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A Small Regulatory RNA, *Rli82*, Is Involved in the Motility and Pathogenicity of *Listeria monocytogenes*

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Abstract

Background: *Listeria monocytogenes* (LM) is a facultative intracellular pathogen that causes food-borne infections in humans and animals. To invade and multiply within host cells, LM utilizes various strategies to precisely modulate its gene expression and to adapt to the *in vivo* environment.
Objectives: To investigate the regulatory roles of *Rli82* sRNA in the motility and pathogenicity of LM EGD-e.
Methods: The *Rli82* gene knock-out mutant strain, LM-Δ*Rli82*, and the complementation strain, LM-Δ*Rli82*/*Rli82*, were constructed using homologous recombination technology, and their motility and virulence, respectively, were determined. Moreover, the potential target mRNA regulated by *Rli82* was predicted using TargetRNA2 software, and then the interaction between the target mRNA and *Rli82* was verified by the two-plasmid reporter system.
Results: The results showed that the motility of LM-Δ*Rli82* was significantly increased at 25°C, facilitated by the production of more flagella than LM EGD-e and LM-Δ*Rli82*/*Rli82*. Furthermore, LD_{50} in LM-Δ*Rli82*-infected mice was significantly increased as compared to LM EGD-e and LM-Δ*Rli82*/*Rli82*, suggesting that the virulence of LM was weakened when the *Rli82* gene was deleted. In addition, the mRNA level of *flaA* was not significantly elevated, but *flaA* protein was significantly higher in LM-Δ*Rli82* than in LM EGD-e and LM-Δ*Rli82*/*Rli82*, suggesting that *Rli82* might modulate the translation of *flaA* mRNA at the post-transcriptional level.
Conclusions: Taken together, our findings for the first time revealed that *Rli82* sRNA might be involved in the modulation of the expression of *flaA* protein, thereby influencing the mobility and pathogenicity of LM.

Keywords: *Listeria monocytogenes*, Regulatory sRNA, *Rli82*, Motility, Pathogenicity

1. Background

Listeria monocytogenes (LM) is an intracellular Gram-positive pathogen causing listeriosis in both humans and various animal species (1). As a ubiquitous food-borne bacterium, LM can infect the host through contaminated food or drinking water. During the process of invasion, LM can express and deploy a variety of virulence factors, thereby breaking through the blood-intestinal, blood-brain, or placental barriers to cause meningitis, miscarriage, and sepsis in humans (2, 3). Since LM poses a great threat to food safety, it has been classified as one of the most important food-borne bacteria by the World Health Organization (WHO) (4). Bacterial small RNA (sRNA) is a class of non-coding RNAs that are usually transcribed within the intergenic region of the bacterial chromosome but do not encode proteins (5). In contrast to regulatory proteins, sRNA allows bacteria to respond rapidly to various environmental conditions during infection (6, 7).

In order to survive and proliferate in hosts, the pathogen can perceive changes in the host's internal environment and regulate the expression of its virulence genes accordingly through various modulators and signaling cascades (8). Among regulatory repertoires, sRNA is now considered an important gene expression regulator at the post-transcriptional level. In recent years,

many studies have reported that sRNA can be involved in cellular metabolism (9), physiological growth, and pathogenicity of bacteria through interacting with target mRNAs, thereby modulating responses to a variety of environmental stresses and facilitating survival in hosts (5). In 2011, Mraheil et al. identified about 150 sRNAs in LM (10) through sRNA transcriptomic analysis during the growth of the bacterium in macrophages. Among them, *Rli82* was one of the identified sRNAs showing significant differential expression. Meanwhile, *Rli82* deletion alters the growth pattern of LM; however, the regulatory function of *Rli82* sRNA remains unknown so far.

2. Objectives

The main purpose of this study was to unveil the role of *Rli82* sRNA on the motility and pathogenicity of LM and to further ascertain the potential regulatory mechanism of this sRNA. To achieve this goal, overlap extension PCR (SOE-PCR) and homologous recombination techniques were employed to construct *Rli82*-deleted and complementation LM strains. Then, the potential target gene regulated by *Rli82* was predicted and verified to provide new insights into the mechanism of sRNA-mediated control of flagellum-related genes in LM.

3. Methods

3.1. Plasmids, Strains, and Culture Condition

The shuttle vectors of pKSV7 and pHT304 were used to generate *Rli82*-deleted and complementation LM strains. EGD-e strain was cultured in Brain Heart Infusion (BHI) Broth (Difco, USA) at 37°C, whereas *Escherichia coli* DH5α and BTH101 strains were cultured in LB (Difco, USA) medium at 37°C.

3.2. Primer Design

The specific primers used in this study were designed based on the LM EGD-e genome sequence deposited in GenBank (accession number: AL591824) using Primer Premier 5.0 software. Table 1 shows the detailed information of the designed primers.

3.3. Generation of Rli82 Gene-Deleted and Complementation Strains

Briefly, the LM EGD-e strain was cultured in BHI at 37°C for 12 h. The genomic DNA of LM was extracted according to the protocol of a bacterial genomic DNA extraction kit (Omega, USA). The upstream and downstream homology arms of the *Rli82* gene were amplified using two pairs of primers (F1 - F2 and F3 - F4). These fragments were then used to generate *Rli82*-deleted mutant strain (Δ*Rli82*) by SOE-PCR. Then, the Δ*Rli82* fragment was cloned into a pMD19-T simple vector (TaKaRa, Japan) to generate pMD19-T-Δ*Rli82*. The pMD19-T-Δ*Rli82* and pKSV7 plasmids were double digested with *Kpn* I and *Hind* III (TaKaRa, Japan), and the target fragments were recovered and ligated with T4 DNA ligase (TaKaRa, Japan) at 16°C to produce the recombinant shuttle plasmid (pKSV7-Δ*Rli82*). After that, pKSV7-Δ*Rli82* was transformed into LM EGD-e competent cells by electroporation (2500 V, 5.0 ms), and positive clones were screened by PCR using F5 - F6 primers.

The positive clones were passaged in BHI medium at a concentration of 10 μg/mL chloramphenicol for 15 generations at 42°C and in chloramphenicol-free BHI liquid medium for 15 generations at 30°C. The obtained recombinant LM-Δ*Rli82* was verified by PCR and sequencing. For the generation of the complementation strain, the *Rli82* gene was amplified in LM EGD-e using 82F-82R primers and cloned into pHT304 plasmid to generate pHT304-*Rli82*. Then, pHT304-*Rli82* was transformed into LM-Δ*Rli82* competent cells by electroporation, and the positive clones were screened on plates containing solid BHI at a concentration of 5 ug/mL of erythromycin. Positive clones were further verified by sequencing to obtain the complementation strain (LM-Δ*Rli82*/*Rli82*) (11).

3.4. Determination of Motility

The motility of LM EGD-e, LM-Δ*Rli82*, and LM-Δ*Rli82*/*Rli82* strains was assayed in the BHI semi-solid medium at 25°C. In brief, individual clones from these strains were harvested and washed three times with 0.01 M PBS buffer (pH = 7.2), centrifuged, and subjected to negative staining with 2% phosphotungstic acid solution (Sigma, USA). The morphological characteristics of these bacteria were observed using transmission electron microscopy (TEM) (HT7700, HITACHI, Japan). The flagella of 50 bacteria per strain were counted.

3.5. Determination of Pathogenicity

Mice were infected with LM EGD-e, LM-Δ*Rli82*, and LM-Δ*Rli82*/*Rli82* by intraperitoneal injections. To determine LD_{50}, bacterial concentration was adjusted to the same level (approximately 10^9 cfu/mL) for all three strains, from which a series of 10-fold dilutions to 10^5 CFU/mL were prepared. Then 6-8-week-old BALB/c mice were divided into 5 groups, and each mouse was injected intraperitoneally with 0.5 mL of the prepared bacterial solution, and 0.01 M PBS (pH = 7.2) was used as the control. After animal infection, mortality was monitored for

Table 1. Primers Used in This Study

Primer Name	Primer Sequence (5'→3')	Product Size (Bp)
F1	GGTACCAAGACACCAGTTCCGTTTA	495
F2	GTTCTGTTATACAGTATCTTTTTGATGACTAAAGTATATA	
F3	TATATACTTTAGTCATCAAAAAGATACTGTATAACAGAAC	316
F4	AAGCTTCCTATTAGAAACACGAGCATTA	
F5	TGCTGTCTTACCAGTAGGCTCA	1625
F6	AAGAAATCAGTGGAAGTAGCCC	
82 F	GGATCCATCCTCCTATAGGCACTTTTTAGTATCTA	70
82 R	AAGCTTATATACCGTACAGAATAACAAGAAGGTAC	
***flaA*-laczF**	AAGCTTTTTGGACAACTTTTCTGTTCA	251
***flaA*-lacz R**	GGTACCGTATTTACTTTCATTTGTGTTTCC	
***flaA* F**	AACAAGCAACTGAAGCTATTGATGAATT	247
FlaA R	TGCGGTGTTTGGTTTGCTTGA	
16sRrna F	CACTGGGACTGAGACACGG	243
16sRrna R	GGACAACGCTTGCCACCTA	
FlaA F	GTCGGATCCATGAAAGTAAATACTAATAT	864
FlaA R	CATCTCGAGTTAGCTGTTAATTAATTGAGT	
GAPDH F	CGGGATCCATGACAGTTAAAGTTGGTATTAA	1011
GAPDH R	CCTCGAGTTATTTAGCGATTTTTGC	

10 consecutive days in the study groups, and LD_{50} was calculated by the Spearman-Kärber method. In parallel, bacterial loads in the liver and spleen were determined in infected mice, and histopathological changes in these organs were observed after HE staining (11).

3.6. Target mRNA Prediction and Verification

The potential genes targeted by *Rli82* were analyzed using TargetRNA2 bioinformatics online software (http://cs.wellesley.edu/~btjaden/TargetRNA2/). To verify the interaction between *Rli82* and target genes, the two-plasmid reporter system based on *E.coli* (BTH101 strain) was employed. Briefly, the recombinant plasmids of pUT18C-*Rli82* and pUT18C-Δ*Rli82* (*Rli82* without the base-pairing region) and pMR-LacZ-target (the 5'-UTR region of *flaA* mRNA) vectors were constructed and co-transformed into *E. coli* BTH101 competent cells. Then, positive clones were cultured on LB agar containing X-gal and IPTG (TaKaRa, Japan) at 37°C for 12 h. Color change in the lawn solution was monitored, and the optical density (OD_{450} nm) of the lawn solution rinsed from the plates was determined.

3.7. Quantitative Real-time RT-PCR

Briefly, LM EGD-e, LM-Δ*Rli82*, and LM-Δ*Rli82*/*Rli82* strains were incubated in the BHI medium at 25°C for 16 h, and total RNA was extracted using Trizol reagent in compliance with the instructions of the provider (Invitrogen, USA). Then, cDNA was synthesized using the AMV Reverse Transcription Kit (TaKaRa, Japan) following its instruction manual. Quantitative real-time RT-PCR (qRT-PCR) was performed on Light Cycler 480 (Roche, Switzerland) using a SYBR Premix Ex Taq™ kit (TaKaRa, Japan). The relative transcription levels of the target genes and *Rli82* were calculated by the $2^{-\Delta\Delta CT}$ method (12). The 16s rRNA gene was employed as an internal reference control.

3.8. Western Blot Analysis

Western blot was performed as previously described (13). Briefly, bacterial protein was extracted using the Bacterial Protein Extraction Kit (Sangon Biotech, China) and analyzed by SDS-PAGE, followed by Western blot analysis using mouse-specific primary antibodies (1: 2000) and HRP-labeled rabbit anti-mouse IgG (Sigma, USA) secondary antibodies (1: 5000). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference protein. Image J software was applied to quantify the protein bands.

3.9. Statistical Analysis

SPSS software (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis of the data. The data were

presented in figures as mean values ± standard deviation (SD) from three independent experiments. The analysis of variance (ANOVA) was used to compare continuous variables, while the chi-square test was employed to analyze categorical variables. A P value $<$ 0.05 was considered to be statistically significant, while P values $<$ 0.01 were considered extremely significant.

4. Results

4.1. Generation of Rli82 Gene-Deleted and Complementation Strains

The deletion mutant (LM-Δ*Rli82*) and complementation (LM-Δ*Rli82*/*Rli82*) strains were successfully constructed and verified by PCR, restriction enzyme digestion, and sequencing (Appendix 1, Appendix 2, and Appendix 3, respectively).

4.2. Effects of Rli82 Gene Deletion on LM Motility

The mean diameter of the colonies formed by LM-Δ*Rli82* was significantly larger than that of LM EGD-e in semi-solid plates (Figure 1A and B), indicating that the motility of LM-Δ*Rli82* was significantly increased. Moreover, LM EGD-e bacteria carried 1.2 flagella per cell on average, whereas 4.3 flagella were counted on average for LM-Δ*Rli82*, showing a significantly higher value in the mutant than in the parental strain (Figure 2).

4.3. Effects of Rli82 Gene Deletion on LM Pathogenicity

The LD50 of LM-Δ*Rli82* was $10^{7.00}$ CFU/mL, which was significantly higher than that of LM EGD-e ($10^{5.56}$ CFU/mL) and LM-Δ*Rli82*/*Rli82* (105.95 CFU/mL) (Appendix 4), suggesting that the virulence of LM EGD-e was significantly decreased when the *Rli82* gene was deleted. Moreover, bacterial loads in the liver and spleen of mice infected with LM-Δ*Rli82* were significantly lower than those of animals infected with LM EGD-e and LM-Δ*Rli82*/*Rli82* (Figures 3A and 4B). Histopathological changes in LM-infected mice revealed distinct necrotic foci, central venous congestion, and infiltration of inflammatory cells in the surrounding obstructive tissue in the liver. Moreover, spleeny nodules in the spleen were enlarged, and tissue congestion was evident. However, these pathological changes were significantly ameliorated in LM-Δ*Rli82* infected mice as compared to LM EGD-e and LM-Δ*Rli82*/*Rli82* infected animals (Figure 4A). LM-Δ*Rli82*-infected mice survived significantly longer and had a significantly higher survival rate compared to those infected with LM EGD-e and LM-Δ*Rli82*/*Rli82* (Figure 4B), suggesting that sRNA *Rli82* deficiency hampered the pathogenicity of LM.

4.4. Potential Target mRNAs Modulated by Rli82

Bioinformatic analyses revealed that *Rli82* was located at positions 910875 - 910944 on the genome of LM EGD-e (accession number: AL591824) (Appendix 5A). Regarding its secondary structure, *Rli82* depicted a linear-shaped neck-loop structure with five loops and four complementary double strands (Appendix 5B). Online software, TargetRNA2, suggested *flaA* mRNA as a potential target for *Rli82* based on a base-complementary segment (-22 $\sim$ - 8 bases) in the 5'-UTR of the mRNA that paired with *Rli82* (15$\sim$1 bases), implying that *flaA* mRNA could be potentially modulated by *Rli82* at the post-transcriptional level (Figure 5A).

4.5. Verification of Interaction Between Rli82 and the Target mRNA

The two-plasmid reporter system based on *E. coli* showed that the bacterial strain co-transformed by pUT18C-*Rli82* and pMR-LacZ-*flaA* formed deeper dark green colonies compared to the strain co-transformed by pUT18C and pMR-LacZ-*flaA*, accompanied by a significant 2-fold increase in the OD450nm of lawn's flushing fluid. Meanwhile, there was a significant difference between the strain co-transformed by pUT18C-*Rli82* and pMR-LacZ-*flaA* and the strain co-transformed by pUT18C-Δ*Rli82* and pMR-LacZ-*flaA* (Figure 5B and C). The results suggested that there was a substantial interaction between *Rli82* and *flaA* mRNA.

4.6. FlaA Gene Expression Analysis

Compared with LM EGD-e and LM-Δ*Rli82*/*Rli82* strains, the mRNA level of the *flaA* gene was not significantly elevated in the LM-Δ*Rli82* strain (P $>$ 0.05). Meanwhile, there was no significant difference in the mRNA level of *Rli82* between LM EGD-e and LM-Δ*Rli82*/*Rli82* (Figure 6A). However, it was revealed that the expression level of *flaA* protein was significantly higher in LM-Δ*Rli82* strain compared to LM EGD-e and LM-Δ*Rli82*/*Rli82* strains (Figure 6B and C), implying that *Rli82* could negatively regulate the gene expression of *flaA*.

5. Discussion

So far, many studies have shown that sRNAs can act on target mRNAs to affect their transcriptional and translational levels (14, 15), whereby sRNAs can be involved in the regulation of the metabolism and virulence of bacteria (7, 9, 16). It is generally accepted that sRNAs regulate target genes' mRNAs in a variety of ways. First, sRNA pairing at the Shine-Dalgarno (SD) region will suppress the binding of ribosomes with the mRNA,

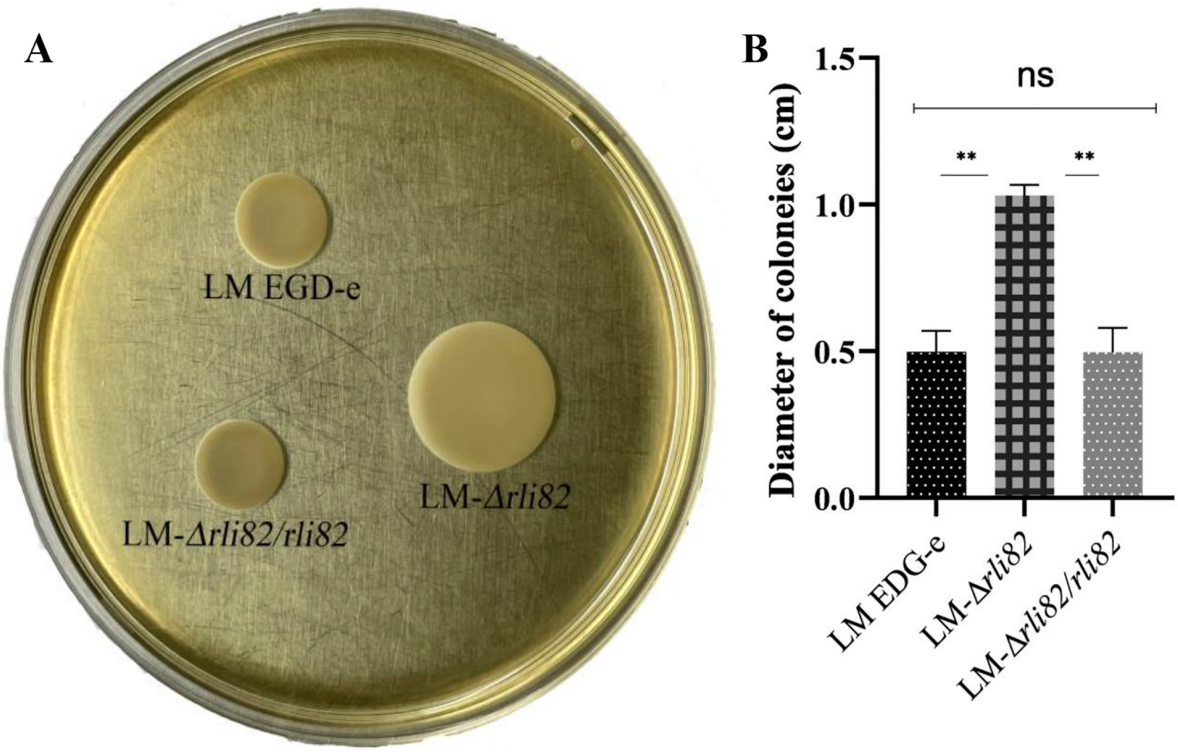

Figure 1. Motility of LM EGD-e, LM-Δ*Rli82*, and LM-Δ*Rli82*/*Rli82*. Strains. (A) Colonies in BHI solid medium; (B) The diameter of bacterial colonies. ns; not significant, *; $P < 0.05$, **; $P < 0.01$

inhibiting the initiation of translation (17). In LM, sRNA LhrA inhibits the translation of lmo0850 by binding to its SD region (18). Second, sRNA can release the SD region, which, under normal conditions, is sequestered in a secondary structure, activating the translation of the target mRNA by ribosomes. In *E. coli*, sRNA Mcas can unlock the secondary structure of flhDC mRNA, thereby releasing the SD sequence and facilitating the translation process (19).

Alternatively, sRNAs may act on the far upstream of the ribosome binding site (RBS) of the target mRNA at its 5'-UTR, which protects the target mRNA from degradation by concealing its RNase E cleavage site. In this case, sRNA may promote the stability of the target mRNA and thus facilitate its translation (20). In *Streptococcus*, sRNA *FasX* in streptococci can bind to ska mRNA and prevent its degradation by RNase E, thereby stabilizing this mRNA and maintaining the translation of ska protein (21). Moreover, sRNA can also bind to a sequence near the ribosome binding site of the target mRNA, competing with 30S ribosomes for this binding site, resulting in the suppression of translation (22, 23). In *Salmonella typhimurium*, sRNA *RyhB* binds to fhlA mRNA and interferes with translation initiation (24). Herein, our experiments, combined with bioinformatics analyses, demonstrated that sRNA *Rli82* might bind to the SD region of *flaA* mRNA, inhibiting the translation of *FlaA* protein, thus playing a vital role in the control of motility and pathogenicity of LM.

Previous studies have proven that the flagellum is closely related to physiological processes such as environmental stress tolerance, motility, and pathogenicity in bacteria (13, 25, 26). Existing studies have shown that the flagellum is composed of three parts, namely the flagellar filament, consisting of the flagellar subunit, hook, and basal body (27). Among these, the flagellar subunit is composed of *flaA* and other proteins (28). It has been shown that the deletion of the *flaA* gene can impair flagellar formation and interfere with the motility of LM (29). Here, target prediction analyses revealed that *Rli82* was capable of complementary pairing with bases at the positions -22 to - 8 in the 5'-UTR of *flaA* mRNA, a site possibly representing the ribosomal binding site (RBS). Furthermore, the motility of the LM-Δ*Rli82* strain was significantly enhanced, which was in agreement with the observation of more flagella in

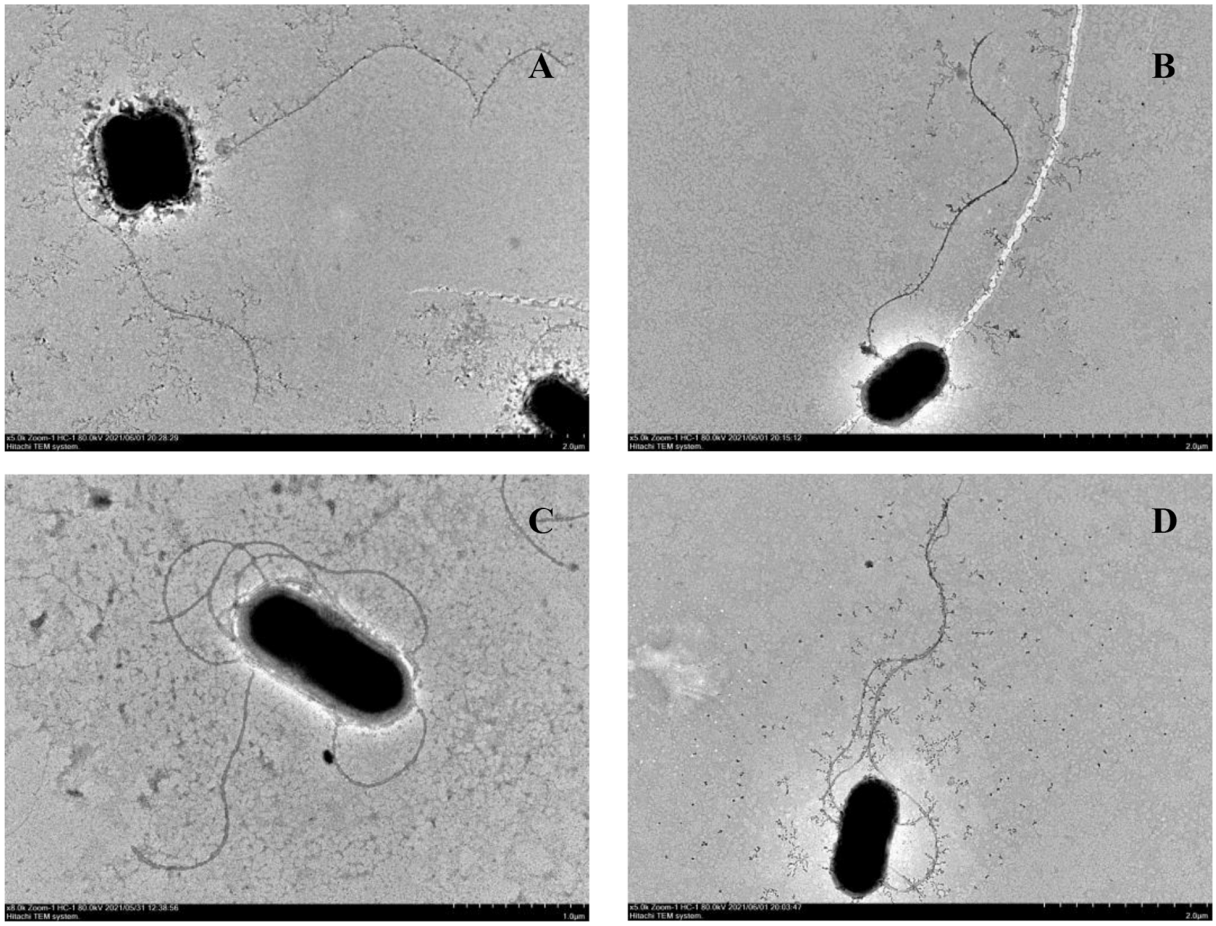

Figure 2. The morphological characteristics of LM EGD-e and LM-Δ*Rli82* strains using transmission electron microscopy (Original magnification, × 5000). A and B: LM EGD-e strain; C and D: LM-Δ*Rli82* strain

LM-Δ*Rli82* than in LM EGD-e. Collectively, combined with the results of bioinformatic analyses, it can be noted that *Rli82* may negatively modulate the expression of *flaA* mRNA by occupying its ribosomal binding site.

It has been proven that the functioning of LM flagella is restricted to temperatures below 37°C due to the opposing activities of the MogR transcriptional repressor and the GmaR anti-repressor (13, 30-32). Once LM enters the host, however, the biosynthesis of flagella is suppressed to help the bacterium evade the host's immune system, thereby facilitating its survival and proliferation *in vivo* (31). Here, our results revealed that LM-Δ*Rli82* could produce more flagella than LM EGD-e and LM-Δ*Rli82*/*Rli82* at 25°C, suggesting a role for sRNA *Rli82* in flagellar formation. Generally, LM maintains strong motility in the extracellular environment at temperatures below 37°C by enhancing the production of flagella, thereby expediting its chemotaxis, biofilm formation ability, and infectivity. However, the underlying mechanisms through which sRNA *Rli82* can modulate flagella formation in LM need to be further elucidated by transcriptomic analyses.

5.1. Conclusions

Taken together, this study demonstrated that sRNA *Rli82* was involved in the motility and pathogenicity of LM via modulating *flaA* mRNA at the post-transcriptional level. This observation provides new insights into sRNA-based modulation of the expression of flagella-related genes in LM.

Supplementary Material

Supplementary material(s) is available here [To read supplementary materials, please refer to the journal website and open PDF/HTML].

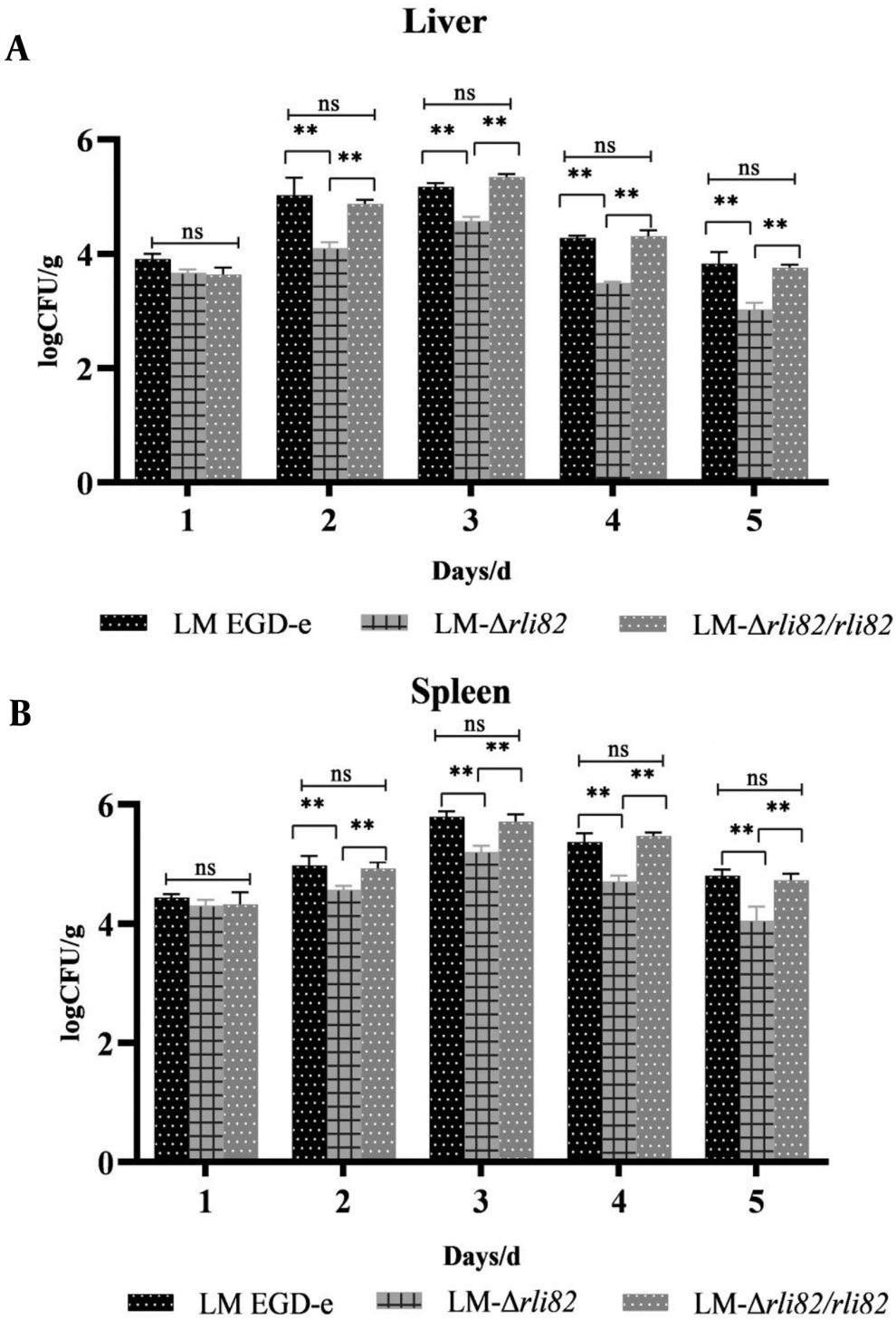

Figure 3. Bacterial hepatic and splenic loads in mice infected by LM. (A) Bacterial load in the liver in LM-infected mice; (B) Bacterial load in the spleen in LM-infected mice. ns; not significant, *; $P < 0.05$, **; $P < 0.01$

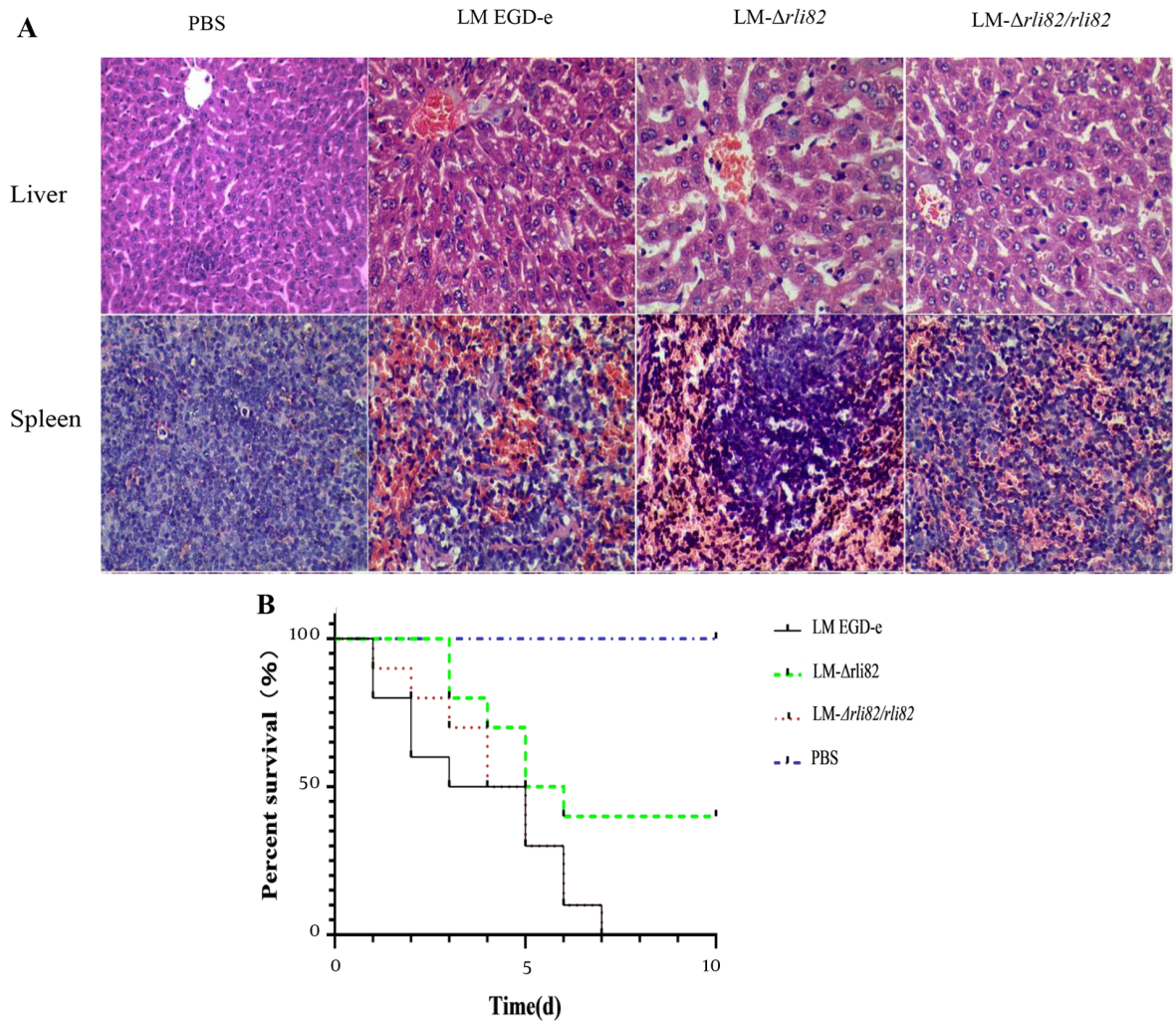

Figure 4. Histopathological changes and survival curves in LM-infected mice. (A) Histopathological changes in the liver and spleen from LM-infected mice; (B) The survival curve of LM-infected mice.

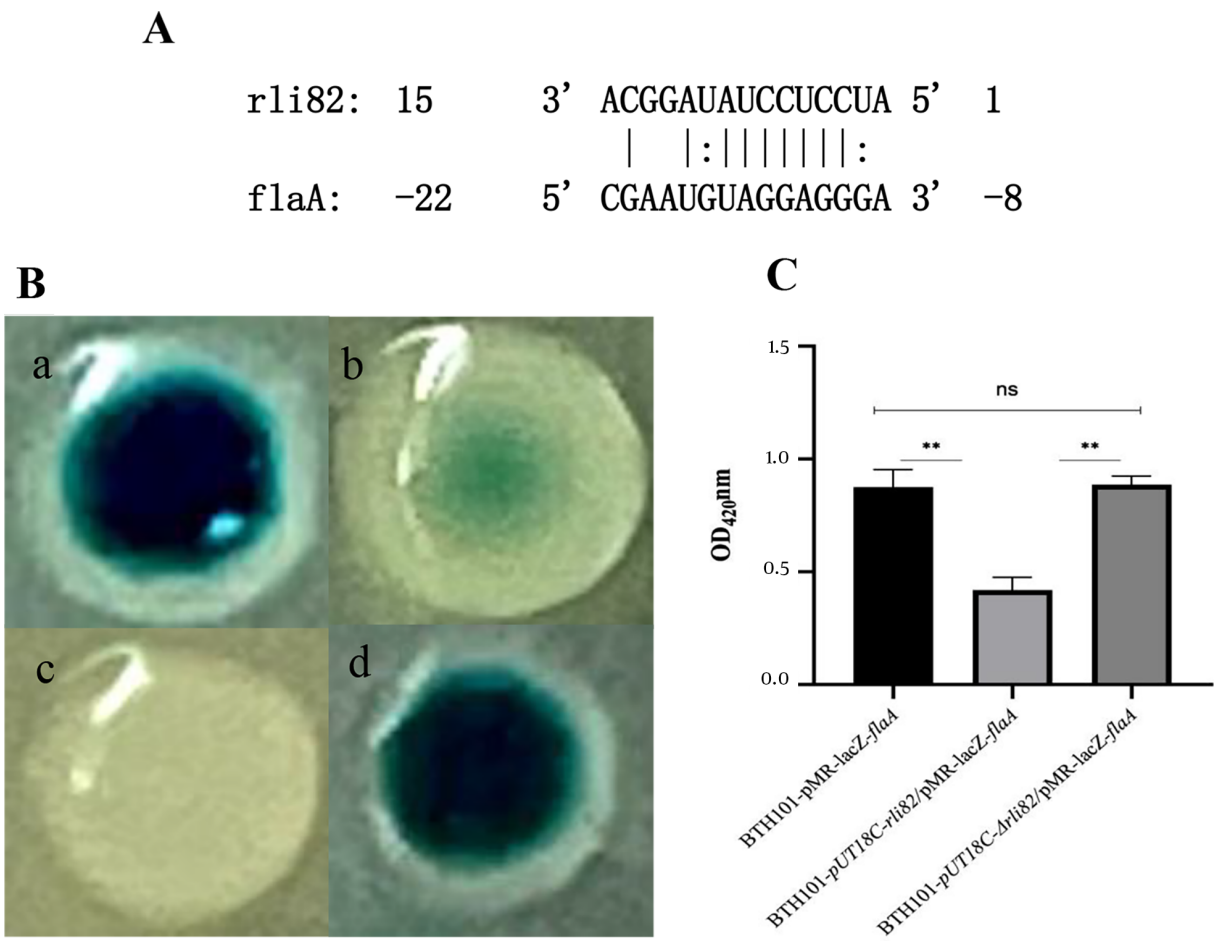

Figure 5. Prediction and verification of the target mRNA of *Rli82*. (A) The predicted target gene of *Rli82*; (B) Colonies of BTH101 (a): BTH101-pUT18C-*Rli82*/pMR-lacZ-*flaA*; (b): BTH101-pUT18C/pMR-lacZ-*flaA*; (c): BTH101-pUT18C/pMR-lacZ; (d): BTH101-pUT18C-Δ*Rli82*/pMR-lacZ-*flaA*); (C): Comparison of OD at 450 nm of the flushing fluid between BTH101-pUT18C-*Rli82*/pMR-lacZ-*flaA*, BTH101-pUT18C/pMR-lacZ-*flaA*, and BTH101-pUT18C-Δ*Rli82*/pMR-lacZ-*flaA* colonies

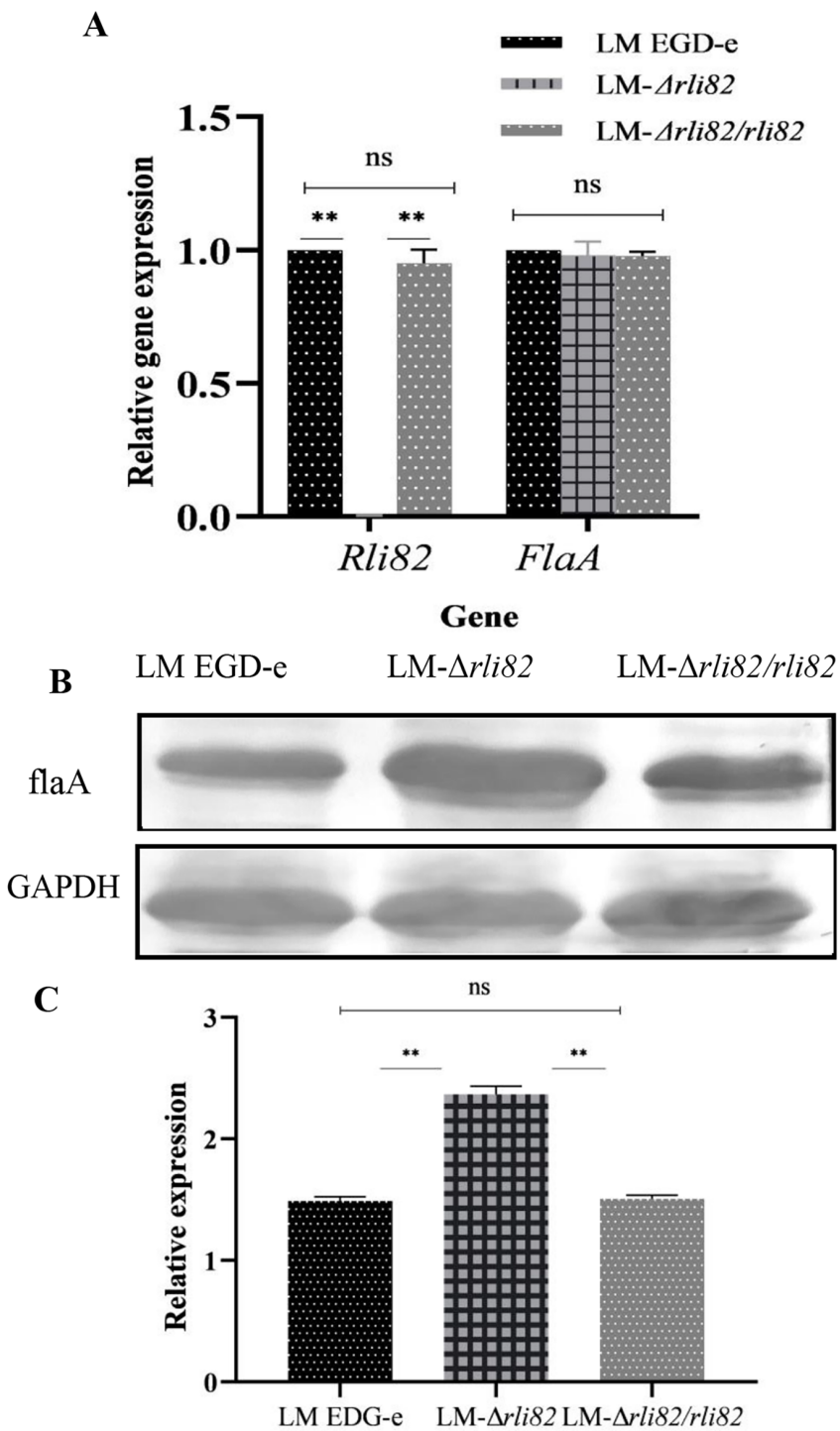

Figure 6. Determination of mRNA and protein level of the target gene regulated by *Rli82*. (A) Relative mRNA level of the *flaA* and *Rli82* genes; (B) Protein expression levels of *flaA* and GAPDH; (C) Quantitative analysis of *flaA* protein by ImageJ software. ns: not significant, *: $P < 0.05$, **: $P < 0.01$

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Footnotes

Authors' Contribution: Study concept and design: Qingling Meng and Jun Qiao; analysis and interpretation of data and drafting of the manuscript: Chunhui Ji, Nengxiu Li, Jian Jiao; critical revision of the manuscript for important intellectual content: Yaoqiang Sun, Xin Huang, Zhiyuan Li, Qingwen Leng, and Xuepeng Cai; statistical analysis: Yufei Zuo, Xiaoxing Huang, and Yaling Li.

Conflict of Interests: The authors have no conflict of interest to declare.

Ethical Approval: Ethical approval for this study was given by the Research and Ethical Committee of Shihezi University (No. A2019186).

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