Jundishapur J Microbiol. 2020 May; 13(5):e95758. doi: 10.5812/jjm.95758.
Published online 2020 May 13. **Research Article**

Effects of Lmo2672 Deficiency on Environmental Adaptability, Biofilm Formation, and Motility of *Listeria monocytogenes*

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Received 2019 July 17; **Revised** 2020 April 14; **Accepted** 2020 April 15.

Abstract

Background: *Listeria monocytogenes* is a food-borne pathogen with strong environmental adaptability. It can survive at high temperatures and in acidic high-salt and other unfavorable stressful environments and consequently form a biofilm.
Objectives: This study aimed to explore the roles of lmo2672 on the environmental adaptability, biofilm formation, and motility of *L. monocytogenes*.
Methods: We analyzed the molecular characteristics of lmo2672 protein, constructed a lmo2672 gene deletion strain of *L. monocytogenes* strain (*L. monocytogenes*-Δ*lmo2672*), using the homologous recombination technique and compared the environmental adaptability, biofilm formation, and motility of *L. monocytogenes*-Δ*lmo2672* with its parental strain *L. monocytogenes* EGD-e. The impacts of *lmo2672* gene deficiency on the transcription of genes associated with flagella formation and the environmental adaptability of *L. monocytogenes* were also determined.
Results: The results showed that the lmo2672 contains two helix-turn-helix (HTH) DNA-binding domains. Compared to *L. monocytogenes* EGD-e, *L. monocytogenes*-Δ*lmo2672* grew significantly slower at different temperatures (37°C and 42°C) under different osmotic pressures (5% and 8% NaCl) and in different media containing 0.3% bile salts, 5 mM H_2O_2, or 1% Triton X-100 ($P < 0.05$). Moreover, LM-Δ*lmo2672* showed a significant decrease in biofilm formation ($P < 0.01$) and motility ($P < 0.05$) at 48 h of culture. Furthermore, the transcriptional levels of regulatory gene *prfA* and flagella-related genes *motA*, *fliP* and *fliE* significantly decreased in LM-Δ*lmo2672* ($P < 0.05$).
Conclusions: In general, these findings indicated that *lmo2672* played critical regulatory roles in the environmental adaptability, biofilm formation, and motility of *L. monocytogenes*, thus providing new insights into the regulatory mechanism of *lmo2672* in *L. monocytogenes*.

Keywords: *Listeria monocytogenes*, AraC, *lmo2672*, Biofilm, Environmental Adaptability, Motility

1. Background

Listeria monocytogenes is an intracellular parasitic zoonotic pathogen causing listeriosis in animals and humans (namely neonates, pregnant women, and immune-compromised individuals) (1, 2). *Listeria monocytogenes* has a strong environmental adaptability and can survive at high temperatures and in acidic high-salt (3, 4) and other unfavorable stressful environments and consequently form a biofilm. Recently, many studies have shown that the adaptability of *L. monocytogenes* to the stressful environment is closely related to its complex regulatory network, in which PrfA (5) and SigmaB (6-8) play an important role in regulating the stress adaptability and virulence of *L. monocytogenes*.

As an important food-borne pathogen, *L. monocytogenes* can infect host cells under the mediation of various pathogenic factors. Until now, many pathogenic factors, including InlA, InlB, ActA, InlC, LLO and phospholipase (PlcA, PlcB) (9-13), have been extensively studied. It is proved that the expression of these virulence factors was regulated by PrfA, sigmaB, and other transcription regulatory factors, resulting in the long-term intracellular survival and proliferation of *L. monocytogenes* (1, 14). *Listeria monocytogenes* can cross the hosts' blood-brain, blood-fetal, and gastrointestinal barrier (15), thereby posing sig-

nificant problems in the food industry.

Many studies have confirmed the significance of transcriptional regulation for *L. monocytogenes* in adapting to different environments (5, 16), including the AraC family, which could exert its functions in both Gram-positive and negative bacteria (17-21). Structurally, the AraC family members contain a conserved helical-turn-helix (HTH) DNA-binding domain (22), which can activate gene transcription and play regulatory roles in adapting to environmental stresses and virulence (18, 19, 21, 23). lmo2672, a novel AraC family regulator, is encoded by the differential gene lmo2672 between virulent and avirulent strains of *L. monocytogenes* (24). However, the biological function of lmo2672 is still unclear.

2. Objectives

The main purpose of this study was to explore the potential roles of *lmo2672* in adverse environment adaptation, biofilm formation, and motility of *L. monocytogenes*. To this end, we constructed a *lmo2672* deletion *L. monocytogenes* strain (LM-Δ*lmo2672*) and compared its adaptability to adverse environmental stresses, biofilm formation, and motility with its parental strain *L. monocytogenes* EGD-e to provide insights into the *lmo2672* mechanism in transcriptional regulation of *L. monocytogenes*.

3. Methods

3.1. Strains and Plasmids

The *L. monocytogenes* EGD-e strain was a gift from Professor W. Goebel at the University of Wurzburg, Germany. The strain was cultured in brain heart infusion broth (BHI) (Sangon, China). *Escherichia coli* DH5α was preserved by the College of Animal Science and Technology at Shihezi University (Shihezi, China) and cultured in LB media (Difco, USA). The temperature-sensitive plasmid pKSV7 was provided by Professor Zhu Guoqiang from Yangzhou University (Yangzhou, China).

3.2. Primers

The primers used for constructing *L. monocytogenes*-Δ*lmo2672* and primers for qRT-PCR analysis of genes associated with environmental adaptation and flagellar movement were designed based on the genome sequence of *L. monocytogenes* EGD-e in GenBank (accession number: AL591824.1) using Primer software version 5.0 (Premier Biosoft International, USA). The 5' ends of F1 and R2 primers were flanked with *Kpn I* and *Pst I* (Takara, Japan) sites and protective bases, respectively (Table 1).

Table 1. List of Primer Sequences in This Study

Primer Names	Nucleotide Sequence (5' → 3')	Size of Amplicons, bp
lmo2672F	ATGGCTAAGCTAGAAACGTT	807
lmo2672R	TCATTGTATATTTGCGACATTT	
F1	GGGGTACC CTGTCATTTTTTCTCCTCCT	638
R1	CAAAGCATTTACGTTTTAAAGAGACCCCCTTTTC	
F2	GAAAAGGGGGTCTCTTTAAAACGTAAATGCTTTG	698
R2	AACTGCAG GCGAATCAAGTCTTTATCTC	
F3	ATAACGTCGCAAGGTGCATG	2309/1502
R3	GGTCCATACAGAAAACCACGA	
F4	ATGATTAATGAATTTGTTTGTA	840
R4	TTAGAGTTTTTCGACAGTCT	
***prfA*-F**	TTAGCGAGAACGGGACCAT	392
***prfA*-R**	TGCGATACCGCTTGAATAG	
***sigB*-F**	TCATCGGTGTCACGGAAGAA	310
***sigB*-R**	TGACGTTGGATTCTAGACAC	
***motA*-F**	TGGAAGAACGTCATGCTGCT	129
***motA*-R**	GTTCGACATTTCGCCCATCG	
***motB*-F**	AATCGCCAAAGAAATCGGCG	130
***motB*-R**	GGCGACACTTAGTTCCCAGT	
***fliP*-F**	TGAATGTGCATGCCGAGAGT	82
***fliP*-R**	ACAAACAGCGCCACACTAGA	
***fliE*-F**	ACCGCGAAAACAGACAATGC	96
***fliE*-R**	TACGGAAGTTTGCGCGTTTG	
***flhA*-F**	ATGAACTCCTGATGCGCCAA	129
***flhA*-R**	GTTGTCGTAGCACCCCTTGA	
16S *rRNA*-F	GATGCATAGCCGACCTGAGA	116
16S *rRNA*-R	TGCTCCGTCAGACTTTCGTC	
***rpoB*-F**	TGCCATTTATGCCAGAC	188
***rpoB*-R**	TTCTTCCACTGTGCTCC	

3.3. Cloning and Molecular Characterization of lmo2672 Gene

In brief, *lmo2672* gene was amplified using *L. monocytogenes* EGD-e genomic DNA as a template and the lmo2672F-lmo2672R primers. After gel purification, the product was ligated with pMD19-T simple vector (TaKaRa, Japan) overnight and transformed into *E. coli* DH5α strain. The positive clones were selected by sequencing. Its encoded protein was analyzed using Expasy software (http://www.expasy.org/tools/) for it's functional domains.

3.4. Construction and Identification of Listeria monocytogenes-Δlmo2672 Strain

The temperature sensitive plasmid pKSV7 was used to generate mutations, and LM-Δ*lmo2672* strain was then constructed by overlap extension (SOE) PCR and homologous recombination techniques. In brief, the upstream and downstream homologous arms of the *lmo2672* were amplified using primer pair F1-R1 as well as primer pair F2-R2, respectively. The PCR products were then gel purified and subjected to the overlap extension PCR using

primer pair F1-R2 to generate the *lmo2672* deletion fragment (Δ*lmo2672*). The fragment was then ligated with pMD19-T simple vector (TaKaRa, Japan) to obtain pMD19-T-Δ*lmo2672* and subcloned into pKSV7 after *Kpn I* and *Pst I* digestion. The obtained pKSV7-Δ*lmo2672* was then transformed into *L. monocytogenes* competent cells via electroporation to generate recombinant LM-Δ*lmo2672* strain. The LM-Δ*lmo2672* was then screened and serially passaged at 42°C in a chloramphenicol-containing BHI agar medium (Sangon, China). The selected LM-Δ*lmo2672* strain was confirmed by PCR and DNA sequencing and further cultured at 37°C in a chloramphenicol-free BHI medium.

3.5. Effect of lmo2672 Gene Deletion on the Adaptability of Listeria monocytogenes to Environmental Stresses

Briefly, *L. monocytogenes* EGD-e and LM-Δ*lmo2672* single colonies were picked and cultured overnight at 37°C. After adjusting OD_{600} nm to 0.5, 50 μL of the cultures were inoculated into 5 mL of the fresh BHI medium and incubated at different temperatures (37°C and 42°C) or in BHI media containing different osmotic pressures (5% NaCl, 8% NaCl), 0.3% bile salts, 5 mM H_2O_2, 1% Triton X-100, respectively. The growth curve of these stains was plotted after measuring OD_{600} values every 2 h. Each experiment was performed in triplicate, and the adaptability of *L. monocytogenes* EGD-e and LM-Δ*lmo2672* to stressful environments was then analyzed, as previously reported (25).

3.6. Effects of lmo2672 Gene Deletion on Biofilm Formation Ability of Listeria monocytogenes

The biofilm formation ability of *L. monocytogenes* was determined using the microplate method. In brief, the overnight cultures of *L. monocytogenes* EGD-e and LM-Δ*lmo2672* were diluted using the fresh BHI medium (Sangon, China) to OD_{600} nm = 0.2, and 200 μL of the diluted culture was inoculated into each well of a 96-well plate. After incubation at 37°C for 12 h, 24 h, and 48 h, respectively, the plate was washed with sterile water to remove the floating bacteria and dried for 45 min. Afterward, 150 μL of 1% crystal violet solution was added into each well and incubated the plate for 30 min. The Biofilm formation was observed under an inverted microscope (LEICA, Germany) and determined, as described by Peng (26). Each experiment was performed three times, with five replicates. The data were expressed as mean ± standard error.

3.7. Effect of lmo2672 Gene Deletion on the Motility of Listeria monocytogenes

In brief, the single colonies of *L. monocytogenes* EGD-e and LM-Δ*lmo2672* were cultured overnight in the BHI medium at 28°C. After being adjusted to the same OD_{600} nm, 1 μL of the cultures were inoculated on the soft BHI agar plate (0.3%). Moreover, a small portion of the cultures were inoculated onto the 0.3% soft agar tube using an inoculation needle and cultured at 28°C for 48 h. The spots formed by the migration of the bacteria on the plate were photographed, and their diameters were measured. Each experiment was repeated three times. The data were expressed as mean ± standard error.

3.8. Determining Transcriptional Levels of Genes Associated with Stressful Environment Adaptability and Flagellar Formation of Listeria monocytogenes

After being cultured at 28°C to OD600 nm ≈ 0.6, the cell pellets of *L. monocytogenes*-Δ*lmo2672* and *L. monocytogenes* EGD-e strains were collected by centrifugation and used to extract total RNA using Trizol (Invitrogen, USA) following the instructions provided by the manufacturer. The cDNA was synthesized using an AMV reverse transcription kit (TaKaRa, Japan) following the procedure provided by the manufacturer. The synthesized cDNA was included as a template for qRT-PCR on the LightCycler 480 (Roche, Switzerland) instrument using the SYBR Premix Ex TaqTM kit (TaKaRa, Japan) to detect the transcriptional levels of two genes associated with environmental stress regulation (namely *prfA* and *sigmaB*) and five genes associated with flagellate (namely *motA*, *motB*, *flhA*, *flip*, and *fliE*). The relative transcriptional levels of the above genes were calculated according to the $2^{-\Delta\Delta CT}$ method using two housekeeping genes *rpoB* as the internal controls. Each experiment was repeated three times. The data were expressed as mean ± standard error.

3.9. Data Analysis

The differences between the *L. monocytogenes*-Δ*lmo2672* and *L. monocytogenes* EGD-e strains were analyzed using GraphPad Prism 5.0 (GraphPad Software, USA). The significance level was set at $P < 0.05$.

4. Results

The *lmo2672* gene is 807 bp in length and encodes 268 amino acid residues (Appendix 1A in Supplementary File). Domain analysis revealed that the C-terminus of the *lmo2672* had a typical helix-turn-helix (HTH) DNA-binding domain (Figure 1A). Tertiary structures predication revealed that the lmo2672 protein consisted of five α-helices, six β-sheets, four β-turns, and random coils (Figure 1B). The amplification performed by the primers F3-R3 showed a 1502 bp in *L. monocytogenes*-Δ*lmo2672* strain (Appendix

1B in Supplementary File). Further amplification by the primers F4-R4 and sequencing the amplified product indicated that the *L. monocytogenes*-Δ*lmo2672* strain was successfully constructed (Appendix S2 in Supplementary File).

The growth of *L. monocytogenes*-Δ*lmo2672* strain at 37°C and 42°C was significantly slower than that of the *L. monocytogenes* EGD-e strain ($P < 0.05$) (Figure 2A and B), indicating that the adaptability of *L. monocytogenes*-Δ*lmo2672* to temperature significantly decreased. Compared with the *L. monocytogenes* EGD-e strain, the *L. monocytogenes*-Δ*lmo2672* strain grew significantly slower in the medium with 5% NaCl ($P < 0.05$) and in the medium with 8% NaCl after 10 h ($P < 0.05$) (Figure 2C and D). The *L. monocytogenes*-Δ*lmo2672* strain did not grow in the BHI medium with 5 mM H_2O_2, showing significantly slower growth when compared with *L. monocytogenes* EGD-e strain ($P < 0.01$) (Figure 2E). Furthermore, compared with the *L. monocytogenes* EGD-e strain, the *L. monocytogenes*-Δ*lmo2672* strain also grew slower in the BHI medium with 1% Triton X-100 during 6 - 12 h ($P < 0.05$) and with 0.3% bile salts during 8 h ($P < 0.01$) (Figure 2F and G).

All the results indicated that the *lmo2672* plays an important role in the adaptation of *L. monocytogenes* to environment stress The biofilm formation ability of the LM-Δ*lmo2672* strain at 12 h and 24 h culturing in BHI medium was not significantly different from that of *L. monocytogenes* EGD-e ($P > 0.05$); however, it was significantly weaker to form biofilm at 48 h than that of *L. monocytogenes* EGD-e ($P < 0.01$) (Figure 3). Testing the soft agar tube showed that the LM-Δ*lmo2672* significantly reduced motility, in comparison to the *L. monocytogenes* EGD-e (Figure 4A), and testing the soft agar plate also showed that spots formed by the LM-Δ*lmo2672* were significantly smaller than those formed by the *L. monocytogenes* EGD-e ($P < 0.05$) (Figure 4B), suggesting that the motility of LM-Δ*lmo2672* was remarkably reduced.

The qRT-PCR analysis showed that, compared to the *L. monocytogenes* EGD-e, the transcriptional levels of two genes associated with the environmental stress regulation and five genes associated with flagellar formation decreased to some extents in the LM-Δ*lmo2672*, among which the transcriptional levels of *prfA*, *motA*, *fliP* and *fliE* decreased more significantly ($P < 0.05$) (Figure 5).

5. Discussion

In this study, we successfully constructed the *lmo2672* gene deletion strain *L. monocytogenes*-Δ*lmo2672* using the homologous recombination technology and compared its growth with its parental strain *L. monocytogenes* EGD-e in different stress environments. The results showed that the *L. monocytogenes*-Δ*lmo2672* grew significantly slower than the *L. monocytogenes* EGD-e at different temperatures and under osmotic pressures in the media containing 0.3% bile salts, 5 mM H_2O_2, or 1% Triton X-100. These results suggest that the lmo2672, a member of AraC transcriptional regulator family, plays an important role in the adaptation of the *L. monocytogenes* to adverse stressful environments.

Many studies have revealed that the AraC family is an important class of regulators in bacteria and encompasses more than 800 members, most of which are transcriptional activators involved in bacterial growth (20, 22, 27). In *E. coli*, SoxS expression is upregulated in response to oxidative stress (28), while OpiA and Robcould enhance the bile acid tolerance (29, 30). However, the lack of axyR, an AraC family regulator, does not affect the adaptation of *L. monocytogenes* to temperature and acid or osmotic stress conditions (31).

Due to strong environmental adaptability, the *L. monocytogenes* could survive under stressful conditions such as food processing, storage, and transportation (14, 32). However, the regulation mechanism of the *L. monocytogenes* in response to stressful environments is not fully understood yet. The existing studies have confirmed that the environmental adaptability of *L. monocytogenes* is regulated by multiple factors such as PrfA and sigmaB (5, 7, 16, 33). In this study, the transcriptional levels of *PrfA* and *sigmaB* in the *L. monocytogenes*-Δ*lmo2672* strain decreased, suggesting that the lmo2672 may exert its regulatory role in stressful environments by regulating the expression of *PrfA* and *sigmaB*. Nevertheless, the molecular mechanism of the *lmo2672* regulating *PrfA* and *sigmaB* genes needs to be further studied.

It is reported that the AraC family members mediate the transcriptional regulation of the genes associated with the biofilm formation in bacteria. Top et al. (34) showed that the deletion of EbrB, an AraC family regulator, decreased the biofilm formation ability of *Enterococcus faecalis*. Rowe et al. (19) showed that Rbf promoted the biofilm formation of *Staphylococcus epidermidis* by regulating the expression of *icaADBC*. Moreover, Camargo et al. (35) confirmed that $SptRS_{Ss}$, the other AraC family regulator, was involved in the biofilm formation of *Streptococcus mutans*. Our results showed that the biofilm formation ability of the LM-Δ*lmo2672* was significantly lower than that of the *L. monocytogenes* EGD-e, indicating that the *lmo2672* contributes to the formation of the *L. monocytogenes* biofilm.

Flagellum is not only a movement organ of bacteria, mediating the propagation of bacteria in the hosts, but also plays an important role in adhesion to the cell sur-

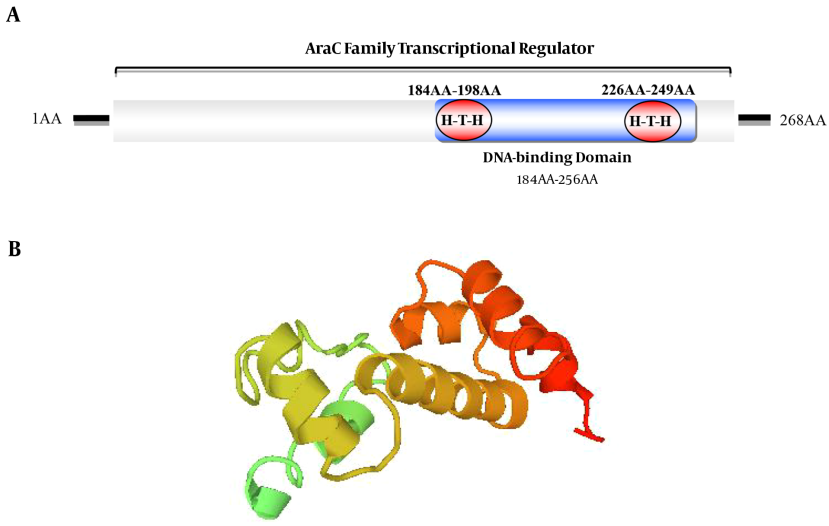

Figure 1. Analyzing molecular characteristics of lmo2672 protein of LM. A, H-T-H DNA binding domains in lmo2672 protein; B, predicting 3D structure of lmo2672 protein.

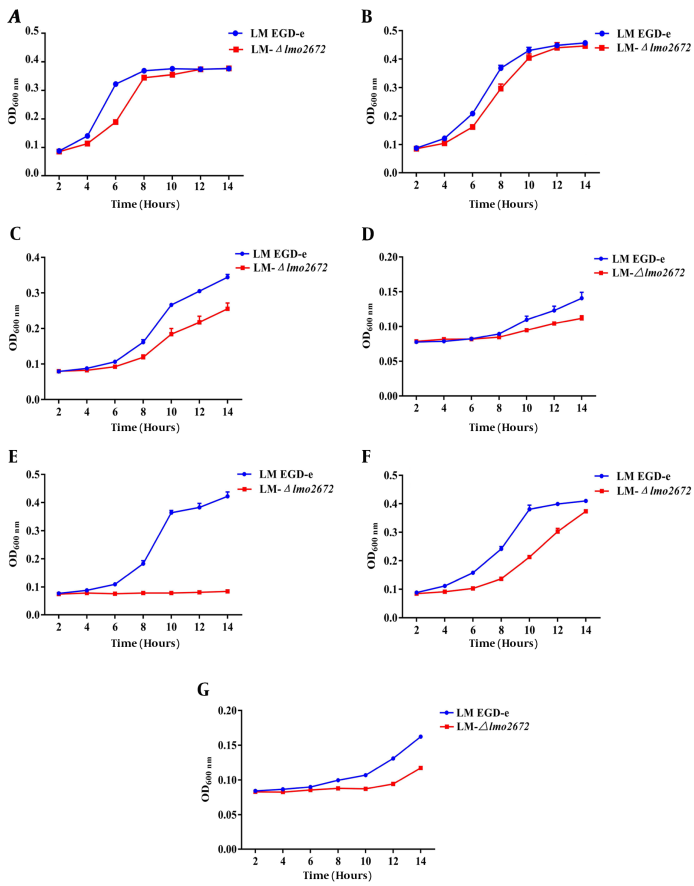

Figure 2. Growth curves of LM EGD-e and LM-Δ*lmo2672* under different environmental stresses. From A to G, Growth curves of LM EGD-e and LM-Δ*lmo2672* at 42°C, 37°C in 5% NaCl, 8% NaCl, 5 mM H_2O_2, 1% Trion X-100, 0.3% bile salts, respectively. Bars indicate the standard error of the mean (SE).

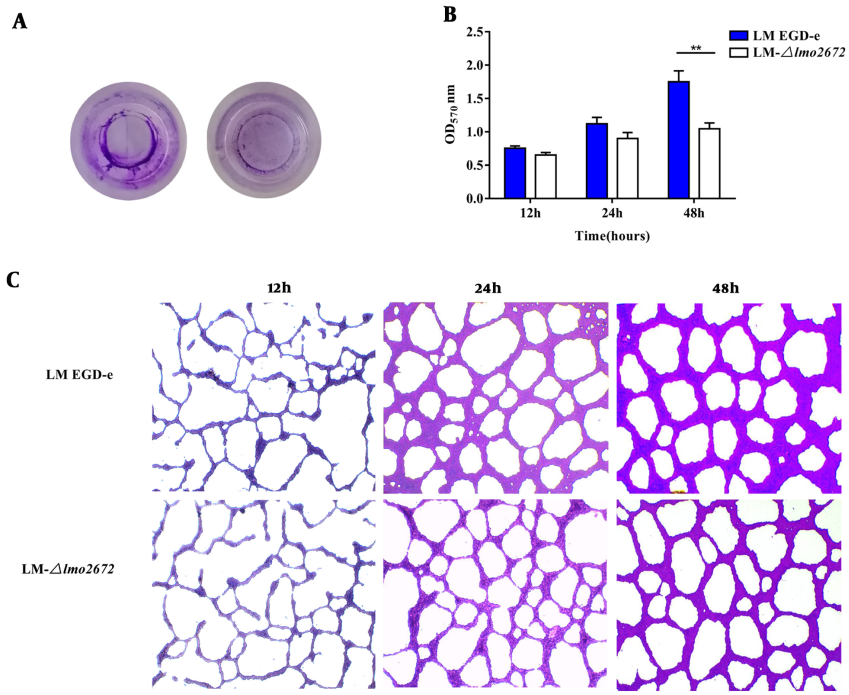

Figure 3. Effects of *lmo2672* gene deletion on biofilm formation of LM. A, Biofilm on plates stained with crystal violet of LM at 48 h; B, biofilm of LM EGD-e and LM-Δ*lmo2672* biofilm determined by OD570 at 12 h, 24 h, and 48 h, respectively; C, formation of biofilm by LM EGD-e and LM-Δ*lmo2672* at 12 h, 24 h, and 48 h, respectively. Bars indicate the standard error of the mean (SE). **, indicates P values < 0.01.

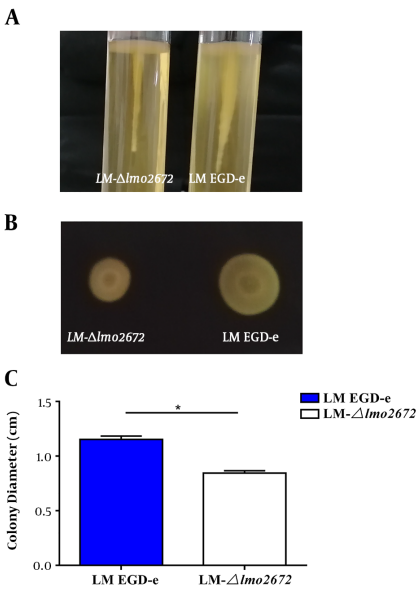

Figure 4. Determination and comparison of the motility of LM EGD-e and LM-Δ*lmo2672*. A, Kinematics in test tubes; B, kinematics in tablets; C, diameter size of bacterial colony in the plate. Bars indicate the standard error of the mean (SE). *, indicates P values < 0.05.

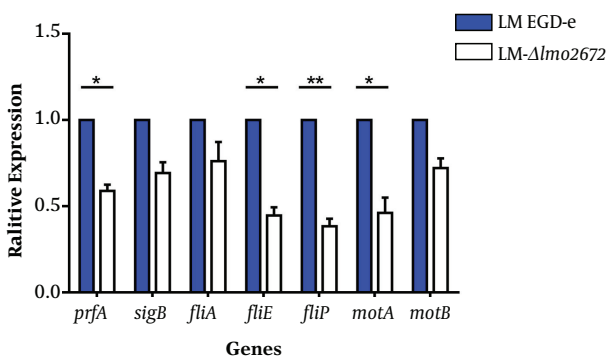

Figure 5. The relative transcriptional levels of the genes related to environmental adaptation and flagellar formation in the LM EGD-e and LM-Δ*lmo2672*. Bars indicate the standard error of the mean (SE). * and **, indicate P values < 0.05 and < 0.01, respectively.

face (36). Studies have revealed that the loss of AraC family member significantly reduced the survival of bacteria and its pathogenicity (17). Gode-Potratz et al. (37) concluded that ExsA, an AraC family transcriptional regulator, could affect the expression of the flagellin gene *flgBL* in *Parasporal vibrio*. Our findings confirmed that the *L. monocytogenes*-Δ*lmo2672* strain significantly decreased motility. Moreover, the expressions of the *motA*, *flip*, and *fliE* significantly decreased in the LM-Δ*lmo2672*, in comparison to *L. monocytogenes* EGD-e, suggesting that *lmo2672* played a regulatory role in the transcription of these three genes.

5.1. Conclusions

In conclusion, we successfully established a *lmo2672* deletion *L. monocytogenes* strain, *L. monocytogenes*-Δ*lmo2672*, and confirmed that *lmo2672*, a member of AraC family, plays important regulatory roles in environmental stress adaptability, biofilm formation and motility of *L. monocytogenes*. These findings in the present study provided new insights into the regulatory mechanism of *lmo2672* in *L. monocytogenes*.

Supplementary Material

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

Acknowledgments

We thank the field staff who provided the samples for this study.

Footnotes

Authors' Contribution: JQ and QM designed the study. JL, MQ, and XZ drafted the manuscript. JL, MQ, XZ, JL, YL, XW, GZ, KZ, and LW performed the experiments and analyzed the data. XC revised the manuscript.

Conflict of Interests: All the authors approve that this manuscript is not simultaneously submitted to another journal for publication. 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.

Funding/Support: This work was supported by the International Science and Technology Cooperation Program of China (no.: 2014DFR31310), the National Key Research and Development Program (no.: 2016YFD0500900), National Natural Science Foundation of China (no.: 31360596, 30960274), and Outstanding Young and Middle-Aged Talent Training Project Of State Key Laboratory For Sheep Genetic Improvement And Healthy Production (no.: SKLSGIHP2017A03)

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