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Deletion of the oligopeptide transporter Lmo2193 decreases the virulence of *Listeria monocytogenes*

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ABSTRACT

Background: *Listeria monocytogenes* is a gram-positive bacterium that causes listeriosis mainly in immunocompromised hosts. It can also cause foodborne outbreaks and has the ability to adapt to various environments. Peptide uptake in gram-positive bacteria is enabled by oligopeptide permeases (Opp) in a process that depends on ATP hydrolysis by OppD and F. Previously a putative protein Lmo2193 was predicted to be OppD, but little is known about the role of OppD in major processes of *L. monocytogenes*, such as growth, virulence, and biofilm formation.

Objectives: To determine whether the virulence traits of *L. monocytogenes* are related to OppD.

Methods: In this study, *Lmo2193* gene deletion and complementation strains of *L. monocytogenes* were generated and compared with a wild-type strain for the following: adhesiveness, invasion ability, intracellular survival, proliferation, 50% lethal dose (LD₅₀) to mice, and the amount bacteria in the mouse liver, spleen, and brain.

Results: The results showed that virulence of the deletion strain was 1.34 and 0.5 orders of magnitude higher than that of the wild-type and complementation strains, respectively. The function of Lmo2193 was predicted and verified as OppD from the ATPase superfamily. Deletion of *Lmo2193* affected the normal growth of *L. monocytogenes*, reduced its virulence in cells and mice, and affected its ability to form biofilms.

Conclusions: Deletion of the oligopeptide transporter Lmo2193 decreases the virulence of *L. monocytogenes*. These effects may be related to OppD's function, which provides a new perspective on the regulation of oligopeptide transporters in *L. monocytogenes*.

Keywords: *Listeria monocytogenes*; oligopeptide permeases; gene deletion; biofilm; virulence

INTRODUCTION

Listeria has over 15 internationally recognized species, among which only *Listeria ivanovii* and *Listeria monocytogenes* are considered to be pathogenic [1-3]. *L. monocytogenes* is a Gram-positive intracellular pathogen originally thought to infect only rabbits and guinea pigs. However, infections in humans were later confirmed, and *L. monocytogenes* was identified as a foodborne pathogen and causative agent of human and livestock listeriosis. *L. monocytogenes* can grow at low temperatures and has strong resistance to environmental stress factors, such as low pH

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Conflict of Interest

The authors declare no conflicts of interest.

Author Contributions

Conceptualization: Honghuan L; Data curation: Honghuan L, Yanjie Q; Formal analysis: Honghuan L; Funding acquisition: Jing W, Xun M; Investigation: Honghuan L; Methodology: Honghuan L, Dongdong D; Project administration: Xun M; Resources: Xun M; Writing - original draft: Honghuan L; Writing - review & editing: Jing W, Xun M.

and high salt concentration. Therefore, *L. monocytogenes* is of high importance in the food and animal husbandry industries [4-6].

Bacterial species can acquire peptides in different ways. Genes for Opp are widely present in Gram-negative and positive bacteria, and it is a multi-subunit protein complex belonging to the ABC transporter family [7]. Opp is usually located in the plasma membrane and its primary function is to capture peptides from the extracellular environment as a source of carbon and nitrogen in plasma. At the genetic level, the five Opp genes encoding the subunits of the transporter are contained in a single operon, OppABCDF [8]. The resulting Opp complex (OppABCDF) is the main transporter of incoming peptides, and acts as an ATP-binding cassette (ABC) transporter. OppABCDF contains five proteins in the assembled complex: OppA, OppB, OppC, OppD and OppF, where each subunit plays different roles in the bacterial transport process. OppB and OppC are homologous membrane integration proteins, which together form a translocation pore. OppD and OppF are two homologous nucleotide binding domains that can facilitate transport by ATP binding and hydrolysis. Finally, OppA is a receptor or substrate binding protein that determines the specificity towards the system's substrate. It has been hypothesized that the *L. monocytogenes* Opp operon also consists of five subunits [9]. In addition to their important role in cell nutrition, peptide transporters may be involved in processes that regulate cell-to-cell signaling, including the regulation of pathogenic bacterial virulence gene expression.

In 2016, Maury used pan-genome technology to analyze genome-wide sequences of 104 *L. monocytogenes* strains, and predicted 15 new putative virulence factors, including Opp that is potentially coded in 2 putative genes: *lmo2192* and *lmo2193*. The RAST annotation of *lmo2192* is OppF, however the function of *lmo2193* is unknown, as no RAST annotation exists [10]. Studies indicate that Opp in *L. monocytogenes* is associated with survival at low temperatures, oligopeptide transport, and virulence.

In this study, *lmo2193* was assigned as OppD, which belongs to the ATPase superfamily. A deletion mutant in *lmo2193* impaired virulence of the LM90SB2 strain both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Bacterial strains, plasmids and cells

Wild-type *L. monocytogenes* strain LM90SB2 of serotype 4b was isolated from sheep with sciatic encephalitis in Xinjiang. The strain was isolated, identified and preserved by the Institute of Animal Science and Technology at Shihezi University. The temperature sensitive shuttle vector pKSV7 was gifted by Zhejiang University, China; and the integration plasmid pIMK2 was gifted by Professor Yin Yuelan of Yangzhou University, China. Primary human brain microvascular endothelial cells (HBMEC) were purchased from Scien Cell Research Laboratories, USA. Mouse brain microvascular endothelial cells (MBMEC) were purchased from Guangzhou Ginio Biotech Co., Ltd. Swine intestinal epithelial cells (SIEC) and the mouse macrophage line RAW264.7 were stored by our laboratory. 6- to 8-week-old BALB/c mice were purchased from the Shihezi University Animal Experimental Center.

Primers

Primers were designed using the gene sequence of the *L. monocytogenes* reference strain EGD-e genome in GenBank (accession number: AL591982), *lmo2193* gene sequence (accession

Table 1. Polymerase chain reaction primers used in this experiment

Name/number	Sequence (5'-3')	Product length (bp)	Target gene
F ₁	<u>A</u> ACTGCAGTGTGGTAACGACCAGCAT 3' (<i>Pst</i> I)	454	Upstream sequence of the <i>lmo2193</i> gene
R ₁	TAAAAGAGAGGTGAGGAAAACGAACAAAGAGAAAATT		
F ₂	AATTTTCTCTTTGTTTCAGITTTCTCACCTCTCTTTA	342	Downstream sequence of the <i>lmo2193</i> gene
R ₂	<u>C</u> GGGATCC-TATGGCTTCATGACTTTAG (<i>Bam</i> H I)		
DF	CATTCACACTCCTTATCGGGT	2,563 (1,486)	Detecting primers
DR	CTTTATTGGGCTTGGTCTTCC		
CF	<u>C</u> GCGGATCCATGGAAAAGCTATTAGAAG (<i>Bam</i> H I)	1,077	<i>lmo2193</i> gene
CR	<u>C</u> CCTCGAGTCATTCTACCTACCCCT (<i>Xho</i> I)		

Underlined values, restriction enzyme cutting site.

number: CAD00271) using Primer 5.0 software. Primers F₁ and F₂ were used to construct the *lmo2193* deletion strain. The detection primers DF and DR were used to amplify a 1486-bp DNA fragment from the genomic DNA of the mutant strain ΔOppD. Recombinant primers CF and CR were used to amplify a 1077-bp target gene fragment. **Table 1** shows information for all primers.

Prediction and analysis of protein structure and function

ORF Finder was used to determine the encoded amino acid sequences and to define potential open reading frames. The functional domains of proteins were predicted using NCBI CDD (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) software.

Determination of ATPase activity

The ATPase activity of Lmo2193 was determined by two methods. Bacterial cells induced for Lmo2193 expression were centrifuged, and the supernatant was used to determine ATPase activity, according to the instructions of a Microorganism ATPase ELISA Kit (Shanghai Yuchun Biological Technology Co., Ltd., China). Purified Lmo2193 protein was quantified according to the instructions of a protein quantitative assay kit (Nanjing Jiancheng Bioengineering Institute, China). The ATPase activity of samples was determined according to the specification of an ultra-micro total ATPase test kit (Nanjing Jiancheng Bioengineering Institute), with ultraviolet spectrophotometry detection at 636 nm and a 1 cm optical path. The concentration dependent ATPase activity was then fitted using the following equation: $T\text{-ATPase}(\text{U/mgprot}) \frac{OD1 - OD2}{OD3 - OD4} \times 0.02 \times 6 \times 7.8 \times 2 \div C$, where T-ATPase is the activity of ATPase, OD1, OD2, OD3 and OD4 are optical density values for the sample, the control, the standard and the blank group, respectively; C (mgprot/mL) is the protein concentration of the test sample.

Mutagenesis and complementation

The upstream and downstream arms of ΔOppD were amplified with F₁, R₁ and F₂, and R₂ primers, respectively. The fusion fragment *lmo2193* was amplified by splicing with overlap extension polymerase chain reaction (SOE-PCR), ligated with the pKSV7 fragment and sequenced. The resulting plasmid, pKSV7Δlmo2193, was transformed by electroporation into LM90SB2 cells. Homologous recombination was carried out in the presence of chloramphenicol at 42°C. The bacterium were subcultured every 12 h, and homologous recombination was detected using Para lateral primers DF and DR every 2 days. Purification of plate grown colonies was performed only after a single 1486-bp band was detected when run on agarose gels and the strain confirmed as correct by sequencing. Recombinant bacteria were incubated in chloramphenicol-free Brain Heart infusion (BHI) liquid medium at 42°C for 15 generations, and then transferred to 37°C for 15–20 passages. DF and DR primers were used for PCR detection of the gene deletion every 4 passages. This determined if the deletion

had stable heritability and detected whether the plasmid was lost every two generations of resistance. The successfully constructed gene deletion strain, as confirmed by sequencing, was named Δ OppD.

The *lmo2193* was amplified by PCR using CF and CR primers. The pIMK2 was ligated overnight at 16°C and transformed into *E. coli* DH5 α . The recombination plasmid was then electroporated into Δ OppD competent cells, which were then cultured at 30°C for 30–36 h. Single colonies were picked and tested in BHI medium containing kanamycin (final concentration 50 μ g/mL). The successful complementation strain, as confirmed by sequencing, was named C Δ OppD.

LM90SB2, Δ OppD and C Δ OppD were activated by streaking onto BHI solid medium. Single colonies were picked after 16–18 h ($OD_{600} \approx 0.4$), and then inoculated into 20 mL BHI liquid medium at 1:100. The cells were cultured in a 37°C shaking incubator, and OD_{600} values were determined in parallel in 3 samples in 96-well plates every 2 h. The growth curves of the three *L. monocytogenes* strains were plotted with OD_{600} as the ordinate and time as the abscissa.

Determination of biofilm formation ability

Biofilms were prepared as previously described, with slight modifications [11-13]. Fresh BHI liquid medium (180 μ L) with 20 μ L culture broth was added to 8 separate wells of a 96-well cell culture plate. Once $OD_{600} \approx 0.2$, the plates were sealed with a sealing membrane and incubated at 37°C. To test the differences in biofilm formation at different culture times, samples were taken at 6, 8, 12, and 24 h, which spans the logarithmic phase of bacterial proliferation. After incubating for 6 h, the culture was removed from each well and wells were washed with phosphate buffer saline (PBS) 3–5 times; plates were then inverted and dried in a 55°C oven for 30 min. One hundred microliters of 1.0% crystal violet was added to each well. The solution was incubated at room temperature for 20 min to stain cells, and then the solution was discarded. The 96-well plate was washed 3 to 5 times with distilled water, inverted in a 55°C oven for 30 min, and then placed under an inverted microscope. Biofilm formations were observed at different time points and images were collected. After observation, 200 μ L 95% EtOH was added to each well before allowing to stand for 30 min to fully decolorize the stain. Values at OD_{600} were then measured using a microplate reader.

Infection of eukaryotic cells and determination of invasion rates and intracellular colonization

For bacterial infection, eukaryotic cell lines were cultured to $\sim 5 \times 10^5$ cells/mL in 24-well plates at 37°C with humidified air and 5% CO₂. *L. monocytogenes* strains were cultured at 37°C for 16–20 h in BHI medium under shaking conditions. Prior to initiating the infection, bacteria were resuspended in cell culture medium at 37°C and added at a multiplicity of infection of 10:1 or 100:1 (bacteria/eukaryotic cell) to eukaryotic cell lines. Prepared bacteria were incubated with eukaryotic cells in a 24-well plate for 1 h. Infected cells were lysed in a solution containing PBS, 0.2% TritonX-100, and 0.25% trypsin to determine the number of cell-associated bacteria (adhesion rate). Infected cells were further incubated in fresh cell culture medium containing 100 mg/mL gentamicin, and after incubation at 37°C for 1 h, the wells were washed three times with 37°C PBS. Viable intracellular bacteria at 2 h were enumerated upon incubation of the infected cells with the PBS, 0.2% TritonX-100, 0.25% trypsin (invasion rate). The culture was continued for 1 h, then DEME medium was added containing 10 mg/mL gentamicin, followed by collection at 2, 4, 6, 8, 10, and 12 h with PBS, 0.2% TritonX-100, 0.25% trypsin solution (intracellular colonization). The number of viable

intracellular *L. monocytogenes* was determined by serial dilution and colony counting on BHI agar plates. The culture was counted for 20–30 hours. These experiments were repeated three times using independently derived clones for each of the strains. Statistical analyses were performed using Student's *t*-test or one-way analysis of variance with Dunnett's post-test.

Virulence

Ninety 6- to 8-week-old BALB/c mice were randomly matched into 15 groups. Bacterial pellets were washed with PBS, resuspended, adjusted to a concentration of 10^9 colony-forming unit (CFU)/mL, and diluted with 5 volumes of PBS to achieve 10^5 CFU/mL. The bacterial cells were injected intraperitoneally into mice, which were then observed for mental changes and deaths over 10 consecutive days after the challenge. Mice mortality was counted and the LD₅₀ was determined using the Karber method [14].

Six- to 8-week-old BALB/c mice were randomly divided into 3 groups with 10 in each group. Each group of mice was injected with 0.5 mL of wild-type, Δ OppD, or Δ OppD inoculum in the peritoneal cavity ($2/3$ LD₅₀). After the challenge, mice were observed for mental changes. Two mice were taken at 24 h, 48 h and 72 h after challenge and CFUs were counted by plating 10-fold serial dilutions of organ homogenates onto BHI agar plates.

Ethics statement

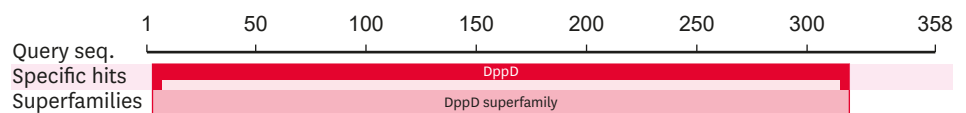
All animal experiments were approved by Care and Use of Animals Center, Shihezi University (IACUC: A2018-094-01). This study was carried out in strict accordance with the Guidelines for the Care and Use of Animals of Shihezi University. Every effort was made to minimize animal pain, suffering and distress and to reduce the number of animal used.

RESULTS

Identification of a new *L. monocytogenes* ATPase

The conserved domain of Lmo2193 was predicted using the NCBI CDD database, results indicating that Lmo2193 belongs to the ATP superfamily (Fig. 1).

A Lmo2193 protein functional domain prediction



B *L. monocytogenes*

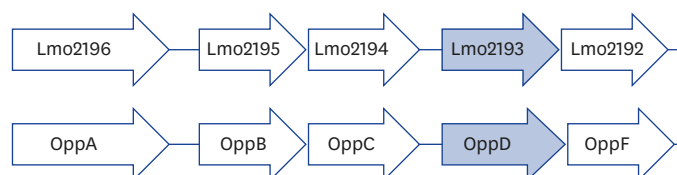


Fig. 1. Prediction of the functional domain of the Lmo2193 protein and the composition of the oligopeptide transporter. (A) Prediction of the functional domain of Lmo2193 protein in *L. monocytogenes*. The arrows indicate gene orientation. (B) Composition of *L. monocytogenes* oligopeptide transporter. Opp, oligopeptide permeases.

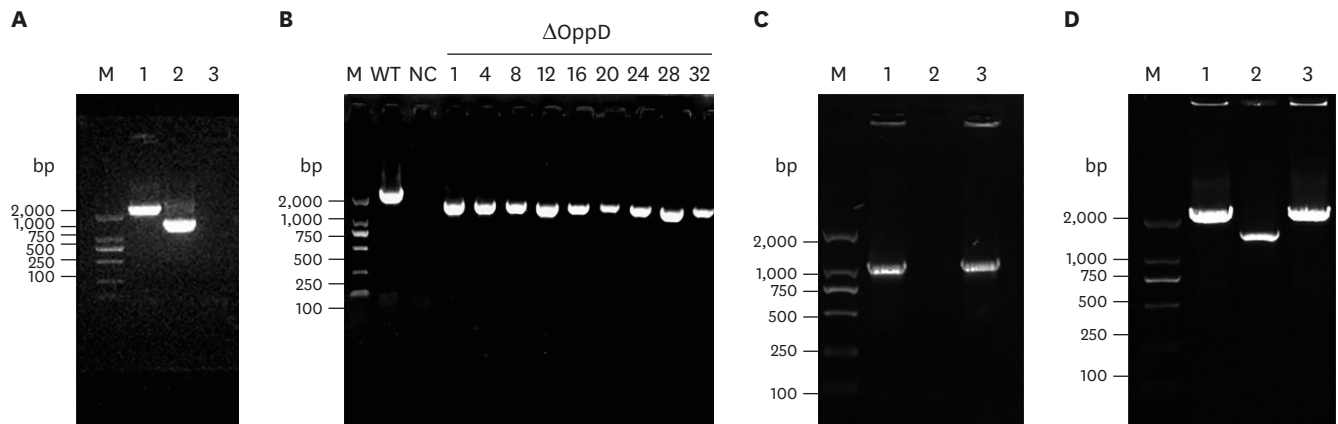


Fig. 2. Gene replacement of OppD. (A) PCR on total DNA isolated from WT and Δ OppD strains using specific primers DF and DR of *Lmo2193*. (B) Analysis of genetic stability of Δ OppD strain by PCR with primers DF and DR. (C) PCR was performed on $C\Delta$ OppD strains to confirm OppD expression in the complemented strain with primers CF and CR. (D) Δ OppD and $C\Delta$ OppD strains were confirmed by PCR using primers DF and DR. M, markers; WT, LM90SB2; Opp, oligopeptide permeases; NC, negative control; PCR, polymerase chain reaction.

Lmo2193 ATPase activity was determined by ELISA. The results demonstrate that *Lmo2193* has ATPase activity of 1.975 U/mL, according to the standard curve equation (**Supplementary Fig. 1**). The ATPase activity of *Lmo2193* was also determined by a colorimetric method, which gave an activity value of 2.247 U/mL. Therefore, these data verified that *Lmo2193* had ATPase activity, is thus OppD.

Identification of mutant strains

Only a 1486-bp fragment was amplified from the wild-type strain when using primers DF and DR on cells cultured in chloramphenicol-free medium for 13 generations at 42°C (**Fig. 2A**) and 36 generations at 37°C (**Fig. 2B**). Sequencing of the amplified products indicated that the strain is genetically stable. Findings also indicated that the *lmo2193* was successfully deleted from the LM90SB2 genome.

A 1077-bp fragment of *lmo2193* was amplified from the LM90SB2 strain using primers CF and CR on cells cultured in the presence of kanamycin (**Fig. 2C**). The presence of the *lmo2193* fragment was detected using primers DF and DR (**Fig. 2D**), and indicated that the gene was successfully integrated into the deletion strain.

The growth trends of wild-type and complementation strains were nearly the same; both exhibited logarithmic growth between 2–8 h. Although the growth of the deletion strain was slower than that of wild-type and complementation strains, it exhibited logarithmic growth at 6–12 h and then reached a stable period (**Fig. 3**).

Determination of biofilm formation ability

Biofilms play an important role in the survival of bacterial populations in their adaptation to environmental stress and antimicrobial agents. Morphologies of biofilms were observed under a microscope. Results showed that a loose mesh state was observed at 6 h, and that a network structure became more obvious over time. At 8 and 12 h, WT and $C\Delta$ OppD exhibited a highly dense and orderly network structure, but the mesh density of Δ OppD was lower than that of WT and Δ OppD. The integrity of the biofilm was less at 24 h than at 8 and 12 h with dispersion and discontinuity visible in the field of view, indicating that the grid of the biofilm has entered the dissipation period (**Fig. 4A**).

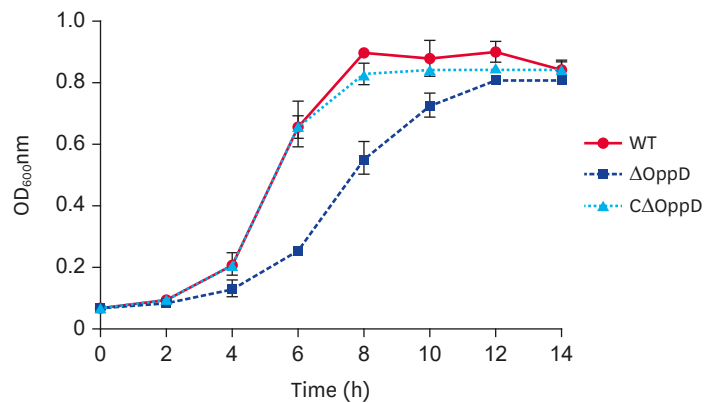


Fig. 3. The growth curves of *L. monocytogenes* in brain heart infusion liquid medium at 37°C. Opp, oligopeptide permeases.

A histogram of the strains' abilities to form biofilms was plotted using OD₆₀₀ data. Results indicate that there was a significant difference in the formation of biofilms between WT and ΔOppD strains at 6 h ($p < 0.01$), but no significant difference between WT and CΔOppD strains ($p > 0.05$). At 8 h, the biofilms formed by the ΔOppD strain were significantly decreased than those of the WT ($p < 0.05$), while there was no significant difference between ΔOppD and CΔOppD ($p > 0.05$). At 12 h, ΔOppD biofilms were significantly less than those of WT and CΔOppD ($p < 0.05$). At 24 h, there was a significant difference between ΔOppD and WT, ΔOppD and CΔOppD, ($p < 0.01$) (**Fig. 4B**). Overall results indicate that the deletion of the *lmo2193* reduced the ability of LM90SB2 to form biofilms.

Cell adhesion and invasion assays

The three strains of *L. monocytogenes* were used to infect RAW264.7, SIEC, MBMEC and HBMEC cells *in vitro* for adhesion and invasion assays. Results indicate that the adhesion and invasion rates of the three strains of *L. monocytogenes* were different. For ΔOppD these rates

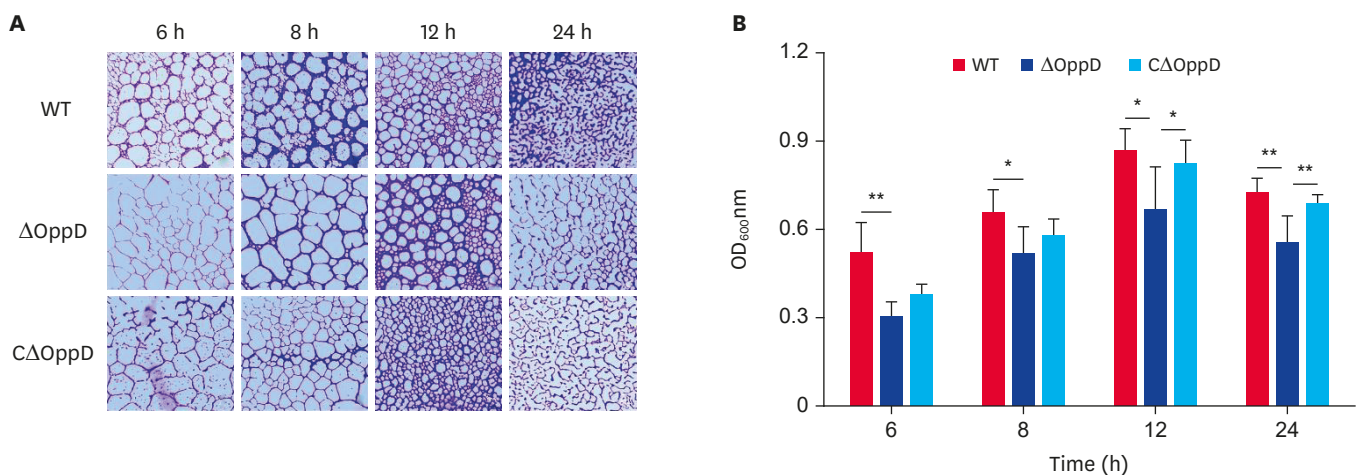


Fig. 4. Biofilm formation by *L. monocytogenes*. (A) Biofilm formed by *L. monocytogenes* at 6, 8, 12 and 24 h, respectively ($\times 200$); (B) Biofilm formation for the three *L. monocytogenes* strains as assessed by measuring OD₆₀₀. The biofilm was stained with 1% crystal violet solution and OD₆₀₀ values were used for normalization to eliminate the effects of the growth rate and cell density. The error bars represent the standard errors of three biological replicates. The p value for the mutant was calculated by analysis of variance. Opp, oligopeptide permeases. * $p < 0.05$, ** $p < 0.01$.

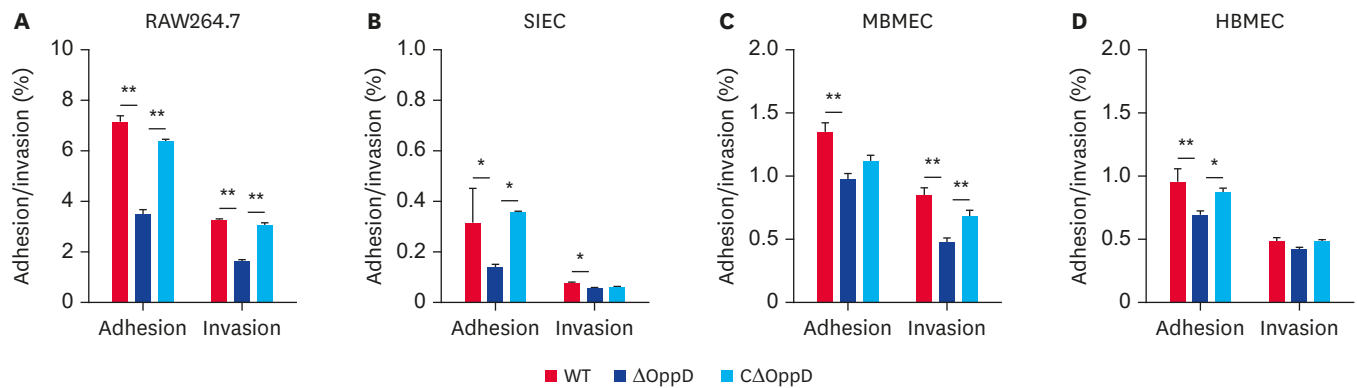


Fig. 5. Role of OppD in *L. monocytogenes* entry. Entry of WT, ΔOppD and CΔOppD strains into RAW264.7, SIEC, MBMEC and HBMEC cells. After 1 h infection, adhesion rates were calculated from the number of cell-associated bacteria and invasion rates were calculated from the number of bacteria that survived 1 h gentamicin incubation. Values are given relative to the adhesion or invasion of the LM90SB2 strain. Values are means ± SD of three experiments run in duplicate. Opp, oligopeptide permeases. * $p < 0.05$, ** $p < 0.01$.

were less than those of WT and CΔOppD. It can be seen that the adhesion and invasion rates of ΔOppD for RAW264.7 cells were significantly less than those of WT and CΔOppD ($p < 0.01$). The adhesion rate of ΔOppD to SIEC cells was significantly less than that of WT and CΔOppD ($p < 0.05$), and the invasion rate of ΔOppD was significantly less than that of WT ($p < 0.05$). The adhesion rate of ΔOppD to MBMEC cells was significantly less than that of WT ($p < 0.01$), and the invasion rate was significantly less in ΔOppD than WT and CΔOppD ($p < 0.01$). Similarly, the adhesion rate of ΔOppD to HBMEC cells was less than that of WT and CΔOppD ($p < 0.05$) but the invasion rate of HBMEC cells had no significant difference between ΔOppD and WT or CΔOppD (Fig. 5). Overall, these results indicate that the deletion of the *lmo2193* could reduce the ability of LM90SB2 to adhere and invade different cells.

Intracellular proliferation assay

The three strains of *L. monocytogenes* were used to infect RAW264.7, SIEC, MBMEC and HBMEC cells *in vitro* for intracellular proliferation assays. The amount of intracellular ΔOppD, WT and CΔOppD increased over time in MBMECs, HBMECs and RAW264.7 cells, but decreased over time in SIECs. In all cultured eukaryotic cells, WT and CΔOppD strains grew faster than the ΔOppD strain (Fig. 6).

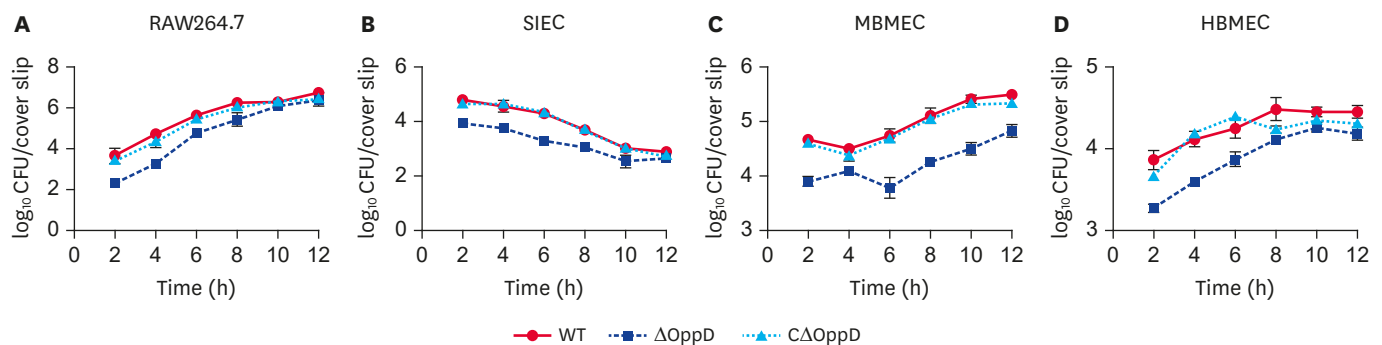


Fig. 6. Intracellular proliferation assay. Entry and intracellular behaviour of the WT, ΔOppD and CΔOppD strains into RAW264.7, SIEC, MBMEC and HBMEC cells. The intracellular number of viable bacteria in various cells were detected every 2 h after the extracellular bacteria were killed by 100 μg/mL of gentamicin. Experiments were repeated three times in duplicate for each cell line. CFU, colony-forming unit; Opp, oligopeptide permeases.

Table 2. Determination of LD₅₀ in mice by three strains of *L. monocytogenes*

Strain	Dilution gradient					LD ₅₀ /(CFU/mL)
	10 ⁹	10 ⁸	10 ⁷	10 ⁶	10 ⁵	
WT	6/6	6/6	4/6	4/6	0/6	10 ^{6.64}
ΔOppD	6/6	3/6	2/6	1/6	0/6	10 ^{7.98}
CΔOppD	6/6	5/6	3/6	2/6	1/6	10 ^{7.14}

LD₅₀, 50% lethal dose; CFU, colony-forming unit; Opp, oligopeptide permeases.

Determination of LD₅₀

Mice were infected with different amounts of each of the three strains of *L. monocytogenes* and LD₅₀ values were calculated. The LD₅₀ of the deletion strain was 10^{7.98} CFU/mL; the LD₅₀ of the wild-type strain was 10^{6.64} CFU/mL; and the LD₅₀ of complementation strain was 10^{7.14} CFU/mL. The virulence of the deletion strain was increased by 1.34- and 0.5- orders of magnitude, respectively (Table 2).

Determination of bacterial load in mice

Mice were injected with either ΔOppD, wild-type or CΔOppD strains. After the bacterial challenge, mice displayed listlessness and anorexia. Anatomical observation revealed that the mice had swelling or congestion in the liver and spleen (data not shown).

Higher counts of viable bacteria were observed in the liver, spleen and brain of wild type and CΔOppD challenged mice respective to those challenged with the ΔOppD deletion mutant at 24 h, 28 h, and 72 h ($p < 0.01$) post-infection (Fig. 7), suggesting that the deletion of *lmo2193* led to decreased virulence of *L.monocytogenes*.

DISCUSSION

L. monocytogenes usually infects individuals with weak immunity. The route of infection is usually food-borne, and a small number of people with mild infection may develop fever-induced gastroenteritis. Pathogens typically activate their virulence factors when they enter the host. *L. monocytogenes* generally utilizes exogenous oligopeptides as a nitrogen source, to sense the environment and control the activity of virulence transcriptional activator, PrfA. Studies have found that PrfA plays an important regulatory role in the balance between the Opp transport system in scavenging activating peptides and inhibiting nutrient peptides; the

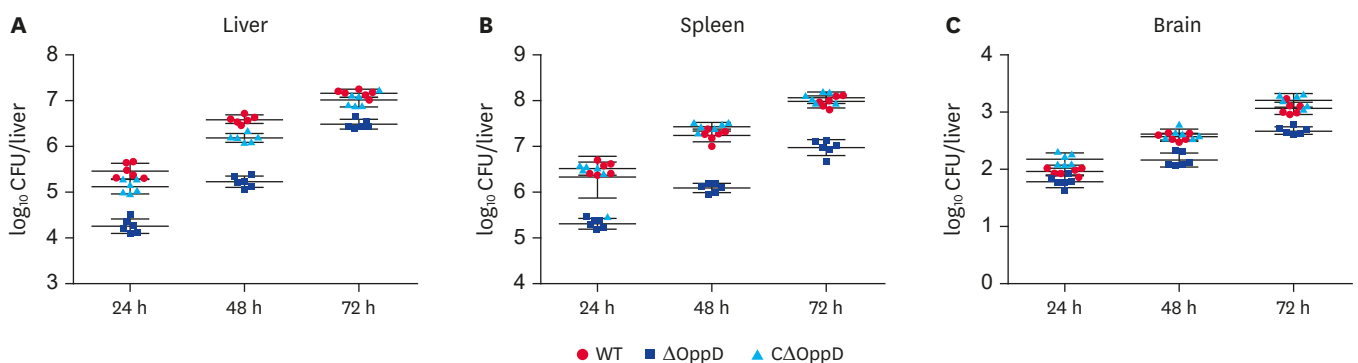


Fig. 7. OppD is required for virulence. Bacterial counts of WT, ΔOppD and CΔOppD strains in the brain, liver and spleen of 6- to 8-week-old BALB/c mice 24, 48 and 72 h after intraperitoneal injection of 5×10^6 CFU. CFU, colony-forming unit; Opp, oligopeptide permeases.

regulatory mechanism is directly involved in the interaction between bacterial virulence and metabolism [15].

ABC transporters are composed of permease proteins, ATPase proteins, and substrate binding proteins and play essential roles in fitness and survival, making them attractive targets for development of new therapies and vaccines. Similar to other bacteria, the *L. monocytogenes* *Opp* gene cluster has two putative permease genes (*OppB* and *OppC*), two putative ATPases (*OppC* and *OppD*), and the *OppA* gene encoding the substrate binding protein. The *Opp* system functions as a nutritional virulence factor for *M. catarrhalis* by mediating the acquisition of limiting nutrients, thus facilitating persistence in the harsh environment of the respiratory tract [16]. The influence of the ATPase activity on the adherence of *M. hominis* to HeLa cells was confirmed [17]. The *OppA* of *Vibrio fluvialis* can reduce the ability to form biofilms, enhance the toxicity to cells, and promote the formation of apoptotic bodies. In severe cases, the cells form debris [8]. Some researchers have developed antiserum to an additional two recombinant proteins, *OppB* and *OppF*, respectively. The *OppB* gene cannot express the full-length mature *OppB* protein and cannot be completed. This is mainly because the protein encoded by the *OppB* gene is a permease, which is toxic to *E. coli* competent [16]. The *X. nematophila* *OppB* mutant strains had altered growth phenotypes, and was capable of initiating and maintaining both mutualistic and pathogenic host interactions [18]. To study the role of *Opp* in *C. pseudotuberculosis*, the Δ *OppD* strain showed impaired growth when exposed to the toxic glutathione peptide, and reduced ability to adhere to and infect macrophages compared to the wild-type [19]. The *OppD* gene in *M. gallisepticum* has the continuous ability to reduce the level of virulence in the body, and suggests that the *OppD* gene may be involved in the regulation of the level of bacterial virulence [20]. In the study of *L. monocytogenes* *Opp*, the *lmo2196* gene was described as the substrate binding protein *OppA* in the ABC transporter [21], which is a very important factor that *L. monocytogenes* depends on for survival in low temperature environments, assisting the organism under these conditions to obtain nitrogen resources, regulate the expression of related virulence factors, and help *L. monocytogenes* survive in macrophages [9]. The *lmo2195* and *lmo2194* genes were described as *OppB* and *OppC*, and function as the permease of the oligopeptide ABC transporter [22], however, research on pathogenicity has not been reported. Maury assumed the *lmo2192* gene function as *OppF* [10]. The *OppD* gene in *L. monocytogenes* has not yet been described or reported.

The oligopeptide transport system *OppABCDF* is one of the main transporters which have functions generally related to the uptake of peptides from the extracellular environment. The oligopeptide transporter contains five subunits and we predicted that the function of the *lmo2193* belongs to the ATPase superfamily. The resultant protein exhibited ATPase activity. Since *lmo2192* functions as *OppF*, we have assigned *lmo2193* as *OppD*. A Δ *OppD* strain of *C. pseudotuberculosis* displayed impaired growth, and reduced adherence and infection of macrophages compared to the wild-type strain [19]. This is because the *OppD* gene encodes the protein that hydrolyzes ATP as a source of energy for peptide internalization. Deletion of *OppD* could lead to the inactivation of the entire transport system, which is a strong indication that *OppD* can regulate virulence levels via nutrient metabolism. In another *OppD* gene deletion study, the *OppD* gene sustained virulence levels of *Mycoplasma gallisepticum* *in vivo* [20]. Our results from *lmo2193* deletion in this experiment are consistent with those of *C. pseudotuberculosis* and *M. gallisepticum*, since *Opp* mutant strains displayed lower virulence and pathogenicity in mice infection assays.

To assess whether the inactivation of the *OppD* gene in *L. monocytogenes* resulted in a decrease in the virulence profile of the mutant strain, invasion rate and intracellular colonization in eukaryotic cells were tested. In the SIEC cell proliferation assay, all three bacterial strains showed a gradual decreasing trend, which may have been caused by the lack of interaction between *L. monocytogenes* in the epithelial barrier, and In1A and mouse E-cadherin, which can impair *L. monocytogenes* entry into epithelial cells [23]. In this experiment, virulence of the *lmo2193* deletion strain was lower than that of wild-type and complementation strains *in vivo* and *in vitro*. This may be related to the function of the Lmo2193 as part of the Opp system in nutrient regulation and transport of oligopeptides. *L. monocytogenes* proliferation was also inhibited by the deletion. To reduce the virulence level, it is speculated that *lmo2193* deletion leads to an insufficient energy supply for bacterial growth, and indirectly reduces the pathogenicity of *L. monocytogenes* due to impaired nutrient metabolism. The results of this experiment suggest that the *lmo2193* reduces *L. monocytogenes* pathogenicity through a nutrient metabolic pathway. Attempts were also made to create a *lmo2192* deletion strain, but the result was unsuccessful. Combined with the measurement of Lmo2192 protein ATPase activity, it was found that of Lmo2192 is twice as high as that of Lmo2193. The author speculates that OppF may provide more energy for bacterial growth, which needs further study.

In the current study, a putative protein Lmo2193 was predicted to be OppD, which may suggest universal efficacy on the role of OppD in major processes of *L. monocytogenes*. To confirm this, the growth, virulence and biofilm formation of *L. monocytogenes* were tested and showed that the deletion of *lmo2193* affected the normal growth of *L. monocytogenes*, reduced its virulence to cells and mice, and affected its ability to form biofilms.

Some reports indicate that biofilms constitute a protected mode of growth that allows survival in a hostile environment [24]. The ability of *L. monocytogenes* to form biofilms can considerably enhance the stress tolerance and, thus, increase the persistence in a hostile environment [25]. To enhance its own survival and proliferation, *L. monocytogenes* adheres to and then invades host cells, ultimately leading to tissue lesions. This process is recognized as an important pathway for bacterial infection [26]. In the current study, both adhesion and invasion rates of *L. monocytogenes* were significantly reduced following the deletion of *lmo2193*, suggesting that *lmo2193* may play an important role in the process of bacteria infection. Intracellular colonization analysis confirmed that *lmo2193* affected the major processes of *L. monocytogenes* associated with the growth analysis, virulence to cells and mice and biofilm formation. These effects may be related to OppD's function, which provides a new perspective on the regulation of oligopeptide transporters in *L. monocytogenes*.

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