Kafkas Univ Vet Fak Derg
26 (2): 269-277, 2020
DOI: 10.9775/kvfd.2019.22932

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Research Article

Effects of RNase III *rncS* Gene Deletion on Stress Response, Biofilm Formation and Virulence of *Listeria monocytogenes*

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Article ID: KVFD-2019-22932 Received: 01.07.2019 Accepted: 15.12.2019 Published Online: 15.12.2019

How to Cite This Article

Wang L, Qiao M, Meng Q, Qiao J, Wu Y, Guo J, Wang X, Li J, Zhang X, Cai X: Effects of RNase III *rncS* gene deletion on stress response, biofilm formation and virulence of *Listeria monocytogenes. Kafkas Univ Vet Fak Derg,* 26 (2): 269-277, 2020. DOI: 10.9775/kvfd.2019.22932

Abstract

The ribonuclease III (RNase III) is an important enzyme system that regulates non-coding RNA (ncRNA) levels. In this study, LM-*ΔrncS* gene deletion strain was investigated by gene overlap extension PCR (SOE-PCR) and homologous recombination techniques. The environmental stress response, biofilm formation and virulence were determined and compared between the deletion strain LM-*ΔrncS* and the parental strain LM EGD-e. When compared with LM EGD-e, the adaptability of LM-*ΔrncS* was significantly reduced (P<0.05) under the stress of 30°C/42°C, pH 9, 5% NaCl, 3.8% ethanol and 0.1% H_2O_2. Biofilm formation ability of LM-*ΔrncS* was significantly lower (P<0.05) than that of LM EGD-e. In LM-*ΔrncS*, the transcription levels of ncRNA *SreA* and *SbrA* genes were significantly decreased (P<0.05). The adhesion rate and invasion rate of LM-*ΔrncS* in RAW264.7 cells were significantly lower (P<0.01) than those of LM EGD-e, and the survival and proliferation of LM-*ΔrncS* in RAW264.7 cells were also significantly decreased (P<0.05). Moreover, the transcription levels of *InlA, hly, prfA* and *SigmaB* gene were significantly lower (P<0.05) than those of LM EGD-e. LD_{50} of LD-*ΔrncS* in BALB/c mice was increased by 1.49 logarithmic orders, and the survival time of the mice was significantly prolonged when compared with LM EGD-e. In addition, the bacterial load in the liver and spleen was markedly decreased, and its pathological damage was also reduced. This study confirmed that RNase III RncS is involved in the regulation of environmental stress response, biofilm formation and virulence in LMd.

Keywords: *Listeria monocytogenes, RNase III rncS, Environmental stress response, Biofilm, Virulence*

Listeria monocytogenes'in RNase III rncS Geninin Silinmesinin Stres Tepkisi, Biyofilm Oluşumu ve Virülansı Üzerine Etkileri

Öz

Ribonükleaz III (RNase III), kodlama yapmayan RNA (ncRNA) seviyelerini düzenleyen önemli bir enzim sistemidir. Bu çalışmada, LM-*ΔrncS* gen silme suşu, gen örtüşme uzatma PCR (SOE-PCR) ve homolog rekombinasyon teknikleri ile incelendi. Çevresel stres tepkisi, biyofilm oluşumu ve virülans düzeyi belirlenerek silme suşu LM-*ΔrncS* ile ana suş LM EGD-e karşılaştırıldı. LM EGD-e ile karşılaştırıldığında, LM-*ΔrncS*'nin adapte olabilirliği 30°C/42°C, pH 9, %5 NaCl, %3.8 etanol ve %0.1 H_20_2 stresi altında önemli ölçüde azaldı (P<0.05). LM-*ΔrncS*'nin biyofilm oluşturma kabiliyeti, LM EGD-e'ninkinden anlamlı olarak daha düşüktü (P<0.05). LM-*ΔrncS*'de, ncRNA *SreA* ve *SbrA* genlerinin transkripsiyon seviyeleri anlamlı derecede azaldı (P<0.05). RAW264.7 hücrelerinde LM-*ΔrncS*'nin adezyon ve invazyon oranı LM EGD-e'ninkinden anlamlı olarak daha düşüktü (P<0.01) ve RAW264.7 hücrelerinde LM-*ΔrncS*'nin hayatta kalması ve çoğalması da önemli ölçüde azaldı (P<0.05). Bunun yanısıra, *InlA, hly, prfA* ve *SigmaB* genlerinin transkripsiyon seviyeleri LM EGD-e'ninkinden anlamlı derecede düşüktü (P<0.05). BALB/c farelerinde LD-*ΔrncS*'nin LD_{50}'si 1.49 logaritmik düzende arttırıldı ve farelerin hayatta kalma süresi LM EGD-e ile karşılaştırıldığında önemli ölçüde uzadı. Ek olarak, karaciğer ve dalaktaki bakteri yükü ve patolojik hasar da belirgin şekilde azaldı. Bu çalışma, RNase III RncS'nin LM'deki çevresel stres tepkisi, biyofilm oluşumu ve virülansın düzenlenmesinde rol oynadığını doğruladı.

Anahtar sözcükler: *Listeria monocytogenes, RNase III rncS, Çevresel stres yanıtı, Biyofilm, Virulans*

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INTRODUCTION

Listeria monocytogenes (LM) is a Gram-positive facultative anaerobic intracellular parasite that infects animals and humans, causing gastroenteritis, meningoencephalitis, abortion, sepsis and other symptoms in animals and immunocompromised populations such as elderly, newborn, and pregnant women, leading to high mortality and serious harm to animal husbandry production and food hygiene and safety [1,2]. As an important food-borne zoonotic pathogen, LM is widely found in nature and can survive in high-salt, hypertonic, low temperature, acidic and oxidative stress environments. It can form biofilms that are resistant to the external environment in animal foods and on the surface of processing containers [3]. Many studies have found that the strong viability and pathogenicity of LM are closely associated to its environmental stress factors and virulence factors [4,5]. To survive in stress conditions, LM has to modulate its transcriptions of related genes constantly to adapt to the different environments. Despite LM possesses many regulatory proteins to modulate gene expression, environmental stress factor Sigma B [4,5], positive regulatory factor PrfA and response regulator VirR [6,7] appear to play predominant roles in LM survival and infection. In recent years, studies have found that non-coding RNAs (ncRNAs) in LM can form complex regulatory networks with regulatory molecules such as PrfA, Sigma B and VirR, which precisely regulate the virulence and stress response-related genes of LM at transcription, post-transcription, and translational levels [6,8].

Ribonuclease III (RNase III) is a conserved RNase that cleaves a double-stranded RNA [9,10], which has been proved to be involved in the regulation of ncRNAs in bacteria [11,12]. However, the role and mechanism through which RNase III regulates the response of LM to environmental stress, biofilm formation and virulence is still unclear. In the present study, the main purpose was to explore the regulatory role of RNase III *rncS* in environmental stress response, biofilm formation and virulence of LM by constructing a RNase III *ΔrncS* gene deletion mutant of LM, which will provide an insight into the regulatory role of RNase III in environmental stress response and the pathogenesis of LM.

MATERIAL and METHODS

Primers

According to the genomic sequence of LM EGD-e registered in GenBank (accession number: AL591824), the specific primers of the *ΔrncS* deletion strain (R1-R6) and the qRT-PCR primers for the detection of virulence and biofilm formation-related genes were designed by Primer 5.0 software (Premier Inc, Canada). The cleavage sites of *Kpn* I and *Pst* I, and protective bases were added to the 5′ ends of the R1 and R4 primers, respectively. The information of these primers is shown in *Table 1*.

Table 1. *Primers used in the study*

Primer Names	Primer Sequences (5′→3′)
R1	GGGGTACCATGAATCAATGGGAAGAAT
R2	GTCCCGTTGAACAATTTCATATTTGTTAAATAGATA
R3	TATCTATTTAACAAATATGAAATTGTTCAACGGGAC
R4	TGCACTGCAGTTATCTGTGTATTAGTTTGTTTAT
R5	ACCACGGAGCGATTTAGC
R6	TTACGAGATTTGTTGGTTCA
SbrA F	CATCTAGATCCATACCCCTAAACTCCCT
SbrA R	AAAAAAAGAGCAGCACCCGAGAGTAC
SreA F	CAAGAAACGTCACTATAGTCACAAAT
SreA R	AAAAAAAGCCTTTCTGCTGATGAG
16s rRNA F	GAGCTAATCCCATAAAACTATTCTCA
16s rRNA R	ACCTTGTTACGACTTCACCCC
SigmaB F	CGCCGAATCAAAGAGTTAGG
SigmaB R	CTTTTTCCCATTTCCATTGCTTC
PrfA F	ACGGGAAGCTTGGCTCTATT
PrfA R	TGCGATGCCACTTGAATATC
hly F	TGCAAGTCCTAAGACGCCA
hly R	CACTGCATCTCCGTGGTATACTAA
inlA F	TGTGACTGGCGCTTTAATTG
inlA R	TCCAATAGTGACAGGTTGGCTA

Generation and Identification of LM-ΔrncS Deletion Strain

The LM EGD-e strain (a gift from W. Goebel of the University of Woodsburg, Germany) was inoculated in brain heart infusion (BHI) broth (Difco, USA) and cultured for 16 h at 37°C. The genomic DNA of the bacteria was extracted according to the instruction of the bacterial genomic DNA extraction kit (Omega, USA). Briefly, using the primer pairs R1-R2 and R3-R4, the upstream and downstream homologous arms were amplified using LM genomic DNA as the template, respectively. The fusion fragment of *rncS* deletion mutation was obtained by SOE-PCR and cloned into pMD19-T simple vector (TaKaRa, Japan) to generate the recombinant plasmid pMD19-T-*ΔrncS*. The pMD19-T-*ΔrncS* and pKSV7 plasmids were digested with *Kpn* I and *Pst* I (TaKaRa, Japan), respectively, and the digested target fragment was ligated to the pKSV7 vector by T4 DNA ligase (TaKaRa, Japan) to generate a recombinant shuttle plasmid pKSV7-*ΔrncS*. Then, pKSV7-*ΔrncS* was electroporated (2.5 kv, 5.0 ms) into LM EGD-e competent cells, and the cells were inoculated on BHI plates containing chloramphenicol (15 μg/mL, Amresco, USA) and cultured. Positive clones were screened by PCR using primers P5-P6. Homologous recombination was performed using the positive clones at 42°C and in the presence of chloramphenicol (15 μg/mL, Amresco, USA). The recombinant strain was screened by PCR and sequencing, and was passed continuously for 20 generations to obtain LM-*ΔrncS* deletion strain.

Effect of rncS Gene Deletion on the Environmental Stress Response of LM

In brief, LM EGD-e and LM-*ΔrncS* were inoculated separately in 1.0 mL BHI liquid medium, cultured at 37°C, transferred to BHI medium (Difco, USA) at a ratio of 1:100 and cultured at 30°C, 37°C, and 42°C with shaking (200 r/min), respectively. Then $OD_{600\ nm}$ value was measured every 1.5 h and the bacterial growth curve was plotted. At the same time, the bacterial solution was inoculated in BHI medium containing 3.8% alcohol, 5% NaCl, 0.1% H_2O_2 at pH 4, 7, or 9. The $OD_{600\ nm}$ value was measured at different times and the growth curve was prepared. The experiment was repeated 3 times.

Effect of rncS Gene Deletion on the Biofilm Formation Ability of LM

Briefly, 200 μL of LM EGD-e and LM-*ΔrncS* bacterial culture were added to a 96-well microplate, and the biofilm was prepared using the method of crystal violet staining as described by Peng [13]. Each sample was divided into 3 groups, with 8 replicates in each group, and the $OD_{570\ nm}$ value was determined by enzyme-linked detector (BIOTEK, USA). Statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad Software, Inc, La Jolla, CA, USA). The morphological structure of the biofilm was observed and photographed under an inverted microscope (LEICA, Germany).

Determination of Cell Adhesion, Invasion and Intracellular Survival and Proliferation

Mouse macrophage RAW264.7 was cultured in Dulbecco's modified Eagle medium (DMEM) (Gibco, Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (Invitrogen, USA) in 6-well plates. The adhesion rate, invasive rate and intracellular bacteria number were determined by the method described by Peng [14]. The experiment was repeated three times.

Detection of Relative Transcription Levels of Biofilm Formation and Virulence-related Genes by qRT-PCR

The transcription levels of biofilm formation genes *(SreA* and *SbrA)* and virulence-related genes *(InlA, hly, prfA* and *SigmaB)* were determined using qRT-PCR according to the method described by Kun [15], respectively. Briefly, total RNA of LM EGD-e and LM-*ΔrncS* strains were extracted by Trizol (Invitrogen, USA) and reverse transcribed into cDNA using AMV reverse transcription kit (TaKaRa, Japan). The biofilm formation genes *(SreA* and *SbrA)* and virulence-related genes *(InlA, hly, prfA* and *SigmaB)* was quantified by qRT-PCR using a LightCycler 480 instrument (Roche, Switzerland). Each sample had 3 replicates, and each experiment was repeated 3 times. The relative transcript levels of biofilm formation genes *(SreA* and *SbrA)* and virulence-related genes *(InlA, hly, prfA* and *SigmaB)* genes were calculated according to the $2^{-\Delta\Delta CT}$ method using the *16s rRNA* as an internal control. The results were statistically analyzed using GraphPad Prism 5.0 software (GraphPad Software, Inc, La Jolla, CA, USA).

Determination of LD_{50} of LM in Mice

The LD_0 and LD_{100} of LM EGD-e and LM-*ΔrncS* in mice were respectively determined by the method described by Peng et al.[14]. Based on the above results, 100 8-week-old BALB/c mice were randomly divided into LM EGD-e group and LM-*ΔrncS* group. Each group consisted of 5 subgroups, with 10 mice in each subgroup. Each mouse received 0.5 mL bacteria culture at different dilutions by intraperitoneal injection. The mice were observed for 7 days. The mortality of the mice was calculated and the LD_{50} of LM EGD-e and LM-*ΔrncS* was determined by Karber method, respectively.

Determination of Mouse Survival Rate

Briefly, thirty 8-week-old BALB/c mice were randomly divided into 3 groups, with 10 mice in each group. Two groups were intraperitoneally injected with 0.5 mL LM EGD-e or LM-*ΔrncS* bacterial solution, and the third group was intraperitoneally injected with 0.5 mL phosphate buffered saline (PBS). The mental state changes of the mice were observed every day for 7 days. The mental state and death in each group were recorded, and the Kaplan-Meier survival curve was prepared.

Bacterial Load in Mouse Organs and Histopathological Analysis

Eight-week-old BALB/c mice were randomly divided into 3 groups, with 7 mice in each group. The three groups of mice were intraperitoneally injected with 0.5 mL of sub-lethal dose of LM EGD-e, LM-*ΔrncS* bacterial solution or PBS buffer. After injection, the liver, spleen and kidney of a mouse in each group were extracted and cultured in BHI medium for bacteria counting. The procedure was repeated 3 times. On the 5th day, the liver, spleen and kidney of a mouse in each group were removed and fixed with 4% formaldehyde solution. After the tissue sections were prepared, histopathological changes of mice tissues were analyzed after hematoxylin and eosin (HE) staining.

Statistical Analysis

All data were statistically analyzed using GraphPad Prism 5.0 software (GraphPad Software, Inc, La Jolla, CA, USA). Continuous variables were compared using *t* test, and categorical variables were analyzed using chi-square test. Data were expressed as mean ± standard deviation (SD). Statistically, $P<0.05$ was considered significantly different, $P<0.01$ was considered extremely significant difference.

RESULTS

The PCR amplification and sequence analysis confirmed that the LM-*ΔrncS* deletion strain was successfully constructed and identified.

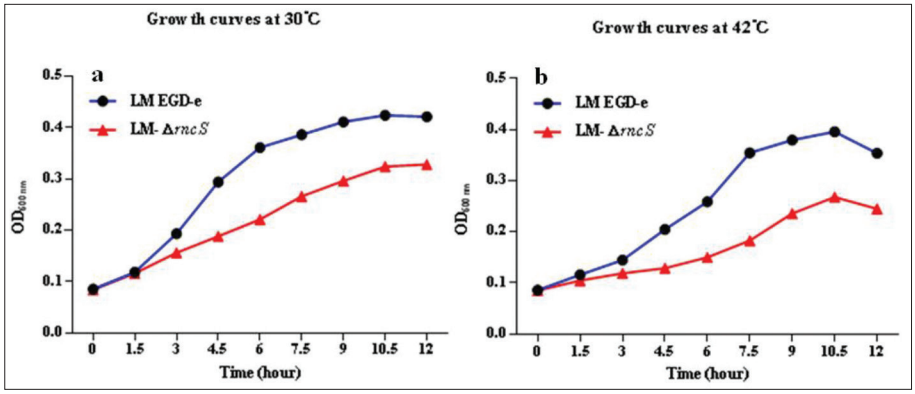


Fig 1. Growth curves of LM EGD-e and LM-△*rncS* at different temperatures. **a:** Growth curves of LM EGD-e and LM-△*rncS* at 30°C; **b:** Growth curves of LM EGD-e and LM-△*rncS* at 42°C

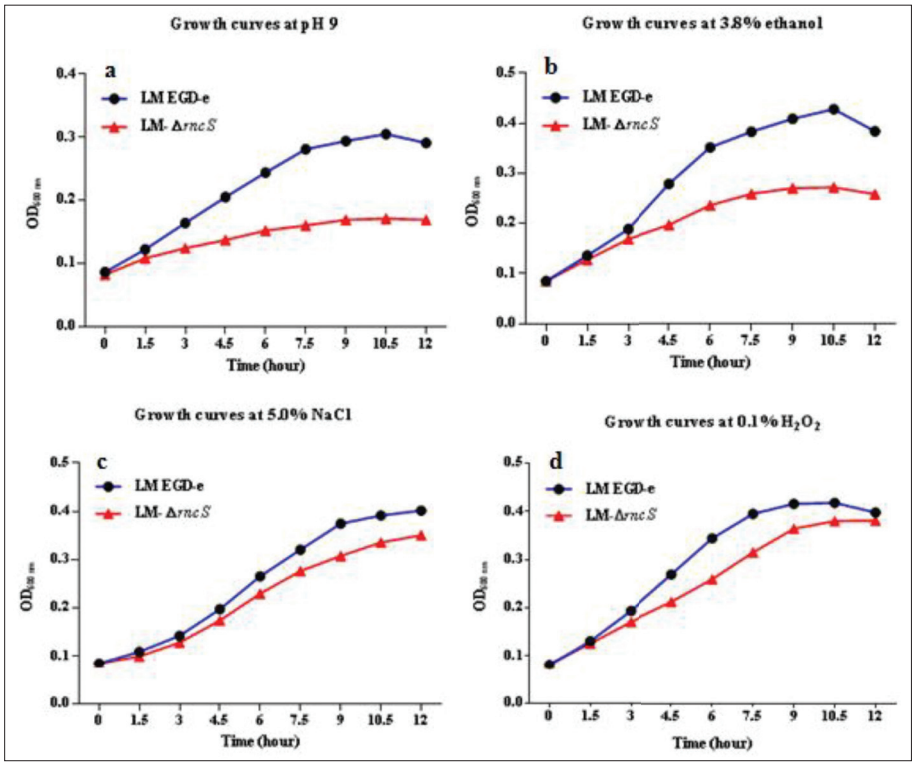


Fig 2. Growth curves of LM EGD-e and LM-△*rncS* at different stress conditions. **a:** Growth curves of LM EGD-e and LM-△*rncS* at pH 9; **b:** Growth curves of LM EGD-e and LM-△*rncS* at 3.8% ethanol; **c:** Growth curves of LM EGD-e and LM-△*rncS* at 5% NaCl; **d:** Growth curves of LM EGD-e and LM-△*rncS* at 0.1% H_2O_2

The growth curve showed that the growth of LM EGD-e and LM-*ΔrncS* was not significantly different at 37°C (P>0.05). However, at 30°C and 42°C, the growth difference between the two was significantly reduced after 4.5 h (P<0.01), indicating that the resistance of LM-*ΔrncS* to low temperature and high temperature was markedly decreased *(Fig. 1a,b)*.

At pH 4, both LM EGD-e and LM-*ΔrncS* were in a growth arrest state; at pH 7, the growth difference between the two bacterial strains was not significant (P>0.05); at pH 9, the growth of LM-*ΔrncS* was dramatically lower than that of LM EGD-e after being cultured for 3 h (P<0.05) *(Fig. 2a)*. Under the condition of 3.8% alcohol, the growth difference between the parental strain and the mutant strain after 4.5 h culture was extremely significant (P<0.01) *(Fig. 2b)*. Under 5% NaCl, the growth of the two strains was significantly different after 7.5 h culture (P<0.05) *(Fig. 2c)*. In the presence of 0.1% H_2O_2, the growth of LM-*ΔrncS* was significantly slower than LM EGD-e (P<0.01) after being cultured between 4.5 and 9 h *(Fig. 2d)*, revealing that the stress response of LM-*ΔrncS* under the conditions of alkali, high salt, alcohol and H_2O_2 is weaker than that of LM EGD-e.

At 24 h and 48 h, both LM EGD-e and LM-*ΔrncS* produced biofilms *(Fig. 3a,b,c,d)*, but the strength of LM-*ΔrncS* biofilm was significantly lower than that of LM EGD-e (P<0.05) *(Fig. 3e)*. The results of qRT-PCR showed that in the absence of *rncS* gene, the transcription levels of *SreA* and *SbrA* genes were decreased at 24 h and were significantly decreased significantly at 48 h (P<0.01) *(Fig. 4)*.

Cell infection assay showed that the adhesion rate and invasion rate of LM-*ΔrncS* in RAW264.7 cells were significantly lower than those of LM EGD-e (P<0.01) *(Fig. 5A,B)*. After infection, in different time periods, the amount of LM EGD-e and LM-*ΔrncS* was first decreased and then increased with time, and the amount of LM-*ΔrncS* was lower than that of

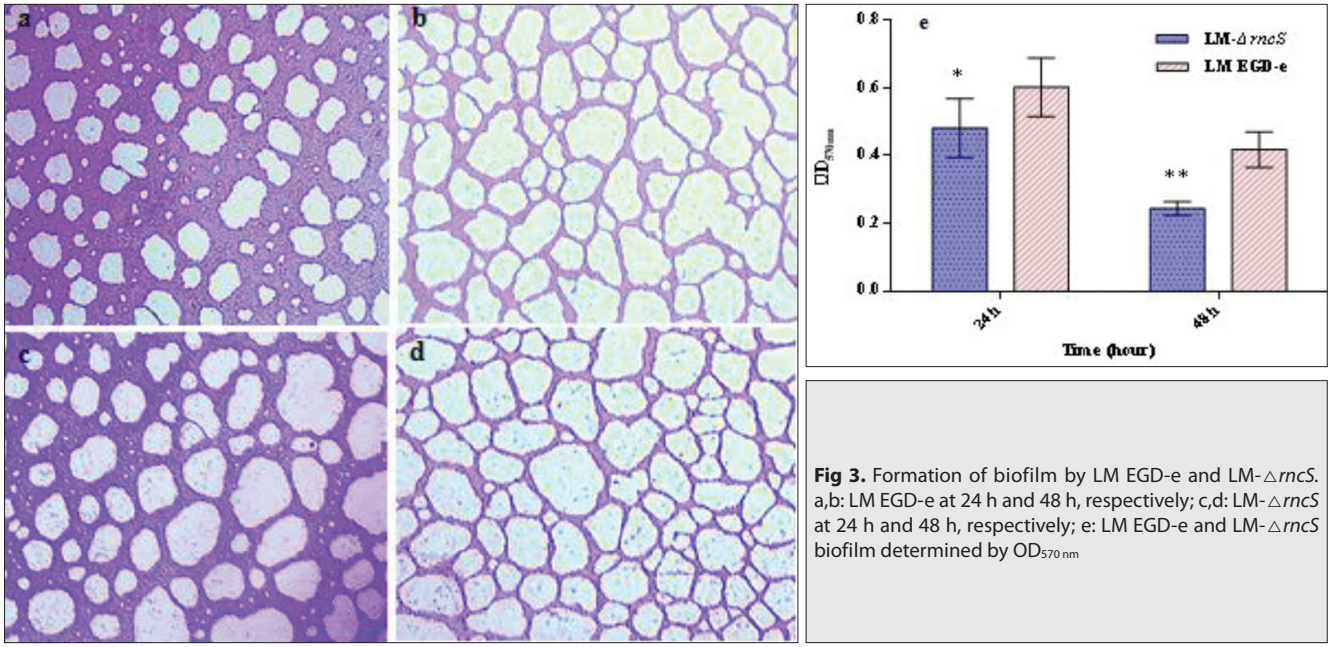


Fig 3. Formation of biofilm by LM EGD-e and LM-△*rncS*. a,b: LM EGD-e at 24 h and 48 h, respectively; c,d: LM-△*rncS* at 24 h and 48 h, respectively; e: LM EGD-e and LM-△*rncS* biofilm determined by $OD_{570\,nm}$

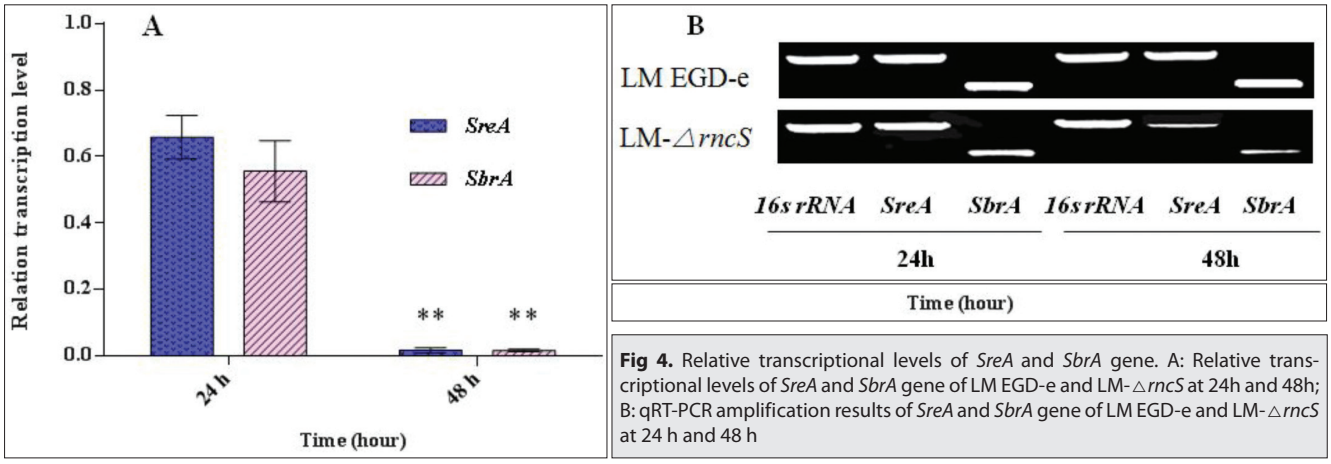


Fig 4. Relative transcriptional levels of *SreA* and *SbrA* gene. A: Relative transcriptional levels of *SreA* and *SbrA* gene of LM EGD-e and LM-△*rncS* at 24h and 48h; B: qRT-PCR amplification results of *SreA* and *SbrA* gene of LM EGD-e and LM-△*rncS* at 24 h and 48 h

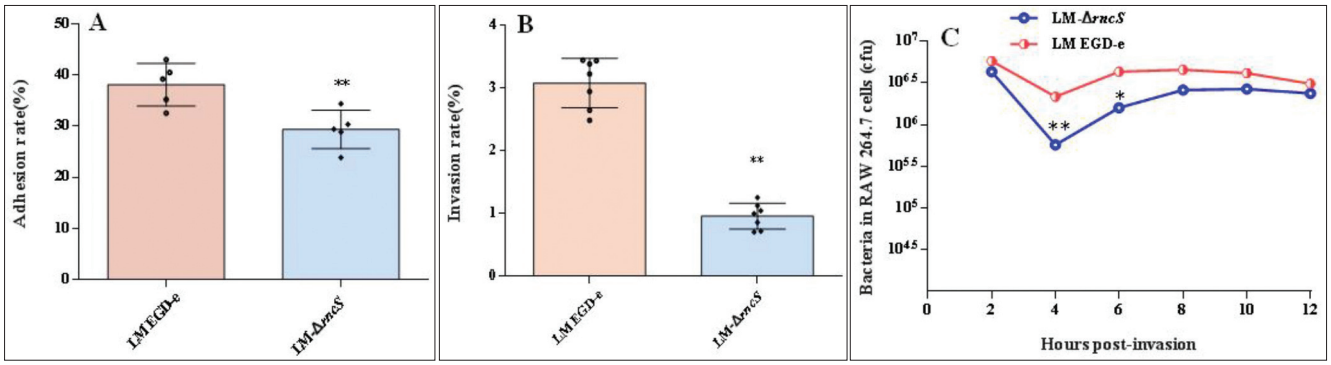


Fig 5. Invasion of LM EGD-e and LM-△*rncS* in RAW264.7 cells. A: LM EGD-e and LM-△*rncS* adhesion rate in RAW264.7 cells; B: LM EGD-e and LM-△*rncS* in RAW264.7 cell invasion rate; C: Bacterial numbers of LM EGD-e and LM-△*rncS* in the RAW264.7 cells after infection at various time

LM EGD-e throughout the whole time course *(Fig. 5C)*. At 2-4 h, the amount of both bacteria strains was decreased rapidly, and the decreasing trend of LM-*ΔrncS* was more obvious (P<0.01). At 4-6 h, both strains increased rapidly, and the difference was significant (P<0.05). Moreover, the transcription levels of *InlA*, *hly*, *prfA* and *SigmaB* gene were significantly lower than those of LM EGD-e (P<0.05) *(Fig. 6)*. These results of cell infection confirmed that *rncS* gene deletion reduced the survival and proliferation of LM EGD-e in RAW264.7 cells.

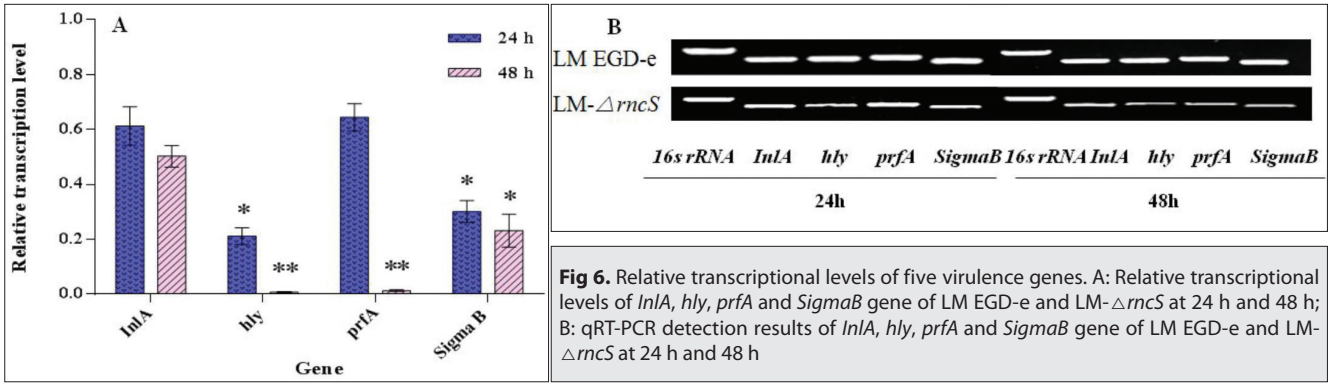
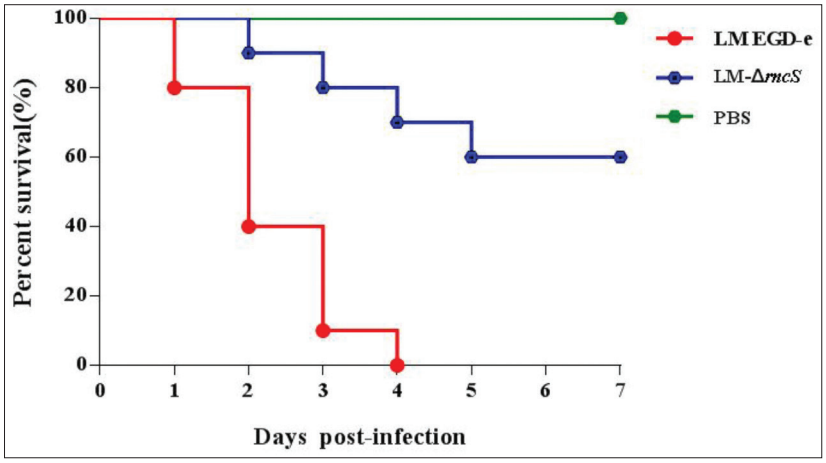


Fig 6. Relative transcriptional levels of five virulence genes. A: Relative transcriptional levels of *InlA*, *hly*, *prfA* and *SigmaB* gene of LM EGD-e and LM-△*rncS* at 24 h and 48 h; B: qRT-PCR detection results of *InlA*, *hly*, *prfA* and *SigmaB* gene of LM EGD-e and LM-△*rncS* at 24 h and 48 h

Fig 7. Survival curves of mice infected by intraperitoneal injection of LM EGD-e and LM-△*rncS*



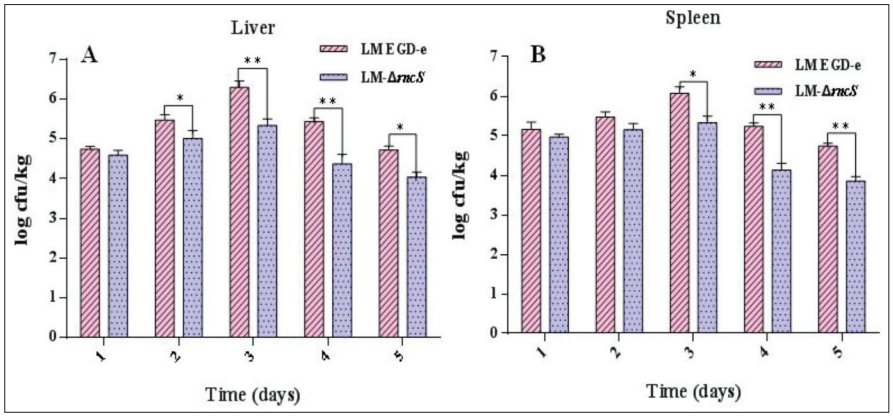


Fig 8. Bacterial load of LM EGD-e and LM-△*rncS* in the livers and spleens of mice infected by intraperitoneal infection. **A:** Bacterial numbers in the livers; **B:** Bacterial numbers in the spleens

Table 2. *LD_{50} measurement results of LM EGD-e and LM-ΔrncS*

Group	LM EGD-e			LM-ΔrncS		
	Dose/cfu	Mortality	LD_{50}	Dose/cfu	Mortality	LD_{50}
1	1.68×10^{6}	(10/10)	$10^{5.56}$	1.12×10^{9}	(10/10)	$10^{7.05}$
2	8.40×10^{5}	(9/10)		1.12×10^{8}	(8/10)	
3	4.20×10^{5}	(6/10)		1.12×10^{7}	(5/10)	
4	2.10×10^{5}	(2/10)		1.12×10^{6}	(2/10)	
5	1.05×10^{5}	(0/10)		1.12×10^{5}	(0/10)	

The LD_{50} of LM EGD-e and LM-*ΔrncS* in BALB/c mice were $10^{5.56}$ cfu and $10^{7.05}$ cfu, respectively *(Table 2)*. Compared with the LD_{50} of LM EGD-e, the LD_{50} of LM-*ΔrncS* increased by 1.49 logarithmic order, which indicated that the virulence of the bacteria was significantly decreased. Compared with LM EGD-e-infected mice, the survival time of LM-*ΔrncS*-infected mice was significantly prolonged *(Fig. 7)*, suggesting that the *rncS* gene has a regulatory effect on the virulence of LM.

After LM EGD-e and LM-*ΔrncS* infection in the liver and spleen, the bacterial load in the liver and spleen of LM-*ΔrncS*-infected mice was lower than that of LM EGD-e-infected mice at different time points of infection.

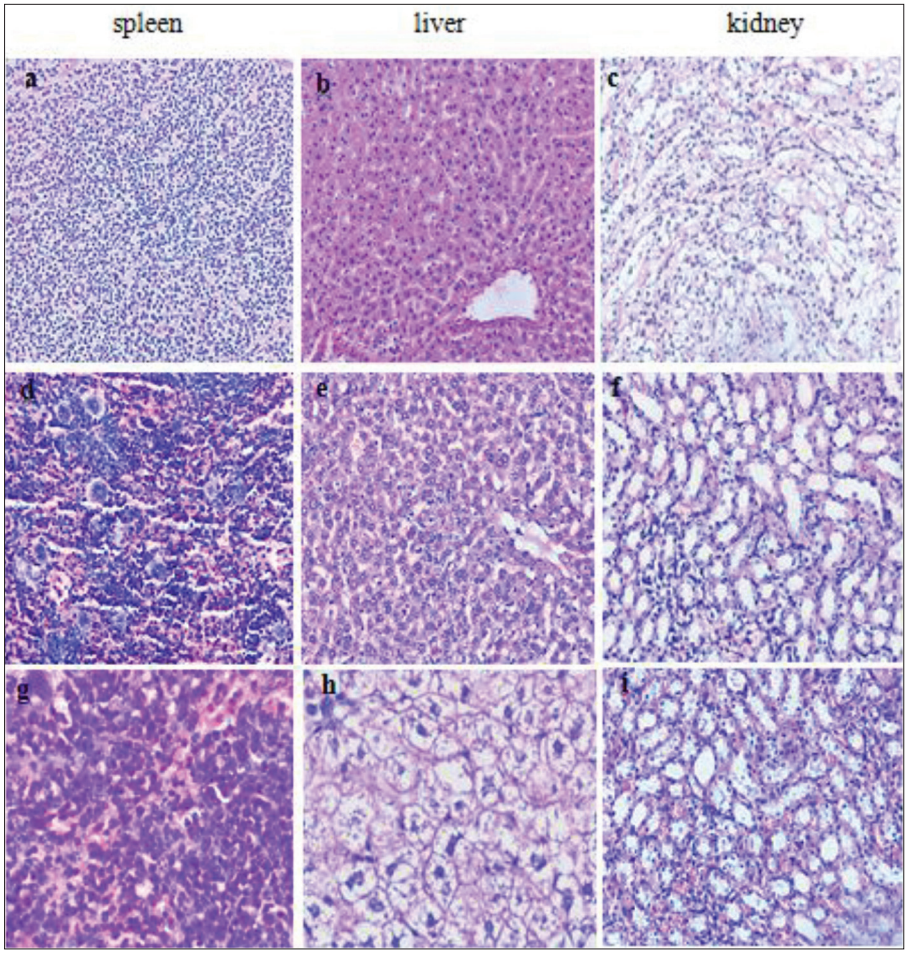


Fig 9. Histopathological examination of mice spleen, liver and kidney (HE×400). **a,b,c:** spleen, liver and kidney of normal mouse; **d,e,f:** spleen, liver and kidney of mice injected with LM-△*rncS*; **g,h,i:** spleen, liver and kidney of mice injected with LM EGD-e

In day 3-5, the bacterial load in the liver and spleen was significantly different between the two groups (P<0.05) *(Fig. 8A,B)*.

Histopathological analysis revealed that compared with the spleen *(Fig. 9a),* liver *(Fig. 9b)* and kidney *(Fig. 9c)* of normal mice, the LM EGD-e-infected mice had partial hepatocyte necrosis, hepatic lobular inflammatory cell infiltration, the tissue structure was unclear and a reticular filamentous structure appeared *(Fig. 9h)*. Transparent degeneration and lymphoid tissue necrosis occurred in spleen reticular fibers, spleen nodules increased in size, intercellular structure was loose, and interstitial volume was widened *(Fig. 9g)*. Renal venular hemorrhage occurred, inflammatory cells appeared, and the interstitial volume was enlarged *(Fig. 9i)*. Compared with the LM EGD-e-infected mice, the pathological changes were obviously alleviated in LM-*ΔrncS*-infected mice *(Fig. 9d,e,f),* indicating that *rncS* gene deletion reduced the virulence of LM.

DISCUSSION

Studies have found that RNaseIII is a highly conserved nuclease that can cleave double-stranded RNA and participates in the regulation of bacterial ncRNA, directly or indirectly affecting the adaptability of bacteria in stressful environments such as weak bases and oxidation [16-18]. Kim et al.[19] found that RNase III is involved in the regulation of low temperature stress response in *E. coli*. Roy and Chanfreau [20] confirmed that degradation of BDF2 mRNA by RNase III under hypertonic conditions resulted in a significant decrease in the growth of the yeast Δbdf1 deletion strain. So far, more than 200 ncRNAs have been found in LM [21]. These ncRNAs may be associated with various life activities such as acid-base tolerance, glucose metabolism and stress response of LM [22-24]. In this study, we confirmed that RNase III is involved in the regulation of stress response of LM to low temperature, high temperature, ethanol, high salt, alkaline and oxidative environment. However, which ncRNAs are required to mediate RNase III regulated environmental stress response of LM is still unclear. RNase III can indirectly affect the formation of biofilm by regulating the expression of some genes in bacteria. Saramago et al.[25] verified that in *R. typhimurium*, RNase III regulates the production of biofilms by regulating the mRNA level of a transcriptional regulator CsgD that regulates the expression of biofilm-associated matrix compounds and the expression of the flagellin *fljB* and *fliC* genes[26]. Kim et al.[27] revealed that RNase III can regulate the formation of *E. coli* biofilm through the RNase III-dependent pathway. Ruiz et al.[28] confirmed in *S. aureus*, the pairing of the biofilm repressor IcaR 3'-UTR with the 5'-UTR provides

a double-stranded RNA substrate for RNase III, which accelerates the decay of icaR mRNA and regulates the formation of biofilms. In addition, Zhao et al.[29] verified that RNase III indirectly regulates the formation of *Salmonella* typhimurium biofilm by catalytically cleaving the 3'UTR of the riboflavin synthase subunit RibE α mRNA to generate a novel ncRNA RibS. Lemon et al.[30] found that prfA plays a role in flagella-mediated movement and maturation of the biofilm after initial surface adhesion, whereas ncRNA *SreA* interacts with the 5'UTR of PrfA mRNA and reduces the stability of prfA transcript or prfA mRNA translation. In addition, *SreA* can also regulate the expression of AgrD [31] and indirectly regulate the adhesion process of biofilms [32]. Studies also revealed that σB regulates the formation of biofilm by regulating the expression of flagellin [33], while RNase III can cleave σB-dependent sRNA *SbrA* [34], suggesting that RNase III indirectly regulates the formation of biofilm. In this study, the phonotype analysis result demonstrated that the biofilm formation ability of LM-*ΔrncS* was significantly lower than that of LM EGD-e, and qRT-PCR further confirmed that the transcription levels of both *SreA* and *SbrA* genes were decreased, suggesting that RNase III may be indirectly involved in the regulation of biofilm formation through biofilm-associated ncRNA *SreA* and *SbrA*-mediated regulation.

Recent studies have also found that RNase III also plays an important regulatory role in the expression of bacterial virulence genes [35]. Darfeuille et al.[11] found in *E. coli* that RNase III mainly cleaves tisAB mRNA after IstR-1 sRNA binding. Furthermore, *in vivo* RyhB sRNA decay depends on RNase III after sRNA pairing with the 5'-UTR of its mRNA target [36]. Abidat revealed that RNase III can directly act on the virulence-related genes of *Salmonella*, and can also bind small non-coding antisense RNA molecules and mRNA target complexes to regulate the expression of virulence genes [25]. Bonnin and Bouloc [37] found that RNase III is involved in the regulation of the expression of cell adhesion factors in *S. aureus*. In this study, cell infection assay result confirmed that the adhesion, invasion and intracellular survival and proliferation of LM-*ΔrncS* in macrophages were significantly decreased. Meanwhile, the transcription levels of *InlA*, *hly*, *prfA* and *SigmaB* gene were significantly lower than those of LM EGD-e. In addition, the animal infection test further confirmed that the survival ability of LM-*ΔrncS* in the liver and spleen was significantly decreased, indicating that the *rncS* gene plays a regulatory role in the virulence of LM. However, which ncRNAs are involved in RNase III-mediated regulation of the virulence in LM require further study of transcriptomics of LM-*ΔrncS* deletion strain.

In conclusion, we for the first time confirmed that RNase III *rncS* is involved in the environmental stress response, biofilm formation and virulence in LM, which provide insights into the regulatory role of RNase III *rncS* in LM environmental adaptability and pathogenicity.

Acknowledgments

We thank the field staff who provided the samples for this study. This work was supported by the national key research and development program (No. 2016YFD0500900), Young and middle-aged leading science and technology innovation talents plan of Xinjiang Corps (No.2016BC001), the International Science & Technology Cooperation Program of China (No. 2014DFR31310), National Natural Science Foundation of China (31360596, 30960274), and Cooperation project of Urumqi-Chongqing in Science and technology (No. Y161220001).

Conflict of Interests Statement

The authors declare that they have no conflict of interest.

Ethical Approval

Ethical approval for this study was given by the Research and Ethical Committee of the Shihezi University.

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