



Full Length Article

Impacts of *lmo2672* Gene Deficiency on Pathogenicity of *Listeria monocytogenes*

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Abstract

Listeria monocytogenes (LM) is an important food-borne pathogen, which is severely harmful to human and animals. In order to explore the roles of *lmo2672* on pathogenicity of LM, a $\Delta lmo2672$ deletion strain was generated using homologous recombination technique, and then the virulence of $\Delta lmo2672$ mutant was analyzed in macrophage cells and mice. The transcription of virulence-related genes was also determined by real-time RT-PCR. Besides, the interaction between *lmo2672* and the upstream DNA sequence of *prfA* gene was investigated by electrophoretic mobility shift assay (EMSA). The results showed that 1) the hemolytic ability of the $\Delta lmo2672$ mutant was significantly weaker than that of wild-type strain LM EGD-e ($P < 0.05$); 2) the invasion and intracellular proliferation in macrophage cells RAW264.7 of LM- $\Delta lmo2672$ were significantly decreased ($P < 0.05$); 3) the 50% lethal dose (LD₅₀) to mice for the $\Delta lmo2672$ mutant was significantly higher than wild-type strain ($P < 0.05$); 4) the bacterial loads of the $\Delta lmo2672$ strain in mouse liver and spleen were significantly less than that of wild-type strain ($P < 0.05$); 5) the transcription levels of virulence-related genes *prfA*, *actA* and *hly* in $\Delta lmo2672$ mutant declined significantly ($P < 0.05$) as compared with LM EGD-e. Furthermore, EMSA confirmed that there existed the interaction between *lmo2672* and the upstream DNA sequence of *prfA* gene. These findings suggested that AraC family transcription regulator *lmo2672* is involved in pathogenicity of LM, which provided an insight into the biological function of *lmo2672* in LM virulent regulation. © 2020 Friends Science Publishers

Keywords: *Listeria monocytogenes*; *lmo2672*; Pathogenicity; AraC family

Introduction

Listeria monocytogenes (LM) is a Gram-positive zoonotic pathogen that infects humans through ingestion of contaminated food (Pérez-Trallero *et al.* 2014; Fagerlund *et al.* 2016), which can cause abortion in pregnant women, meningoencephalitis in infants, gastroenteritis and other fatal illnesses (Lotfollahi *et al.* 2017). Likewise, livestock population is mainly infected after fed with LM-contaminated feed, leading to abortion and a high mortality rate of 20–30% (Lomonaco *et al.* 2015; Boqvist *et al.* 2018; McDougal and Sauer 2018; Yao *et al.* 2018). What's more, as an important food-borne pathogen, LM can survive in a variety of complex environmental stress conditions, such as low temperature, high temperature, high concentration of salt and low pH, causing serious harm to food safety and animal husbandry (Colagiorgi *et al.* 2017; Boqvist *et al.*

2018; Chaturongakul *et al.* 2008; Hurley *et al.* 2019).

As a facultative intracellular pathogen, the process of LM infection requires the participation of many virulence factors and genes, such as internalin A (inlA), internalin B (inlB), adhesion protein (LAP), phospholipase C (plcA and plcB), listeriolysin O (LLO) and actin polymerizing factor (actA) (Chen *et al.* 2017; Drolia *et al.* 2018). However, the expression of these virulence factors is regulated by PrfA, SigB and other regulators, enabling LM to survive and reproduce in complex intracellular environments (O'Byrne and Karatzas 2008; Heras *et al.* 2011).

So far, many studies have found that AraC family proteins play important roles in regulating virulence, multiple drug resistance and metabolic reactions in bacteria (Gallegos *et al.* 1997; Bailey *et al.* 2010). *lmo2672*, as a member of AraC family protein, is encoded by a novel differential gene *lmo2672* between virulent and avirulent

strains of LM (Roche *et al.* 2008; Hadjilouka *et al.* 2016). However, the biological function of *lmo2672* is still unclear. The purpose of this study is to explore the role of *lmo2672* on pathogenicity by construction of $\Delta lmo2672$ deletion strain and its comparison with that of the wild-type strain, which would provide insights into the biological function of *lmo2672* in LM virulent regulation.

Materials and Methods

Strain and plasmid

LM EGD-e strain (serotype 1/2a) was kindly donated by Dr. W. Goebel, University of Woodsburg, Germany. A temperature-sensitive plasmid pKSV7 was a gift from Professor Zhu Guoqiang of Yangzhou University. *Escherichia coli* (*E. coli*) DH5 α (TaKaRa, Japan) and BL21 (DE3) (TaKaRa, Japan) were grown in Luria-Bertani (LB) broth (Difco, USA). LM strain was cultured in brain-heart infusion (BHI) broth (Difco, USA) or tryptic soy agar (TSA) plate (Difco, USA) at 37°C

Primer design and synthesis

Eleven pairs of primers were designed by Primer Premier 5.0 (Premier Inc., Canada) according to the genome sequence of LM EGD-e in GenBank (accession number: AL591824.1) (Table 1). The primers were synthesized by Huada Biotechnology Co., Ltd. (Beijing, China).

Generation of LM- $\Delta lmo2672$ deletion strain

A $\Delta lmo2672$ deletion strain was constructed using homologous recombination technique. Briefly, the DNA fragments containing upstream and downstream regions flanking the *lmo2672* gene in LM EGD-e strain were amplified using PCR with primers F1/F2 and F3/F4, respectively. The PCR products were recovered and the deletion fragment of the *lmo2672* gene was generated using overlap extension PCR (SOE-PCR). The deletion fragment was then cloned into the plasmid pKSV7 to generate pKSV7- $\Delta lmo2672$. The recombinant shuttle plasmid (pKSV7- $\Delta lmo2672$) was introduced into a competent strain LM EGD-e by electroporation (2.5 kV, 100 Ω and 25 μ F). Then, the transformed bacteria were selected on BHI agar (Difco, USA) with chloramphenicol (12.5 μ g/mL) (Amresco, USA) at 42°C for several passages. The recombinant strain LM- $\Delta lmo2672$ was further screened by PCR with the primer pair F7/F8, and PCR product was sequenced for molecular identification.

Assay of hemolytic activity

In brief, overnight cultures of wild-type strain and $\Delta lmo2672$ mutant were diluted 1:10 in fresh medium, respectively, and 100 μ L of the diluted bacterial suspension

was cultivated on tryptic soy agar (TSA) plate (Difco, USA) with 5% sheep red blood cells (SRBCs) and incubated at 37°C for 24 h. The size of transparent hemolytic ring around the colony was recorded. The hemolytic activity was determined as previously reported with slight modifications (Alonzo *et al.* 2009). The optical density at 600 nm (OD₆₀₀) was measured and normalized for each strain (OD₆₀₀ = 0.5), followed by the addition of 1 mL PBS (pH=5.6) containing SRBCs, then 30 min of incubation at 37°C. The mixtures were then centrifuged to pellet unlysed cell and the hemoglobin absorbance in the supernatants was measured at 543 nm.

Macrophage adhesion, invasiveness and intracellular survival assays

Briefly, mouse macrophages cells RAW264.7 were seeded in the 6-well plates (Gibco, USA) at 37°C in 5% CO₂. Wild-type strain and $\Delta lmo2672$ mutant were collected at the mid-log phase, respectively. Then washed three times with PBS (pH=7.2) and re-suspended in DMEM (Gibco, USA). The cells were subjected to infection with the wild-type strain and $\Delta lmo2672$ mutant at a multiplicity of infection (MOI) of 10:1. After infection, the cells were washed three times with PBS, and lysed with 500 μ L 0.1% Triton X-100 (Amresco Inc., USA) for 10 min. Then the bacteria in the lysate were counted. The adhesion rate, invasion rate and survival rate were calculated as previously reported (Zhang *et al.* 2013). All the infections were performed three replicates.

Mouse virulence assay

The overnight cultures of wild-type strain and LM- $\Delta lmo2672$ were plated on BHI agar to calculate the colony number. Three groups of Six-week-old BALB/c mice, 10 mice per group, were inoculated intraperitoneally with 200 μ L different dilutions of bacteria (10⁵, 10⁶, 10⁷, 10⁸, and 10⁹ CFU/mouse, respectively), one group was inoculated with 200 μ L sterile PBS (pH=7.2). Then mice were assessed 10 days after inoculation and recorded for the number of deaths daily and the LD₅₀ was calculated using the Karber method. Besides, according to the death of mice in the group of 10⁷ CFU injection dose, the Kaplan-Meier survival curve was drawn. Meanwhile, the mice were divided into three groups randomly and intraperitoneally injected with 2 $\times$ 10⁵ CFU of LM EGD-e, LM- $\Delta lmo2672$ and sterile PBS (pH=7.2), respectively. Then, the spleens and livers of mice were homogenized, and bacteria loads were determined through enumeration of CFU. On the fifth day after infection, the organs were fixed in formalin and tissue sections were prepared for histopathological analysis.

Transcriptional analysis of virulence genes

Briefly, LM- $\Delta lmo2672$ and LM EGD-e were incubated to the logarithmic growth phase. At this point, bacteria were harvested by centrifugation, and total RNA were extracted

for using Trizol agent (Invitrogen, USA). Reverse transcription into cDNA was performed using the AMV reverse transcription kit (TaKaRa, Japan). The qRT-PCR was performed using the reversely transcribed cDNA using SYBR Premix Ex Taq™ kit (TaKaRa, Japan) according to the manufacturer's instructions on LightCycler 480 instrument (Roche, Swiss). qRT-PCR analysis was conducted to determine the effects of *lmo2672* on the transcription of genes associated with virulence, *sigB*, *prfA*, *hly*, *actA* and *inlB* genes were determined using the $2^{-\Delta\Delta CT}$ method with the housekeeping gene *rpoB* as internal reference.

Electrophoretic mobility shift assay (EMSA)

Electrophoretic mobility shift assay (EMSA) was carried out to further confirm the interaction between *lmo2672* and the upstream DNA sequence of *prfA* gene (containing promoter P1 and P2). Briefly, the full length *lmo2672* gene was cloned into pET32a vector, and then the recombinant plasmid (pET32a-*lmo2672*) was transformed into DE3 for the expression under the induction of 0.5 mM IPTG for 8 h at 37°C. The recombinant protein *lmo2672* was purified using Ni-NTA affinity chromatography (Invitrogen, USA). The promoter fragment of *prfA* gene was generated by PCR and purified using a Gel Extraction Kit (Omega, USA). The *lmo2672* protein was mixed with 100 ng DNA in 20 μ L of the gel-shift binding buffer (100 mM NaCl, 100 mM Tris-HCl, 1 mM DTT, 10% glycerol). Meanwhile, Bovine serum albumin (BSA) was used as negative control. After incubation at 25°C for 30 min, the sample was analyzed by 8% non-denaturing polyacrylamide gel electrophoresis, then the polyacrylamide gel was stained with ethidium bromide (EB) and visualized under ultraviolet light.

Statistical analysis

One-way analysis of variance was performed using GraphPad Prism software, version 5.0 (GraphPad Software Inc., USA). The analyses were expressed as the mean $\pm$ standard error of the mean. The statistical comparison between different variables was done using $P < 0.05$ and $P < 0.01$ as level of significance.

Results

Using the flanking primer pair F5/F6, a 1502 bp fragment was generated from LM- Δ *lmo2672*. Compared with the wild-type strain LM EGD-e, 807 bp was lost from LM- Δ *lmo2672* (Supplementary Fig. 1A). After 20 passages, a fragment of expected size was amplified by PCR (Supplementary Fig. 1B). Sequence analysis showed that the *lmo2672* gene was successfully deleted (Supplementary Fig. 2).

The hemolytic ring (Fig. 1A, Fig. 1B) and erythrocytolysis (Fig. 1C, Fig. 1D) of LM- Δ *lmo2672* were

smaller or significantly weaker than these of wild-type strain ($P < 0.05$), indicating that the deletion of *lmo2672* reduces the hemolytic ability of LM, which is consistent with the results of transcription level.

Compared with wild-type strain LM EGD-e, there was no significant impacted adhesion rate of Δ *lmo2672* mutant ($P > 0.05$) (Fig. 2A), but the invasion rate was significantly lower ($P < 0.01$) (Fig. 2B). Within 12 h after infection, the proliferation capability to RAW 264.7 cells in Δ *lmo2672* mutant strain was significantly lower than that in wild-type strain LM EGD-e ($P < 0.01$) (Fig. 2C), suggesting that *lmo2672* gene deficiency can reduce the ability of LM infection.

Compared with wild-type strain LM EGD-e, the LD₅₀ of Δ *lmo2672* mutant increased by 6.3 times ($P < 0.05$) (Fig. 3A). The average survival time of mice infected with Δ *lmo2672* mutant was significantly increased ($P < 0.05$) (Fig. 3B). The bacterial load of LM- Δ *lmo2672* was significantly less than that of wild-type strain ($P < 0.05$) in liver (Fig. 3C), while, the bacterial load was significantly less than that of wild-type strain ($P < 0.05$) in spleen (Fig. 3D), suggesting that the gene deletion of *lmo2672* significantly reduced survival and reproduction ability of wild-type strain in liver and spleen.

Compared with normal mice (injected with PBS) (Fig. 4E, Fig. 4F), histopathological changes in liver of the mice injected with wild-type strain and Δ *lmo2672* mutant were characterized by vacuoles in hepatocytes, degeneration and necrosis, and hemorrhagic necrosis, while the structure of splenic cord disappeared with hemorrhage and lymphocyte infiltration. Compared with wild-type strain LM EGD-e (Fig. 4A and Fig. 4B), however, the pathological changes of liver and spleen in LM- Δ *lmo2672* infected mice were significantly reduced (Fig. 4C and Fig. 4D).

The qRT-PCR analysis revealed that *lmo2672* deletion down-regulated the expression of five genes associated with virulence. Compared with wild-type strain, the transcription levels of virulence genes *prfA*, *hly* and *actA* in Δ *lmo2672* mutant except *inlB* and *sigB* were significantly lower ($P < 0.05$) (Fig. 5).

The recombinant protein *lmo2672* could bind to upstream DNA sequence of *prfA*, (Fig. 6, Supplementary Fig. 3), which implied that there was an interaction between *lmo2672* and the upstream DNA sequence of *prfA* gene.

Discussion

AraC family transcription regulators (AFTRs) have been shown to be an important class of regulators in bacteria. They are generally composed of 200 and 300 amino acids arranged in two characteristic domains: one is the conserved DNA binding domain at the C-terminal (CTD), the other is the variable N-terminal domain (NTD) (Gallegos *et al.* 1997; Yang *et al.* 2011), which are necessary for *in vivo* transcriptional activation (Porter and Dorman 2002).

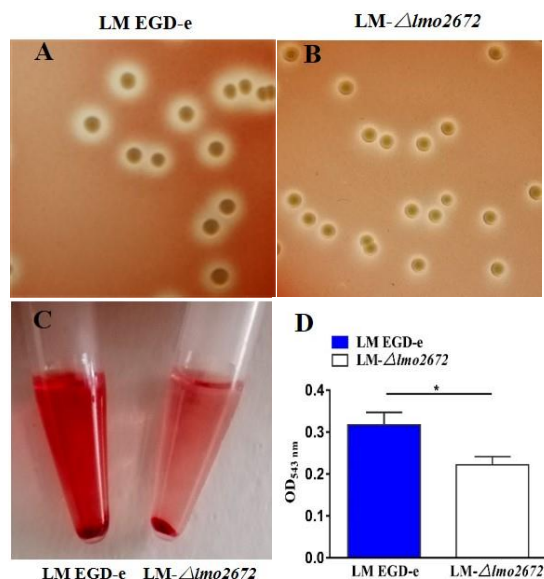


Fig. 1: Determination of hemolytic activity of wild-type strain LM EGD-e and LM-Δlmo2672

(A) and (B): LM EGD-e and LM-Δlmo2672 cultured on sheep blood agar for 24 h, respectively. (C): Hemolytic activity of mutant and LM-Δlmo2672. (D): OD_{543 nm} value of the supernatant was measured. Error bars represent the mean ± standard error (SE) of triplicate experiments. Stars indicate *P*-values < 0.05 (*)

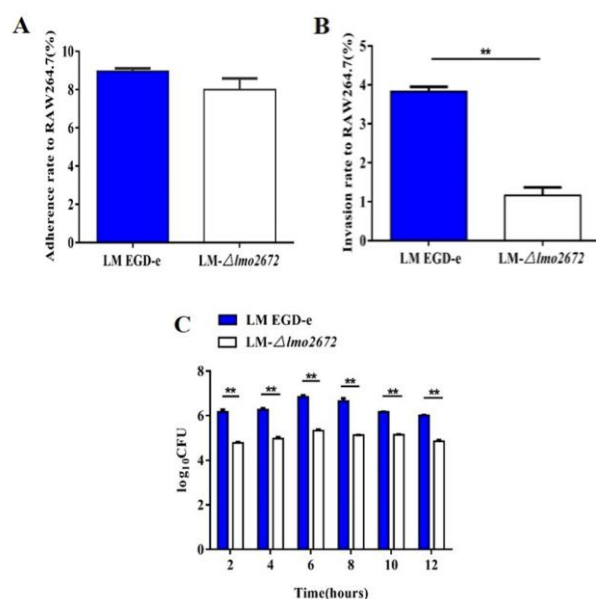


Fig. 2: Determination of adhesion, invasion and replication of wild-type strain LM EGD-e and LM-Δlmo2672 in RAW 264.7 cells

The macrophage RAW 264.7 is infected with bacterial suspension to a MOI of 10 bacteria per cell

(A): Adhesion rates of wild-type strain LM EGD-e and Δlmo2672 mutant; (B): Invasion rates of wild-type strain LM EGD-e and Δlmo2672 mutant; (C): The number of bacteria in live in different time. Error bars represent the mean ± standard error (SE) of triplicate experiments. Stars indicate *P*-values < 0.01 (**)

Furthermore, most members of the AraC family have been demonstrated to be involved in virulence regulation in many

bacteria such as *Vibrio cholerae* (Champion et al. 1997; Krukoni and DiRita 2003), *Citrobacter rodentium* (Kelly et al. 2006; Hart et al. 2008; Santiago et al. 2016) and *Shigella flexneri* (Koppolu et al. 2013; Martino et al. 2016). Garrity-Ryan et al. confirmed that the virulence of *Yersinia pseudotuberculosis* could be significantly reduced by inhibiting or knocking out the transcriptional regulatory genes of the AraC family (Gallegos et al. 1997; Martin and Rosner 2001; Garrity-Rya et al. 2010). However, the role of AraC family member lmo2672 in pathogenicity of LM is still unclear. Here, the impacts of lmo2672 gene deficiency on LM pathogenicity were investigated using LM-Δlmo2672 deficiency strain, erythrocytolysis, macrophage infection and mouse infection assays. The results showed that the Δlmo2672 deletion strain has significantly reduced erythrocytolysis, adhesion, invasion and intracellular proliferation of macrophage RAW264.7, and significantly lowered virulence to mice, suggesting that lmo2672 is involved in pathogenicity of LM.

LM can survive in complex environment inside hosts, which is due to its complex gene regulatory network (O'Byrne and Karatzas 2008; Heras et al. 2011; Guariglia-Oropeza et al. 2014). It has been shown that the AraC family transcription factors regulate the expression of bacterial genes (Bailey et al. 2010). Recently, Rowe et al. found that AraC-type regulator Rbf influences the *Staphylococcus epidermidis* biofilm formation by regulating the expression of the *icaADBC* gene, and subsequently influences its drug resistance and virulence (Rowe et al. 2016). Fang et al. found that BfvR directly or indirectly regulate the expression of *psaABC* and *psaEF* to inhibit the pathogenicity of *Y. pestis* in mice (Fang et al. 2018).

In this study, the qRT-PCR found that Δlmo2672 mutant repressed the expression of three deletion of virulence genes (*prfA*, *actA*, *hly*). PrfA is the member of the Crp/Fnr transcription regulator families in LM (Hall et al. 2016), which can directly or indirectly regulate the expression of many virulence genes in LM (Heras et al. 2011). ActA (actin aggregation factor) is involved in the cell adhesion and invasion of LM (Bruhn et al. 2007; Hadjilouka et al. 2018), which promotes the aggregation of actin to drive LM through the cytoplasm into adjacent cells (Milohanic et al. 2003; Hamon et al. 2012; Brenner et al. 2018). Hly encodes virulence factor LLO, enabling LM to escape from the phage of host cells and replicate inside cells (Chen et al. 2017). This study showed the significant decrease of *hly* gene transcription level is consistent with the phenotype of reduced hemolytic activity. Besides, the transcription levels of *prfA* and *actA* in LM-Δlmo2672 are significantly reduced, which is consistent with the results of LM macrophage cell and mouse infection assays. In addition, the interaction between lmo2672 and the upstream sequence of *prfA* gene was also confirmed by EMSA.

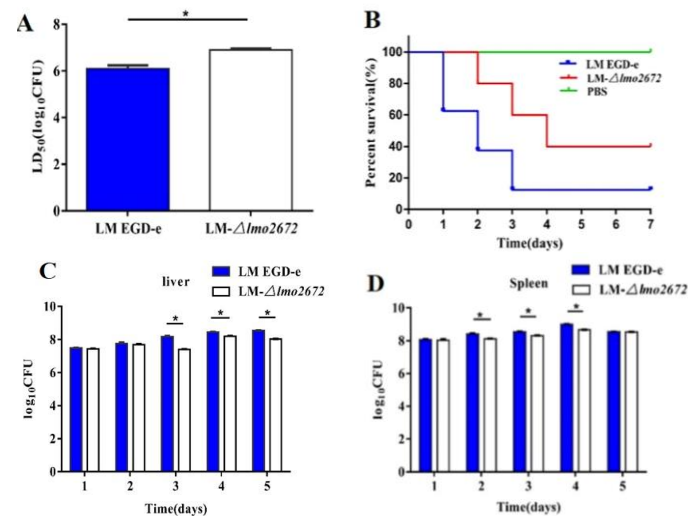


Fig. 3: Effects of *lmo2672* gene deletion of LM on virulence in mice (A): The LD₅₀ of wild-type strain LM EGD-e and LM-Δ*lmo2672*; (B): Survival curves of mice infected by intraperitoneal injection of wild-type strain LM EGD-e and LM-Δ*lmo2672*; (C): Bacteria count in liver; (D): Bacteria count in spleen. Error bars represent the mean ± standard error (SE) of triplicate experiments. Stars indicate *P*-values < 0.05 (*)

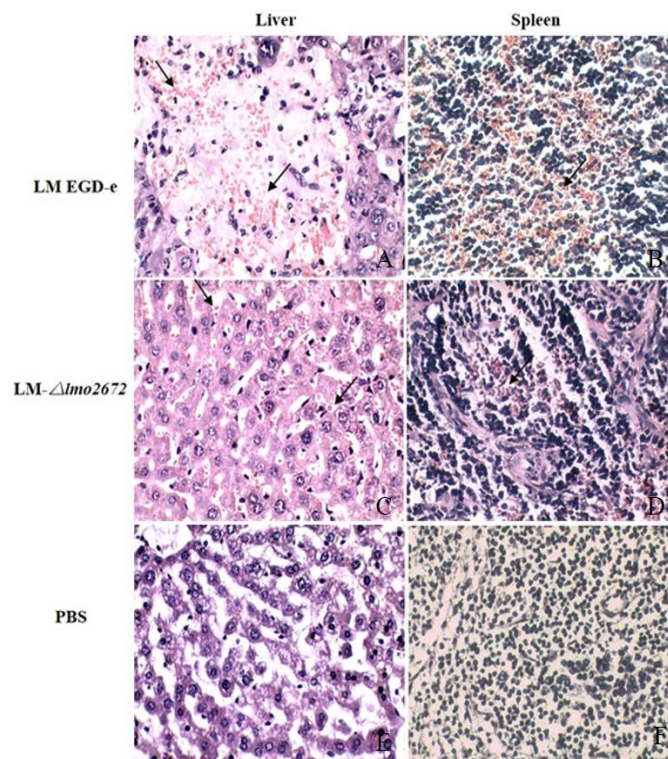


Fig. 4: Histopathological analysis of liver and spleen of mice infected by wild-type strain LM EGD-e and LM-Δ*lmo2672* (HE staining, × 400) (A) and (C): The liver of the mice injected with LM EGD-e and LM-Δ*lmo2672*. Hepatocytes showed vacuol-like appearance, degeneration necrosis and hemorrhagic necrosis (indicated by arrows); (B) and (D): The spleen of the mice injected with LM EGD-e and LM-Δ*lmo2672*. The structure of splenic cord disappeared with hemorrhage and lymphocyte infiltration (indicated by arrows); (E) and (F): Liver and spleen tissue's slices of mice inoculated with PBS (control)

Conclusion

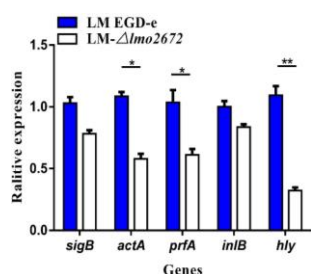
We for the first time demonstrated that *lmo2672* gene deficiency could reduce the virulence of LM, indicating that *lmo2672* was involved in the pathogenicity of LM, which provided an insight into the biological function of *lmo2672* in LM virulent regulation.

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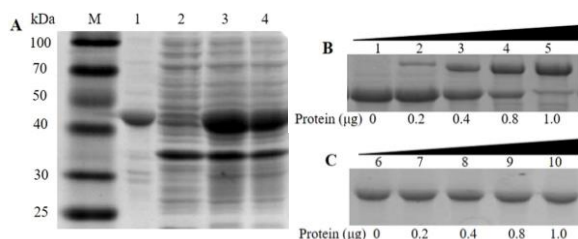
We thank Dr. W. Goebel and Professor Zhu Guoqiang who provided the samples for this study. This work was supported by the national key research and development program (No. 2016YFD0500900), National Natural Science Foundation of China (31360596, 30960274), Young and

Table 1: List of primer sequences used in this study

Target gene	Primer name	Nucleotide sequence (5'→3')	Product size (bp)
lmo2672-L	F1	GGGGTACCCTGTCATTTTTTCTCCTCCT	638
	F2	CAAAGCATTACGTTTAAAGAGACCCCTTTTC	
lmo2672-R	F3	GAAAAGGGGTCTCTTTAAACGTAAATGCTTTG	698
	F4	AACTGCAG GCGAATCAAGTCTTTATCTC	
lmo2672-I	F5	ATAACGTCGCAAGGTGCATG	2309/1502
	F6	GGTCCATACAGAAAACACGGA	
lmo2672-E	F7	ATGATTAATGAATTTGTTTGTA	840
	F8	TTAGAGTTTTTCGACAGTCT	
rpoB	rpoB-F	TGCCATTTATGCCAGAC	188
	rpoB-R	TTCTTCCACTGTGCTCC	
prfA	prfA-F	TTAGCGAGAACGGGACCAT	392
	prfA-R	TGCGATACCGCTTGAATAG	
actA	actA-F	GGCGAAAGAGTCAGTTGC	492
	actA-R	GTTGGAGGCGGTGGAAAT	
hly	hly-F	TGTAAACTTCGGCGCAATC	462
	hly-R	TAAGCAATGGGAACCTCTG	
sigB	sigB-F	TCATCGGTGTCACGGAAGAA	310
	sigB-R	TGACGTTGGATTCTAGACAC	
inlB	inlB-F	TATGCAGCATGGCTTGTAACC	200
	inlB-R	GTTCTTGCAGAGATGGCACG	
prfA-EMSA	prfA-1	AGTATATCTCCGAGCAACCTCG	243
	prfA-2	GTCTCATCCCCCAATCGTT	

**Fig. 5:** Determination of transcriptional levels of virulence related genes in LM EGD-e and LM-Δlmo2672

Error bars represent the mean $\pm$ standard error (SE) of triplicate experiments. Stars indicate P -values < 0.05 (*) and < 0.01 (**)

**Fig. 6:** Analysis of the interaction between lmo2672 and the upstream DNA sequence of prfA gene using EMSA

(A): Analysis of expressed lmo2672 by SDS-PAGE

Lane 1, Purified lmo2672 protein; lane 2, Expression of pET32a vector in *E. coli* BL21(DE3); lanes 3–4, Expression of recombinant lmo2672 in *E. coli* BL21(DE3)

(B) The interaction between lmo2672 and the upstream DNA sequence of prfA gene
Lane 1–5, The upstream DNA sequence of prfA gene incubated with an increasing protein lmo2672;

(C) The interaction between BSA and the upstream DNA sequence of prfA gene

Lane 6–10: an increasing BSA (unrelated protein) binds to the upstream DNA sequence of prfA gene as negative control; Shifted bands are indicated by arrow heads

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