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RESEARCH ARTICLE

A *Trans*-encoded sRNA rli34 is Involved in Virulence Modulation in *Listeria monocytogenes*

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ABSTRACT

The sRNA is an important regulatory molecule that plays a key role in virulence and environmental adaptive responses in bacteria. To explore the role of sRNA rli34 in virulence of *Listeria monocytogenes* (*L. monocytogenes*), here, the molecular features of *trans*-encoded sRNA rli34 and its intracellular and extracellular expression profiles were characterized. Furthermore, the effects of *rli34* gene deletion on virulence were investigated. Meanwhile, target genes regulated by *rli34* were predicted and analyzed and rli34-mRNA interactions were validated using a bacterial dual plasmid reporter system based on *E. coli*. The results showed that rli34 was *trans*-encoded sRNA and highly conserved in *L. monocytogenes*, and its intracellular expression level was increased by 10.94-fold as compared to extracellular condition. Moreover, compared with LM EGD-e and LM-*Δrli34*-*rli34*, the adhesion, invasion, intracellular survival and cell inhibition of *LM-Δrli34* on RAW264.7 cells were significantly declined ($P<0.05$). Furthermore, there were significant differences in bacterial loads in both liver and spleen ($P<0.01$), and the virulence of LM-*Δrli34* was significantly dampened. Bioinformatics analysis revealed that rli34 could complementarily pair with 5'-UTR (-279 to -270) bases of wall teichoic acids (WTAs) glycosyltransferase (gtcA) mRNA, whereby it promoted the expression of *gtcA* gene. These findings suggested that this novel *trans*-encoded sRNA rli34 may facilitate the glycosylation of cell WTAs by targeting *gtcA* mRNA, thereby precisely modulating the virulence of *L. monocytogenes*. This study provided new insights into the mechanisms of *trans*-encoded sRNAs in virulence modulation in *L. monocytogenes*.

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INTRODUCTION

Listeria monocytogenes (*L. monocytogenes*) is a Gram-positive bacterium that causes listeriosis in humans and a variety of animals. As a ubiquitous pathogenic agent in nature, *L. monocytogenes* can invade the gastrointestinal tract through contaminated food or drinking water and breach the epithelial barrier through various cellular types: M-cells, goblet cells and enterocytes (Farber and Peterkin 1991; Drolia and Bhunia 2019; Quereda *et al.*, 2021). After then, *L. monocytogenes* can intracellularly replicate in body organs, resulting in the life-threatening encephalitis and meningitis, with the high mortality rate (usually 20~30%) among immunocompromised individuals (Matereke and Okoh, 2020; Osek *et al.*, 2022). Meanwhile, *L. monocytogenes* can survive in harsh environments such as extreme pH, high salt, low temperature and oxidative stress (Matle *et al.*, 2020; Lourenco *et al.*, 2022) and thus facilitates its resistance to a variety of disinfectants (Lecuit, 2020). Since this zoonotic pathogen has posed severe threats to public health, *L. monocytogenes* is recognized as one of the four major foodborne pathogenic bacteria by World Health Organization (WHO) (Farber and Peterkin, 1991).

As a facultative intracellular pathogen, *L. monocytogenes* possesses a sophisticated infection and survival strategy to invade host cells and evade the immune responses. Initially, *L. monocytogenes* achieves infection by utilizing various virulence factors such as internalins, listeriolysin O (LLO), phospholipase (PLC), actin assembly-inducing protein (ActA) and other proteins like LAP (Lecuit 2020; Quereda *et al.*, 2021). Subsequently, *L. monocytogenes* may survive and propagate within the intercellular space, thereby evading the immune system of host. Hence, the infection and intracellular survival of *L. monocytogenes* is closely related to the complex regulatory expression of virulent factors it owns (Nitzan *et al.*, 2017;

Dutta and Srivastava, 2018). Existing studies have revealed that various regulators (PrfA, SigmaB, VirR, MogR, as well as regulatory small RNAs, etc) consisted of a complex regulatory network in *L. monocytogenes*, which that precisely modulated the expression of its virulence-related genes at the transcriptional, post-transcriptional or translational levels (Stincone *et al.*, 2021), respectively. Among these regulators, sRNAs play critical roles in regulating gene expression through complementary base-pairing with target mRNAs, thus enabling *L. monocytogenes* to rapidly adapt to the intercellular environments. To date, more than 150 sRNAs have been identified in LM, however, the biological functions of most sRNAs remain unexplored yet. The sRNA rli34 is a newly identified *trans*-encoded sRNA in *L. monocytogenes*, which is paired with messenger RNAs potentially directly involved in virulence (Toledo-Arana *et al.*, 2009), however, its specific molecular characteristics and biological roles required further study.

The main purpose of this study was to explore the molecular characteristics of *trans*-encoded sRNA rli34, identify its potential target genes and further unveil the regulatory roles and mechanism in *L. monocytogenes* virulence. Our findings provide new insights into the understanding of regulatory mechanisms of *trans*-encoded sRNA in virulence of *L. monocytogenes*.

MATERIALS AND METHODS

Primers, plasmids, strains and growth conditions: According to the genome sequence of *L. monocytogenes* EGD-e deposited in GenBank (accession number: AL591824), the various primers were designed using Primer Premier 5.0 software (Table 1). The plasmids pHoss1, pHT304, pUT18C and pMR-LacZ were purchased from BioVecto NTCC. The strains DH5α and BTH101 were grown in Luria-Bertani broth at 37°C, while *L. monocytogenes* was grown in brain heart infusion broth with 180 rpm shaking at 37°C.

Intracellular and extracellular expression profiles of sRNA *rli34*: Total RNA of *L. monocytogenes* EGD-e was extracted from extracellular culture and LM-infected RAW264.7 cells by Trizol. Then, *L. monocytogenes rli34* gene was amplified by PCR using cDNA as template. After that, the real-time quantitative RT-PCR (qRT-PCR) was employed to profile *intra*- and *extra*-cellular expression levels of *rli34* gene.

Construction of *LM-Δrli34* deletion and complementary strains: The LM-*Δrli34* strain was constructed using homologous recombination system as previously described (Abdelhamed *et al.*, 2015). Briefly, the fusion fragment of *rli34* gene deletion was obtained by SOE-PCR. Then, pHoss1-*Δrli34* (2.5kv, 5.0ms) was electro-transformed into LM EGD-e competent cells. The positive clones were subjected to homologous recombination at 42℃ and erythromycin resistance (10μg/mL), and thus the *rli34* gene deletion strain LM-*Δrli34* was obtained. For the generation of complementation strain, pHT304-*rli34* vector was electro-transformed into LM-*Δrli34* competent cells, and then positive colonies were screened and verified by sequencing.

Cell infection experiments: LM EGD-e, LM-*Δrli34* and LM-*Δrli34-rli34* were cultured to logarithmic growth phase and added into 6-well microplates at the ratio of 1: 100 (macrophage: bacteria). The rates of adhesion, invasion and intracellular proliferation of RAW264.7 cell were examined according to the method reported by Wang *et al*. (2017), respectively. Meanwhile, Cell Counting Kit-8 (CCK-8) assay was used to test the cytotoxic activity of the compound against RAW264.7 cells (Kuang *et al.*, 2020).

Determination of *L. monocytogenes* virulence in mice: LM EGD-e, LM-*Δrli34* and LM-*Δrli34-rli34* bacterial solutions were injected intraperitoneally at 0.5mL/each for 7 days, and their LD_{50} on mice was determined using the Karber method. In addition, the Kaplan-Meier survival curve was plotted according to the morbidity and mortality in infected mice. Moreover, bacterial loads in liver and spleen from mice inoculated with sublethal doses were determined, respectively, and organ sections were prepared and subjected to HE staining for the observation of pathological changes.

Molecular characteristics and potential target gene of sRNA *rli34*: Molecular characteristics of sRNA rli34 were bioinformationally analyzed using the software Softberry, fruitfly, RNAfold, respectively. In addition, phylogenetic tree based on *rli34* gene was constructed using MEGA10.0 software (NJ method, Bootstrap for 1 000). The potential rli34-regulated target genes were predicted using online software TargetRNA2 (http://cs.wellesley.edu/~btjaden/TargetRNA2/) and IntaRNA (http://rna.informatik.uni-freiburg.de/IntaRNA/) (Wright *et al.*, 2014).

Verification of the interaction between rli34 and target Mrna: The bacterial dual plasmid reporter system was used to detect the interaction of *rli34* with its regulatory target genes. Briefly, Put18C-*rli34*, Put18C-*Δrli34* and Pmr-LacZ-gtcA recombinant vectors were constructed, respectively. The recombinant vectors were electro-transferred into *E. coli* BTH101 competent cells, coated with LB nutrient agar containing Kan^r (100mg/Ml) and Amp^r (100mg/Ml), and screened for positive clones. Subsequently, the obtained bacteria were cultured on LB medium containing X-gal and IPTG, incubated at 37°C for 12 h. The color change of the moss was observed, and then the moss was suspended in phosphate buffer (PBS), followed by the measurement of its OD_{470nm} value. Additionally, the Mrna level of target gene *gtcA* was determined using Qrt-PCR.

Analysis of the protein level of target gene: Western blot assay was performed as described previously (Shahid *et al.*, 2021) with some modifications. Briefly, Western blot was performed using mouse anti-gtcA protein antibody as primary antibody (1: 3 000) and goat anti-mouse IgG-HPR (1:5000) as secondary antibody. Meanwhile, the glyceraldehyde-3-phosphate dehydrogenase (GapA) was used as internal reference protein to analyze the effects of rli34 on the expression of target gene *gtcA*. And then, ImageJ 1.8.0 software was applied to quantify the protein bands of Western blot.

Table 1: Primers used in the study

Primer names	Primer sequences （5'→3'）
rli34 F	ATAAAAAGTGAATACTGCTTCATA
rli34 R	TAGATTTATTCTTACACAGTATGAGT
R1	CGGGGTACCTAGATTGAAATGAAAGAGGCAGG
R2	CCTTAAAACTGGCTTTATTGCAATCAATTTTTCTTCGT
R3	ACGAAGAAAAATTGATTGCAATAAAGCCAGTTTTAAGG
R4	AAACTGCAGGTCGTCAACTTGCATCATTTCTACA
P1	CCCAAGCTTATAAAAAGTGAATACTGCTTCA
P2	GGAATTCTAGATTTATTCTTACACAGTATGAG
16S rRNA F	GAGCTAATCCCATAAAACTATTCTCA
16S rRNA R	ACCTTGTTACGACTTCACCCC
P3	GGATCCGATTGAAATGAAAGAGGCAGGA
P4	GGTACCTTCTATTAGCTCTCTTTTCCTT
P5	CCAAGCTTTTCTTATACGACATTGCC
P6	GGGGTACCCTCTCCGTTA TATTCTTCT
P7	GGATCCGATTGAAATGAAAGAGGCAG
P8	AGATATTACTCATACTCACATTCTTTAAATCTAAATAAAGCC
P9	GGCTTTATTTAGATTTAAAGAATGTGAGTATGAGTAATATCT
P10	GGGGTACCCTCTCCGTTA TATTCT
P11	ATGAACAAAATAAGAAAAT
P12	AAGTACAAGG ACAATTACA

Statistical analysis of data: Independent t-tests were used to analyze differences between two groups, and one-way analysis of variance (ANOVA) was used to analyze differences between multiple groups. All data were expressed as mean ± standard deviation (SD). A value of $P<0.05$ was considered as significant difference, while $P<0.01$ was recognized as extra significant difference.

RESULTS

Expression profiles of sRNA *rli34* gene under intra- and extracellular conditions: The sRNA *rli34* gene have been amplified by TR-PCR, and the sRNA rli34 gene size was 84 bp (Fig. S1), which was consistent with the theoretical value, indicating that the *rli34* gene was successfully amplified from LM EGD-e by RT-PCR amplification and sequence determination. As shown in Fig. 1, the intracellular transcriptional level of *rli34* gene was up-regulated 10.94-fold as compared with extracellular culture ($P<0.05$) (Fig. 1).

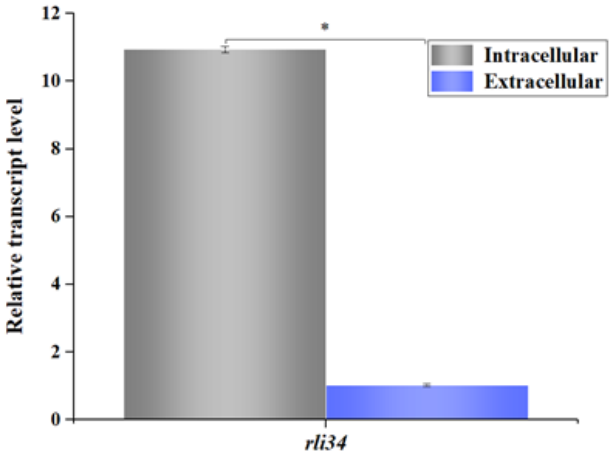

Fig. 1: Intracellular and extracellular expression profiles of *rli34* gene of *L. monocytogenes 16S rRNA* gene was used as an internal reference gene. Error bars represent the standard deviation of three biological replicates. Independent t-tests were used to analyze differences between intracellular and extracellular expression level of *rli34* gene (* p-value < 0.05).

Deficiency of *rli34* gene impaired the cell adhesion, invasion and intracellular survival: The LM-*Δrli34* deletion strain and the complementary strain LM-*Δrli34-rli34* were successfully generated and verified by PCR amplification and sequencing (Fig. S2a, b and c). To probe the regulatory role of rli34 during infection of macrophage, the adhesion, invasion and intracellular proliferation of RAW264.7 were examined by bacterial cell counts. As shown in Fig. 2, the adhesion, invasion, intracellular survival and cell inhibition (Fig. 2) of LM-*Δrli34* on RAW264.7 cells were significantly declined ($P<0.05$) as compared with LM EGD-e and LM-*Δrli34-rli34*. The results indicated that *rli34* gene deletion reduced the viable proliferation ability of LM in RAW264.7 cells.

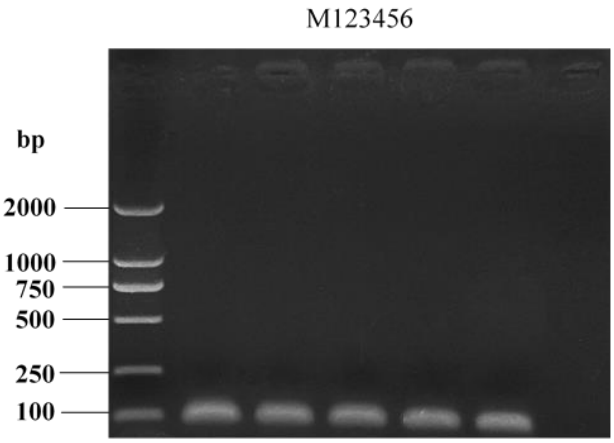

Fig. S1: Amplification of LM *rli34* gene by RT-PCR. M: DNA marker DL-2000 (2000, 1000, 750, 500, 250, 100 bp); 1-5: Amplified products of *rli34* gene by RT-PCR; 6: Negative control.

Deficiency of *rli34* gene reduced *L. monocytogenes* virulence in mice: To assess the role of rli34 in virulence of *L. monocytogenes*, we compared the lethality of different strains of LM-infected mice. The results showed that the LD_{50} of LM-*Δrli34* was elevated by 1.76 and 1.56 logarithmic orders of magnitude as compared with LM EGD-e and LM-*Δrli34-rli34* (Table S1). As shown in Fig. 3, mice in the LM - *Δrli34* infection group survived significantly longer (Fig. 3a). There were significant differences in bacterial loads in both liver and spleen ($P<0.01$) (Fig. 3b and c). Moreover, we further investigated the pathological changes in the livers, spleens and kidneys

Table S1: Determination of LD_{50} of LM EGD-e, LM-*Δrli34* and LM-*Δrli34-rli34* in mice

Group	LM EGD-e			LM-*Δrli34*			LM-*Δrli34-rli34*		
	Dose/cfu	Mortality	LD_{50}	Dose/cfu	Mortality	LD_{50}	Dose/cfu	Mortality	LD_{50}
1	1.68×10^{6}	(10/10)	$10^{5.56}$	2.1×10^{9}	(10/10)	$10^{7.32}$	2.68×10^{6}	(10/10)	$10^{5.76}$
2	8.40×10^{5}	(9/10)		2.1×10^{8}	(8/10)		1.34×10^{6}	(8/10)	
3	4.20×10^{5}	(6/10)		2.1×10^{7}	(5/10)		6.20×10^{5}	(7/10)	
4	2.10×10^{5}	(2/10)		2.1×10^{6}	(2/10)		3.10×10^{5}	(2/10)	
5	1.05×10^{5}	(0/10)		2.1×10^{5}	(0/10)		1.52×10^{5}	(0/10)	

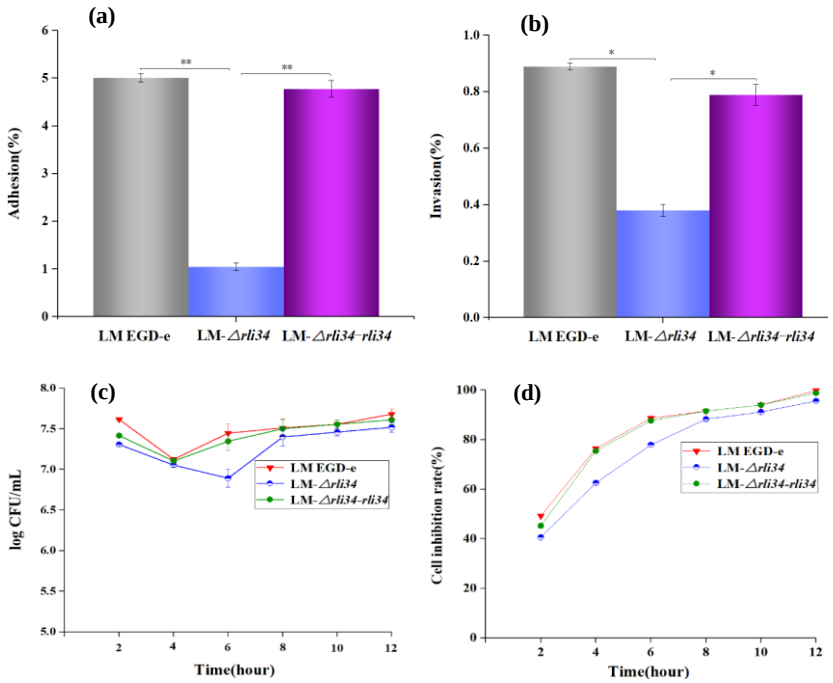

Fig. 2: Determination of cell adhesion and invasion of different strains of *L. monocytogenes:* **(a)** Adhesion rate of LM EGD-e, LM-*Δrli34* and LM-*Δrli34-rli34* in RAW264.7 cells. (**b)** Invasion rate of LM EGD-e, LM-*Δrli34* and LM-*Δrli34-rli34* in RAW264.7 cells. (**c)** Bacterial numbers in RAW264.7 cells infected by LM EGD-e, LM-*Δrli34* and LM-*Δrli34-rli34* at various periods. (**d)** Inhibition rate of LM EGD-e, LM-*Δrli34* and LM-*Δrli34-rli34* in the RAW264.7 cells after infection at various time. All data are expressed as the means ± SD (n = 3). The differences between strains were assessed by one-way ANOVA (*p-value <0.05, **p-value <0.01).

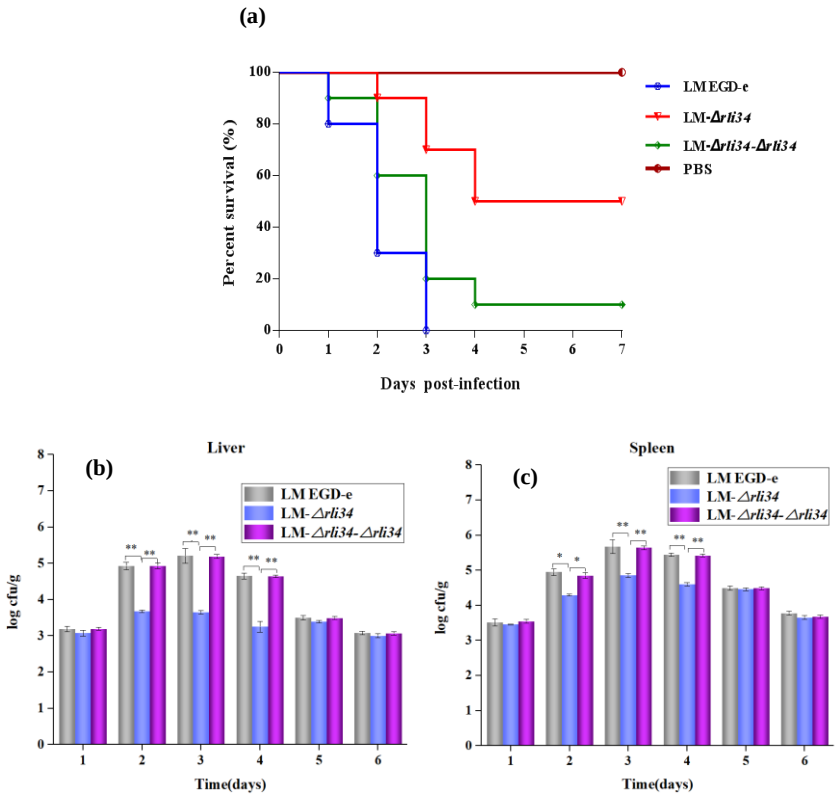

Fig. 3: Survival curves and bacterial loads in mice infected by different strains of *L. monocytogenes:* **(a)** Survival curves. (**b)** Bacterial loads in livers. (**c)** Bacterial loads in spleens. Results are represented as means ± SD of three experiments and expressed as log CFU/g. The differences between strains were assessed by one-way ANOVA (*p-value <0.05, **p-value <0.01).

of different LM-infected mice. The results showed that pathological damages in livers, spleens and kidneys were significantly reduced in LM-*Δrli34* (Fig. 4), indicating that *rli34* gene deletion suppressed the pathogenicity of *L. monocytogenes* in mice.

Molecular characteristics of sRNA *rli34:* Sequence analysis showed that *rli34* gene was located on the intergenic region between LM EGD-e *lmo0777* and *lmo0778* gene, with a size of 84bp. Furthermore, an *argR* transcription factor binding site was predicted in the promoter sequence

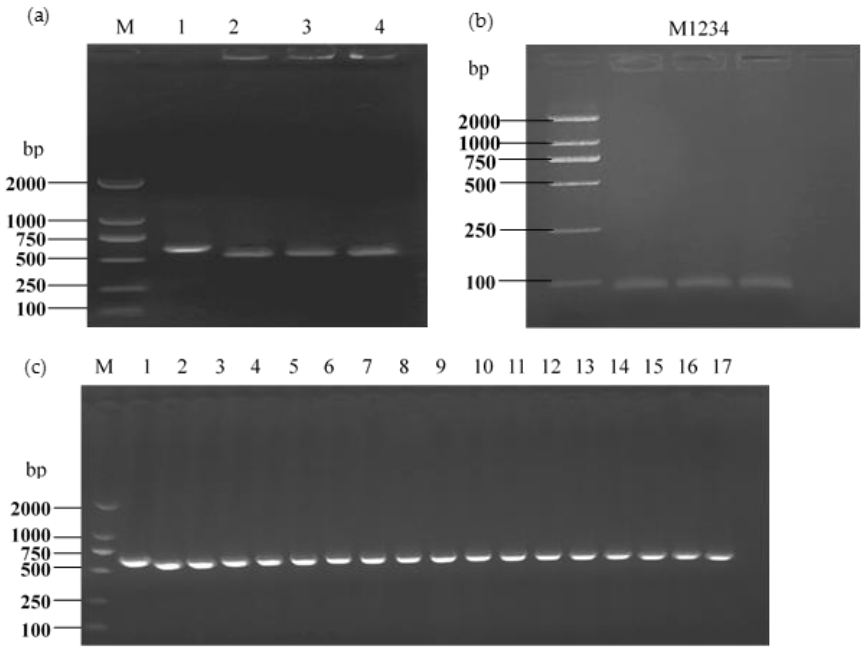

Fig. S2: Construction of LM-*Δrli34* deletion and complementary strains. (a) Screening and identification of recombinant LM-*△rli34* by PCR. M: DNA marker DL-2000 (2000, 1000, 750, 500, 250, 100 bp); 1: LM EGD-e strain (positive control); 2-4: Amplified products of recombinant LM-*△rli34* by PCR. (b) Screening and verification of complement strain LM-*△rli34-rli34* by PCR. M: DNA marker DL-2000 (2000, 1000, 750, 500, 250, 100 bp); 1-3: Amplified products of complement strain LM-*△rli34-rli34* by PCR; 4: Negative control. (c) Analysis of genetic stability of LM-*△rli34* by PCR. M: DNA marker DL-2000 (2000, 1000, 750, 500, 250, 100 bp); 1: LM EGD-e strain (positive control); 2-17: PCR Identification of LM-*△rli34* of 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30 generation.

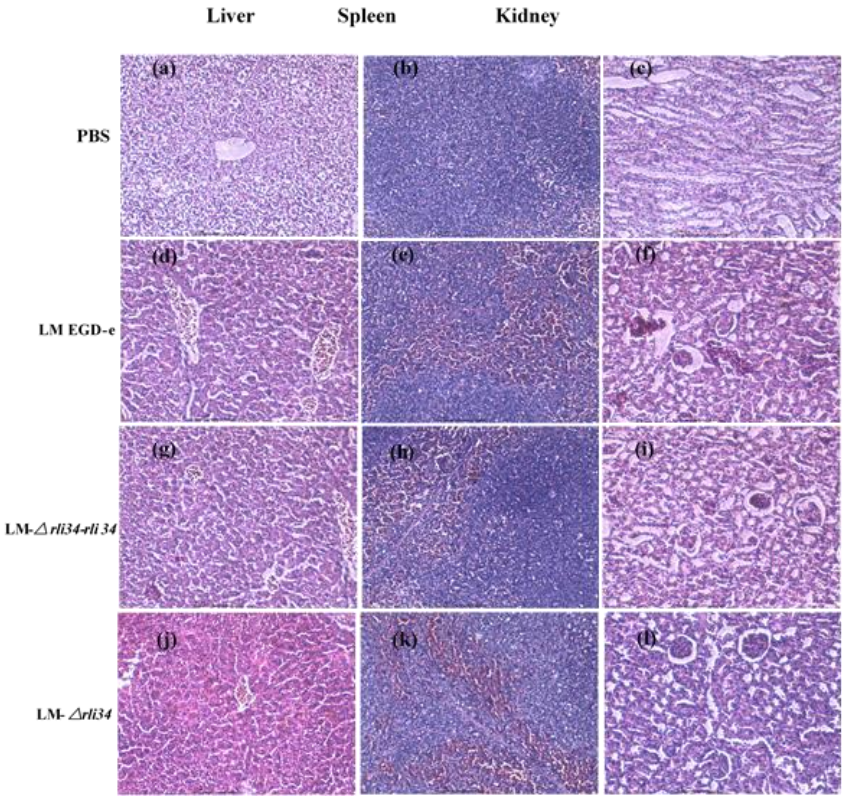

Fig. 4: Histopathological examination of liver, spleen and kidney mice infected by *L. monocytogenes* (HE×400, scale bars, 100 μm) **(a)**, **(b)** and **(c)** liver, spleen and kidney from mice injected with PBS. (**d**), **(e)** and (**f**) liver, spleen and kidney from mice infected by LM EGD-e. (**j**), **(k)** and (**l**) liver, spleen and kidney from mice infected by LM-*△rli34*. (**g**), **(h)** and (**i**) liver, spleen and kidney from mice infected by LM-*△rli34-rli34*.

of *rli34* gene, suggesting that the expression of sRNA *rli34* was regulated by *argR* transcription factor (Fig. S3a). Genetic evolutionary analysis showed that the *rli34* gene was highly conserved in *L. monocytogenes* serotypes 1/2a, 1/2c, 3a, 3c and 4b (Fig. S3b).

Rli34 can modulate the expression of target gene *gtcA*: As shown in Fig. 5, *in silico* prediction of secondary structure showed that *rli34* was likely to fold as a three stem-loop structure (Fig. 5a). It was found that *rli34* could complementarily pair with *gtcA* mRNA 5'-UTR (-279 to -270) by combined prediction of TargetRNA2 and IntaRNA software (Fig. 5b). Moreover, to further verify the interaction between Rli34 and gtcA mRNA, a dual plasmid reporter system based on *E. coli* was employed, and then the pUT18C-rli34 and pMR-LacZ-gtcA plasmids were successfully constructed and verified by PCR and double enzyme digestion, respectively (Fig. S4). As shown in Fig. 5, the moss of *E. coli* strain co-transformed by pUT18C-*rli34* and pMR-LacZ-*gtcA* displayed darker blue on the plates containing X-gal as compared with these strains of *E. coli* transformed by pUT18C, pMR-LacZ-*gtcA* or its co-transformation, and the $OD_{470\ nm}$ values of bacterial suspensions differed significantly ($P<0.05$). While there is no difference between the strain co-transformed by pUT18C-pMR-LacZ-gtcA and the strain co-transformed by pUT18C-Δrli34-pMR-LacZ-gtcA (Fig. 5c and d), suggesting that Rli34 can interact with the mRNA of target

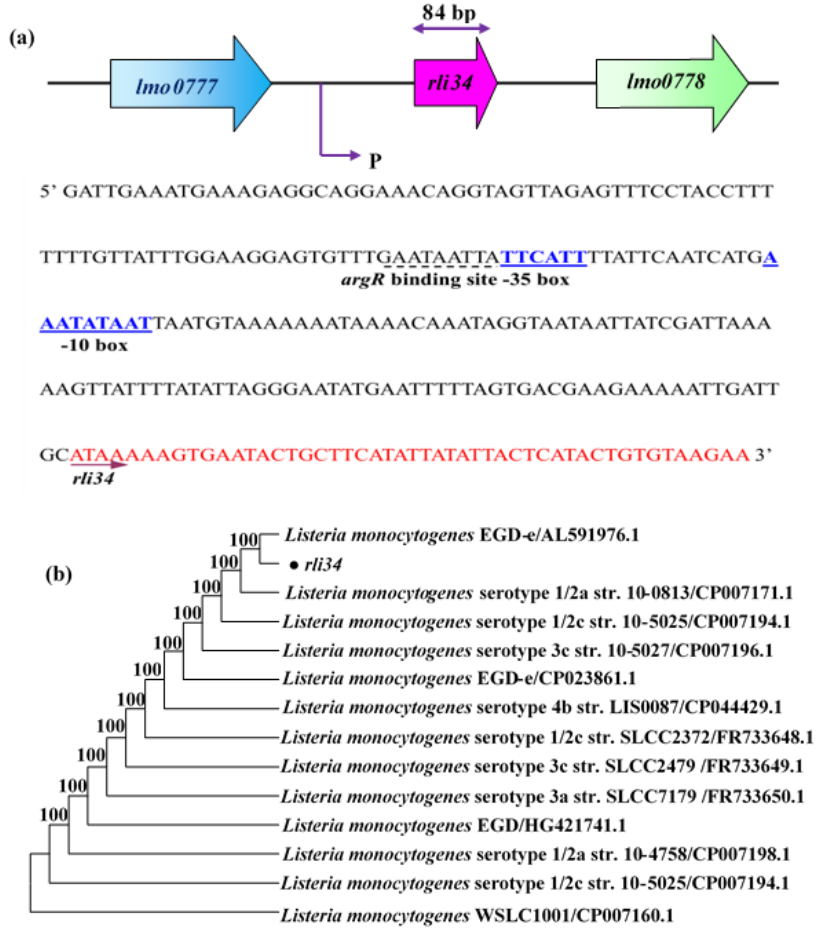

Fig. S3: Molecular characterization of *rli34* gene and its phylogenitic analysis. (a) Genetic location of *rli34* gene in the genome of LM EGD-e; (b) Phylogenitic analysis of *rli34* gene of different serotypes of LM

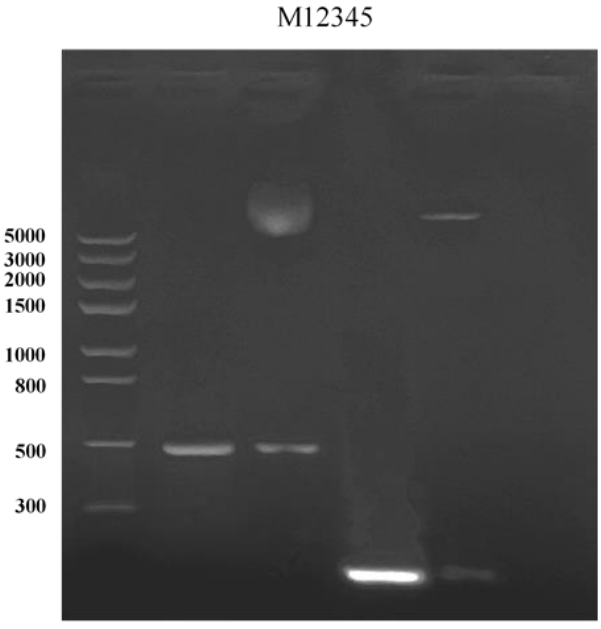

Fig. S4: Screening and identification of pMR-LacZ-*gtcA* and pUT18C-*rli34* using PCR and restriction enzyme digestion. M: DNA marker DL-5000 (5000, 3000, 2000, 1500, 1000, 800, 500, 300 bp); 1: Identification of pUT18C-*rli34* by PCR; 2: pUT18C-*rli34* digested by restriction enzyme; 3: Verification of pMR-LacZ-*gtcA* by PCR; 4: pMR-LacZ-*gtcA* digested by restriction enzyme; 5: Negative control.

gene *gtcA*. What's more, compared with LM EGD-e and LM-*Δrli34-rli34*, the mRNA and protein levels of target gene *gtcA* were significantly lower (P<0.05) in LM-*Δrli34* (Fig. 5e and f), respectively, indicating that *rli34* exerts positive regulatory roles on expression of *gtcA* gene.

DISCUSSION

Small RNAs (sRNAs) are a class of non-coding RNAs of 40-500 nucleotides in length, which are widely distributed in Gram-negative and positive as important regulator (Ponath *et al.*, 2022), which consist of a complex regulatory network to facilitate the invasion, intracellular reproduction and survival, virulence regulation and immune escape in bacteria (Nitzan *et al.*, 2017; Liu *et al.*, 2022; Raad *et al.*, 2022). Based on the differences of position relationship and pairing status, sRNAs are classified into two categories, *cis*-encoded (*cis*-acting) and *trans*-encoded (*trans*-acting), respectively (Dutta and Srivastava, 2018). Typically, *cis*-encoded sRNA, the heterogeneous groups of regulatory transcripts, is fully complementary paired with its target mRNA, which is often derived from reverse transcription of complementary templates of the same gene or the overlapping 5′-UTRs and 3′-UTRs of adjacent genes. By contrast, the *trans*-encoded sRNA is not adjacent to its target mRNA and is not fully complementary paired with its target mRNA, which may regulate multiple target mRNAs. sRNAs may bind to target gene mRNAs through complementary base pairing, whereby its exert regulatory functions at the post-transcriptional or translational level (Nitzan *et al.*, 2017).

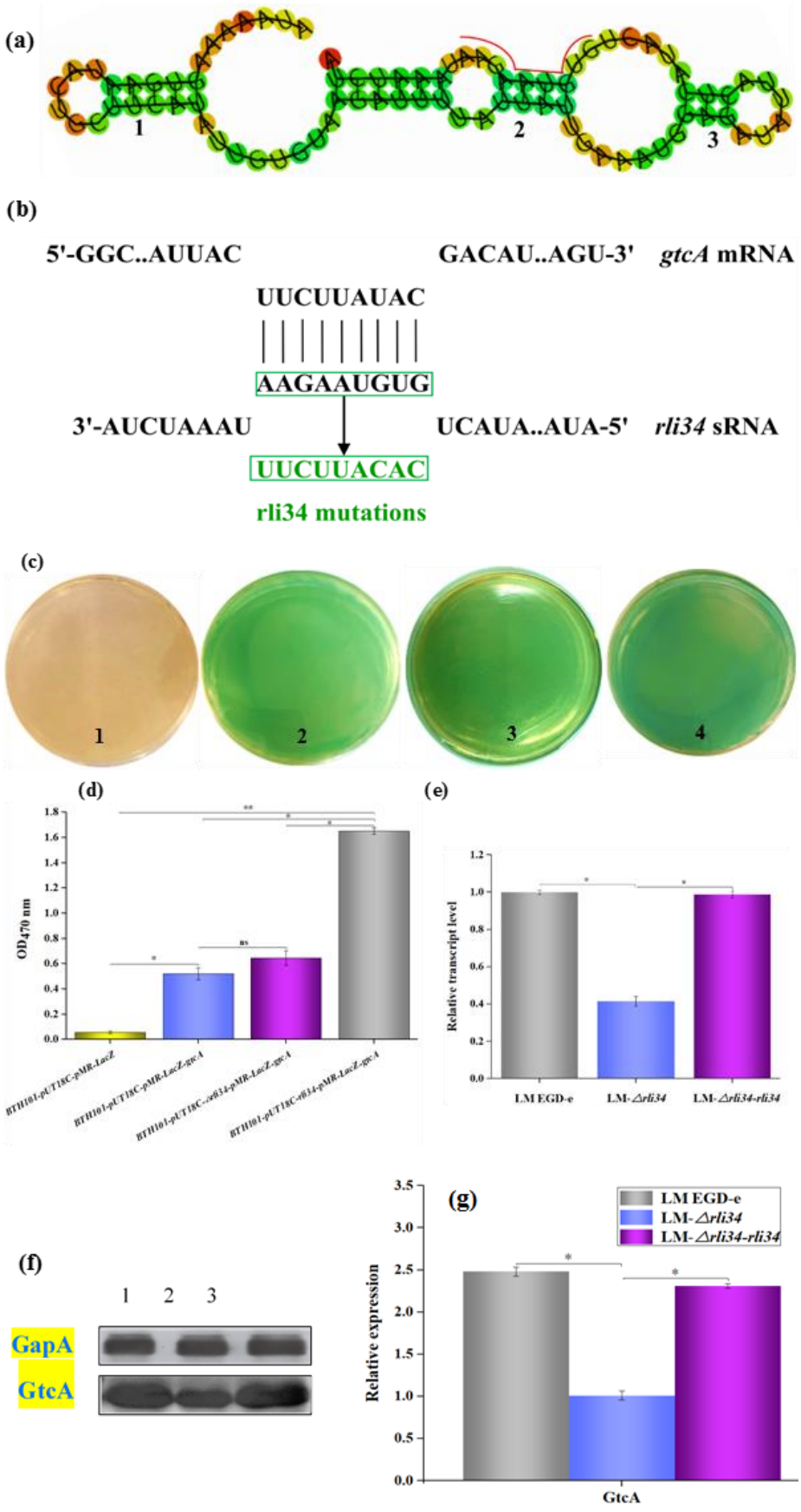

Fig. 5: Verification of interaction between sRNA *rli34* and target gene *gtcA* using dual plasmids reporter system: **(a)** Secondary structure of sRNA *rli34* (3 stem-loop structures, and red line represents base-paring region). **(b)** The predicted target gene regulated by *rli34* (the nucleotide mutations in green box for the sRNAs). **(c)** Bacterial lawn of BTH101 (1. BTH101 co-transformed by pUT18C and pMR-LacZ; 2. BTH101 co-transformed by pUT18C and pMR-LacZ-gtcA; 3. BTH101 co-transformed by pUT18C-Δrli34 and pMR-LacZ-gtcA; 4. BTH101 co-transformed by pUT18C-rli34 and pMR-LacZ-gtcA). **(d)** Bacterial suspension determined by $OD_{470\ nm}$. **(e)** Determination of the mRNA level of *gtcA* genes by qRT-PCR. **(f)** Determination of the expression of GtcA protein by Western blot (1. LM EGD-e; 2. LM-*Δrli34*; 3. LM-*Δrli34-rli34*).

Currently, many sRNAs have been proved to play important roles in the regulation of glucose metabolism, environmental stress, motility, biofilm formation, virulence, drug resistance and population sensing in bacteria (Cameron *et al*., 2019). The novel rli34 is a newly identified *trans*-encoded sRNA in *L. monocytogenes* (Toledo-Arana *et al.*, 2009), however, its role of in virulence still remains unclear. Here, the sRNA rli34 was found to be significantly more expressed in RAW264.7 cells than that of extracellular condition, and thus was hypothesized that *rli34* may be involved in the infection and intracellular survival in *L. monocytogenes*.

Bioinformatics analysis revealed that the wall teichoic acids (WTAs) glycosyltransferase (gtcA) gene was one of the potential target genes regulated by rli34. Teichoic acid is an important wall polymer produced in Gram-positive bacteria, which is covalently linked to peptidoglycan to form WTAs (Percy and Grundling, 2014; Sumrall *et al.*, 2020). Several studies have confirmed that WTAs glycosylation plays an important role in colonization, invasion and virulence in bacteria (Winstel *et al.*, 2015; Brauge *et al.*, 2018; Bellich *et al.*, 2022). In *L. monocytogenes*, cell WTAs glycosylation have been shown to be involved in adhesion with mammalian cells through its surface proteins (Ortega *et al*., 2014; Yin *et al*., 2019; Thomasen *et al*., 2022). In the present study, it was confirmed that the ability of cell adhesion, invasion and intracellular proliferation of LM-*Δrli34* was significantly weakened. Furthermore, the virulence of LM-*Δrli34* had significantly reduced in mice, suggesting that *rli34* can indirectly influence the virulence of *L. monocytogenes via* regulating the expression of *gtcA* gene.

It is generally believed that sRNAs can regulate the stability or translation of target mRNAs at the post-transcriptional or translational level by pairing with target mRNA, thereby regulating gene expression and affecting a variety of physiological functions in bacteria (Ortega *et al.*, 2014). To identify the rli34-gtcA mRNA interaction, this study used a bacterial dual plasmid reporter system to confirm that rli34 can bind to the gtcA mRNA 5'-UTR. The results of qRT-PCR and Western blot confirmed that the deficiency of *rli34* gene down-regulated the expressions of the target gene *gtcA* at mRNA and protein levels. The molecular mechanism of regulation is presumed to be that rli34 may stabilize gtcA mRNA by binding to 5'-UTR of its mRNA, which prevents the degradation of gtcA mRNA by RNase J1, and thus facilitating the expression level of *gtcA* gene. This finding was similar to the regulatory mechanisms underlying described in other bacteria (Dutta and Srivastava, 2018; Anand *et al*., 2022), which highlighted that there existed the similar regulatory mechanisms for *trans*-encoded sRNAs in prokaryotes.

Conclusions: This study revealed for the first time that sRNA rli34 can directly modulate the glycosylation of *L. monocytogenes* WTAs by targeting *gtcA* mRNA, thereby indirectly exerting its regulatory roles in modifying the virulence, which provided new insights into the molecular mechanisms of *trans*-encoded sRNAs in virulence regulation in *L. monocytogenes*.

Authors contribution: Qingling Meng and Jun Qiao designed the research. Lixia Wang conducted the research. Zhiyuan Li collected and analyzed the data. Lixia Wang and Xuepeng Cai wrote the manuscript. Xianzhu Xia helped in the preparation of the manuscript and was responsible for the revision and correction of the text. All authors read and approved the final version of the manuscript.

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