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# The role of *Listeria monocytogenes* cell wall surface anchor protein LapB in virulence, adherence, and intracellular replication

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ABSTRACT

Lmof2365_2117 is a *Listeria monocytogenes* putative cell wall surface anchor protein with a conserved domain found in collagen binding proteins. We constructed a deletion mutation in *lmof2365_2117* in serotype 4b strain F2365, evaluated its virulence, and determined its ability to adhere and invade colonic epithelial cells and macrophages. In A/J mice, colonization of liver was significantly higher for F2365 than for F2365Δ2117. The ability of F2365Δ2117 to adhere to Caco-2 cells was significantly lower than F2365. The mutant also showed impaired ability to replicate in intestinal epithelial cell and murine macrophages relative to wild type F2365. Lmof2365_2117 contributed to *L. monocytogenes* attachment to catfish fillets. Because of its role in adherence to Caco-2 cells, we designated Lmof2365_2117 *Listeria* adhesion protein B (LapB). The carboxy terminus of LapB is similar to a domain in collagen binding proteins, but our results show that *L. monocytogenes* does not bind collagen.



## 1. Introduction

*Listeria monocytogenes*, a gram positive, facultative pathogen, is responsible for listeriosis and has the highest mortality rate among foodborne pathogenic bacteria [1]. *L. monocytogenes* isolates vary in pathogenic potential, and only a few serotypes (4b, 1/2a, 1/2b, 1/2c) account for >96% of listeriosis cases in humans. *L. monocytogenes* strain F2365 was isolated from a cheese product in 1985 in California, USA, during an outbreak of listeriosis [2].

Cell wall surface proteins participate in bacterial adherence, motility, protection from phagocytosis, and enzymatic activity [3]. Approximately 4.7% of the *L. monocytogenes* genome encodes predicted surface proteins (133 total), which is higher than most other bacteria [4]. Among these are some previously identified virulence factors such as internalin A (InlA) and internalin B (InlB), which participate in pathogen adhesion and invasion [5,6]. They have LPXTG motif (InlA) and LRR domains (InlA and InlB) that serve as cell wall anchors. Another cell wall protein, ActA, stimulates accumulation and polymerization of actin and helps in mobility of *L. monocytogenes* from cell to cell during infection [7]. Peptidoglycan hydrolase cell wall anchor proteins also have established roles in listerial virulence [8]. *Listeria* adhesion protein (LAP) was identified as a surface protein that contributes to adherence to Caco-2 cells and intestinal infection [9].

Lmof2365_2117 is a putative cell wall surface anchor protein with a conserved domain found in collagen binding proteins. We selected this protein for investigation because a gene encoding an orthologous protein from serovar 1/2a strain EGD-e (Lmo2085) is significantly upregulated in both the intravacuolar and intracytosolic compartments in a murine macrophage cell line [10]. Lmof2365_2117 does not have an ortholog in nonpathogenic *Listeria innocua* CLIP11262 [10], but it has an orthologous protein in nonpathogenic *L. monocytogenes* serovar 4a strain HCC23 (LmHCC_0465). In the current study, we constructed an in-frame deletion mutation in the *lmof2365_2117* gene to determine the protein's effect on collagen binding and virulence, which was evaluated using a mouse model and using murine macrophage and human colonic epithelium cell lines. Results indicated that the mutant had attenuated virulence in the host and had impaired ability to adhere and replicate intracellularly, but *L. monocytogenes* does not bind collagen.

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## 2. Materials and methods

### 2.1. Ethics statement

All mice experiments were conducted in accordance with a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at Mississippi State University.

### 2.2. Bacterial strains and growth conditions

*L. monocytogenes* serotype 4b strain F2365 was originally isolated from a Mexican style soft cheese in California in 1985 [2]. *L. monocytogenes* was grown in brain heart infusion (BHI) broth or agar plates at 37 °C, and *E. coli* was grown in Luria-Bertani (LB) media. Antibiotics used were ampicillin (100 μg/ml for *E. coli*) and erythromycin (300 μg/ml for *E. coli*; 5 μg/ml for *L. monocytogenes*).

### 2.3. Construction and complementation of deletion mutant

Overlap extension PCR was used to construct a cloned *lmof2365_2117* deletion [11]. Primers A (TAAATCTGTATTTTGCCTCAC) and B (GCTTTCTCCCCCTTTTTCGCCCTA) were used to amplify an upstream 620 base pair (bp) region flanking *lmof2365_2117*. Primers C (TAGGGCGAAAAAGGGGGAGAAAGCAAAGCAAATACAAAAAAATTCCAAGGTTA) and D (AGGGGGAATTTTACGAGAAGTTCA) amplified a downstream 620 bp region flanking the gene. Mixture of the two products and reamplification using primers A and D generated a 1689 bp in-frame deletion. The amplified gene deletion was cloned into TOPO® TA (Invitrogen) and subcloned into pAUL-A [12], which is a temperature sensitive vector. The recombinant plasmid was transferred into F2365 by electroporation (100 Ω, 25 μF and 2.5 kV) and selected on BHI plates with erythromycin. Resulting colonies were passed three times on BHI with erythromycin at 42 °C for 48 h each to select for integration, and then colonies were grown in BHI up to 8 h to allow plasmid excision. Colonies were checked for subsequent gene deletion using PCR and confirmed using DNA sequencing.

For complementation, the *lmof2365_2117* gene was amplified from strain F2365 genomic DNA on a 1686 bp fragment with primers A (AAAGAGCTCGAAAAAGGGGGAGAAAGCAT) and B (AAAGTCGACTAACCCGCTAGCTACGACAA). The amplicon was digested with SacI and SalI and cloned into pPL2 integration vector [13] digested with the same enzymes, and the resulting plasmid was designated pPL2-2117. Electrocompetent F2365Δ2117 mutant strain was transformed with pPL2-2117, and positive transformants were selected in BHI with chloramphenicol (10 μg/ml). Chromosomal integration in the resulting complemented strain (designated F2365Δ2117::pPL2-2117) was confirmed by PCR.

### 2.4. Growth kinetics

Growth kinetics of F2365 and F2365Δ2117 in BHI broth at 37 °C was compared. 25 ml broth cultures were inoculated to an optical density at 600 nm ($OD_{600}$) of 0.1 from overnight cultures, and $OD_{600}$ was measured every hour up to 9 h. Growth curves were performed in triplicate.

### 2.5. Mouse virulence assay

To assess the virulence of F2365Δ2117, a mouse virulence assay was used as described previously [14] with minor modifications. Overnight cultures of F2365 and F2365Δ2117 were grown to an $OD_{540}$ of 1.35, and 1 ml of the bacterial broth was pelleted by centrifugation and resuspended in 1 ml phosphate buffered saline (pH 7.2). Serial dilutions were prepared, and bacterial densities were quantified by plate counting. Four groups of 6–8 week old female A/J mice (Jackson Laboratory), 5 mice per group, were inoculated intraperitoneally with 100 μl aliquots of appropriate dilutions of bacteria ($1.67 \times 10^2$, $1.67 \times 10^3$, $1.67 \times 10^4$, and $1.67 \times 10^5$ CFU/mouse). One group of 5 mice was inoculated with 100 μl sterile saline, and another group was not injected. The mice were observed twice a day for mortalities and signs of illness. On the seventh day after infection, all mice were euthanized, and their livers were aseptically removed. The organs were weighed, homogenized in 1 ml 0.05% Triton X-100, diluted, spread on BHI plates, and incubated at 37 °C for 48 h to determine CFU/gram in each liver.

### 2.6. Cell lines and assays

Human colon carcinoma enterocyte-like epithelial cells (Caco-2) were obtained from ATCC. Caco-2 cells were grown in Eagle's Minimum Essential Medium (MEM) supplemented with 20% heat inactivated fetal bovine serum (FBS). J774A.1 cells, a murine macrophage cell line, were grown in DMEM complete medium supplemented with 10% heat inactivated FBS. All the cell lines used in this study were grown at 37 °C and 5% $CO_2$.

### 2.7. Adhesion assay

Two days prior to the assay, Caco-2 cells were seeded in 12 well plates with approximately $10^5$ cells per plate. On the day of assay, cells were washed with PBS, and fresh prewarmed media was added to the wells. Overnight cultures of F2365, F2365Δ2117, and F2365Δ2117:pPL2-2117 were adjusted to an $OD_{600} = 1.0$, and approximately $10^6$ bacteria were added to each well to yield a MOI of 10:1. After infection the plates were briefly centrifuged for 45 s and incubated for 30 min. Cells were washed five times with PBS and lysed using 500 μl of cold 0.1% Triton X-100. The resulting suspensions were diluted, spread on BHI agar, and grown at 37 °C for 48 h to obtain colony counts. All infections were performed in duplicate, and three replicates were performed for each infection.

### 2.8. Invasion assay

Caco-2 cells were infected as described in the adhesion assay and incubated 2 h. Cells were washed twice with PBS, and fresh media containing gentamicin (1 μg/ml) was added to kill extracellular bacteria. At 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24 h post-infection, cells were washed twice with PBS and lysed using 500 μl of cold 0.1% Triton X-100 and sonication (Fisher Scientific Sonic Dismembrator Model 100, setting 3, 3 pulses, 5 s each). The resulting suspension was diluted, spread on BHI, and grown at 37 °C for 48 h. All infections were performed in duplicate, and three replicates were performed for each infection.

J774A.1 cells were seeded onto 12 well plates two days prior to the assay with approximately $10^6$ cells per well. Approximately $1 \times 10^7$ *L. monocytogenes* CFU per well were used for infection (MOI of 10:1). Following 1 h incubation, the cells were washed twice with PBS, and fresh media containing gentamicin (1 μg/ml) was added to kill extracellular bacteria. At appropriately 1, 2, 3, 4, 5, 6, and 7 h post-infection, the cells were washed twice with PBS and lysed using 500 μl of cold 0.1% Triton X-100. The resulting suspension was diluted, spread on BHI agar, and grown at 37 °C for 48 h for CFU determinations. All infections were performed in duplicate, and three replicates were performed for each infection.

### 2.9. Catfish fillet attachment

Specific-pathogen-free channel catfish fingerlings were