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

Swetha Reddy, Ali Akgul, Attila Karsi, Robert W. Wills, and Mark L. Lawrence*

College of Veterinary Medicine, Mississippi State University, Mississippi State, MS 39762, USA

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*Corresponding author. Tel.: +1 662 325 1205; fax: +1 662 325 1193.

E-mail address: lawrence@cvm.msstate.edu

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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 *L. 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.

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

Listeria 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 ºC, 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 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 ºC for 48 hours each to select

for integration, and then they grown in BHI up to 8 hours to allow plasmid excision. Colonies were checked for subsequent gene deletion using PCR and confirmed using DNA sequencing.

2.4. Growth kinetics

Growth kinetics of F2365 and F2365Δ2117 in BHI broth at 37^{o}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 hours. 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 [13] 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 x 10^2, 1.67 x10^3, 1.67 x 10^4, and 1.67 x 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^{o}C for 48 hours 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 ºC 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 and F2365Δ2117 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 seconds and incubated for 30 minutes. 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 37ºC for 48 hours to obtain colony counts.

2.8. Invasion assay

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

J774A.1 cells were seeded onto 12 well plates two days prior to the assay with approximately 10^6 cells per well. Approximately 1 x 10^7 *L. monocytogenes* CFU per well were used for infection (MOI of 10:1). Following 1 hour incubation, the cells were washed twice with PBS, and fresh media containing gentamycin (1 µg/ml) was added to kill extracellular bacteria. At appropriately 1, 2, 3, 4, 5, 6, and 7 hours 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 ºC for 48 hours for CFU

determinations. All infections were performed in duplicate, and three independent assays were performed for each cell line.

2.9. Catfish fillet attachment

Specific-pathogen-free channel catfish fingerlings were euthanized by bath immersion in 400 mg/ml tricaine methanesulfonate. Using a 6 mm biopsy punch, muscle samples were aseptically collected, weighed, and placed in 1.5 ml sterile centrifuge tubes. Strains F2365 and F2365Δ2117 were grown to mid log phase (OD_{600} = 0.6-0.8) and diluted in sterile PBS. Bacteria (0.5 – 1 $x10^9$ CFU) were added to each muscle sample in 500 µl total volume. Attachment was conducted at 30 ^oC for 30 min, and unattached bacteria were removed by washing samples three times with 1 ml room temperature PBS followed by either 1) manually inverting tubes ten times, or 2) placing tubes on a rocker shaker for 30 min at room temperature. After washing, muscle samples were homogenized in 250 µl PBS by a hand held tissue homogenizer, and 750 µl PBS was added to the homogenate. The number of bacteria attached was determined by serial dilution and plate counting. Each experimental trial included 10 replicates, and the experimental trial was repeated eight times.

2.10. Collagen binding assay

Microtiter trays (Immulon 4HBX from Thermo scientific) were coated overnight with 10 µg ml^{-1} collagen type 1 (Sigma) in 0.1 M acetic acid at 4 ºC [14, 15]. Control wells were coated with 1% BSA. The wells were washed with PBS, and 5% BSA (100 µl, room temp, 1.5 hrs) was used to prevent nonspecific binding of bacteria. Overnight cultures of *L. monocytogenes* strains F2365 and F2365Δ2117 were adjusted to an OD_{600} 1.0, pelleted by centrifugation, and resuspended in PBS. Bacterial suspension (100 µl per well) was added to each well, and plates were incubated at 37^oC for 2 hrs with shaking every 30 minutes. The wells were washed thoroughly with PBS + 0.01% Tween 20 to remove unattached bacteria and reduce hydrophobic interactions. Bacteria were stained with 0.42% crystal violet in water for 10 min. After removing excess dye with PBS washes, 7% acetic acid (100 µl) was added to dissolve the

dye. Absorbance at 550 nm (A_{550}) was recorded using a Thermo Max Microtitre plate reader (Molecular Devices). To determine if other listerial strains are capable of binding collagen, representative strains [16] from different serotypes were tested (Table 1). *Staphylococcus aureus* strain MW2 was used as a positive control, and *Staphylococcus aureus* strain DU5883 was used as negative control for the assay. Two biological replicates were used for the assay, and assays were performed in triplicate for each biological replicate.

2.11. Statistical analysis

Mean separation analysis was conducted to compare treatments in the mouse virulence assay at each dose ($\alpha = 0.05$) using PROC GLIMMIX (SAS institute, 2008). Histograms were used to visually assess if the outcome, colony forming units (CFU) per gram of tissue, were normally distributed using PROC UNIVARIATE in SAS for Windows 9.3 (SAS Institute, Inc., Cary, NC). For samples where no bacterial counts were observed, we used the lower limit of detection to obtain mean values for analysis [17]. Although the raw data were generally right skewed, the data was found to be approximately normally distributed following $\log_{10}$ transformation.

The same procedure was used to assess normality of the adhesion and invasion assays of Caco-2 and J774A.1 cell lines. A mixed model analysis was conducted using PROC MIXED in SAS for Windows for the $\log_{10}$ CFU/ml outcome. A model was fit with fixed effects of strain, time, and strain by time interaction for the invasion assays. A model was fit with strain as the only fixed effect for the study of adhesion of the Caco-2 cell line. Plate identity was included as a random effect with compound symmetry covariance structure in all models. Comparisons between levels of significant fixed effects were made using least squares means. For significant interactions between time and strain, differences in least square means between strains were assessed at each time point using the SLICE option in the LSMEANS statement. An alpha level of 0.05 was used to determine statistical significance for all tests.

For the catfish muscle attachment assay, normality of CFU/g of tissue data was checked by the same procedure. When CFU/g data were not normally distributed, $\log_{10}$ transformation was applied, and

log_{10} transformed data were used in subsequent mixed model analyses using PROC MIXED to determine the effect of wash, bacteria, and their interaction on CFU/g. Day of the experiment was included as a random effect using a compound symmetry covariance structure.

3. Results

3.1. Construction of deletion mutant

An in-frame deletion of *lmof2365_2117* was generated in F2365 to avoid any polar effects on downstream genes *lmof2365_2118* (DNA binding protein) and *lmof2365_2119* (MATE efflux family protein). The resulting F2365Δ2117 strain had a deletion of 562 amino acids, which was verified by PCR using primers that anneal to flanking regions of the chromosome (data not shown). The deletion was further confirmed by DNA sequencing. Growth kinetics demonstrated that the deletion did not affect the growth rate of F2365Δ2117 compared to F2365 (data not shown).

3.2. Mouse virulence assay

On day 6 post-infection, two mice from the highest dose F2365 treatment group were euthanized for humane reasons, and on day 7 post-infection, all remaining mice were euthanized. At all doses except for $1x10^4$ CFU/mouse, colonization in liver (CFU/g tissue) was significantly higher (>10-fold difference) for mice infected with F2365 compared to F2365Δ2117 (Figure 1).

3.3. Adherence and invasion

The role of Lmof2365_2117 in cellular adhesion and invasion was determined using the colonic epithelium cell line Caco-2 and the murine macrophage cell line J774A.1. F2365Δ2117 had significantly decreased ($p<0.0001$) adherence to Caco-2 cells compared to F2365. Bacterial quantities (CFU/ml) were $1.18x10^6 \pm 8.55x10^4$ and $6.19\ x10^5 \pm 2.83x10^4$ for F2365 and F2365Δ2117, respectively. Lmof2365_2117 influenced intracellular replication of strain F2365 in Caco-2 cells (Figure 2). F2365 had significantly higher intracellular concentrations than F2365Δ2117 at 2 hours post-infection, but intracellular F2365

concentrations dropped so that it had significantly lower concentrations than F2365Δ2117 at 6 and 8 hours post-infection. F2365 then replicated faster than F2365Δ2117 so that intracellular concentrations were significantly higher for F2365 at 10 and 12 hours post-infection. Later time points did not show significant difference between the wild type and mutant strains. In macrophages, intracellular concentrations of F2365 were significantly higher than F2365Δ2117 at 1 hour, 3 hours, and 4 hours post-infection, after which there were no significant differences between strains (Figure 3).

3.4. Catfish fillet attachment

To determine whether Lmof2365_2117 could contribute to *L. monocytogenes* persistence on food, we compared attachment of F2365 and F2365Δ2117 to catfish fillets. There was a significant ($P < 0.01$) difference in attachment of F2365 and F2365Δ2117 to catfish muscle tissue (2.498×10^4 CFU/g of tissue versus 1.875×10^4 CFU/g of tissue, respectively). Wash procedure had no effect on the outcome, and wash procedure and bacteria did not show any interaction. Therefore all eight experimental trials were included together in the statistical analysis.

3.5. Collagen binding assay

F2365 and F2365Δ2117 had no significant collagen binding compared to negative control. To determine whether this was strain specific, we performed collagen binding assays with listerial strains from several serotypes (Table 1), and our results showed that *L. monocytogenes* does not have significant collagen binding.

4. Discussion

The genome of F2365 encodes a cell wall surface anchor family protein, Lmof2365_2117, with a predicted molecular mass of 60.68 KDa and an estimated isoelectric point of 5.06. The *lmof2365_2117* gene is flanked by *lmof2365_2116* and *lmof2365_2118*, which encode a predicted phosphotransferase enzyme family protein and a putative DNA-binding protein, respectively. Lmof2365_2117 is annotated as

a cell wall surface anchor family protein in most listerial serotypes, and it is annotated as a peptidoglycan bound protein in most serotype 1/2a strains. Orthologs of Lmof2365_2117 are present in *Bacillus cereus*, *Staphylococcus aureus*, *Streptomyces* species, and Flavobacteriaceae species. The 562 amino acid protein encoded by *lmof2365_2117* contains a signal peptide followed an amino terminal domain with no detectable homology to non-listerial proteins. The carboxy end of the protein has three Cna-B (collagen binding B) domains and a LPXTG motif-containing domain at the carboxy terminus.

Our results indicated that Lmof2365_2117 contributes to virulence of *L. monocytogenes*. Colonization of liver was significantly decreased in the F2365Δ2117 mutant compared to the parent strain. The abundance of bacteria in the liver can be correlated to virulence in *L. monocytogenes* [17], and in the current study, colonization of liver was significantly higher for F2365 compared to the mutant strain for almost all doses.

Adhesion of F2365Δ2117 to Caco-2 cells was significantly lower than F2365, demonstrating that the protein contributes to adhesion to enterocytes. Lmof2365_2117 also has sequence similarity with a carboxypeptidase regulatory-like domain and a peptidase-associated domain, both of which are involved in protein folding [18]. Because of its role in adherence to Caco-2 cells, we designated Lmof2365_2117 as *Listeria* adhesion protein B (LapB). Interestingly, LapB significantly contributed to *L. monocytogenes* attachment to muscle tissue from channel catfish fillets (33% reduction in catfish muscle attachment in F2365Δ2117 compared to F2365), suggesting the protein could contribute to persistence in some types of food. However, there are likely other *L. monocytogenes* components that also contribute to catfish fillet attachment, and other food types need to be tested to determine if the effect is limited to catfish muscle.

In *Staphylococcus*, surface anchor proteins containing a collagen binding domain are necessary for adhesion and entry into host cells [19, 20]. Furthermore, collagen binding domain protein (*cna*) deficient mutants of *Staphylococcus* species are less virulent in animal models than their parental wild type strains [3]. The Cna protein sequence in *Staphylococcus* has a nonrepetitive collagen binding A region and a repetitive B region. The binding of Cna_A to collagen is independent of the Cna_B region [21]. The B region has a beta sandwich structure and does not show affinity towards collagen; its

function is to serve as a stalk to facilitate binding of bacteria to collagen through the A region [21, 22]. Depending on the strain, B region has one to four repeat units, and each unit has two domains, D1 and D2. Currently six listerial LPXTG proteins containing repeats related to the D domain of Cna_B (Pfam PF05738) have been identified; four of these six proteins studied show similarity with collagen binding proteins (Lmo0159, Lmo0160, Lmo2178, and Lmo2576) [23]. However, the role of these listerial Cna_B repeat regions in collagen adherence and virulence has not been elucidated. The C-terminal Cna_B region in LapB shows similarity to the B region of collagen binding surface protein Cna from *Staphylococcus aureus*. Therefore, LapB appears to have a stalk domain, but the binding affinity of its amino terminus is not known.

Due to the presence of the Cna_B region in LapB, we performed a collagen binding assay to determine if the protein mediates collagen adhesion. We found that *L. monocytogenes* F2365 and F2365Δ2117 did not show affinity towards collagen, indicating that LapB is apparently not involved in collagen binding. Therefore, although LapB has a stalk structure similar to the Can_B region, the protein's function may not be in binding [24]. However, LapB has a LPXTG-motif at its C-terminus, so the protein is clearly surface anchored [24]. Further work is needed to determine the enzymatic or binding activity of LapB.

L. monocytogenes initiates infection via the intestinal route [25, 26]. Interestingly, F2365Δ2117 had a different pattern of intracellular replication in Caco-2 cells compared to F2365 parental strain. F2365 had significantly higher initial intracellular concentration than F2365Δ2117 at 2 hours post-infection, but intracellular concentrations then decreased between 2 to 6 hours post-infection. A similar trend was seen in invasion assays conducted with Caco-2 cells using strains F9599 (unidentified serotype) and F7243 (serotype 4b) [27]. By contrast, F2365Δ2117 did not have an initial transient infection at 2 hours post-infection; rather, intracellular concentrations increased at each time point and peaked at 12-16 hours post-infection. For F2365, intracellular bacterial quantities peaked at 12 hours post-infection and were significantly higher than F2365Δ2117 at this point. Overall, the differences in intracellular replication between F2365 and F2365Δ2117 in Caco-2 cells were minor, suggesting LapB plays a more

important role in cellular adhesion than intracellular replication. It is possible that the differences between F2365 and F2365Δ2117 in intracellular concentrations were due to the effects on adhesion.

In the macrophage cell line, F2365 and F2365Δ2117 had similar trends, with the most notable difference being that F2365 reached intracellular concentrations that were approximately 10-fold higher than F2365Δ2117 at 3-4 hours post-infection. Therefore, although LapB contributes to optimal listerial intracellular replication, it is not required for intracellular survival and replication.

In the current study, we showed that cell wall surface anchored protein LapB (Lmof2365_2117*)* is required for full virulence of *L. monocytogenes*. F2365Δ2117 does not show a difference in growth rate in broth culture when compared with the parent strain, but the mutant is significantly attenuated in the mouse model. LapB contributes significantly to the ability of F2365 to adhere to colonic epithelium cell line Caco-2, and it has a lesser role in intracellular replication. LapB participates in catfish fillet attachment, and it also contributes to optimal intracellular replication in macrophages. Although LapB has a domain similar to collagen-binding proteins, *L. monocytogenes* does not have significant binding to collagen. Based on presence of conserved domains, the protein is anchored to the cell wall and appears to have a domain that forms a stalk-like structure in its carboxy terminus. Further work is needed to determine the enzymatic activity or binding affinity of LapB and the mechanism by which it contributes to virulence and adherence.

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Figure Legends

Figure 1. Tissue concentrations of *L. monocytogenes* F2365 and F2365Δ2117 in liver. Tissues were collected on day 7 post-infection, homogenized, diluted, spread on BHI agar plates, and allowed to grow for 24 - 48 hours at 37 ºC to determine CFU/gm for each organ. Each tissue concentration is the mean of five mice; standard error is indicated by error bars. Statistically significant differences between F2365 and F2365Δ2117 are indicated by *.

Figure 2. Invasion of Caco-2 cell line by F2365 and F2365Δ2117. Cells were infected with bacterial suspension to a MOI of 10 bacteria per cell for 2 hours, washed two times with PBS, and incubated in media containing 100 μg/ml of gentamycin. At different time points, cells were washed, lysed in cold PBS with .01% Triton-X100 and sonication, and spread on BHI agar plates. Each bar represents the mean of six independent replicates; standard error is indicated by error bars. Statistically significant differences between F2365 and F2365Δ2117 are indicated by *.

Figure 3. Invasion and replication of F2365 and F2365Δ2117 in murine macrophages. Cells were infected with bacterial suspension to a MOI of 10 bacteria per cell for 1 hour. Each bar represents the mean of six independent replicates; standard error is indicated by error bars. Statistically significant differences between F2365 and F2365Δ2117 are indicated by *.

Table 1. Collagen binding assay results

Strain	**Serotype**	**Source**	**Mean absorbance***	**Standard deviation**
EGD-e	1/2a	Guinea pig	0.072	0.0005
RM3158 (J1-169)	1/2b	Human blood	0.057	0.005
RM3017	1/2c	Blood	0.071	0.012
HCC7	1	Catfish brain	0.0686	0.007
RM3162 (M1-004)	3a	Human hip	0.063	0.004
RM3836	3b	Beef frank	0.059	0.013
RM3027	3c	Chicken	0.075	0.027
HCC25	4a	Catfish kidney	0.085	0.004
RM3177	4b	Human	0.059	0.013
ATCC 19116	4c	Chicken	0.087	0.002
ATCC 19117	4d	Sheep	0.062	0.008
RM2218	4e	Oyster	0.066	0.005
L. innocua CLIP 11262	6a	Corn	0.063	0.0097
F2365	4b	Mexican Cheese	0.081	0.03
F2365Δ2117			0.073	0.005
Staphylococcus aureus strain MW2 (Positive control)			1.23	0.027
Staphylococcus aureus strain DU5883 (Negative control)			0.089	0.017

*Mean absorbance at λ550 following 2 hr incubation at 37 ºC in microtiter plates coated with collagen type 1 and stained with crystal violet.