Appl. Environ. Microbiol. doi:10.1128/AEM.01006-13

Title: Characterization of a Potential Virulence Factor in *Listeria monocytogenes* Associated with an Attachment to Fresh Produce

Dongryeoul Bae†, Keun Seok Seo, Ting Zhang, and Chinling Wang *

Department of Basic Sciences, College of Veterinary Medicine, Mississippi State University,

Running title: The attachment of *Listeria monocytogenes* on leafy vegetables

* Corresponding author.

E-mail: wang@cvm.msstate.edu

Phone: (662) 325 1683; Fax: (662) 325 1031

Mailing address: P.O. Box 6100, Department of Basic Sciences, College of Veterinary Medicine, Mississippi State University, Mississippi State, MS 39762

†Current address: Division of Microbiology, National Center for Toxicological Research, U.S. Food and Drug Administration, 3900 NCTR Rd., Jefferson, AR 72079

Key words: *Listeria*, surface proteins, attachment, cellulose, lettuce leaf, spinach, cantaloupe

ABSTRACT

A study to determine the attachment of *L. monocytogenes* serotype 4b strain F2365 on vegetables and fruits was conducted. In initial study, we screened 32 genes encoding surface proteins and lipases of the strain to find highly expressed genes on lettuce leaves. Results showed that transcription levels of LMOf2365_0413, LMOf2365_0498, LMOf2365_0859, LMOf2365_2052, and LMOf2365_2812 were significantly up-regulated on lettuce leaves. *In silico* analysis showed that LMOf2365_0859 contains a putative cellulose binding domain. Thus, we hypothesized that this gene may be involved in an attachment to vegetables and named *Listeria* cellulose-binding protein (*lcp*). *lcp* mutant (Δ*lcp*) and *lcp* complement (F2365::pMAD:*cat*:*lcp*) strains were generated by the homologous recombination. The attachment ability of a wild type (WT), Δ*lcp*, and a complemented strain to lettuce leaves was evaluated, indicating that the attachment of the Δ*lcp* to lettuce was significantly less than the WT and the complemented strain. Similar results were observed in baby spinach and cantaloupe. Fluorescence microscopy and field emission scanning microscopy analysis further support these findings. Binding ability of *L. monocytogenes* to cellulose was determined using cellulose acetate-coated plate. Results showed that a binding ability of Δ*lcp* was significantly lower than that of wild type. Combined, these results strongly suggest that LCP plays an important role in an attachment to vegetables and fruits.

INTRODUCTION

Listeria monocytogenes is a life-threatening food borne pathogen that attributed to an estimated rates of hospitalization (94.0 %) and mortality (15.9 %) in all food-borne illnesses (23). This pathogen is found in a natural environment and food and has an ability to survive under extreme conditions such as high acidity, low temperature, high osmolarity, and high hydrostatic pressure (1, 3, 10, 11). Most cases of human listeriosis have been linked to the consumption of ready-to-eat (RTE) products contaminated with *L. monocytogenes* serotype 1/2a, 1/2b, and 4b among the 13 serotypes (14, 27). Numerous studies have been conducted on the adhesion, invasion, and/or virulence regulation of *L. monocytogenes* in animal hosts and their derived food products (7, 9, 13). In particular, the roles of virulence and surface proteins (i.e. SigB, PrfA, ActA, InlA, InlB, InlC, InlH, or LPXTG family) of *L. monocytogenes* on pathogenesis have been well characterized in different hosts and cell types. These studies demonstrated that *L. monocytogenes* utilized a specific host-parasite interaction that is mediated by a specific interaction between listerial surface proteins and host cell receptors (8, 17, 19, 21, 22, 25).

The concern about the prevalence of *L. monocytogenes* in RTE foods has escalated due to food-borne outbreaks. Especially, the consumption of fresh or minimally-processed vegetables has been increasing every year. *L. monocytogenes* also has been detected on raw or minimally processed vegetables, such as cabbage, broccoli, bean sprouts, cucumber, lettuce, peppers, and potatoes in many countries (5). Furthermore, outbreaks of human listeriosis associated with ingestion of celery, tomatoes, lettuce, and shredded cabbage contaminated with the pathogen have been reported (15, 24). Importantly, a recent deadly outbreak of human listeriosis that led to 30 deaths and 1 miscarrage was caused by cantaloupes contaminated with *L. monocytogenes.* (http://www.cdc.gov/listeria/outbreaks/cantaloupes-jensen-farms/index.html). These studies suggest that the prevention of human listeriosis caused by the ingestion of vegetables is an important challenge in the food industry and public health.

Despite of the increase in consumption of vegetables and outbreaks of human listeriosis associated with their ingestion, very limited study has been done regarding the survival, growth, and interaction of *L. monocytogenes* on vegetables compared to animal hosts and their derived RTE foods. Interestingly, we found that the transcription levels of genes encoding listerial surface proteins were highly increased when *L. monocytogenes* was cultured on vegetables. Of interest, one of the up-regulated genes (LMOf2365_0859) contains a putative cellulose binding domain. We named this gene as *Listeria* cellulose binding protein (*lcp*) and investigated the role of LCP in attachment to vegetables and fruits.

MATERIALS AND METHODS

Bacterial strains and growth condition

L. monocytogenes serotype 4b F2365 (wild type), *lcp* deletion mutant (Δ*lcp*), and complemented F2365::pMAD:*cat*:*lcp*) strains were grown overnight in brain-heart infusion (BHI) broth (Difco Laboratories, Detroit, MI). For inoculums, overnight cultures were washed twice with PBS and resuspended in PBS. *Escherichia coli* DH5α were grown in Luria-Bertani (LB) broth.

Vegetable and fruit preparation

Fresh iceberg lettuce, bagged baby spinach, and cantaloupes were purchased from a local retail grocery, stored at 4°C and used within two days. The adaxial side of approximately five inner leaves of iceberg lettuce (5 × 5 cm for quantitative real time PCR or 1 × 1 cm) and baby spinach (1 × 1 cm) was used. Cantaloupe skin (1 × 1 cm) was cut into thin pieces. The leaf pieces were washed with sterile phosphate buffered saline (PBS, pH 7.4, Invitrogen, Gland Island, NY) three times before inoculation. Data from the samples that were negative for bacterial culture were used.

Quantitative real time PCR

Fresh iceberg lettuce was inoculated with a wild type F2365 strain (1.55×10^7 CFU), and maintained in Whirl-Pak bags (Nasco, Fort Atkinson, WI) at 4°C for up to 16 h. Parallel cultures in PBS were used as controls. The reason the PBS was chosen as a control because the number of the bacterial cells grown on vegetable surface was very similar to that of strain grown in PBS up to 24 h at 4°C. The bacteria on lettuce leaves were gently washed using 25 ml of PBS. Supernatant containing unattached bacterial cells was discarded. Finally, the bacterial cells from inoculated lettuce were collected at 8 h and 16 h by vigorously vortexing using a Mini Vortex Mixer (VWR, Radnor, PA). Total RNA was purified using Trizol (Invitrogen, Carlsbad, CA) and Lysing Matrix B tube (MP Biomedicals, Solon, Ohio) as described previously (4). The concentration of total RNA was measured using a Nanodrop ND1000 UV-Vis spectrophotometer (Nonodrop Technologies, Wilmington, DE) and agarose gel electrophoresis**.** cDNAs were synthesized from 1 μg of the total RNA from bacteria grown on lettuce leaves or control cells using a cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) as per manufacturer’s instructions. Primers specific to surface proteins were designed using Primer Express Version 3.0 software (Applied Biosystems, Supplementary Table 1, S1). Quantitative real time PCR (qRT-PCR) were performed with Power SYBR® Green PCR Master Mix

(Applied Biosystems) and 400 nM of forward and reverse primers in a 25 µl final reaction volume using a Mx3005P Real-Time PCR System (Stratagene Inc., La Jolla, CA) under temperature cycles as follows; initially incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 15 sec. *gap* gene was used as a reference to normalize the data. The relative transcription of the target genes in F2365 attached on lettuce compared to the control (PBS culture) were calculated by using the ΔΔCt method as described previously (4).

***In silico* analysis**

The protein domains of LMOf2365_0859 (accession number: Q721X5) were predicted using a NCBI 3D molecular structure database with the protein GenInfo Identifier number 46907073. The prediction of the protein domains is available at http://www.ncbi.nlm.nih.gov/Structure/cblast/cblast.cgi. ClustalW2 software provided from the European Molecular Biology Laboratory's European Bioinformatics Institute (EMBL-EBI) and ESPript 2.2 (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi) were used to align the amino acid sequences of a cellulose binding domain (CBD) with those of Endoglucanase D of *Clostridium cellulovorans,* which show similar homolog sequences as generated by the National Center for Biotechnology Information (NCBI) database.

Construction of *lcp* deletion mutant

A *lcp* deletion mutant was generated by allelic replacement as described previously with slight modifications (2). Briefly, a chloramphenicol resistant gene, coding for chloramphenicol acetyltransferase (cat), was amplified from pMK4 (26) using catF/R primers (S1), inserted to pMAD *Sal*I and *Eco*RI sites, and formed pMAD_*cat*. Furthermore, regions flanking LMOf2365_0859 (*lcp*) gene were amplified using 0859UF/R and 0859DF/R primers (S1), then inserted to pMAD_*cat* *Bam*HI and *Sal*I and *Eco*RI and *Bgl*II sites, respectively, generated pMAD_*lcp*. The pMAD_*lcp* was transformed into *L. monocytogenes* F2365 competent cellsusing electroporation. The transformation mixture was plated on BHI agar plate containing chloramphenicol (10 µg/ml) or erythromycin (5 µg/ml) and incubated at 43°C overnight as the first integration. The generated intergrant as a merodiploid (F2365::pMAD:*cat*:*lcp*) was used as a complemented strain. To promote the second integration, a single colony was transferred to BHI broth and cultured overnight. The culture was plated on BHI agar contained with chloramphenicol and erythromycin, respectively. The second integrants were resistant to chloramphenicol and erythromycin. The *lcp* deleted mutant (Δ*lcp*) that was resistant to chloramphenicol, but susceptible to erythromycin was selected. The *lcp* deleted intergrant (Δ*lcp*) was confirmed using PCR with 0859F/R primers. Supplementary Table S1 shows strains and plasmids used in this study.

Vegetables and fruit attachment assay

Vegetable leaves and cantaloupe skins were inoculated with the wild type, Δ*lcp*, and the complemented strains to make 1 × 10^5 CFU/cm^2 and incubated for 2 h at room temperature. Inoculated samples were washed three times with PBS by gentle vortex. Supernatant containing unattached bacteria was discarded. Inoculated samples were homogenized using a mortar and pestle and the number of attached bacteria was determined by a standard plate count. The % adherence was calculated as (the number of bacteria adhered to vegetables/the number of bacteria in the inocula) × 100.

Field emission scanning electron microscopy (FESEM) analysis

The leaves were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at 4°C after the bacterial inoculation as described above. The fixed leaves were then rinsed, post fixed in 2% osmium tetraoxide in 0.1 M sodium cacodylate buffer, dehydrated in a graded ethanol series, and dried using critical point drying method in a Polaron Critical Point Dryer (Quorum Technologies, Newhaven UK). Dried specimens were mounted on aluminum stubs with carbon adhesive, and coated with platinum using an ES150T ES sputter coater (Electron Microscopy Science, Hatfield, PA). The attachment of WT, Δ*lcp,* and F2365::pMAD:*cat*:*lcp* strains was detected using a JEOL JSM-6500F scanning electron microscope (JEOL USA, Peabody, MA) at 5kv.

Fluorescence microscopy analysis

The wild type, mutant, and complemented strains were labeled with 5-(and -6)- carboxy-fluorescein diacetate succinimidyl ester (CFSE, 5 nM in final concentraton) (Molecular Probes, Eugene, OR) as described previously (16). The lettuce leaves inoculated with labeled bacteria treated as described in attachment assay were mounted with Vectashield Mounting Medium

(Vector Labs, Burlingame, CA) on glass slides. Attached bacteria were observed using a fluorescence microscope (Nickon, Tokyo, Japan) with FITC filter at 20 × magnification.

Cellulose binding assay

Ninety-six-well plates (Nalge NuncInternational, Rochester, NY) were coated with 1% (w/v) cellulose acetate (Sigma- Aldrich, St. Louise, MO) dissolved in glacial acetic acid according to the method of Wierzba et al (28). Cellulose acetate-coated wells were washed with PBS three times using an orbital shaker (Lab-Line Instruments, Inc., Melrose Park, IL). Bacterial suspensions (about 1.0×10^6 CFU) in 100 μl of PBS were added in each well and incubated for 16 h at room temperature. After washing three times with PBS using a 3-D rotator (Lab-Line Instrument Inc.), attached bacteria were stained with 0.5% (w/v) crystal violet solution (BD Biosciences, Sparks, MD). After washing three times with PBST (PBS containing 0.05% Tween 20), the OD of each well was measured at 590 nm using a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA).

Statistical Analysis

Statistical significance of qRT-PCR data were analyzed by Student's *t*-test. Attachment and cellulose binding results were analyzed by ANOVA test. All data were analyzed using SAS version 9.1.3 program (SAS Institute, Cary, NC).

RESULTS

Transcription of surface protein and lipase genes and *in silico* analysis

To identify transcriptional changes in response to an attachment to lettuce, transcription levels of 32 genes encoding listerial surface proteins and lipases were measured after incubation for 8 and 16 h, respectively. qRT-PCR results showed that transcription of five genes (LMOf2365_0413, LMOf2365_0498, LMOf2365_2052, LMOf2365_0859, and LMOf2365_2812) were up-regulated (Fig. 1). *In silico* analysis showed that LMOf2365_0859 contains a putative CBD (at position 20-144 aa), 7 Bacterial Ig-like domains (Big_3), and LPXTG motif (a conserved sorting signal domain at carboxyl-terminal) (Fig. 2). A putative CBD found in LMOf2365_0859 was similar to a CBD of Endoglucanase D found in *Clostridium cellulovorans*, which acts to bind to cellulose, a major component of plant cell wall. The sequence and structure alignment was generated (Fig. 2). Most of *Listeria* spp. seems to have LCP(s) based on the pan-genomic sequence analysis (Supplementary Fig.1, S2). Supplementary Figure 2 (S3) showed that F2365 seems to have two LCP paralogs (LMOf2365_0859 and _1974) with high amino acid sequence identity (>60%). The identity matrix grouping of different serotypes with the threshold of the 90% identity is shown in Supplementary Figure 3 (S4). Data revealed that LCPs within the same serotype showed >99% identity. In particular, LCPs from *L. monocytogenes* serotype 4b strains showed >98% amino acid sequence identity, but < 86% identity with other serotypes.

Generation of *lcp* deletion mutant and bacterial growth

In order to determine the role of LCP in an attachment to vegetables, a *lcp* deletion mutant (Δ*lcp*) and a complemented strain (F2365::pMAD:*cat*:*lcp*) were generated (Fig. 3). A deletion mutant and complemented strains were confirmed by PCR with primers (0859F/R) annealed to the deletion region of a mutant strain, showing a specific amplification product from the wild type and complemented strains and not from a deletion mutant (Fig. 4a). We determined if a deletion of *lcp* gene affects bacterial growth kinetics in BHI broth. Results showed that bacterial growth of the WT strain in BHI was not different than those of Δ*lcp* and F2365::pMAD:*cat*:*lcp* (Fig. 4b).

The role of LCP on attachment to lettuce leaves

The most abundant components of plant cell wall are cellulose, hemicellulose, pectin, and lignin. We speculated that LCP plays an important role in an attachment to vegetables due to the possession of a putative CBD. To test this possibility, fresh iceberg lettuce leaves were inoculated with 1×10^5 CFU/cm^2 of the WT, Δ*lcp*, or F2365::pMAD:*cat*:*lcp* . Results showed that % of adherence by the WT (2.97% ± 0.37) was significantly higher than that by the Δ*lcp* (0.3% ± 0.05) (Fig. 5a, $p < 0.001$). The F2365::pMAD:*cat*:*lcp* showed similar % of adherence (3.03% ± 0.14) to the WT. In consistence with bacterial count data, fluorescence microscope analysis showed more WT bacteria (green dots) than the Δ*lcp*. The F2365::pMAD:*cat*:*lcp* showed the same amount of bacteria as the WT (Fig. 5b). FESEM analysis showed that the WT bacteria were evenly distributed and attached to the surface of leaves, and there is no any preferential attachment site on the leaves (Fig. 5c). The results strongly indicate that LCP may be an important adherence factor to vegetable leaves.

The role of LCP in an attachment to baby spinach and cantaloupe

The attachment interaction between LCP and vegetable leaf was further characterized if LCP is a generalized attachment factor of *L. monocytogenes* to the surface of plants. Baby spinach leaves and cantaloupe skins were used. The WT strain showed the percentage (%) attachment to baby spinach leaves was 6.62% ± 2.59, whereas the numbers of Δ*lcp* attached to spinach leaves was significantly lower than that of the WT strain, showing 0.64% ± 0.13 (Figure 6a). Of interest, overall attachment of WT, Δ*lcp*, and F2365::pMAD:*cat*:*lcp* strains to cantaloupe skins was 19.17% ± 4.05, 3.25% ± 0.88, and 17.12% ± 2.59, respectively (Figure 6b).

Cellulose binding assay

In order to determine the mechanism by which *L. monocytogenes* attaches to vegetables and fruit through interaction between LCP and cellulose, cellulose binding assay was performed using 1% (w/v) cellulose acetate-coated 96-well plates. Attached bacteria were quantified by staining with 0.5% (w/v) crystal violet, followed by measuring absorbance at 590 nm (OD590). Results showed that the OD590 value of the WT (0.189 ± 0.014) was significantly higher than that of Δ*lcp* (0.110 ± 0.005) (Fig. 7).

DISCUSSION

The consumption of vegetables has annually increased, and the prevalence of *L. monocytogenes* in raw and RTE vegetables has been shown to be high when compared to other RTE foods (5). Even though vegetables have been considered as vehicles for listeriosis, there is no clear evidence that *L. monocytogenes* can survive and/or grow on leafy vegetables. Furthermore, studies for the attachment or colonization of *L. monocytogenes* on vegetables at the molecular level have been less conducted. Thus, this study tried to elucidate the function of gene encoding a listerial surface protein involved in adherence on the surface of vegetables and fruits. In order to discover listerial genes associated with adhrence on vegetable leaves, genes encoding surface proteins with LPXTG motif and lipases were screened using qRT-PCR. Of the up-regulated genes, LMOf2365_0859, *lcp*, was targeted for a carbohydrate-protein interacton based on mRNA expression level and a functional protein domain. LMOf2365_0859 deletion mutant showed significant decrease in an attachment to iceberg lettuce. Thus, we further characterized *lcp* as a generalized attachment factor to vegetables and fruits.

In silico analysis shows that LMOf2365_0859 contains a putative cellulose binding domain (CBD), 7 Bacterial Ig-like domains (Big_3), and LPXTG motif (a conserved sorting signal domain at carboxyl-terminal). Most listerial surface proteins containing LPXTG motif have Big_3 domains, which may interact with carbohydrates on the surface of host cells (6). Interestingly, the NCBI CBLAST module presents that LCP has a similar sturcture and amino acid sequences to a CBD of the Endoglucanase D in *Clostridium cellulovorans* (S2). Previous studies on the degradation of plant cell wall have conducted for an interaction between bacterial cellulosome (i.e. extracellular enzyme complexes) and plant cell wall (12). Cellulosome consists of scaffolding proteins and degrading enzymes associated with carbohydrate degradation. A CBD, a component of scaffolding protein, has been found in various aerobic or anaerobic bacteria and fungi. Therefore, the degradation of plant cell wall by the activity of cellulosome in various microorganisms may imply that the CBD in *L. monocytogenes* has also a potential binding property to the surface of vegetable leaves or fruits.

The difference in attachment ability between the wild type and Δ*lcp* at 4C was significant but the difference was approximate two-fold (data not shown) in comparison to a 10-fold change at 22C. The growth of Δ*lcp* or F2365::pMAD:*cat*:*lcp* was not different from the WT, indicating that the deletion of *lcp* gene did not affect bacterial growth under an optimal conditions. The attachment assay of listerial strains on fresh iceberg lettuce, spinach, and cantaloupe skin showed that the % attachment of the WT strain was much higher in cantaloupes than in leafy vegetables. It may be related to a physical difference by a rough surface or a component of cantaloupe skin compared to the content of dietary fiber (including cellulose) in iceberg lettuce (1.25 g fiber/100 g leaf) and spinach (2.33 g fiber/100 g leaf) (www.ars.usda.gov/Services/docs.htm?docid=22114) (20). In addition, a recent study has demonstrated that the numbers of *Salmonella typhimurium* attached on romaine lettuce varied in the different regions and with ages of the leaves (18). Therefore, bacterial attachment or colonization can be considered to be mediated by different compositions of plant tissue or cell wall components in various vegetables, sites, or ages.

The use of merodiploid as a complement strain in this study has several benefits than the plasmid complementation. Firstly, the merodiploid strain has a single copy of the wild-type gene. Therefore, there is no concern about over-complementation which often observed in the plasmid based complementation method making multiple copies of gene complementation. Secondly, the merodiploid strain has a single copy of the wild-type gene at the same location as the wild type strain. Therefore, if there is a regulatory element near by the wild type gene, it would most likely to be regulated as in the wild type strain.

The pan-genomic sequence analysis revealed that most of *Listeria* spp. except for a few species such as *L. grayi* seem to have the LCP(s). All *L. monocytogenes* strains seem to have LCP, regardless of the serotype (S2). Therefore, LCP seems to be a core gene existing in all *L.*

monocytogenes strains, implicating its functional importance. Previous studies have shown that a LCP ortholog, lmo0842 from *L. monocytogenes* EGD-e, was associated with a virulence or adherence to a human cell (6). However, LCPs have not been reported in other *L. monocytogenes* strains or serotypes that functions as the LMOf2365_0859. This is the first report elucidating the functional involvement of LCP(s) in *L. monocytogenes* as an attachment factor to the surface of vegetables and fruits. Figures 5, 6, and 7 showed that Δ*lcp* strain still bound to the surface of vegetable leaves, nevertheless the adhesive ability of LCP to vegetables or cellulose was disrupted by the deletion of *lcp* in F2365 strain. F2365 has two LCP paralogs (LMOf2365_0859 and _1974) as shown in figures S3 and S4 and both of the genes were up-regulated (Fig. 1). Based on the identity matrix of LCP paralogs, *L. monocytogenes* strains have low gene redundancy (one or two copies per genome) in the genomes. The partial adhesive ability of Δ*lcp* to the surface of vegetable leaves or cellulose may be due to functional redundancy between the LCP paralogs in F2365 strain.

For cellulose binding assay, we coated 96-well plates with commercially available cellulose or its derivatives such as cellulose acetate, methyl cellulose, and cellulose microcrystalline. Except for cellulose acetate, all cellulose derivatives yielded a high background of non-specific crystal violet staining or were detached from the wells during washing steps, consequently generating inconsistent results. 96-well plates coated with 1% (w/v) cellulose acetate dissolved in glacial acetic acid was given the satisfactory results as described by Wierzba et al (28). Binding assay data, showing the interaction between a listerial surface protein encoded by *lcp* and cellulose, a major component of plant cell walls, suggest that LCP may have a strong binding activity to the surface of vegetables. The OD values of all the strains in wells coated with cellulose acetate were consistent. In addition, the OD values of all the strains in the coated wells were significantly higher than that of all strains in the wells without coating (data not shown), indicating an interaction of listerial surface proteins with cellulose acetate. It is noteworthy that the fold difference in OD590 between the wild type and Δ*lcp* (approximately 1.72 times higher in a wild type strain) was less than the % attachment difference between them (10 times higher in the wild type strain), suggesting LCP might interact with other plant cell wall components such as semicellulose.

In conclusion, LCP harboring a CBD may play a major role in an attachment to vegetables and fruits. Additionally, cantaloupes may be considered as a potent vehicle for transmitting *L. monocytogenes*.

ACKNOWLEDGMENTS

This work was supported by grants from USDA/ARS (#58-6402-7-230) and the Mississippi Agriculture and Forestry Experiment Station. We thank Dr. Stephen Pruett for his valuable review and suggestions. We also thank Ms. Amanda Lawrence for FESEM and imaging analyses.

REFERENCES

1. **Ananth, V., J. S. Dickson, D. G. Olson, and E. A. Murano.** 1998. Shelf life extension, safety, and quality of fresh pork loin treated with high hydrostatic pressure. J Food Prot **61:**1649-1656.

2. **Arnaud, M., A. Chastanet, and M. Debarbouille.** 2004. New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, gram-positive bacteria. Appl Environ Microbiol **70:**6887-6891.

3. **Bae, D., C. Liu, T. Zhang, M. Jones, S. N. Peterson, and C. Wang.** 2012. Global Gene Expression of *Listeria monocytogenes* to Salt Stress J Food Prot **75:**906-912.

4. **Bae, D., M. R. Crowley, and C. Wang.** 2011. Transcriptome analysis of *Listeria monocytogenes* grown on a ready-to-eat meat matrix. J Food Prot **74:**1104-1111.

5. **Beuchat, L. R.** 1996. Pathogenic microorganisms associated with fresh produce. J. Food Protect **59:**204-216.

6. **Bierne, H., and P. Cossart.** 2007. *Listeria monocytogenes* surface proteins: from genome predictions to function. Microbiol Mol Biol Rev **71:**377-397.

7. **Burnett, S. L., Mertz, E. L., Bennie, B., Ford, T., and Starobin, A.,.** 2005. Growth or survival of *Listeria monocytogenes* in ready-to-eat meat products and combination deli salads during refrigerated storage. J. Food Sci. **70:**m301-m304.

8. **Chaturongakul, S., and K. J. Boor.** 2006. SigmaB activation under environmental and energy stress conditions in *Listeria monocytogenes*. Appl Environ Microbiol **72:**5197-5203.

9. **Chaturongakul, S., S. Raengpradub, M. Wiedmann, and K. J. Boor.** 2008. Modulation of stress and virulence in *Listeria monocytogenes*. Trends Microbiol **16:**388-396.

10. **Chen, H.** 2007. Temperature-assisted pressure inactivation of *Listeria monocytogenes* in turkey breast meat. Int J Food Microbiol **117:**55-60.

11. **Cole, M. B., M. V. Jones, and C. Holyoak.** 1990. The effect of pH, salt concentration and temperature on the survival and growth of *Listeria monocytogenes*. J Appl Bacteriol **69:**63-72.

12. **Doi, R. H., and A. Kosugi.** 2004. Cellulosomes: plant-cell-wall-degrading enzyme complexes. Nat Rev Microbiol **2:**541-551.

13. **Drevets, D. A., M. J. Dillon, J. S. Schawang, N. Van Rooijen, J. Ehrchen, C. Sunderkotter, and P. J. Leenen.** 2004. The Ly-6Chigh monocyte subpopulation transports Listeria monocytogenes into the brain during systemic infection of mice. J Immunol **172:**4418-4424.

14. **Goulet, V., C. Jacquet, P. Martin, V. Vaillant, E. Laurent, and H. de Valk.** 2006. Surveillance of human listeriosis in France, 2001-2003. Euro Surveill **11:**79-81.

15. **Ho, J. L., K. N. Shands, G. Friedland, P. Eckind, and D. W. Fraser.** 1986. An outbreak of type 4b Listeria monocytogenes infection involving patients from eight Boston hospitals. Arch Intern Med **146:**520-524.

16. **Joyce, S. A., and C. G. Gahan.** 2010. Molecular pathogenesis of *Listeria monocytogenes* in the alternative model host Galleria mellonella. Microbiology **156:**3456-3468.

17. **Kazmierczak, M. J., M. Wiedmann, and K. J. Boor.** 2006. Contributions of *Listeria monocytogenes* sigmaB and PrfA to expression of virulence and stress response genes during extra- and intracellular growth. Microbiology **152:**1827-1838.

18. **Kroupitski, Y., R. Pinto, E. Belausov, and S. Sela.** 2011. Distribution of *Salmonella typhimurium* in romaine lettuce leaves. Food Microbiol **28:**990-997.

19. **Marino, M., M. Banerjee, R. Jonquieres, P. Cossart, and P. Ghosh.** 2002. GW domains of the *Listeria monocytogenes* invasion protein InlB are SH3-like and mediate binding to host ligands. EMBO J **21:**5623-5634.

20. **Nutrient Data Laboratory, A. R. S., US Department of Agriculture.** 2011. USDA Nutrient database for standard reference, release 24. Washinton, DC: US Department of Agriculture,.

21. **Roberts, A. J., S. K. Williams, M. Wiedmann, and K. K. Nightingale.** 2009. Some *Listeria monocytogenes* outbreak strains demonstrate significantly reduced invasion, *inlA* transcript levels, and swarming motility in vitro. Appl Environ Microbiol **75:**5647-5658.

22. **Sabet, C., M. Lecuit, D. Cabanes, P. Cossart, and H. Bierne.** 2005. LPXTG protein InlJ, a newly identified internalin involved in *Listeria monocytogenes* virulence. Infect Immun **73:**6912-6922.

23. **Scallan, E., R. M. Hoekstra, F. J. Angulo, R. V. Tauxe, M. A. Widdowson, S. L. Roy, J. L. Jones, and P. M. Griffin.** 2011. Foodborne illness acquired in the United States--major pathogens. Emerg Infect Dis **17:**7-15.

24. **Schlech, W. F., 3rd, P. M. Lavigne, R. A. Bortolussi, A. C. Allen, E. V. Haldane, A. J. Wort, A. W. Hightower, S. E. Johnson, S. H. King, E. S. Nicholls, and C. V. Broome.** 1983. Epidemic listeriosis--evidence for transmission by food. N Engl J Med **308:**203-206.

25. **Suarez, M., B. Gonzalez-Zorn, Y. Vega, I. Chico-Calero, and J. A. Vazquez-Boland.** 2001. A role for ActA in epithelial cell invasion by *Listeria monocytogenes*. Cell Microbiol **3:**853-864.

26. **Sullivan, M. A., R. E. Yasbin, and F. E. Young.** 1984. New shuttle vectors for *Bacillus subtilis* and *Escherichia coli* which allow rapid detection of inserted fragments. Gene **29:**21-26.

27. **Swaminathan, B., and P. Gerner-Smidt.** 2007. The epidemiology of human listeriosis. Microbes Infect **9:**1236-1243.

28. **Wierzba, A., U. Reichl, R. F. Turner, R. A. Warren, and D. G. Kilburn.** 1995. Adhesion of mammalian cells to a recombinant attachment factor, CBD/RGD, analyzed by image analysis. Biotechnol Bioeng **46:**185-193.

Table 1.

Strains and plasmids used in this study

Strain or plasmid	**Description**	**Reference or source**
Strains		
E. coli DH5α	Cloning host	Invitrogen
L. monocytogenes F2365	Wild type of serotype 4b strain	this study
Δ*lcp*	LMOf2365_0859 deletion mutant strain, Cm^r	this study
F2365::pMAD:*cat*:*lcp*	Complementation of LMOf2365_0859 deletion mutant strain, Er^r, Cm^r	this study
Plasmids		
pMK4	Shuttle vector (5.585 kb) harboring *bla* and *cat*, Ap^r, Cm^r	(26)
pMAD	Temperature sensitive shuttle vector (9.666 kb), Er^r	(2)
pMAD_*cat*	pMAD derivative containing *cat*, Er^r, Cm^r	this study
pMAD_*lcp*	pMAD_*cat* derivative allowing deletion of LMOf2365_0859, Er^r, Cm^r	this study

FIGURE LEGENDS

Figure 1. The transcription levels of genes encoding listerial surface proteins and lipases. The expression levels of 32 genes encoding surface proteins and lipases in *L. monocytogenes* colonized on lettuce leaf was measured at 8 and 16 h after incubation. LMOf2365_0413, LMOf2365_0498, LMOf2365_2052, LMOf2365_0859, and LMOf2365_2812 were up-regulated at the time points. The *gap* was used as a control gene. Transcription levels were expressed as log2. Data were obtained from three independent experiments using triplicate RNA samples per each experiment ($n = 9$). Data were analyzed by Student's *t*-test. Bars represent SEM.

Figure 2. *In silico* analysis for the up-regulated genes of *L. monocytogenes* on lettuce leaf. JCVI annotation file, KEGG database, and NCBI CBLAST module were used to select a candidate gene for the listerial attachment on lettuce leaf. Database obtained from CBLAST showed that LCP (2027 aa) contains a putative CBD (at position 20-144 aa), 7 Bacterial Ig-like domains (Big_3), and LPXTG motif (a). The surface protein has amino acid sequences similar to a CBD of Endoglucanase D from *Clostridium cellulovorans*. ClustalW2 and ESPript 2.2 softwares were used to generate the sequence alignment (b).

Figure 3. Construction of in-frame deletion *lcp* mutant. The PCR products from adjacent the 5′ and 3′ flanking regions of LMOf2365_0859 were amplified. The generated up- and down-DNA fragments were digested with *BamH*I and *Sal*I and *EcoR*I and *Bgl*II, respectively. Each DNA fragment with pMAD_*cat* digested with the same restriction enzymes was ligated.

pMAD_*lcp* was transformed into *L. monocytogenes* F2365. The recombinant plasmid was incorporated into the chromosome of F2365 by 1st homologous recombination at 43°C. F2365 retaining chromosome incorporated with pMAD_*lcp* was subcultured at 30°C to select deletion mutant mediated through 2nd homologous recombination. The complementation of F2365::pMAD:*cat*:*lcp* was generated after first incorporation of the recombinant plasmid into the chromosome of F2365.

Figure 4. Confirmation of *lcp* deletion mutant and bacterial cell growth. Wild type, Δ*lcp* and complemented strains were confirmed by PCR using primers (0859F/R) designed from a region of deleted gene. The size of PCR product is 180 bp (a). Wild type, Δ*lcp*, and F2365::pMAD:*cat*:*lcp* strains were grown in BHI broth for 24 h at 37 °C with 180 rpm. The growth kinetics for bacterial strains was measured at 2, 4, 8, 16, and 24 h by a standard plate count. Data were obtained from three independent experiments using duplicate bacterial samples per each experiment ($n = 6$). Data were analyzed by ANOVA. Bars represent SEM (b).

Figure 5. Attachment assay and the detection for all strains on lettuce leaves. Attached wild type, Δ*lcp*, and F2365::pMAD:*cat*:*lcp* strains on lettuce leaves were homogenized and the homogenates were plated on BHI agar plates. Data were obtained from three independent experiments using triplicate bacterial samples per each experiment ($n = 9$). The difference in the percentage of attached bacteria to total bacterial numbers inoculated on lettuce leaves was analyzed by ANOVA. Bars represent SEM (a). The symbol (*) represents a significant difference ($P < 0.05$) between wild type/complement and Δ*lcp*. All strains labeled with CFSE (5

nM) revealed under fluorescent microscope (Nickon, Tokyo, Japan) at 20× magnification with FITC filter (b). Attached WT, Δ*lcp,* and F2365::pMAD:*cat*:*lcp* strains were observed under a JEOL JSM-6500F scanning electron microscope (JEOL USA, Peabody, MA) at 5kv (c).

Figure 6. The attachment of *L. monocytogenes* strains on spinach and cantaloupe. Baby spinach leaves and cantaloupe skins inoculated with the wild type, Δ*lcp* or F2365::pMAD:*cat*:*lcp* were homogenized and the homogenates were plated on BHI agar plates. Data were obtained from three independent experiments using triplicate bacterial samples per each experiment (n = 9). The difference in the percentage of attached bacteria to total bacterial numbers inoculated on spinach leaves (a) and cantaloupe skins (b) was analyzed by ANOVA. Bars represent SEM. The symbol (*) represents a significant difference ($P < 0.05$) between wild type/complement and Δ*lcp*.

Figure 7. The comparion of the binding ability of wild type, Δ*lcp* or F2365::pMAD:*cat*:*lcp* strains to cellulose. 96-well plates were coated with 1% (w/v) cellulose acetate. The adhesion of *L. monocytogenes* to cellulose acetate using 0.5% (w/v) crystal violet was measured using a microplate reader at OD590. Three independent experiments with triplicate bacterial samples per each experiment were used. The difference in OD values was analyzed by ANOVA. The symbol (*) represents a significant difference ($P < 0.05$) between type/complement and Δ*lcp*.

FIGURES

Fig 1.

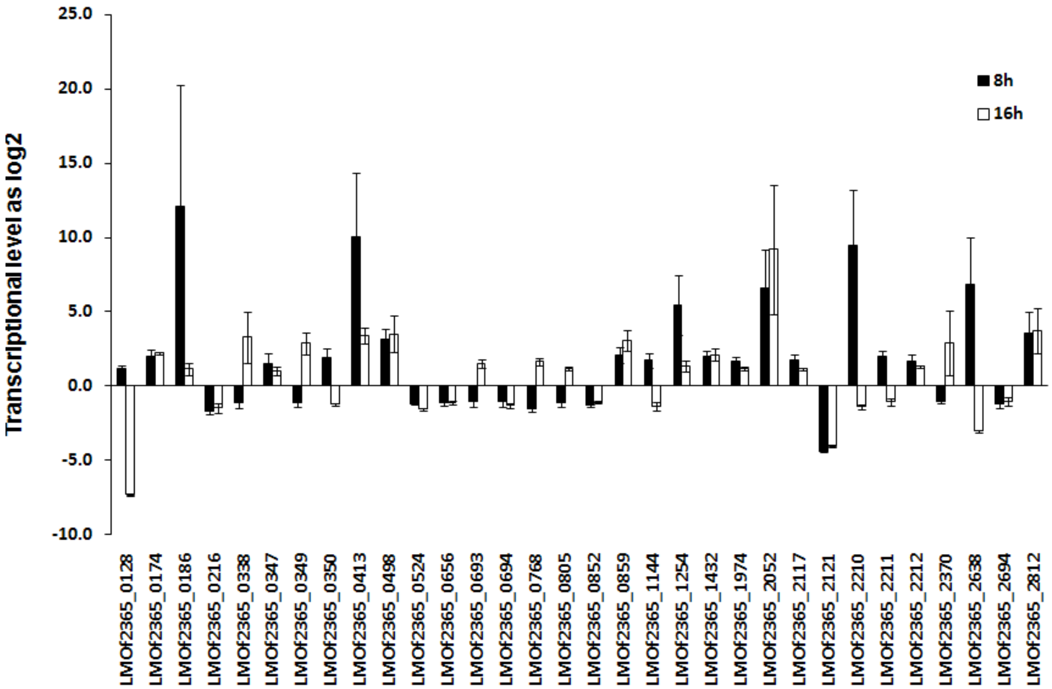


Fig 2.

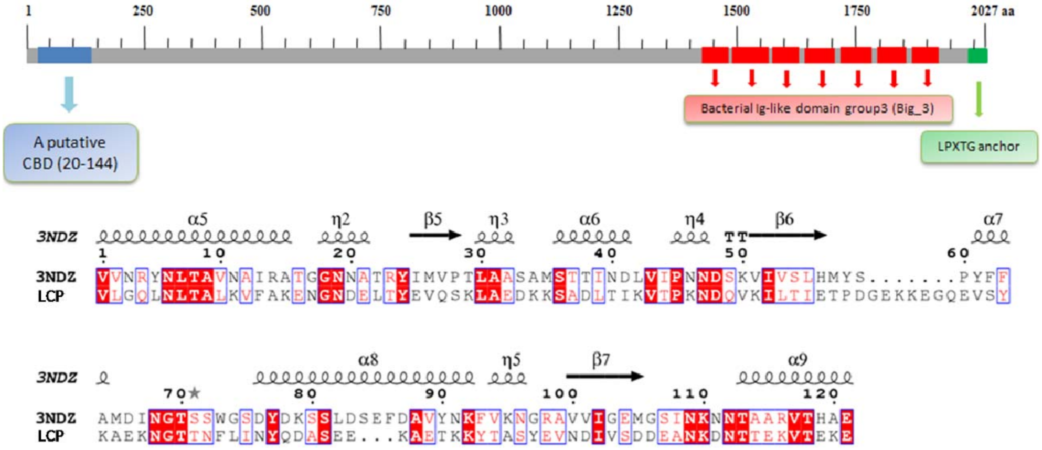

1
250
500
750
1000
1250
1500
1750
2027 aa
A putative CBD (20-144)
Bacterial Ig-like domain group3 (Big_3)
LPXTG anchor
3NDZ
α5 η2 β5 η3 α6 η4 TT β6 α7
1 10 20 30 40 50 60
3NDZ VVNRYNLTAVNAIRATGGNNATRYIMVPTLAASAMSTTINDLVIPNNDSKVIVSLHMYS.......PYFF
LCP VLGQLNLTALKVFAKENGNDELTYEVQSKLAEDKKSADLTIKVTPKNDQVKILTIETPDGEKKEGQEVSY
3NDZ
α8 η5 β7 α9
70 80 90 100 110 120
3NDZ AMDINGTSSWGSDYDKSSLDSEFDAVYNKFVKNGRAVVIGEMGSINKNNTAARVTHAE
LCP KAEKNGTTNFLINYQDASEE...KAETKKYTASYEVNDIVSDDEANKDNTTEKVTEKE

Fig 3.

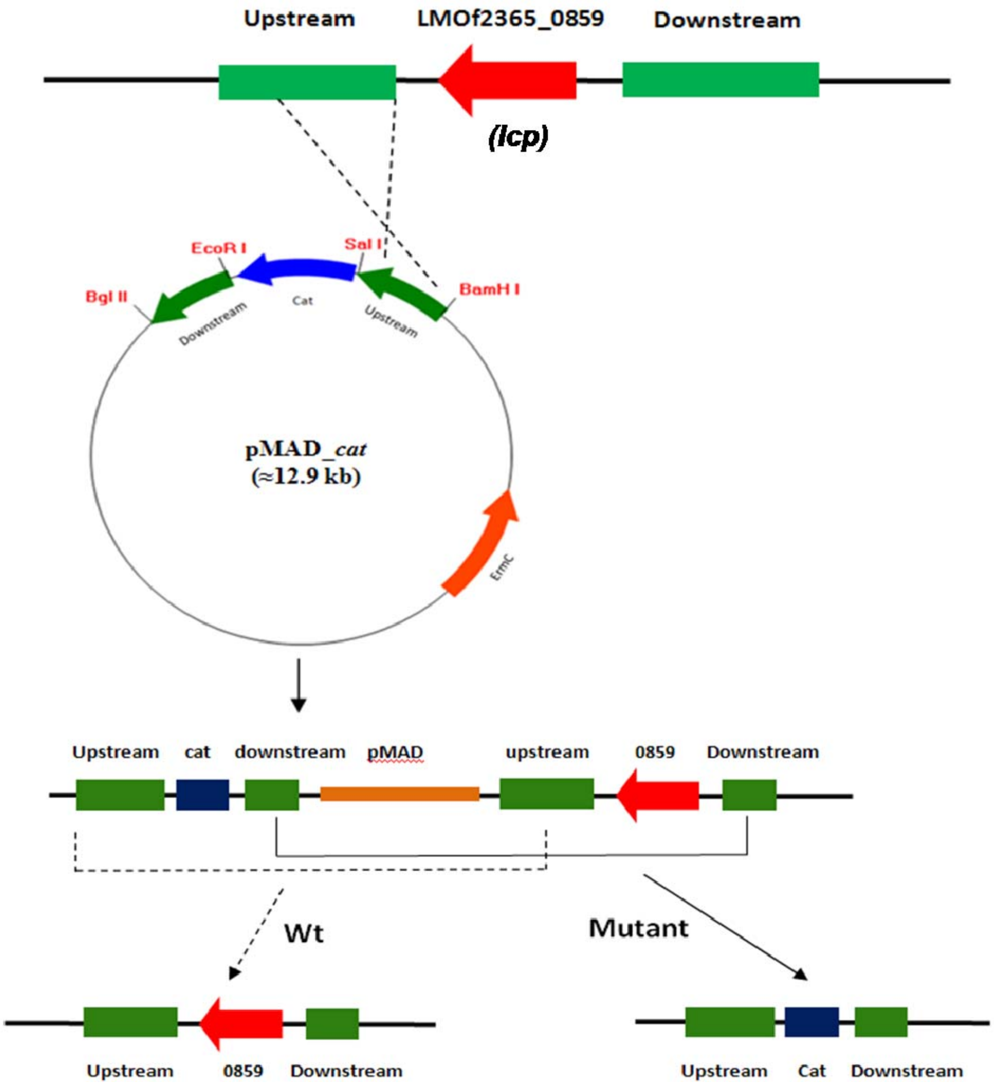

Upstream
LMOf2365_0859
Downstream
(lcp)
EcoR I
Sal I
Bgl II
BamH I
Cat
Downstream
Upstream
pMAD_cat
(≈12.9 kb)
ErmC
Upstream
cat
downstream
pMAD
upstream
0859
Downstream
Wt
Mutant
Upstream
0859
Downstream
Upstream
Cat
Downstream

Fig 4.

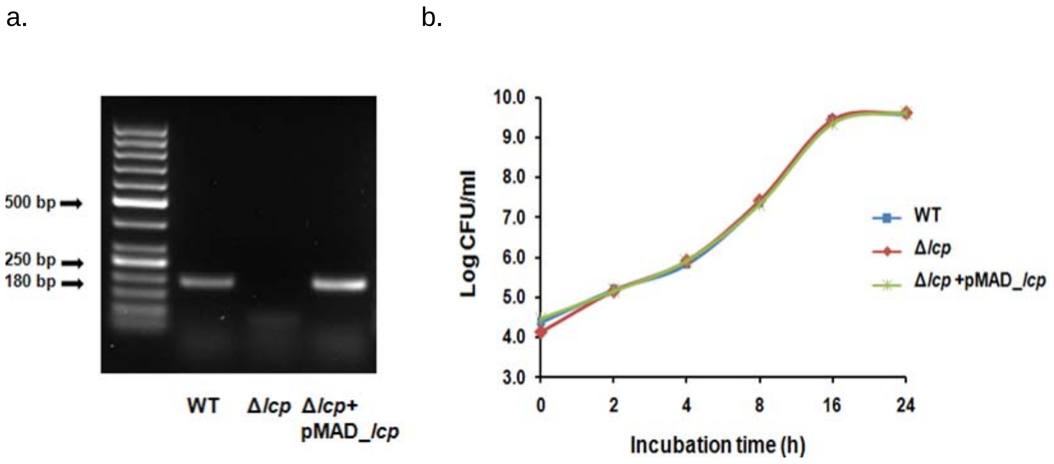

a.
b.
500 bp
250 bp
180 bp
WT
Δ*lcp*
Δ*lcp*+ pMAD_*lcp*
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
Log CFU/ml
0
2
4
8
16
24
Incubation time (h)
WT
Δ*lcp*
Δ*lcp* +pMAD_*lcp*

Fig 5.

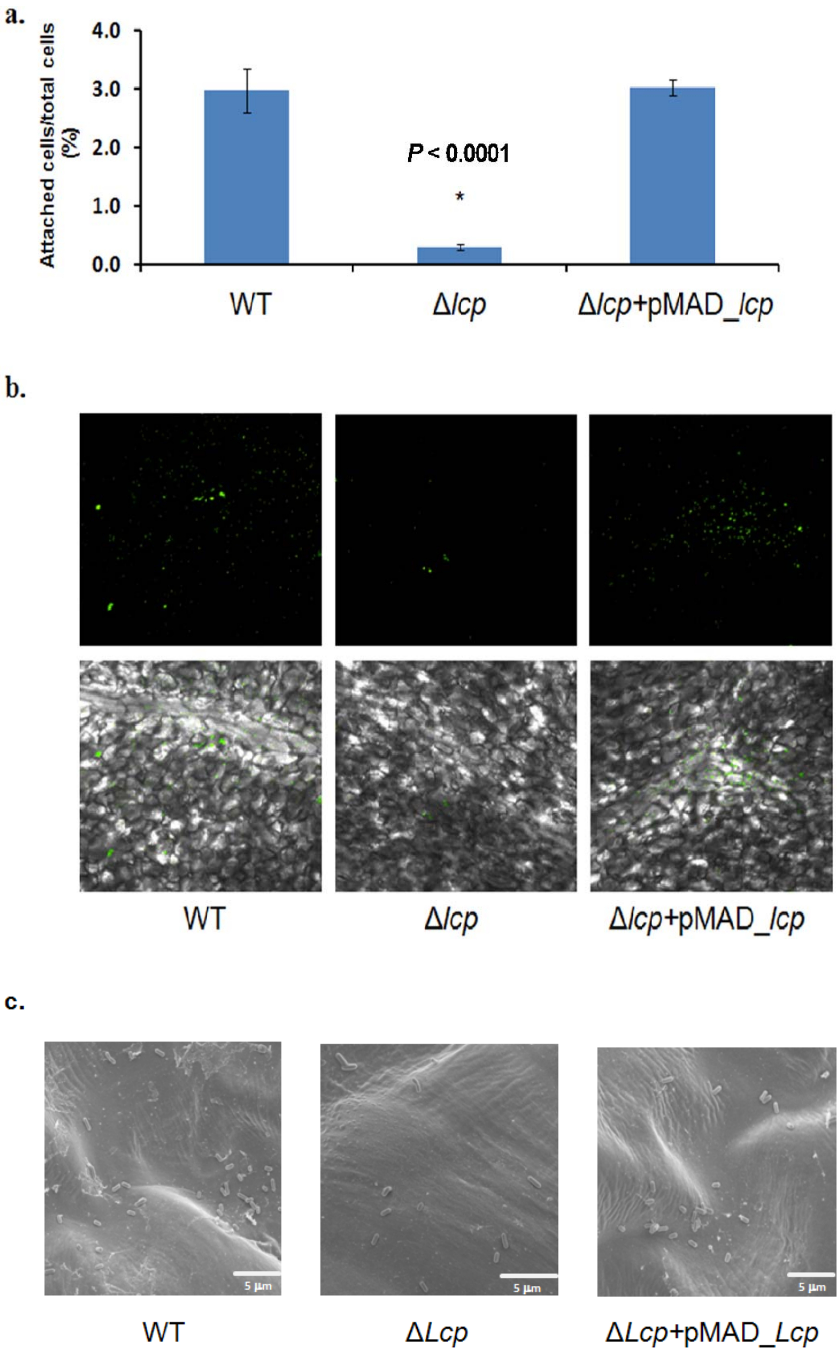

a.
Attached cells/total cells (%)
4.0
3.0
2.0
1.0
0.0
P < 0.0001
*
WT
Δ*lcp*
Δ*lcp*+pMAD_*lcp*
b.
WT
Δ*lcp*
Δ*lcp*+pMAD_*lcp*
c.
5 µm
5 µm
5 µm
WT
Δ*Lcp*
Δ*Lcp*+pMAD_*Lcp*

Fig 6.

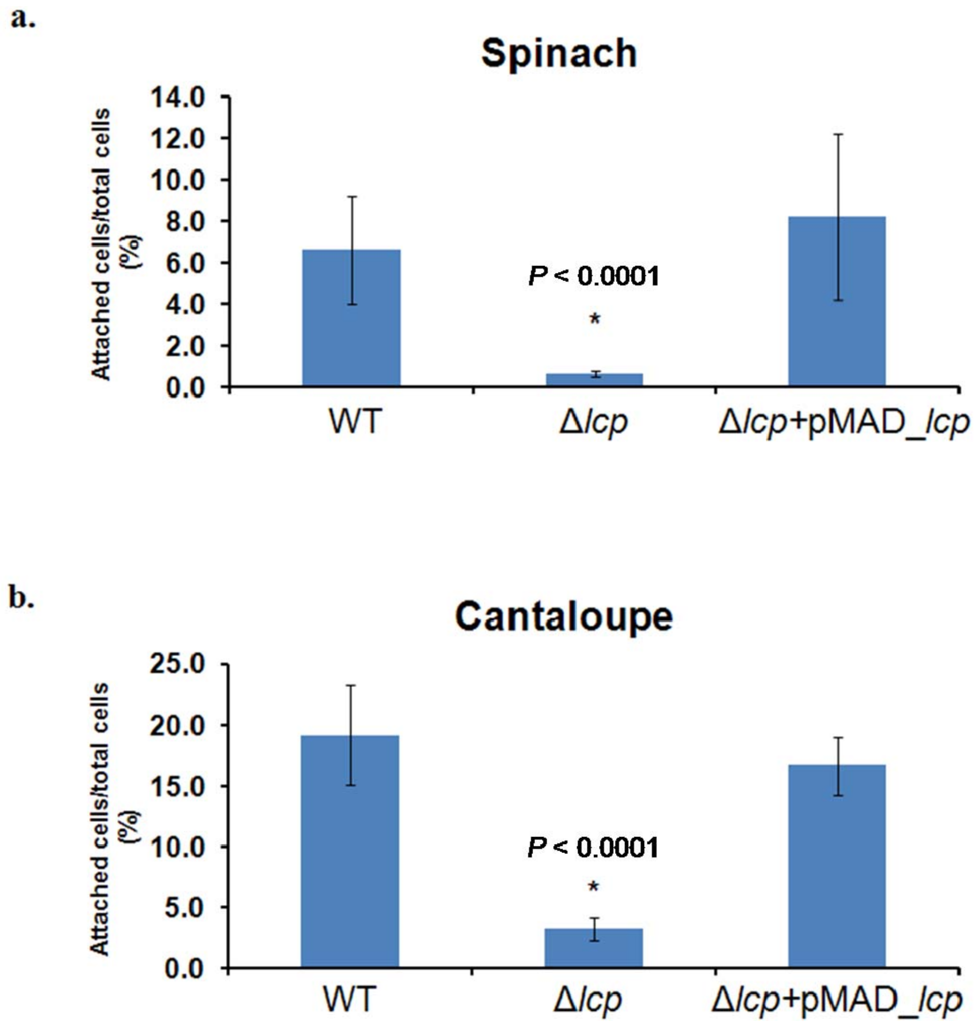
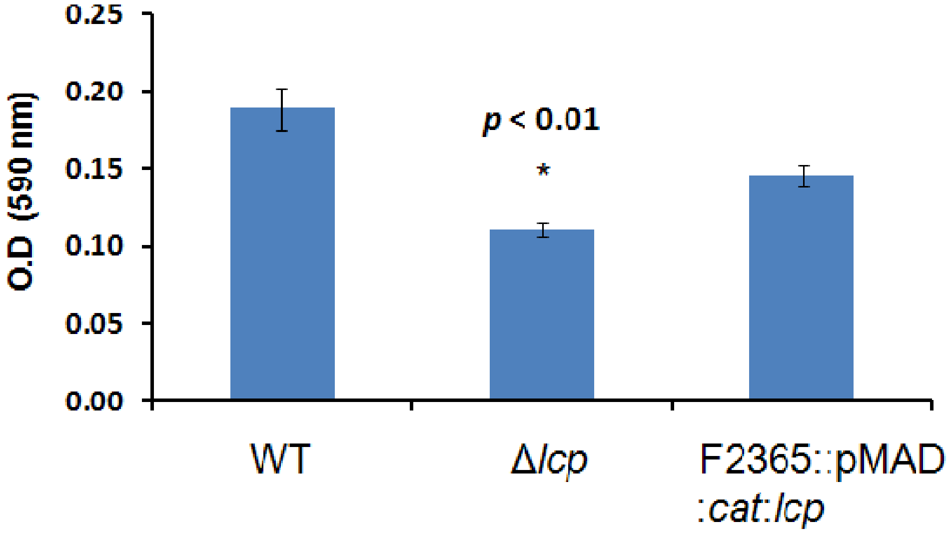

a.
Spinach
Attached cells/total cells (%)
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
$P < 0.0001$
*
WT
Δ*lcp*
Δ*lcp*+pMAD_*lcp*
b.
Cantaloupe
Attached cells/total cells (%)
25.0
20.0
15.0
10.0
5.0
0.0
$P < 0.0001$
*
WT
Δ*lcp*
Δ*lcp*+pMAD_*lcp*

Fig 7.


O.D (590 nm)
0.25
0.20
0.15
0.10
0.05
0.00
p < 0.01
*
WT
Δlcp
F2365::pMAD
:cat:lcp