicals), and then the clones were sequenced and analyzed. Five clones were sequenced for each RACE analysis, and the farthest 5' or 3' end was considered the 5' or 3' end of the sRNA.

1.8. Construction of an EsR240 mutant and its complementary strain

The EsR240 mutant strain was constructed as described by Gao et al. with minor modifications (Gao et al., 2014). The fused fragment (1144 nucleotides; modified by *Xba*I and *Sac*I) of the upstream and downstream regions of EsR240 was synthesized chemically by Genewiz (Suzhuo, China). The fragment was ligated into the suicide vector, pHM5. The resulting recombinant plasmid was introduced into *E. coli* SM10λ*pir*. Transconjugants were selected on LB containing polymyxin and ampicillin, and their sequences were confirmed by PCR using the F1-F2 primers (Table 1). Deletion mutants were obtained by selecting for resistance to sucrose (15% wt/vol) and screening for sensitivity to ampicillin (100 μg/ml). The identity of the EsR240 mutant was confirmed by sequencing and PCR, using the EsR240 primers (Table 1).

The fragment amplified by PCR with EsR240 primers was cloned into the pACYC184 plasmid to generate a recombinant plasmid. The recombinant plasmid was transformed into *E. coli* strain DH5α and sent to Sunshine Biotechnology (Nanjing, China) for sequencing. The plasmid then was transformed into the EsR240 mutant strain by an electric shock method. The sequence of the transformant was confirmed by PCR using the C-EsR240 primers (Table 1). The EsR240 complementary strain was named EsR240Com strain.

1.9. Bacterial survival in vitro

The survival rates of the wild type ET13, EsR240 mutant and EsR240Com strains under various conditions of environmental stress were determined according to the report by Cheng et al (Cheng et al., 2010). The strains were first grown in the usual LB broth or LB broth (100 μg/ml ampicillin) to mid-log phase (OD_{600} = 1.0) at 37 °C, then equal aliquots of the bacteria culture were continuously incubated at 37 °C for 2 h in LB broth or LB broth (100 μg/ml ampicillin) with 0.5 M H_2O_2, 0.5 M NaCl or 0.1 M NaCl, or at pH 4.0 or pH 7.0, with another aliquot grown as a control in the usual LB broth. After treatment, bacteria were diluted and plated on usual LB plates or LB plates (100 μg/ml ampicillin) to determine their viability. The rates of bacterial survival were expressed as colony numbers in an experimental group/colony numbers in a control group X 100%.

1.10. Bacterial survival and replication in macrophages

The survival of bacteria was assessed in RAW264.7 macrophages as described by Gao et al (Gao et al., 2016b). Briefly, the cells were infected with the mid-log wild type or mutant bacteria at a multiplicity of infection (MOI) of 100:1. After designated time intervals (2, 4, 6 h) post-infection, the macrophages were lysed with 1% Triton X-100 for 10 min, then the solutes were diluted, spread onto LB agar plates, and the plates were incubated at 37 °C overnight. The colony-forming units (cfus) per milliliter of viable intracellular bacteria were counted on each of the plates.

1.11. RNA extraction and quantitative RT–PCR (qRT-PCR)

After wild type ET13 and its EsR240 mutant strains were cultured in LB (pH = 7.2) to an OD_{600} of 0.8 at 37 °C, the bacteria were treated with LB (pH = 4.0) or LB (pH = 6.3) for 2 h. Total RNAs were isolated from these bacteria using a RNA Extraction Kit (TaKaRa, Dalian, P.R. China). The primers RS06220, RS06965, RS01475, RS03715, RS03265, RS11955, RS01115, RS05140, RS15050 and RS09555 (Table 1) were used during qRT-PCR to find the target genes of EsR240. Using a SYBR ExScript qRT-PCR kit (Takara, Dalian, China), qRT-PCR was performed in an ABI 7300 real-time detection system (Applied Biosystems Company, USA). Bacterial 16S rRNA was used as a control. The $2^{-\triangle\triangle CT}$ method (Livak and Schmittgen, 2001) was used to compare the mRNA levels in wild type and mutant bacteria.

1.12. LD_{50} and the bacterial loads from infected mouse tissues

A murine model of *E. tarda* infection was used to examine the virulence of the wild type strain and its EsR240 mutant (Janda et al., 1991). Two groups of BALB/c mice were intraperitoneally infected with 200 μl doses of successive 5-fold dilutions of the bacterial suspension of the wild type strain (5×10^6, 2.5×10^7, 5×10^7, 2.5×10^8, 5×10^8, 2.5×10^9 and 5×10^9 cfu/ml) or the EsR240 mutants (5×10^7, 2.5×10^8, 5×10^8, 2.5×10^9, 5×10^9, 2.5×10^{10} and 5×10^{10} cfu/ml). The mice were monitored twice daily for 4 weeks. The 50% lethal dose (LD_{50}) values were determined using the SPSS software.

The bacterial loads of various mouse organs were determined after mice were injected intraperitoneally with 200 μl containing 5×10^6 cfu/ml of the wild type or the EsR240 mutant strains. At various times post-infection (1, 3, 5, 7, 9 or 11 days), mice from each group were sacrificed and various organs (kidney, spleen, lungs and liver) were removed. The organs were prepared as tissue homogenates in 1 ml sterile PBS/organ. A series of dilutions of the homogenates were plated onto LB plates and incubated at 37 °C overnight. The bacterial colony forming units (CFUs) per organ were counted on LB plates

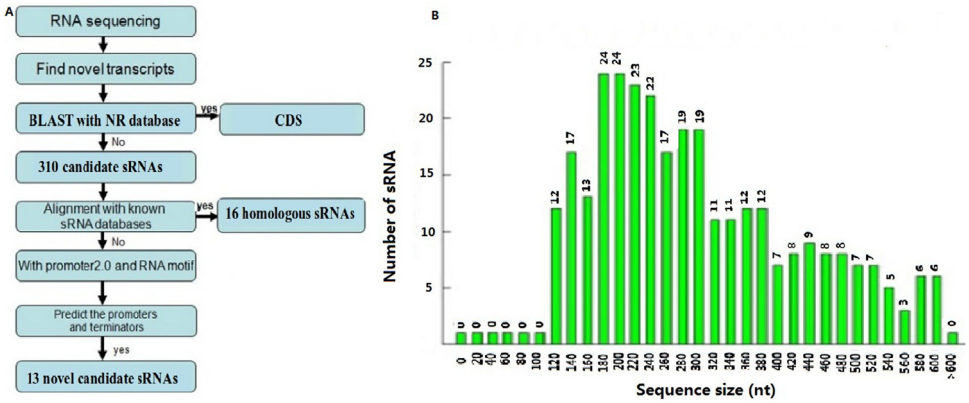

Fig. 1. The candidate sRNAs found in *E. tarda*.
(A) The procedure used to find novel sRNAs in the ET13 strain. (B) The numbers and sizes of 310 candidate sRNAs found in the chromosomes of ET13 as determined by RNA-Seq.

1.13. Statistical analysis

Probability (*P*) values were determined by Student's *t*-test, and analyses were performed using the GraphPad Prism (version 6) software. Values < 0.05 were considered statistically significant. The qRT-PCR data and LD_{50} values were analyzed using two-tailed tests and the SPSS software, respectively.

2. Results

2.1. Characterization of potential sRNAs with the help of RNA-Seq and bioinformatics software

The procedure used to find novel sRNAs is shown in Fig. 1A. The genome sequence of ET13 was used to identify putative untranslated regions (UTRs) of mRNAs that were included in the set of potential sRNAs. A total of 5838 UTRs were selected according to their length and degree of coverage (> 2). New transcripts were found from some UTRs located in the intergenic regions that were from 100 bp at the 5′-end upstream of the gene to 100 bp at the 3′-end downstream of the gene. After these new transcripts were mapped using the non-redundant (NR) proteins library to remove genes sequences, 310 transcripts remained as candidate sRNAs that were absent from the libraries. The lengths of these candidate sRNAs were generally between 180 bp and 300 bp, but a few were up to 600 bp (Fig. 1B). After the 310 candidate sRNAs were compared with the data from the sRNAMap, sRNATarbase, and SIPHT sRNA libraries, 284 potential new sRNAs remained. Thirteen potential sRNAs with Rho-independent terminators at their 3-ends and promoters at their 5-ends were found when these 284 sRNAs were examined with Promoter 2.0 to predict their promoters and RNA Motif to predict their terminators. The 13 potential sRNAs were named EsR77, EsR128, EsR139, EsR150, EsR169, EsR190, EsR213, EsR214, EsR240, EsR255, EsR261, EsR285 and EsR289, respectively. The transcription start and end points, and the predicted lengths of these candidate sRNAs are shown in Table 2. More than half of the sRNA regions (9 of 13) were in the sense chain, with only 4 of 13 located in the antisense chain. These sequences do not seem to encode small peptides as they lack appropriate start and stop codons.

2.2. Experimental validation of eight sRNAs by RT-PCR

As an intracellular bacterial pathogen, *E. tarda* can survive and replicate in host phagocytes, where they likely encounter different stresses, such as oxidative stress, low pH, osmotic stresses, and limited nutrition (Weiss and Schaible, 2015). We therefore investigated the expression profiles of 13 putative sRNAs under these stresses to explore their roles in the intracellular survival of *E. tarda*. Only eight sRNAs (EsR77, EsR128, EsR139, EsR150, EsR169, EsR213, EsR214, and EsR240) were transcribed. It is possible that the other 5 putative sRNAs may be transcribed under other conditions. Importantly, differences in the expression levels of the 8 sRNAs (EsR77, EsR128, EsR139, EsR150, EsR169, EsR213, EsR214, and EsR240) were found following exposure to the different stresses (Fig. 2A–L). The transcription levels of three sRNAs (EsR128, EsR139, and EsR240) were all highly induced under these stress conditions.

Table 2
The sizes and positions of 13 candidate sRNAs in the chromosome of the ET13 strain.

Candidate sRNA ID	Chromosome	Start	End	Length (bp)	Chain
EsR77	NZ_AFJG01000031.1	41773	42364	592	+
EsR128	NZ_AFJG01000060.1	11814	12314	501	+
EsR139	NZ_AFJG01000066.1	14983	15552	570	+
EsR150	NZ_AFJG01000078.1	9347	9771	425	+
EsR169	NZ_AFJG01000090.1	43847	44288	442	+
EsR190	NZ_AFJG01000098.1	32229	32684	456	–
EsR213	NZ_AFJG01000110.1	6716	7122	407	–
EsR214	NZ_AFJG01000110.1	17717	18164	448	–
EsR240	NZ_AFJG01000134.1	3580	4175	596	–
EsR255	NZ_AFJG01000143.1	1531	2087	557	+
EsR261	NZ_AFJG01000147.1	2516	2988	473	+
EsR285	NZ_AFJG01000181.1	9107	9619	513	+
EsR289	NZ_AFJG01000184.1	12288	12860	573	+

2.3. Experimental verification of the novel EsR240

The BLAST program provides a prediction of the level of conservation of a specific sRNA with known genome sequences for microorganisms with a calculation of their sequence homology. Our results showed that the sequence of EsR240 in the ET13 strain was 99–100% homologous to the sequences in other strains of *E. tarda*, 85–91% homologous to the sequences in other species of *Edwardsiella* and 71–79% homologous to the sequence in other genuses of *Enterobacteriaceae* (data not shown). The conservation of EsR240 accorded with the characteristic of sRNA.

Northern blot hybridization was employed to verify that EsR240 was expressed in the ET13 strains under both logarithmic and stationary phases. For each putative EsR240 region, a 3'-end DIG-labeled RNA probe was prepared for the most highly conserved portion of the sequence. The results showed that EsR240 formed a single copy transcript in the chromosomes of the ET13 strain (Fig. 3A). The precise start and end points of EsR240 were determined using 5'and 3'RACE. The results showed that the length of EsR240 was 596 nt, and it was located

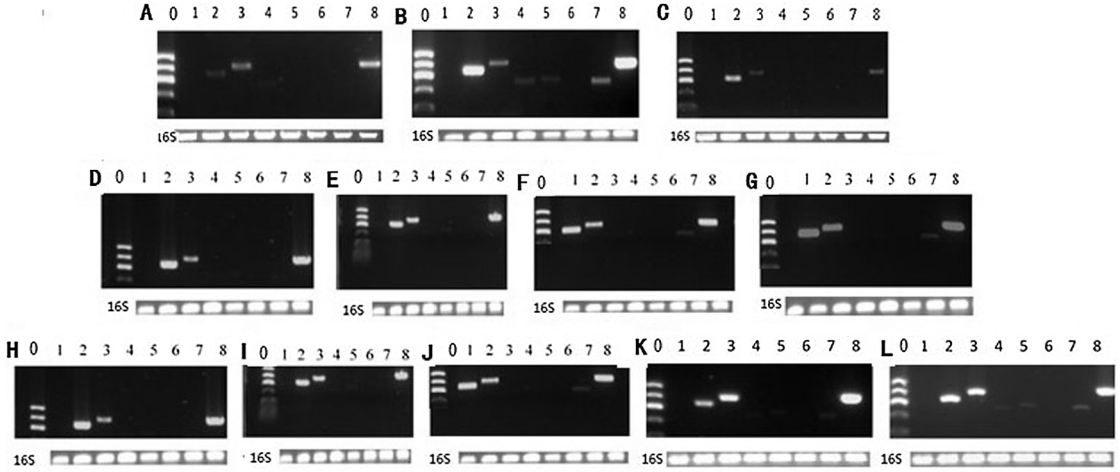

Fig. 2. The transcription levels of the candidate sRNAs of *E. tarda* cultured under different conditions as determined by RT–PCR.
(A–L): RNAs were isolated from mid-log ET bacteria under different conditions: LB at pH = 6.3 (A); LB at pH = 4 (B); GEM broth (C); LB containing different concentrations of NaCl (100 mM, 200 mM, 300 mM or 400 mM)(D–G); LB containing different concentrations of H_2O_2 (50 mM, 100 mM, 200 mM, 300 Mm or 400 mM)(H–L). The transcription levels of the 8 sRNAs from the ET13 strain were examined by RT-PCR. Lane O: 1500 bp DNA marker; lanes 1-8: EsR77, EsR128, EsR139, EsR150, EsR169, EsR213, EsR214, and EsR240.

between the upstream gene encoding 50S ribosomal protein L2 and the downstream gene encoding 30S ribosomal protein S3 (Fig. 3B). As the transcriptional direction of EsR240 was opposite those of the upstream and/ downstream genes, it was believed to be independently transcribed. The EsR240 information had been deposited in the GenBank Data Library under accession number MH794252.

By searching 200 bp upstream of the start site of the EsR240 using the BPRORM it was found that EsR240 has -10 and -35 promoter sites and corresponding TF binding sites. The secondary structure of EsR240 was examined by the RNA Fold program (Fig. 3C). By searching downstream of the end site of EsR240 using the ARNold program, it was noted that EsR240 has Rho-independent terminators at the 3' end.

2.4. Decreased survival exhibited by EsR240 mutants under stress conditions

To study the possible role of EsR240 in the intracellular survival of the ET13 strain within macrophages, we compared the survival rates of the EsR240 mutant with the rates of the wild type and EsR240Com strains under different stress conditions. The survival rates of the wild type grown in GEM (nutritional limitations), LB of pH 4.0 (acid stress), LB with 0.1 M H_2O_2 (oxidative stress), or LB with 0.5 M NaCl (high osmolarity) were higher than the rates of the EsR240 mutant bacteria cultured under the same conditions ($P < 0.05$), and were near to the rates of the EsR240Com strain ($P > 0.05$) (Fig. 4A). Thus, EsR240 appears to modulate a wide range of responses to environmental stresses.

Fig. 4B shows that as the duration of infection progressed, the cfus of the bacteria (EsR240 mutant and wild type) cultured on LB plates all increased (Fig. 4B). When the incubation time was extended 2 h, the intracellular cfus of the wild type bacteria were slightly higher than those of the mutants ($P > 0.05$). When the infection time was extended 4 or 6 h, the cfus of the wild type and the EsR240Com strains increased more significantly compared to the mutant strains ($P < 0.05$), and there was no important difference between the EsR240Com and the wild type strains ($P > 0.05$). These findings suggest that EsR240 may affect the intracellular replication rate of *E. tarda* within macrophages.

2.5. EsR240 regulates the virulence of ET13

The normal route of infection for *E. tarda* ET13 strain is via the oral route, but the bacteria were relatively non-virulent in the Balb/c mice used in our study. Interestingly, when the intra-peritoneal (i.p.) route of

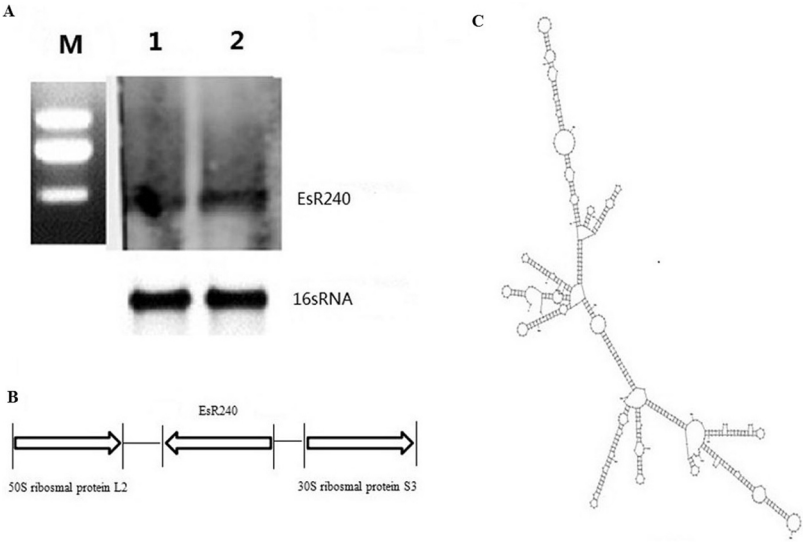

Fig. 3. The expression, direction and structure of EsR240 of *E. tarda.*
(A) Northern blotting was used to determine the position and copy numbers of EsR240 in the chromosomes of the ET13 strain. Total RNA was extracted from the ET13 strain grown in LB (pH 6.3) from the logarithmic (1) and stationary (2) phases (M) 600bp DNA marker. (B) RACE was used to find the direction of transcription and the position of EsR240 within the chromosomes of the ET13 strain. (C) The secondary structure of EsR240 was predicted by RNA Fold program.

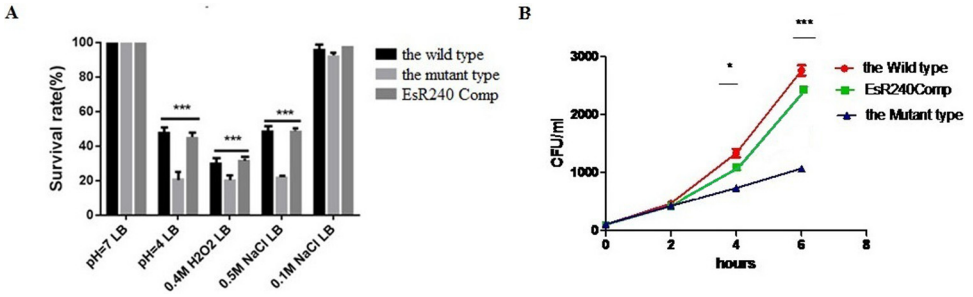

Fig. 4. Comparison of the differences in the survival rates between the wild type ET13、EsR240 mutant and EsR240Comp strains in vitro and within macrophages.
(A) Comparison of the survival rates of the wild type ET13 strains grown in LB or LB (100 μg/ml ampicillin) at pH 7.0, LB at pH 4.0 (acid stress), LB containing 0.4 M H_2O_2 (oxidative stress), LB containing 0.1 M NaCl (high osmolarity) and LB with 0.5 M NaCl (high osmolarity) with the rates of the EsR240 mutant and EsR240Comp strains cultured under the same conditions. (B) Comparison of the survival rates of the wild type, its EsR240 mutant and EsR240Comp strains within macrophages. Note: *$P < 0.05$.

Table 3
The LD_{50} values of the wild stain and the EsR240 mutant.

Inject the wild CFUs /ml	Wild injected animals Mortuus/Survival	Inject the mutant CFUs/ml	Mutant injected animals Mortuus/Survial
2.5×10^{10}	7/0	2.5×10^{11}	7/0
5×10^{9}	7/0	5×10^{10}	6/1
2.5×10^{9}	7/0	2.5×10^{10}	6/1
5×10^{8}	7/0	5×10^{9}	5/2
2.5×10^{8}	6/1	2.5×10^{9}	3/4
5×10^{7}	4/3	5×10^{8}	0/7
2.5×10^{7}	1/6	2.5×10^{8}	1/6
5×10^{6}	0/7	5×10^{7}	0/7
Total	39/17	Total	28/28
LD_{50}	1.17×10^{7}		7.94×10^{7}

infection was used, the virulence of the EsR240 mutant was 6.79-fold lower than the wild type bacteria based on the LD_{50} (Table 3). These data suggested that EsR240 plays an important role in regulating the virulence of ET13 of *E. tarda*.

When the bacterial loads of the organs in mice following i.p. infection were examined, it was apparent that there was a defect in the ability of the mutant to disseminate to the spleen and liver compared with the wild type bacteria (Fig. 5). The bacterial loads of these tissues in the mice infected with the wild type bacteria were significantly higher than in the mice infected with the mutants ($P < 0.05$). Similar findings were obtained for the liver at 3 days post-infection ($P < 0.01$). The significance of the differences disappeared after day 3. Together, these data and the LD_{50} values suggest that EsR240 may be required during the early period of i.p. infection with *E. tarda*.

2.6. Prediction and experimental verification of the target genes of EsR240

Because many bacterial sRNAs act as post-transcriptional regulators by base-pairing with their target mRNAs, we focused the possible target genes of EsR240 that might be involved in the survival and virulence of *E. tarda*. The IntaRNA program was used to predict the target genes of EsR240. A total of 3309 genes were identified, which had predicted free energy values ranging from -4.65 to -25.07. We selected the ten target genes with the minimum absolute free energy for further study (Table 4). These target genes were hypothesized to be involved in bacterial metabolism, transport, and/or virulence.

The transcription levels of these top 10 predicted target genes in the wild type strain were compared with the levels in EsR240 mutant strain

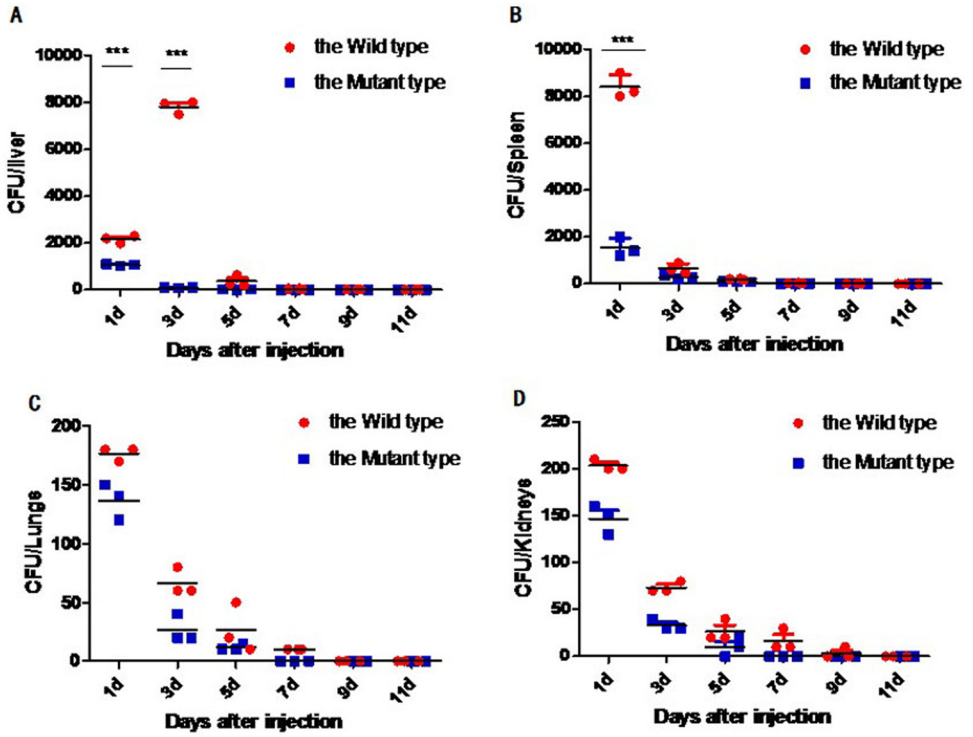

Fig. 5. Comparison of the bacterial loads from select tissues of mice infected by the wild type strain with those from mice infected with the EsR240 mutant strain.
Every mouse was infected intraperitoneally with 1×10^{6} CFU of the wild type ET13 or its EsR240 mutant strains. The bacterial loads of the mouse livers (A), spleens (B), lungs (C) and kidneys (D) were determined 1, 3, 5, 7, 9 and 11 days post-infection. A series of dilutions of the organ homogenates were plated onto LB plates and incubated overnight. The bacterial colony forming units (cfus) per organ were counted on LB plates. Each individual symbol on the histogram represented an individual mouse. Note: *$P < 0.05$, **$P < 0.01$.

Table 4
Prediction of the top ten candidate target genes for EsR240 based on the absolute minimum free energy for binding.

Target RNA	energe	Product
ETATCC_RS06220	−25.0749	sodium:proton antiporter
ETATCC_RS06965	−22.8715	glycoside hydrolase family 2
ETATCC_RS01475	−21.8492	tail fiber protein
ETATCC_RS03715	−21.344	polyribonucleotide nucleotidyltransferase
ETATCC_RS03265	−21.0144	FtsH protease modulator YccA
ETATCC_RS11955	−20.5016	phage tail tape measure protein
ETATCC_RS01115	−20.3678	hypothetical protein
ETATCC_RS05140	−20.3174	FtsX-like permease family protein
ETATCC_RS15050	−19.8219	ribonuclease HI
ETATCC_RS09555	−19.6077	ABC-F family ATPase

by qRT-PCR. There were differences in the expression of 6 of these target genes (ETATCC_RS 06965, ETATCC_RS 01475, ETATCC_ RS 03715, ETATCC_RS 01115, ETATCC_RS 05140 and ETATCC_RS 15050) between the strains grown in the LB at pH 6.3 ($P < 0.05$) (Fig. 6A). All 6 of these genes were up-regulated by EsR240. However, 9 of these top target genes were differentially expressed between the strains grown in LB at pH 4.0 ($P < 0.01$) (Fig. 6B), which was similar to the pH in the phagosomes within macrophages (Weiss and Schaible, 2015). For this reason, it was theorized that EsR240 plays different regulatory roles in response to various environmental stimuli. ETATCC_RS11955 may be not regulated by EsR240. Out of these 9 genes, most genes were up-regulated by EsR240. These target genes may encode ABC-F family ATPase, FtsH protease modulator YccA, Na^+ and H^+ antiporters, FtsX-like permease family protein, glycoside hydrolases or various other proteins. Therefore, EsR240 may positively regulate its target genes in *E. tarda* to allow it resist the acidification and maintain intracellular survival within host macrophages.

3. Discussion

To the best of my knowledge several days ago, only one study of sRNA has been reported in *E. tarda,* where 10 sRNAs were identified by RT-PCR, but their functions haven't been explored (Sun et al., 2017). But now, I just know the work of Sun et al. (2017) used *E. tarda* strain S08, which based on 16S sequence is actually *E. piscicida* (Buján et al., 2018; Reichley et al., 2017). Fortunately, based on nucleotide sequences available in GenBank, my isolate ET13 is bona fide *E. tarda*. Therefore, we found sRNAs in *E.tarda* genome for the first time.

While there has been a rapid increase in the discovery of bacterial sRNAs over the last few years, the identification of their corresponding mRNA targets and the study of sRNAs function have proceeded more slowly. By using bioinformatic and RNA Seq predictions, we found a total of 13 putative novel candidate sRNAs in *E. tarda*. Among them, the transcripts of 8 candidate sRNAs were verified by RT-PCR (albeit under limited conditions). The other 5 non-verified candidate sRNAs may be transcribed under other conditions. Of note, different expression levels of the 8 sRNAs were found under various stress conditions. One novel sRNA, EsR240, was confirmed to be located in the chromosome of ET13 and was found to be independently transcribed from the flanking ORFs by Northern blotting and RACE. Based the location of the sRNAs in IGRs and the sequence conservation between species (Vogel and Wagner, 2007; Wassarman et al., 2001; Waters and Storz, 2009), as well as the existence of promoters and Rho-independent terminators in IGRs, we hypothesize that EsR240 is a kind of trans-encoded sRNA.

We found that the transcript levels of EsR240 were increased under oxidative stress, low pH, high osmolarity and limited nutrition. The expression of wild type EsR240 was also associated with better survival in macrophages with nutrition limitation, acid stress, oxidative stress, and high osmolarity. EsR240 also affected the intracellular replication rate of *E. tarda* within macrophages and increased the bacterial virulence. These effects were apparently mediated by several target genes involved in bacterial survival within host macrophages and virulence.

ETATCC_RS06220 encodes Na^+ and H^+ antiporters, such as NhaA, which are localized in the middle of the membrane and are responsible for Na^+ and H^+ homeostasis in cells (Padan, 2014). The unique structure of NhaA contributes to the cation binding site and allows rapid conformational changes. ETATCC_RS06965 encodes a glycoside hydrolase. Ma et al. identified a novel glycoside hydrolase family 12 (GH12) protein, XEG1 (Ma et al., 2015). This protein acts as an important virulence factor during *P. sojae* infection, but is also a pathogen-associated molecular pattern (PAMP) in soybean and solanaceous species, where it can trigger host defense responses, including cell death (Ma et al., 2015). ETATCC_RS01475 encodes a tail fiber protein. The first step in bacteriophage infection is recognition of and binding to the host receptor, which is mediated by the phage receptor binding protein (RBP), a tail fiber (Washizaki et al., 2016). ETATCC_RS03715 encodes a polynucleotide phosphorylase (PNPase, encoded by pnp), which is generally thought of as an enzyme dedicated to RNA metabolism (Carzaniga et al., 2017). The pleiotropic effects of PNPase deficiency are thought to include altered processing and turnover of mRNAs and sRNAs, which in turn leads to aberrant gene expression.

ETATCC_RS03265 encodes FtsH protease modulator YccA. FtsH is a membrane-bound and ATP-dependent zinc-metalloproteinase of *Escherichia coli*(*E. coli*), which is involved in degradation of some of uncomplexed integral membrane proteins and short-lived cytoplasmic proteins (Akiyama, 1999). yccA, encodes a protein whose *E. coli* homologue modulates the activity of membrane protease FtsH (Akiyama et al., 1998).Its mutation was more sensitivity to aminoglycoside of antibiotics, alkaline pH, NaCl, and other compounds (Hinz et al., 2011).

ETATCC_RS05140 encodes FtsX-like permease family protein. FtsE is predicted to bind ATP and hydrolyse it, and FtsX is predicted to be an integral membrane protein. FtsE is one of the earliest cell division proteins that assembles along with FtsX at the mid-cell site during cell division in *Escherichia coli* (Schmidt et al., 2004).Both these proteins are

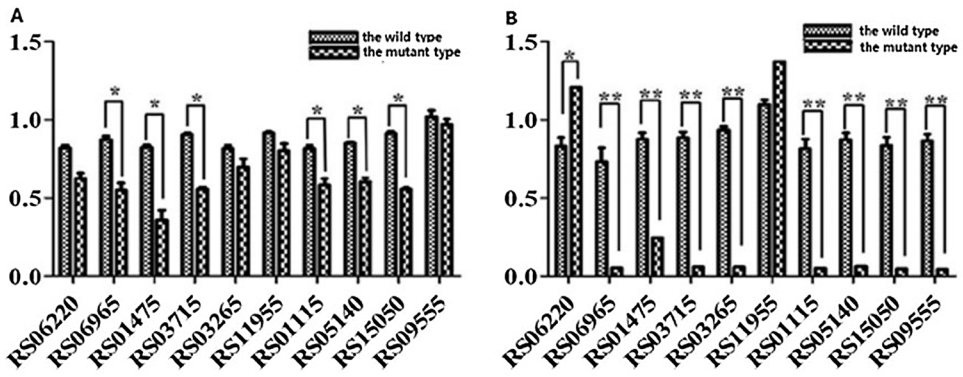

Fig. 6. Comparison of the transcription levels of the target genes between the wild type strain and EsR240 mutant strain under different growth conditions as determined by qRT-PCR. RNAs were isolated from the wild type ET13 and EsR240 mutant strain grown in LB at pH = 6.0 (A) pH = 4.0 (B). The transcriptional levels of the ten putative target genes of EsR240 were analyzed by qRT-PCR. Note: $^*P < 0.05$, $^{**}P < 0.01$.

highly conserved across diverse bacterial genera and are predicted to constitute an ABC transporter type complex (Schmidt et al., 2004). The FtsEX:PcsB complex forms a molecular machine that carries out peptidoglycan hydrolysis during normal cell division of the major respiratory pathogenic bacterium, *Streptococcus pneumoniae(pneumococcus*) (Bajaj et al., 2016).

ETATCC_RS15050 encodes a functional RNase H. Lmo1273 is a kind of RNase H in *L. monocytogenes*, which has been linked with amino acid uptake. Strikingly, inactivation of lmo1273 provoked a strong attenuation of virulence in a mouse model, and the mutation affected the pathogen's intracellular multiplication and cell-to-cell spread in cell culture models (Bigot et al., 2009). ETATCC_RS09555 encodes a membrane-associated ATPase. PotA is a membrane-associated ATPase that is a component of the spermidine-preferential uptake system in prokaryotes. It plays an important role in normal cell growth by regulating the cellular polyamine concentration (Sugiyama et al., 2014).

Overall, these findings of the nuclease mapping suggest that EsR240 regulates target genes that are involved in either virulence or bacterial energy generation and metabolism, which allow *E. tarda* to adapt to adverse environments and survive within macrophages. However, further validation of these findings will be needed to confirm these hypotheses.

In summary, we identified eight novel sRNAs in *E. tarda* and systematically analyzed one of them, EsR240, which was found to modulate the pathogen's intracellular survival within macrophages. This provides significant insights that will be useful for unraveling the sRNA-mediated regulatory networks in *E. tarda.*

Acknowledgment

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