RESEARCH ARTICLE

Involvement of a putative ATP-Binding Cassette (ABC) Involved in manganese transport in virulence of *Listeria monocytogenes*

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Abstract

*Listeria monocytogenes* is a foodborne pathogen and the causative agent of listeriosis, a disease associated with high fatality (20–30%) and hospitalization rates (>95%). ATP-Binding Cassette (ABC) transporters have been demonstrated to be involved in the general stress response. In previous studies, in-frame deletion mutants of the ABC transporter genes, LMOI2365_1875 and LMOI2365_1877, were constructed and analyzed; however, additional work is needed to investigate the virulence potential of these deletion mutants. In this study, two *in vitro* methods and one *in vivo* model were used to investigate the virulence potential of in-frame deletion mutants of ABC transporter genes. First, the invasion efficiency in host cells was measured using the HT-29 human cell line. Second, cell-to-cell spread activity was measured using a plaque forming assay. Lastly, virulence potential of the mutants was tested in the *Galleria mellonella* wax moth model. Our results demonstrated that the deletion mutant, ΔLMOI2365_1875, displayed decreased invasion and cell-to-cell spread efficiency in comparison to the wild-type, LMOI2365, indicating that LMOI2365_1875 may be required for virulence. Furthermore, the reduced virulence of these mutants was confirmed using the *Galleria mellonella* wax moth model. In addition, the expression levels of 15 virulence and stress-related genes were analyzed by RT-PCR assays using stationary phase cells. Our results showed that virulence-related gene expression levels from the deletion mutants were elevated (15/15 genes from ΔLMOI2365_1877 and 7/15 genes from ΔLMOI2365_1875) compared to the wild type LMOI2365, indicating that LMOI2365_1875 may be required for virulence. Furthermore, the reduced virulence of these mutants was confirmed using the *Galleria mellonella* wax moth model. In addition, the expression levels of 15 virulence and stress-related genes were analyzed by RT-PCR assays using stationary phase cells. Our results showed that virulence-related gene expression levels from the deletion mutants were elevated (15/15 genes from ΔLMOI2365_1877 and 7/15 genes from ΔLMOI2365_1875) compared to the wild type LMOI2365, suggesting that ABC transporters may negatively regulate virulence gene expression under specific conditions. The expression level of the stress-related gene, *clpE*, also was increased in both deletion mutants, indicating the involvement of ABC transporters in the stress response. Taken together, our findings suggest that ABC transporters may be used as potential targets to develop new therapeutic strategies to control *L. monocytogenes*.
Introduction

*Listeria monocytogenes*, a Gram-positive foodborne pathogen, is an important public health concern since it can cause listeriosis associated with a mortality rate of approximately 20 to 30% in animals and humans [1]. Listeriosis outbreaks have been associated with the consumption of contaminated food products, which include ready-to-eat (RTE) meats and dairy and more recently fresh produce [2–4]. *L. monocytogenes* is also commonly found in the environment, and it is difficult to eliminate this pathogen from food processing facilities since it is able to survive under harsh conditions such as low pH and high salt [5].

Listeriosis occurs primarily in immunocompromised individuals, including pregnant women, the newborn, and the elderly [1]. *L. monocytogenes* virulence involves adhesion and invasion to host cells, escape from vacuoles, intracellular growth, and cell-to-cell spread [6]. The activity of well-characterized virulence factors is involved in each stage of the process [7], and many genes linked to each stage have been identified [8,9]. The *prfA* gene encodes a transcriptional regulator that turns on transcription of several virulence genes, including *hly*, *plcA*, *plcB*, and *inlA* [10,11]. A transcriptional regulator, encoded by the *sigB* gene positively regulates transcription of stress-related genes, including *clpC* and *clpE* [12]. Several genes involved in adhesion are *actA*, *ami*, *fbpA*, and *flaA*, and internalin A and B (*inlA* and *inlB*) that facilitate invasion into mammalian cells [13]. The *hly* gene encodes for listeriolysin O, a pore-forming toxin, which in combination with the action of two phospholipases, *plcA* and *plcB*, is responsible for escape of *L. monocytogenes* from vacuoles. Intracellular motility and cell-to-cell spread involve the action of the *actA* and *iap* genes [6].

In recent years, an infection model using larvae of the greater wax moth, *Galleria mellonella*, has been shown to be a promising model to assess virulence of numerous human pathogens, including *L. monocytogenes* [14–16]. Advantages of this model are its low cost, easy manipulation, ethical acceptability, and the capability to incubate larvae at 37˚C, which is human body temperature and is a prerequisite for the optimal expression of various key virulence factors in *L. monocytogenes* [17]. Most importantly, the innate immune system of *G. mellonella* resembles that of mammals, with enzymes, reactive oxygen species, and antimicrobial peptides necessary against protection from bacterial infection [14]. In addition, the *G. mellonella* model has also been successfully utilized to explore cadmium resistance in *L. monocytogenes* [18], as well as comparison of the transcriptomes from different isolates [19].

ATP-Binding Cassette (ABC) transporters serve as major transport systems in bacteria [20]. More than 30 copies of different ABC transporters are found in the *L. monocytogenes* genome [21]. Typically, an ABC transporter consists of several subunits, including a nucleotide-binding domain, a transmembrane domain, and/or a solute-binding domain [22]. ABC transporters can be used as targets in the development of antibacterial vaccines and therapies [23]. In addition to transport, some ABC transporters have been demonstrated to be involved in virulence. For example, an ABC transporter that is associated with resistance to antimicrobial peptides contributes to the virulence of *Salmonella* [24].

Liu and Ream [25] showed that *LMOj2365_1875* (ABC transporter, manganese-binding protein), *LMOj2365_1876* (manganese ABC transporter; permease protein), and *LMOj2365_1877* (manganese ABC transporter; ATP-binding protein) were highly induced in milk at 4˚C; however, this ABC transporter operon was inhibited in RTE meats [26]. Magnesium is the potential substrate for this transporter, and it is also present in other *L. monocytogenes* strains [27,28]. To our knowledge, it is not under control of key *L. monocytogenes* transcriptional regulators such as SigB. Previous studies have shown that the in-frame deletion mutants *ΔLMOj2365_1875* and *ΔLMOj2365_1877* had no overall growth defects in Brain Heart Infusion (BHI) medium, but were sensitive to salt, acid, and nisin, indicating that
LMOf2365_1875 and LMOf2365_1877 may be involved in the general stress response [21]. However, there have been no studies on the virulence potential of these deletion mutants. In this paper, we tested the virulence potential of the two in-frame deletion mutants of ΔLMOf2365_1875 and ΔLMOf2365_1877 to gain insight into the possible role of the ABC transporter during infection of the human host.

Manganese is involved in bacterial virulence [22,29]. For example, acquisition of Mn (II) is required for intracellular survival and replication of Salmonella enterica serovar Typhimurium in macrophages and for virulence in vivo [30]. Since LMOf2365_1875 encodes for a putative ABC transporter, manganese-binding protein, we hypothesized that manganese transport may be blocked in ΔLMOf2365_1875; therefore, virulence was reduced in L. monocytogenes. While this hypothesis needs further testing, it is supported by the following lines of evidence. Manganese also plays an important role in streptococcal virulence [31]. An ABC transporter named MtsA that is involved in manganese transport in Streptococcus pyogenes was related to virulence since a deletion mutant resulted in attenuated virulence [32]. MtsA shares 98% similarity with LMOf2365_1877(AAT04647.1) and 72% similarity with LMOf2365_1875 (AAT04645.1). In addition, an Agrobacterium tumefaciens mutant with a manganese transport deficiency had attenuated virulence in plants [33]. Similarly, iron acquisition is also required for virulence in L. monocytogenes since an ABC transporter mutant impaired in heme uptake displayed decreased virulence [34].

Materials and methods
Bacterial strains and cell line culture conditions
L. monocytogenes strain F2365 (isolated from Mexican-style soft cheese) [35] was used in the current study since its genome is fully sequenced and annotated [28]. L. monocytogenes F2365, L. monocytogenes Scott A, L. innocua, and two isogenic deletion mutants (ΔLMOf2365_1875 and ΔLMOf2365_1877) of the parent strain LMOf2365 (Table 1) stored at -80˚C as glycerol stocks were streaked onto BHI agar (Sigma-Aldrich St. Louis, MO) and incubated at 37˚C for overnight prior to performing each experiment. The human colon adenocarcinoma cell line HT-29 (ATCC, Manassas, VA, USA) was maintained as described previously [36].

Cell invasion assays
HT-29 cells (ATCC HTN-38) were used to determine the virulence of the Listeria strains [37]. L. monocytogenes strains (L. monocytogenes Scott A, isogenic deletion mutants ΔLMOf2365_1875 and ΔLMOf2365_1877, and the parental LMOf2365) and L. innocua were used for the invasion assays performed as described previously [36]. In brief, HT-29 cells were grown in 24-well tissue culture plates for 5 days to obtain almost confluent monolayers. Strains

| Bacterial strains | Reference/source |
|------------------|------------------|
| L. monocytogenes F2365 wild-type serotype 4b strain, genome sequenced | [28] |
| L. monocytogenes Scott A | Gift from R. D. Joerger (University of Delaware) |
| L. innocua | *ERRC collection |
| ΔLMOf2365_1875, 1875deletion | [21] |
| ΔLMOf2365_1877, 1877deletion | [21] |

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of *L. monocytogenes* (*L. monocytogenes* Scott A, isogenic deletion mutants ΔLMOf2365_1875 and ΔLMOf2365_1877, and the parental LMOf2365) and *L. innocua* were grown to log-phase (OD₆₀₀nm ~0.3) at 37˚C. HT-29 cell monolayers incubated in medium without antibiotics for 24 h were infected for 1 h at 37˚C with 10⁷ CFU bacterial cells in 300 μl BHI medium (Becton Dickinson and Co., Sparks, MD). The cell monolayers were washed with DMEM and incubated in DMEM containing gentamicin (100 μg/ml) for 1.5 h at 37˚C. HT-29 cell monolayers were gently washed three times with phosphate buffered saline (pH 7.4) and then disrupted with 1 ml cold sterile water (4˚C). Viable bacteria were counted after plating serial dilutions onto TSA. The results were expressed as the percentage of CFU recovered after 2 h relative to the number of bacterial cells deposited per well. Three independent experiments were performed for each strain.

**Plaque forming assays (PFAs)**

Strains of *L. monocytogenes* (*L. monocytogenes* Scott A, isogenic deletion mutants ΔLMOf2365_1875 and ΔLMOf2365_1877, and the parental LMOf2365) and *L. innocua* were used for PFA assays, which were performed using HT-29 cells as described previously [36,37]. In brief, confluent HT-29 cell monolayers were incubated in medium without antibiotics for 24 h. The log-phase *Listeria* cells (described above) were used to infect HT-29 cell monolayers with a dilution series of 10² to 10⁷ CFU/ml cells per well, and then incubated for 2 h at 37˚C. After removing the bacterial suspensions, cell monolayers were washed with DMEM and incubated in DMEM containing 100 μg/ml of gentamicin for 1.5 h. Each well was then covered with DMEM with 0.5% agarose containing 10 μg/ml gentamicin. After solidification, 400 μl of the same liquid medium were added to the top of the agar to prevent starvation. Tissue culture plates were incubated for 3 days at 37˚C under 5% CO₂ (v/v). Enumeration of formed plaques was performed using an inverted microscope. The results were expressed as log numbers of plaques per 10⁷ CFU/ml deposited per well. Experiments were performed in duplicate and repeated twice for each strain.

**G. mellonella injection and mortality assay**

The assessment of virulence of *L. monocytogenes* strains in this study was conducted using the *Galleria* larvae model, described in our previous work [16]. *L. monocytogenes* strains were grown overnight at 37˚C in BHI broth and on BHI agar plates. The overnight liquid cultures in BHI broth were washed twice and serially diluted with phosphate buffered saline (PBS). Appropriate dilutions were plated onto BHI agar and incubated for 24 h at 37˚C to obtain the CFU count. Colony counts were used to calculate the bacterial inoculum for *Galleria* infection. A set of 20 *Galleria* larvae of the similar size (approximately 200–300 mg), light-colored, with a good motility, were inoculated with appropriate dilutions of *L. monocytogenes* in the PBS (Fisher BioReagents), for final concentrations of 10⁶ and 10⁵ CFU/larva. Inoculated larvae were incubated at 37˚C and monitored for mortality and phenotypic changes, including changes in color, motility, dryness, and pupation for a period of seven days. For each treatment, the number of dead larvae was recorded daily for up to seven days. From these data, percent mortality was calculated. Each trial included one set of ten uninoculated larvae and one set of ten larvae inoculated with sterile 0.85% saline solution. One group of uninoculated larvae served as a control for adaptation of *Galleria* larvae to 37˚C, while a second group served as a “manipulation” control. Experiments were conducted with three independent trials.

**RT-PCR analysis of virulence and stress-related genes**

Strains of *L. monocytogenes* (deletion mutants ΔLMOf2365_1875 and ΔLMOf2365_1877, as well as the wild type LMOf2365 parent strain) (Table 1) were inoculated in 5 ml of BHI and
grown with agitation (200 rpm) for 12 h at 37°C. Total RNA was isolated from the above strains of \textit{L. monocytogenes} as previously described \cite{25}. Primers targeting 15 genes related to virulence and stress response (Table 2) were designed as previously described \cite{36}. The \textit{spoG} housekeeping gene was used as an internal control (Table 2). cDNA synthesis and real-time PCR analysis conducted in this study were described in our previous work \cite{25}. Reactions without reverse transcriptase were used as negative controls. RT-PCR assays were performed three times for each strain.

**Statistical analysis**

Data collected from this study were analyzed using the Student’s t test of the Statistical Analysis Software (SAS Institute Inc., Cary, NC) for paired comparison with \( P < 0.05 \) considered significant.

**Results and discussion**

Deletion mutant \( \Delta LMO2365_1875 \) displayed reduced invasion and cell-to-cell spread activities in the HT-29 cell line

Previous studies identified mutants of an ABC transporter responsible for oligopeptide transport in \textit{L. monocytogenes} that were defective in host infection \cite{44}. Since ABC transporters are

| GENE | Forward primer sequences (5'-3') | Role in virulence | Product size (bp) |
|------|----------------------------------|-------------------|------------------|
| actA | F: AAGAGTGAACGGAGAGGTT<br>R: TCACCTAGGGCATCAAATTCC | Adhesion, invasion, vacuole lysis, and intracellular motility \cite{6} | 121 |
| ami  | F: GTAAACCTTCGGATGACTCT<br>R: CTGAATAGGACACCCCTGA | Adhesion \cite{6} | 100 |
| clpC  | F: GTAAACCTTCGGATGACTCT<br>R: CTGAATAGGACACCCCTGA | stress response \cite{38} | 100 |
| clpE  | F: CAGAGACATCACACGAGCA<br>R: TCACCGGTATTTCCCTCAATT | stress response \cite{39} | 141 |
| \( \beta \)pa | F: GCAGTGGAGGTTGGATGAAAG<br>R: AGCTAGTTCTTGGCGGATTT | adhesion \cite{6} | 126 |
| \( \beta \)la | F: CCGAGAAGGGTATGAGAG<br>R: ATGGAATGATTTTCTGGTC | adhesion \cite{40} | 127 |
| hly  | F: GAATGCAATTTCGAGCCTAA<br>R: ATGGATGAGTTTTTGCTTGC | Lysis of vacuoles \cite{41} | 133 |
| iap  | F: GAAAAAACGGCGGCCACAG<br>R: CTGTTGGGGCTTTTGGGCT | Invasion and actin-based activity \cite{42} | 109 |
| inlA  | F: ATGGGATTGGCGAGCAGATA<br>R: CGGAAGGTGGTGGTTCGTTCA | Invasion \cite{6} | 143 |
| inlB  | F: ATCCATTCCCCCAACTCTGG<br>R: GATGGGGTGGGTTAGTGTTCC | Invasion \cite{6} | 140 |
| lap  | F: ATCCCACTCTTCCTAAACACTTG<br>R: CTGGGAAATTTGGAAACCATTGC | Adhesion \cite{43} | 133 |
| plcA  | F: AAGACGAGCAAAACAGCAG<br>R: CTGGTCTACGTTTCCGGAG | Vacuole lysis \cite{6} | 100 |
| plcB  | F: ATCCATTCCCCCAACTCC<br>R: CGGTGGAATTTGGAAACCATTGC | Vacuole lysis \cite{6} | 117 |
| pyrA  | F: CGCAAGAGCCTTTAGCATCT<br>R: ATGGAATGATTTTCTGGTC | Transcriptional regulator, virulence \cite{12} | 127 |
| sigB  | F: TCAGCAGGCTTTCCCAAGGTA<br>R: TGAGACGGTAATTTCCCTGATA | Transcription factor, stress response \cite{12} | 127 |
| spoG  | F: TGACGTGAATTTCCCTGATA<br>R: TCAGCAGGCTTTCCCTGATA | Internal control gene \cite{25} | 147 |

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membrane proteins, they may be involved in the adhesion of *L. monocytogenes* to human host cells. To understand if \( \text{LMOf2365}_{-1875} \) and \( \text{LMOf2365}_{-1877} \) are involved in causing host infection, cell invasion and plaque forming *in vitro* assays using HT-29 cell monolayers were employed to test the virulence potential of each deletion mutant. As shown in Fig 1, *L. monocytogenes* Scott A expressed the highest invasion (1.6 log\(_{10}\) CFU/well), and *L. monocytogenes* F2365 (LMOf2365) also had a high invasion efficiency (0.4 log\(_{10}\) CFU/well). Both *L. monocytogenes* F2365 and *L. monocytogenes* Scott A belong to serotype 4b strains, which is the serotype most often associated with outbreaks of listeriosis. The adhesion and invasion efficiency of \( \triangle \text{LMOf2365}_{-1875} \) was lower compared to the LM Scott A strain (with a p-value of 0.03), which is consistent with the fact that \( \triangle \text{LMOf2365} \) has a truncated \( \text{inlB} \) gene that is involved in adhesion and invasion [45]. Non-pathogenic *L. innocua*, used as a negative control, showed no invasion. The deletion mutant strain \( \triangle \text{LMOf2365}_{-1877} \) had a slightly higher invasion efficiency (0.7 log\(_{10}\) CFU/well) (with a p-value of 0.12) compared to the wild type strain (LMOf2365).

**Fig 1. Invasion of *Listeria* strains in HT-29 cells.** HT-29 cell monolayers were incubated with *Listeria* strains grown to stationary phase for invasion assays. Viable intracellular bacteria were counted after plating serial dilutions on BHI agar plates. The results were expressed as log numbers of CFU recovered relative to the number of bacterial cells (10\(^7\)) deposited per well. Each experiment was conducted in duplicate and repeated three times. Significant differences from parental wild type LMOf2365 strain are shown (*, p-value < 0.05; **, p-value < 0.01).

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The other in vitro assay for *Listeria* virulence was based on the ability of strains to form plaques on HT-29 monolayers. *L. monocytogenes* F2365 formed a higher number of plaques (approximately $3.9 \log_{10}$ pfu/well) in comparison to the two mutants but a lower number compared to Scott A (with a p-value of 0.05) (Fig 2). In contrast, no plaques were visible with the non-pathogenic *L. innocua* strain. $\Delta$LMO2365_1875 formed 71% lower number of plaques ($2.8 \log_{10}$ pfu/well) compared to the wild type (with a p-value of 0.0002) whereas there was a smaller difference in plaque forming ability between $\Delta$LMO2365_1877 ($3.4 \log_{10}$ pfu/well) and the wild type LMO2365 ($3.9 \log_{10}$ pfu/well) (with a p-value of 0.002). Examining all of the data, results from the invasion and plaque forming virulence assays demonstrated that the deletion mutant $\Delta$LMO2365_1875 displayed some weakness in invasion and intracellular cell-to-cell spread in HT-29 monolayers, suggesting that LMO2365_1875 may be required for *L. monocytogenes* virulence.

The $\Delta$LMO2365_1875 and $\Delta$LMO_1877 showed reduced virulence in the *G. mellonella* model

The *Galleria mellonella* insect larvae model has been successfully utilized to assess virulence properties of various *L. monocytogenes* isogenic mutants [16]. In this work we studied two
isogenic deletion mutants, $\Delta LMO2365_{-1875}$ and $\Delta LMO2365_{-1877}$ and compared them with the parent strain $LMO2365$. Our results (Fig 3A and 3B) showed that both mutant strains, regardless of the inoculated dose ($10^6$ or $10^5$ CFU/larva), exhibited reduced mortality, hence lower virulence potential, compared to the parent strain $LMO2365$. At the dose of $10^6$ CFU/larva (Fig 3A), virulence of the deletion mutant $\Delta LMO2365_{-1875}$ was lower than that of the $LMO2365$ parental strain over the first 96 h of the monitoring period, after which the virulence potential of both strains appeared to be similar. The difference in the first 96 h was not statistically significant. On the other hand, the deletion mutant $\Delta LMO2365_{-1877}$, expressed a significantly lower mortality rate/virulence potential compared to both $LMO2365$ and $LMO2365_{-1875}$.

**Fig 3.** Comparison of mortality rates of deletion mutants $\Delta LMO2365_{-1875}$ and $\Delta LMO2365_{-1877}$ with wild type $LMO2365$ in *G. mellonella* using $10^6$ CFU/larva (A) and $10^5$ CFU/larva (B). * Significant difference from both parental strain and $\Delta LMO2365_{-1875}$. ** Significant difference from parental strain. Data presented are the averages of three independent experiments.

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ΔLMOF_1875 throughout the monitoring period of infected larvae. We have observed a similar trend at the dose of 10^5 CFU/larva (Fig 3B). Although the difference in mortality rates between the parental strain and ΔLMOF2365_1875, as well as the mutants themselves, was not significant, the difference between the parent strain LMOF2365 and LMOF2365_1877 was significant (Fig 3B). It appears that the mutant LMOF2365_1877 had an increased effect on virulence compared to ΔLMOF2365_1875 as evidenced by lower mortality. This effect is especially expressed at the higher inoculum dose. Previous work [16] showed that L. monocytogenes isogenic mutants of prfA, hlyA, virR and virS had a significant effect on mortality in the Galleria model while inlA and inlB had marginal effects, which correlates with the known effects of these genes on Listeria virulence.

The expression levels of virulence and stress-related genes were elevated in ΔLMOF2365_1875 and ΔLMOF2365_1877 under stationary phase

To determine the gene expression levels in ΔLMOF2365_1875 and ΔLMOF2365_1877 under stationary-phase conditions, 15 genes related to virulence and stress response [46] were chosen for real-time PCR assays. All of the virulence-related genes were up-regulated in ΔLMOF2365_1877 in comparison to the wild type parental LMOF2365 strain (Table 3), indicating that LMOF2365_1877 negatively regulated virulence and stress gene expression under stationary phase growth conditions. Although the expression level of pfrA, the major virulence regulator in L. monocytogenes, was relatively higher (21.4-fold) in ΔLMOF2365_1875 compared to the wild type parental strain LMOF2365, expression levels of the genes (actA, plcA, plcB and hly) regulated by pfrA were not up-regulated in ΔLMOF2365_1875. On the other hand, the expression levels of other virulence-related genes (ami, inlA, inlB and fbpA) were up-regulated. Our previous studies indicated that stationary phase cells of the deletion mutants (ΔLMOF2365_1875 and ΔLMOF2365_1877) were more resistant to multiple stress conditions [21], indicating that they may contribute to the general stress response. The gene expression levels of three stress-related genes (sigB, clpC, and clpE) were tested using RT-PCR assays. As shown in Table 3, the expression levels of clpC were moderately elevated (6.8 and 10.1-fold) in ΔLMOF2365_1875 and ΔLMOF2365_1877, respectively. The increased levels of stress-related gene, clpC, expression confirmed our previous observation that these deletion mutants may contribute to general stress. In addition, the expression levels of clpE and sigB were also elevated (6.8 and 4.0-fold, respectively) in ΔLMOF2365_1877.

In this study, the deletion mutants showed reduced virulence in terms of invasion and cell-to-cell spreading ability; however, a number of virulence genes showed increase expression under stationary-phase growth. This seems to be contradictory, but the gene expression experiments were not performed under conditions that would occur during infection. It is likely that the virulence gene expression levels were repressed due to catabolite repression under stationary-phase growth conditions; however, these genes were de-repressed in ΔLMOF2365_1875 and ΔLMOF2365_1877.

|         | actA | ami | lap | inlA | inlB | lap | fbpA | plcA | hly | pfrA | clpC | clpE | sigB | flaA | plcB |
|---------|------|-----|-----|------|------|-----|------|------|-----|------|------|------|------|------|------|
| LMOF2365a | 1    | 1   | 1   | 1    | 1    | 1   | 1    | 1    | 1   | 1    | 1    | 1    | 1    | 1    | 1    |
| ΔLMOF2365_1875 | -1.4 | 19.6| 1.2 | 18.3 | 11.2 | -1.0| 5.6  | 1.1  | -3.2| 21.4 | 6.9  | 1.1  | -1.4 |
| Δ LMOF2365_1877 | 8    | 25.6| 10.6| 30.7 | 11.8 | 4.0 | 12.3 | 8.0  | 7.7 | 1.9  | 10.1 | 6.8  | 12.7 |

aThe expression levels of the genes in the mutant strains were normalized to that in wild-type LMOF2365 strains.
b Numbers are average values from three independent experiments.

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We did not perform the complementation experiments for the deleted genes because these deletions are in frame (21), and which by design assures non-interference of other genes at the transcription level. A complementation experiment may not provide any additional information. In addition, gene complementation in Listeria, whether by plasmid or by an integration vector, do not exactly mimic the wild type situation because of the difference in genetic machinery involved in complementation and the topology of the complemented gene.

**Conclusions**

The virulence potential of the deletion mutants, ΔLMOf2365_1875 and ΔLMOf2365_1877, was assessed using both *in vitro* (invasion and plaque forming ability) and *in vivo* (G. mellonella insect model) assays. Our study showed for the first time that LMOF2365_1875 encoding for a manganese-binding protein of an ABC transporter might be required for virulence. In the G. mellonella model, decreased mortality of the deletion mutant ΔLMOf2365_1877 also indicates a possible role in the virulence potential of *L. monocytogenes*. In addition, the gene expression levels of *L. monocytogenes* virulence and stress-related genes were elevated in ΔLMOf2365_1877 under normal laboratory growth conditions. Targeting virulence factors could be a promising approach to develop new strategies against resistant microorganisms.

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**References**

1. Norton DM, Braden CR. Foodborne listeriosis. In: Ryser ET, Marth E, editors. Listeria, Listeriosis and Food Safety. CRC Press, Boca Raton, FL; 2007. pp. 305–356.
2. Farber JM, Peterkin PI. *Listeria monocytogenes*, a food-borne pathogen. Microbiol Rev. 1991; 55: 476–511. https://doi.org/10.1128/mr.55.3.476-511.1991 PMID: 1943998
3. Fleming DW, Cochi SL, MacDonald KL, Brondum J, Hayes PS, et al. Pasteurized milk as a vehicle of infection in an outbreak of listeriosis. N Engl J Med. 1985; 312: 404–407. https://doi.org/10.1056/NEJM198502143120704 PMID: 3918263
4. Garner D, Kathariou S. Fresh produce-associated listeriosis outbreaks, sources of concern, teachable moments, and insights. J Food Prot. 2016; 79:337–44. https://doi.org/10.3389/fcimb.2014.00105 PMID: 26818997

5. Kathariou S. Listeria monocytogenes virulence and pathogenicity, a food safety perspective. J Food Prot. 2002; 65:1811–1829. https://doi.org/10.4315/0362-028X-JFP-15-387 PMID: 12430709

6. Camejo A, Carvalho F, Reis O, Leitão E, Sousa S, Cabanes D. The arsenal of virulence factors deployed by Listeria monocytogenes to promote its cell infection cycle. Virulence. 2011; 2(5):379–94. https://doi.org/10.4161/viru.2.5.17703 PMID: 21921683

7. Pizarro-Cerdá J, Cossart P. Subversion of cellular functions by Listeria monocytogenes. J Pathol. 2006; 208: 215–223. https://doi.org/10.1002/path.1888 PMID: 16362984

8. Portnoy DA, Chakraborty T, Goebel W, Cossart P. Molecular determinants of Listeria monocytogenes pathogenesis. Infect Immun. 1992; 60:1263–1267. https://doi.org/10.1128/iai.60.4.1263-1267.1992 PMID: 1325141

9. Vázquez-Boland JA, Kuhn M, Berche P, Chakraborty T, Domínguez-Bernal G, Goebel W, et al. Listeria pathogenesis and molecular virulence determinants. Clin Microbiol Rev. 2010; 14:584–640. https://doi.org/10.1128/CMR.000085 PMID: 11432815

10. Chakraborty R, Kidd KK. Response. Science. 1992 Feb 28; 255(5048):1052–1053. https://doi.org/10.1126/science.255.5048.1052-b PMID: 1126/science.255.5048.1052-b PMID: 17817769

11. Ireton K, Payrastre B, Cossart P. The Listeria monocytogenes protein InlB is an agonist of mammalian phosphoinositide 3-kinase. J Biol Chem. 1999; 274:17025–17032. https://doi.org/10.1074/jbc.274.24.17025 PMID: 10358053

12. Hu Y, Oliver HF, Raengpradub S, Palmer ME, Orsi RH, Wiedmann M, et al. Transcriptional and phenotypic analyses suggest a network between the transcriptional regulators HrcA and sigmaB in L. monocytogenes. Appl Environ Microbiol. 2007; 73:7981–7991. https://doi.org/10.1128/AEM.01281-07 PMID: 17965207

13. Carvalho F, Sousa S, Cabanes D. How Listeria monocytogenes organizes its surface for virulence. Front Cell Infect Microbiol. 2014; 4:48. https://doi.org/10.3389/fcimb.2014.00048 PMID: 24809022

14. Ramarao N, Leroux C, Lereclus D. The insect Galleria mellonella as a powerful infection model to investigate bacterial pathogenesis. J Vis Exp. 2012 Sep 12; 12(9): e0184557. https://doi.org/10.3791/4392 PMID: 28898264

15. Johansson J, Mandin P, Renzoni A, Chiaruttini C, Springer M, Cossart P. An RNA thermosensor controls expression of virulence genes in Listeria monocytogenes. Cell. 2002; 110:551–561. https://doi.org/10.1016/s0092-8674(02)00905-4 PMID: 12230973

16. Parsons C, Lee S, Jayeola V, Kathariou S. Novel cadmium resistance determinant in Listeria monocytogenes. Appl Environ Microbiol. 2017; Feb 15; 83(5): e02580–16. https://doi.org/10.1128/AEM.02580-16 PMID: 27986731

17. Lee BH, Garmyn D, Gal L, Guérin C, Guillier L, Rico A, et al. Exploring Listeria monocytogenes transcriptomes in correlation with divergence of lineages and virulence as measured in Galleria mellonella. Appl Environ Microbiol. 2019; 85(21), e01370–19. https://doi.org/10.1128/AEM.01370-19 PMID: 31471303

18. Tanaka KJ, Song S, Mason K, Pinkett HW. Selective substrate uptake: the role of ATP-binding cassette (ABC) importers in pathogenesis. Biochim Biophys Acta Biomembr. 2018 Apr; 1860(4):868–877. https://doi.org/10.1016/j.bbamem.2017.08.011 PMID: 28847505

19. Liu Y, Ceresa M, Gunther IV NW, Pepe T, Cortesi ML, Fratamico P. Construction of Listeria monocytogenes mutants with in-frame deletions in putative ATP-Binding Cassette (ABC) transporters and analysis of their growth under stress conditions. J Microbiol Biochem Technol. 2012a; 4:141–146. https://doi.org/10.4172/1948-5948.1000085

20. Lewis VG, Ween MP, McDevitt CA. The role of ATP-binding cassette transporters in bacterial pathogenicity. Protoplasma. 2012; 249:919–942. https://doi.org/10.1007/s00709-011-0360-8 PMID: 22246051
24. Eswarappa SM, Panguluri KK, Hensel M, Chakravortty D. The yejABEF operon of Salmonella confers resistance to antimicrobial peptides and contributes to its virulence. Microbiology. 2008; 154( Pt 2):666–678. https://doi.org/10.1099/mic.0.2007/01114-0 PMID: 18227269
25. Liu Y, Ream A. Gene expression profiling of Listeria monocytogenes strain F2365 during growth in ultrahigh-temperature-processed skim milk. Appl Environ Microbiol. 2008; 74:6859–6866. https://doi.org/10.1128/AEM.00356-08 PMID: 18806004
26. Bae D, Crowley MR, Wang C. Transcriptome analysis of Listeria monocytogenes grown on a ready-to-eat meat matrix. J Food Prot. 2011; 74:1104–1111. https://doi.org/10.4315/0362-028X.JFP-10-508 PMID: 21740712
27. Glaser P, Frangeul L, Buchrieser C, Rusniok C, Amend A, Baquero F, et al. Comparative genomics of Listeria species. Science. 2001 Oct 26; 294(5543):849–52. https://doi.org/10.1126/science.1063447 PMID: 11679669
28. Nelson KE, Fouts DE, NGOs, RJ, RT, KL, CN, BJ, JE et al. Whole genome comparisons of serotype 4b and 1/2a strains of the food-borne pathogen Listeria monocytogenes reveal new insights into the core genome components of this species. Nucleic Acids Res. 2004; 32:2386–2395. https://doi.org/10.1093/nar/gkh562 PMID: 15115801
29. Papp-Wallace KM, Maguire ME. Manganese transport and the role of manganese in virulence. Annu Rev Microbiol. 2006; 60:187–209. https://doi.org/10.1146/annurev.micro.60.080805.142149 PMID: 16704341
30. Boyer E, Bergevin I, Malo D, Gros P, Cellier MF. Acquisition of Mn (II) in addition to Fe(II) is required for full virulence of Salmonella enterica serovar Typhimurium. Infect Immum. 2002; 70(11):6032–42. https://doi.org/10.1128/IAI.70.11.6032-6042.2002 PMID: 12379679
31. Eijkkamp BA, McDevitt CA, Kitten T. Manganese uptake and streptococcal virulence. Biometals. 2015; 28:491–508. https://doi.org/10.1007/s10527-015-9826-5 PMID: 25652937
32. Janulczyk R, Ricci S, Björck L. MtsABC is important for manganese and iron transport, oxidative stress resistance, and virulence of Streptococcus pyogenes. Infect Immum. 2003; 71:2656–2664. https://doi.org/10.1128/IAI.71.5.2656-2664.2003 PMID: 12704140
33. Heindl JE, Hibbing ME, Xu J, Natarajan R, Buechlein AM, Fuqua C. Discrete responses to limitation for iron and manganese in Agrobacterium tumefaciens: influence on attachment and biofilm formation. J Bacteriol. 2015; 198(5):816–829. https://doi.org/10.1128/JB.00668-15 PMID: 26712936
34. Jin B, Newton SM, Shao Y, Jiang X, Charbit A, Klebba PE. Iron acquisition systems for ferric hydroxamate, haemin and haemoglobin in Listeria monocytogenes. Mol Microbiol. 2006; 59:1185–1198. https://doi.org/10.1111/j.1365-2958.2005.05015.x PMID: 16430693
35. Linnan MJ, Mascella L, Lou XD, Goulet V, May S, Salminen C, et al. Epidemic listeriosis associated with Mexican-style cheese. N Engl J Med. 1988; 319:823–828. https://doi.org/10.1056/NEJM198809293191303 PMID: 3137471
36. Liu Y, Yoo BB, Hwang CA, Suy O, Sheen S, Khosravi P, Huang L. LMOI2365_0442 encoding for a fructose specific PTS permease IIA may be required for virulence in L. monocytogenes strain F2365. Front Microbiol. 2017a; 8:6111. https://doi.org/10.3389/fmicb.2017.01611 PMID: 28900418
37. Roche SM, Gracieux P, Milohanic E, Albert I, Virlogeux-Payant I, Témoin S, et al. Investigation of specific substitutions in virulence genes characterizing phenotypic groups of low-virulence field strains of Listeria monocytogenes. Appl Environ Microbiol. 2005; 71:6039–6048. https://doi.org/10.1128/AEM.71.10.6039-6048.2005 PMID: 16204519
38. Nair S, Milohanic E, Berche P. ClpC ATPase is required for cell adhesion and invasion of Listeria monocytogenes. Infect Immum. 2000. 68:7061–7068. https://doi.org/10.1128/IAI.68.12.7061-7068.2000 PMID: 11083831
39. Nair S, Freheli C, Nguyen L, Escuyer V, Berche P. ClpE, a novel member of the HSP100 family, is involved in cell division and virulence of Listeria monocytogenes. Mol Microbiol. 1999; 31:185–196. https://doi.org/10.1046/j.1365-2958.1999.01159.x PMID: 9987121
40. Dons L, Eriksson E, Jin Y, Rottenberg ME, Kristensson K, Larsen CN, Bresciani J, Olsen JE et al. Role of flagellin and the two component CheA/CheY system of Listeria monocytogenes in host cell invasion and virulence. Infect Immum. 2004; 72:3237–3244. https://doi.org/10.1128/IAI.72.6.3237-3244.2004 PMID: 15165625
41. Portnoy DA, Jacks PS, Hinrichs DJ. Role of hemolysin for the intracellular growth of Listeria monocytogenes. J Exp Med. 1988; 167:1459–1471. https://doi.org/10.1084/jem.167.4.1459 PMID: 2833557
42. Pilgrim S, Kolb-Müller A, Gentschev I, Goebel W, Kuhn M. Deletion of the gene encoding p60 in Listeria monocytogenes leads to abnormal cell division and loss of actin-based motility. Infect Immum. 2003; 71:3473–3484. https://doi.org/10.1128/IAI.71.6.3473-3484.2003 PMID: 12761132
43. Pandiripally VK, Westbrook DG, Sunki GR, Bhunia AK. Surface protein p104 is involved in adhesion of Listeria monocytogenes to human intestinal cell line, Caco-2. J Med Microbiol. 1999; 48: 117–124. https://doi.org/10.1099/00222615-48-2-117 PMID: 9989638

44. Schauer K, Geginat G, Liang C, Goebel W, Dandekar T, Fuchs TM. Deciphering the intracellular metabolism of Listeria monocytogenes by mutant screening and modelling. BMC Genomics. 2010; 11:573. https://doi.org/10.1186/1471-2164-11-573 PMID: 20955543

45. Nightingale KK, Milillo SR, Ivy RA, Ho AJ, Oliver HF, Wiedmann M. Listeria monocytogenes F2365 carries several authentic mutations potentially leading to truncated gene products, including inIB, and demonstrates atypical phenotypic characteristics. J Food Prot. 2007; 70: 482–488. https://doi.org/10.4315/0362-028x-70.2.482 PMID: 17340887

46. Olesen I, Vogensen FK, Jespersen L. Gene transcription and virulence potential of Listeria monocytogenes strains after exposure to acidic and NaCl stress. Foodborne Pathog Dis. 2009; 6:669–680. https://doi.org/10.1089/fpd.2008.0243 PMID: 19580450