Identification and Expression Characterization of Surface Proteins for the Detection and Isolation of *Listeria monocytogenes*

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Abstract

Listeria monocytogenes causes a serious foodborne illness (listeriosis) with a fatality rate of about 30% in susceptible individuals (1). Timely identification of foods and food processing environments carrying this deadly bacterium is crucial for implementing effective interventions but remains a practical challenge due to the complexity of test samples, low level of bacterial contamination, and the ubiquity and the genetic diversity of *Listeria* isolates. The purpose of this work was to identify and assess surface proteins of *L. monocytogenes* that can serve as diagnostic biomarkers for pathogen isolation and detection using antibody-based methods. Bioinformatics analysis of 130 putative surface proteins encoded by the genome of *L. monocytogenes* F2365 (serotype 4b) revealed four uncharacterized proteins with extensive amino acid sequences unique to *L. monocytogenes*. These proteins did not contain identifiable PrfA-controlled promoter elements. The four proteins were expressed at the transcriptional level *in vitro*, as demonstrated by RT-PCR, but only one of the four proteins, LMOf2365_0639, was detected on the cell surface by immunofluorescence microscopy (IFM) using rabbit polyclonal antibodies (PAbs) raised against corresponding recombinant proteins. Transcription start site mapping and promoter prediction analysis provided evidence that the *LMOf2365_0639* gene was expressed under the control of a sigma B factor-dependent promoter, an alternative sigma factor involved in stress response. Non-gel based proteomics analysis of *L. monocytogenes* surface proteins identified 36 surface proteins in at least one of the three trials performed. IFM with PAbs raised against each of the five candidate surface proteins identified from the proteomics study revealed a strong fluorescence signal on the surface of live *L. monocytogenes* cells with LMOf2365_0148 specific PAbs, indicating a good level of expression of this protein. These results suggested the potential of the surface proteins LMOf2365_0639 and LMOf2365_0148 as diagnostic biomarkers for *L. monocytogenes*.

Thirty-five and 24 monoclonal antibodies (MAbs) were developed against purified recombinant LMOf2365_0639 and LMOf2365_0148, respectively. Three MAbs against LMOf2365_0639 and five MAbs against LMOf2365_0148 were selected and evaluated for their potential in *L. monocytogenes* detection and isolation based on the observation that these MAbs recognized the highest number of the 53 *L. monocytogenes* isolates and the lowest number of the 10 other *Listeria* species isolates tested. None of these MAbs reacted with the four foodborne pathogens (*Campylobacter jejuni*, *Samonella enterica* serovar Typhimurium, *Escherichia coli* O157:H7 and *Bacillus cereus*) tested. All three MAbs to LMOf2365_0639 were specific for lineage I and II isolates of *L. monocytogenes* commonly found in clinical and food isolates respectively and recognized the N-terminal region of LMOf2365_0639. Anti-LMOf2365_0148 MAbs were reactive to lineage I and lineages III *L. monocytogenes* isolates commonly found in clinical and animal isolates respectively. Both LMOf2365_0639 and LMOf2365_0148 were expressed in standard enrichment culture conditions according to Health Canada's MFHPB-30 and MFHPB-07 methods. In addition, MAbs against LMOf2365_0148 could specifically isolate live *L. monocytogenes* by immunomagnetic separation even in a mixture of *L. monocytogenes* and non-target *L. innocua*. The dissociation constants of the MAbs capable of capturing *L. monocytogenes* ranged from 2.58×10^{-8} M to 8.87×10^{-10} M.

In conclusion, two novel surface proteins LMOf2365_0639 and LMOf2365_0148 were identified, were shown to be expressed in *L. monocytogenes* grown in standard selective enrichment cultures, and can be explored as surface biomarkers for the isolation and detection of *L. monocytogenes* with specific MAbs developed in this study.

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List of Abbreviations

A

ABTS- 2,2'-Azino-Bis-(3-Ethyl-Benzthiazoline-6-Sulfonic Acid)
ACN- Acetonitrile
ALOA- Agar Listeria According to Ottaviani and Agosti

B

BHI- Brain Heart Infusion
BSA- Bovine Serum Albumin

C

CAM- Compendium of Analytical Methods
cDNA- Complementary Deoxyribonucleic Acid
CFIA- Canadian Food Inspection Agency
CFU- Colony-Forming Unit

D

DNA– Deoxyribonucleic Acid
dNTP- Deoxynucleotide Triphosphates
DTT- dithiothreitol

E

ELISA- Enzyme-Linked Immunosorbent Assay

F

Fab- Fragment Antigen Binding
FB- Fraser Broth
Fc- Fragment (Crystallisable)
FfH- Fifty-Four Homolog
FPLC- Fast Protein Liquid Chromatography
FRB- Fraser Broth with Ferric Ammonium Citrate

G

GO- Gene Ontology
GW Modules- Glycine-Tryptophan Modules

I

IFA- Incomplete Freunds Adjuvant

IFM- Immunofluorescence Microscopy
Ig- Immunoglobulin
iap- Invasion-Associated Protein
IMS- Immunomagnetic Separation
InlA- Internalin A
InlB- Internalin B

L

LB- Luria-Bertani
LEB- *Listeria* Enrichment Broth
LLO- Listeriolysin O
LPM Agar- Lithium Chloride Phenylethanol Moxalactam Agar
LPXTG Motif- Leucine-Proline-Any Amino Acid-Threonine-Glycine Motif
Lgt- Diacylglycerol Transferase
LRB- *Listeria* Repair Broth
LRS- Listeriosis Reference Service
LTA- Lipoteichoic Acid
LsyM Domain- Lysine Motif Domain

M

MAb- Monoclonal Antibody
MALS- Multi-Angle Light Scattering
MFB- Modified Fraser Broth
MLA- McBride Listeria agar
MS- Mass Spectrometry
MLEE- Multilocus Enzyme Electrophoresis
MLST- Multilocus Sequence Typing
mRNA- Messenger Ribonucleic Acid

N

NML- National Microbiology Laboratory

O

OXA- Oxford Agar
OD- Optical Density

P

PAb- Polyclonal Antibody
PBP- Penicillin Binding Protein
PBS- Phosphate-Buffered Saline
PCR- Polymerase Chain Reaction
PFGE- Pulse-Field Gel Electrophoresis

PC-PLC- Phosphatidyl-Choline-Specific Phospholipase C
PI-PLC- Phosphotidyl-Inositol-Specific Phospholipase C

R

RACE- Rapid Amplification of cDNA Ends
RTE- Ready-to-Eat
RT-PCR- Real Time-Polymerase Chain Reaction
RNA- Ribonucleic Acid
rRNA- Ribosomal Ribonucleic Acid

S

scFv- Single-Chain Variable Fragment
SDS- Sodium Lauryl Sulfate
SDS-PAGE- Sodium Lauryl Sulphate- Polyacrylamide Gel Electrophoresis
Sec- Secretion Apparatus
SPI- Signal Peptidase I
SPII- Signal Peptidase II
SPR- Surface Plasmon Resonance
SRP- Signal Recognition Particle

T

TA- Teichoic Acid
TFA- Trifluoroacetic Acid
TSS- Transcription Start Site

U

USDA- U.S. Department of Agriculture
UVM- University of Vermont Media
UVM1- University of Vermont Media 1
UVM2- University of Vermont Media 2

W

WxL Domain- Tryptophan-Any Amino Acid-Leucine Domain

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Chapter 1: Introduction

1.1 Research Rationale

Listeria monocytogenes causes a serious foodborne human illness referred to as listeriosis. Although listeriosis is rare when compared to other foodborne illnesses, it has the highest fatality rate among all foodborne pathogens (2, 3). Susceptible individuals include those with weak or suppressed immunity, the elderly, newborns and pregnant women (4). *L. monocytogenes* is widely distributed in the environment (5, 6), and therefore can easily enter the food chain through contaminated fresh produce, milk, and other animal products (7). *L. monocytogenes* contamination is a major concern in Ready-to-Eat (RTE) foods that require no heating. *L. monocytogenes* contamination in RTE foods is a serious health hazard to susceptible individuals (8) as this pathogen has the ability to grow at refrigeration temperatures. Due to the ubiquity of *L. monocytogenes* in nature and the severe consequences of listeriosis in susceptible individuals, *L. monocytogenes* detection in foods and food-processing environments is an important safeguard for public health. Selective enrichment culture is often necessary to amplify *L. monocytogenes*, found in trace amounts (7), to detectable levels for *L. monocytogenes* detection (9-11). Following selective enrichment culture, rapid detection methods such as PCR (12) have been developed to shorten the time for *L. monocytogenes* detection. However, sample preparation remains a bottleneck in rapid *L. monocytogenes* detection methods. PCR inhibitors in foods (13), selective enrichment cultures (14) and genetic materials from background microflora (15) can confound PCR results. Immunological methods may provide some solutions to current detection drawbacks. Immunomagnetic separation in which antibodies are used to capture *L. monocytogenes* can enrich and purify the pathogen from food samples or selective enrichment cultures for subsequent molecular detection. In addition, antibody-based detection methods such

as enzyme-linked immunosorbent assay (ELISA) which are resistant to sample complexity can be used in *L. monocytogenes* detection in foods (16). Since *L. monocytogenes* has at least 12 serotypes (17) and is the only predominant human pathogen among several other *Listeria* species, antibodies that are specific for *L. monocytogenes* and capable of binding to the surfaces of a wide range of *L. monocytogenes* serotypes are not currently available. Moreover, diagnostic antibodies must also detect *L. monocytogenes* in selective enrichment cultures which is important for the successful application of immunomagnetic separation and antibody-based detection methods such as ELISA. Most *L. monocytogenes* antibodies developed recognize undefined targets which may result in cross-reactivity. These antibodies are either specific to a few serotypes (18, 19), cross-reactive to other non-pathogenic *Listeria* species (20-24) or cannot detect *L. monocytogenes* grown in selective enrichment cultures (25-28). Identification of suitable surface biomarkers for *L. monocytogenes* may allow for the development of monoclonal antibodies (MAbs) used in the isolation and detection of this deadly pathogen.

1.2 Hypothesis

The research interest was to identify surface proteins as diagnostic biomarkers for *L. monocytogenes* isolation and detection. While certain *L. monocytogenes* virulence proteins are specific to *L. monocytogenes*, the expression of these virulence proteins are attuned to intracellular conditions within host cells (29, 30) and are under the direct regulation of the transcription factor PrfA (31). Weak or no expression of such virulence factors in culture conditions (26) indicates that such virulence proteins may not be suitable for *L. monocytogenes* detection and isolation. There are 133 surface proteins encoded by the genome *L. monocytogenes* (strain EGD-e) (32). With 12 annotated *Listeria* genomes available (33), it is possible to compare surface protein sequences of *L. monocytogenes* with those of other *Listeria* species to identify

candidates that have extensive regions conserved among *L. monocytogenes* strains and are variable among other *Listeria* species. This thesis research was based on the hypothesis that surface proteins that are not directly regulated by the key virulence transcription factor PrfA (31), but well conserved among *L. monocytogenes* strains can serve as biomarkers for *L. monocytogenes* isolation and detection.

1.3 Research Objectives

The research aim was to identify and characterize surface proteins of *L. monocytogenes* that can serve as diagnostic biomarkers for *L. monocytogenes* isolation and detection.

Specific aims included:

1) Identify surface protein biomarkers of *L. monocytogenes* using sequence comparison and proteomics approaches.
2) Assess expression and surface localization of candidate surface proteins using polyclonal antibodies.
3) Develop and characterize MAbs to potential surface protein biomarkers.
4) Assess the expression of potential surface protein biomarkers under standard selective enrichment culture conditions integral to current *Listeria* detection.
5) Assess the ability of MAbs to capture live *L. monocytogenes*.

Chapter 2: Literature Review

2.1 *Listeria monocytogenes*

2.1.1 Characteristics

L. monocytogenes is a Gram-positive, facultative anaerobic, non-spore forming, rod-shaped bacterium (34, 35). This organism, present in decaying plant material as a saprophyte (36), is widely distributed in the environment but also acts as an intracellular pathogen (4). *L. monocytogenes* has been recovered from soil, vegetable matter, silage, sewage and fecal matter of healthy animals and humans (5, 6). *L. monocytogenes* can grow at temperatures of 0-45 ^{o}C, at high salt concentrations (10%) and at pH of 4.4-9 (37). *Listeria* species are motile at 10-25 ^{o}C (36).

2.1.2 Taxonomy

The genus *Listeria* consists of a group of low CG content bacteria in the phylum of Firmicutes and is closely related to the genera *Bacillus*, *Clostridium*, *Enterococcus*, *Streptococcus* and *Staphylococcus* (36). Members of the genus *Listeria* are *L. monocytogenes, L. ivanovii, L. innocua, L. seeligeri, L. welshimeri, L. grayi* and the recently described *L. marthii, L. rocourtiae, L. fleischmanii* sp. nov., *L. wihenstephanensis* sp. nov., *L. floridensis* sp. nov., *L. aquatic* sp. nov., *L. cornellensis* sp. nov., *L. riparia* sp. nov., *L. grandensis* sp. nov., *L. booriae* sp. nov. and *L. newyorkenesis* sp. nov. (38-43). Only *L. monocytogenes* and *L. ivanovii* are pathogenic. *L. monocytogenes* is pathogenic to humans while *L. invanovii* is mainly an animal pathogen but has been reported to cause gastroenteritis in humans (44).

Phylogenetic analysis of rRNA genes and other genes indicate that *L. monocytogenes* and *L. innocua* form one group while *L. welshimeri*, *L. seeligeri* and *L. ivanovii* form another group.

Within the latter group, *L. seeligeri* and *L. ivanovii* are more closely related to each other than to *L. welshimeri. L. grayi* is distantly related to *L. monocytogenes, L. innocua, L. welshimeri, L. seeligeri* and *L. ivanovii* (45).

Listeria species may have evolved from a pathogenic ancestor. Comparative genomic analysis of the pathogenic species (*L. monocytogenes*) versus non-pathogenic species (*L. welshimeri* and *L.seeligeri*) revealed genomic reduction resulting from deletions within *L. welshimeri* and *L. seeligeri* genomes. For instance, the chromosomal locus that consists of six virulence factors key to the intracellular life cycle is not found within non-pathogenic *Listeria* species except *L. seeligeri.* Gene insertion within the chromosomal locus of *L. seeligeri* disrupted gene function within the chromosomal locus (36).

2.1.3 Subtyping

There are many subtyping methods for *L. monocytogenes* isolates that provide varied degrees of discrimination. Subtyping is necessary to identify relationships between isolates. In addition, subtyping is useful for identifying the source of infection and tracking the infection in outbreak and sporadic cases (46).

Serotyping was the first method developed for subtyping *L. monocytogenes* isolates. Serotyping is based on the use of high quality antisera specific for somatic (O) and flagellar (H) antigens of *Listeria.* Strains of *Listeria* species are divided into serotypes according to which O antigens and H antigens are detected by high quality antisera. There are at least 12 serotypes: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e and 7 (17). Only three serotypes, 1/2a, 1/2b and 4b, cause 95% of human infections (46). Serotyping has poor discriminating power compared to other subtyping methods.

Molecular subtyping methods have been developed for *L. monocytogenes* and include multilocus enzyme electrophoresis (MLEE), DNA macro-restriction analysis by pulse-field gel electrophoresis (PFGE) and DNA-sequence-based methods such as multilocus sequence typing (MLST) and ribotyping.

MLEE examines the mobility of cytoplasmic enzymes. Variations in enzyme mobility are considered as different electromorphs (46). The combination of an electromorph set makes up an electrophoretic type. Although MLEE is useful in evolutionary studies, its discrimination ability is not enough for use in epidemiological investigations (46). In addition, the required equipment and the involved procedure limit its use in epidemiological studies (46).

PFGE is widely used in subtyping of *L. monocytogenes* isolates for listeriosis cluster detection, epidemiologic investigation and monitoring food production facilities (47). This method is used internationally for subtyping *L. monocytogenes* (47). PFGE changes the orientation of the electric field periodically to separate large fragments of genomic DNA (> 40 kb) cut by infrequently cutting restriction enzymes (46).

MLST is a subtyping method based on the sequences of complete or partial genes of housekeeping enzyme loci as well as certain virulence genes (46). Virulence genes and intergenic regions between virulence genes are more variable and provide more discrimination between strains than housekeeping genes (48).

Ribotyping is a type of Southern hybridization analysis. *Listeria* genome DNA is digested and probed with 16S and 23S rRNA specific probes (46). This method detects possible restriction fragment length polymorphism associated with the ribosomal operon. Ribotyping is commonly used for phylogenetic studies and long-term epidemiology studies (46).

2.1.4 Lineage Designation

There are three lineages among *L. monocytogenes* isolates (49). *L. monocytogenes* subtyping studies such as MLEE (50, 51), partial sequencing of virulence genes (52, 53), PFGE (54), ribotyping (55-57) and genomic microarray (58) demonstrate that *L. monocytogenes* serotypes can be grouped into three lineages. Lineage I consists of *L. monocytogenes* serotypes 1/2b, 4b, 3b, 4d and 4e (59). Lineage II consists of *L. monocytogenes* serotypes 1/2a, 1/2c, 3a and 3c (59). Lineage III consists of *L. monocytogenes* serotypes 4a and 4c (59).

Within lineage I, serotype 1/2b and 3b strains are clustered into one group while 4b strains are clustered into another group (60). Serotype 4b may have evolved from serotype 1/2b, the ancestral serotype of lineage I. Serotype 4d and 4e strains are genetically and antigenically similar to 4b but are relatively rare among clinical and food isolates of *L. monocytogenes* (59). Certain 4b strains, based on DNA-based analysis, are clustered into lineage III along with serotypes 4a and 4c strains. These serotype 4b stains have lost or undergone pronounced divergence of two genes conserved and specific to serotype 4b strains (61).

Lineage I and II isolates are associated with human listeriosis, while lineage III isolates are more common in animals with clinical symptoms (59). There is a high prevalence of lineage I isolates, mainly serotype 4b, in clinical cases as opposed to serotype 1/2c isolates of lineage II which are under-represented in clinical isolates (49). Interestingly, it is possible that the over-representation of lineage I isolates, particularly serotype 4b isolates, is due to the presence of an intact Internalin A (InlA). A study of serotype 1/2c isolates revealed these isolates commonly harbour an InlA, a key virulence factor involved in host cell entry, while no serotype 4b isolates carry *inlA* premature stop codon alleles (62). In addition, the putative virulence attenuation

within lineage II may be characteristic of not only the serotype but also the source of the strain. Although only 63% of serotype 1/2a food isolates had full-length InlA, 97% of serotype 1/2a clinical isolates had full-length InlA (62).

Lineage II isolates are more frequently recovered from food and food processing plants than lineage I isolates (49). Many researchers believe that lineage II has a better capacity to grow and survive in food and food-related environments and to persist in food processing plants. Long-term (12 years) persistence of a lineage II strain has been documented. A serotype 1/2a isolate which caused a case of sporadic human listerosis in 1988 was also responsible for a multi-state outbreak in 2000 (63). In addition, the prevalence of lineage II in food samples may be due to a bias in lineage II detection. Some lineage II isolates were observed to out-compete lineage I isolates in the UVM medium commonly used in culture enrichment of *L. monocytogenes* in food and environmental samples (64).

2.1.5 Pathogenesis

L. monocytogenes is a foodborne pathogen. After ingestion of contaminated food, the primary entrance point is the gastrointestinal tract. *L. monocytogenes* predominantly invades and translocates through the small intestine at the apical tips of intestinal villi (65), villus epithelial folds and junctions between globlet cells (66). After cellular internalization, *L. monocytogenes* releases from the phagocytic vacuole, multiples and spreads between cells (4). *L. monocytogenes* primarily colonizes the liver and the spleen via blood or lymph. Although initial tissue colonization is rapid, the incubation period between ingestion of contaminated food to symptoms of invasive listeriosis is 20-30 days (4). The majority of *L. monocytogenes* cells are eliminated by resident macrophages in the liver and spleen with the help of cells from innate and adaptive

immunity (4). In individuals with weak or compromised immune systems, uncontrolled *L. monocytogenes* proliferation occurs in the liver resulting in colonization of secondary target organs such as the brain and placenta (4). Severely immunocompromised hosts also suffer from septicaemia (4).

2.1.6 Listeriosis Manifestation

There are two forms of listeriosis: non-invasive and invasive. Non-invasive listeriosis can develop in any population when foods highly contaminated with the bacteria ($>10^3$ colony forming units (CFUs)/g) are consumed. The average incubation time in otherwise healthy individuals is 24 hours and manifests as febrile gastroenteritis (67).

Invasive listeriosis can be life-threatening. Although invasive listeriosis is rare with respect to other foodborne illnesses, it accounts for 3.8% of foodborne disease hospitalization and 27.6% of foodborne disease deaths (3). It occurs in the elderly, pregnant women and individuals with weak or compromised immune systems. Invasive listeriosis frequently occurs in non-pregnant adults with at least one underlying illness such as heart disease, corticosteroid therapy, cancer, renal disease, diabetes and HIV infection (68). Non-pregnant patients who acquire invasive listeriosis commonly suffer from meningitis and bacteremia (4).

Most cases of listeriosis during pregnancy occur in healthy women. The infected mother experiences non-specific flu-like symptoms while the fetus develops systemic infection resulting in miscarriage, stillborn or premature birth of an infant with septicaemia and meningitis (69). Although antibiotic treatment of the mother, with early detection, can cure the infant of listeriosis, non-specific symptoms of the disease makes diagnosis difficult (69). The flu-like symptoms experienced by pregnant women are associated with the bacteremic phase of the

infection and is the optimal time for blood tests (69). Hence, all febrile episodes during pregnancy should be assessed with blood cultures (69).

Neonatal infection is serious and often fatal. In early-onset neonatal listeriosis, the fetus acquires the infection *in utero* through the placenta from the bloodstream of the mother (69). Illness occurs at birth or shortly after, within the first week of life. Between 45-70 % of neonatal listeriosis is early onset (69). Symptoms include respiratory distress, fever and neurological abnormalities. Less commonly, abscesses in multiple internal organs can develop (69). Late onset neonatal listeriosis occurs at least one week after birth (69). Infants are often born from pregnancies without complications. Infants that have late onset neonatal listeriosis more frequently suffer from meningitis (69). Unlike the early onset disease, the transmission of bacteria in late onset neonatal listeriosis is less clear. The transmission of late onset neonatal listerosis can be transplacental, acquired during passing through the birth canal or contact with an external source (69)

2.1.7 Foodborne Listeriosis and Control

The ubiquity of *L. monocytogenes* makes its introduction into foods an unavoidable reality. Most raw food products likely harbour *Listeria* in low numbers (7). Fish, shellfish and vegetable may be contaminated with *Listeria* by soil or water. Meat and poultry may be contaminated by contact of the carcasses with feces during slaughter (7). *Listeria* in Ready-to-Eat foods may be due to contamination of the production environment (7). The high fatality rate of listeriosis makes surveillance and government regulations to reduce the threat of listeriosis necessary. Past listeriosis outbreaks has improved our understanding of its control. It has become evident that active surveillance, use of standardized subtyping methods, stringent regulation on

Ready-to-Eat foods, especially for the consumption by susceptible individuals, and public education are means of reducing the frequency of listeriosis.

Listeriosis outbreaks are difficult to track due to the low frequency of listeriosis cases. In addition, due to centralized food-processing with wide distribution ranges, listeriosis cases can be geographically dispersed. In 1981, a listeriosis outbreak in Canada due to contaminated coldslaw first established *L. monocytogenes* as a foodborne pathogen (70). The recognition, that food was the primary vehicle for *L. monocytogenes* infection, allowed for subsequent investigation of many listeriosis outbreaks and sporadic cases. In early listeriosis cases, surveillance and identification of cases associated with listeriosis were limited. In 1985, 142 cases of listeriosis were documented in Los Angeles County, California (71). Detection of the outbreak was only possible due to a focused cluster of cases from a single ethnic group which sought medical attention at a single facility (71). This case underscored the need for surveillance as most food manufacturing facilities have wide geographic distributions. In the United States, an active surveillance program in which public health officials routinely contact personnel at all clinical laboratories and acute-care hospitals was established after the 1985 outbreak to better estimate incidence of laboratory-confirmed cases of listeriosis. In 2000, it became mandatory to report listeriosis cases to public health officials (47). These measures may lead to quicker identification of listeriosis clusters and response to limit the magnitude of infection (47).

Due to the dispersed distribution of processed food products, the use of subtyping methods is essential in tracking many listeriosis outbreaks. Standardized PFGE, a highly discriminatory method, is employed by a network of public health and other regulatory laboratories in the United States and Canada, as well as in other countries (47).

Ready-to-Eat foods, that require no heating to eliminate contaminating pathogens, are particularly hazardous to susceptive individuals. Foods such as deli meats and cheese are frequently implicated vehicles of *L. monocytogenes* infection (Table 2-1). Environmental contamination at the processing facility which can occur at multiple steps of food processing (e.g. raw material, after heat inactivation and in storage) were implicated as causes of listeriosis outbreaks (72, 73). In addition, certain contaminated Ready-to-Eat foods which harbour *L. monocytogenes* may allow the pathogen to grow to disease-inducing levels during cold storage. Moreover, food hygiene is especially important to susceptible individuals as evidence by a listeriosis outbreak in a hospital resulting in six deaths (74).

Government policies have been adapted to reduce the risks of ready-to-eat foods. In 2011, Canada updated its policy on *L. monocytogenes* in Ready-to-Eat foods (8). Updates included identifying high and low risk Ready-to-Eat foods, encouraging the use of post-lethality treatments and *Listeria* growth inhibitors and establishing environmental monitoring programs which included sampling surfaces for *Listeria* species that contact products before final packaging and end product testing for *L. monocytogenes*. In addition, strategic education of susceptible individuals and their care-givers were also outlined. Similar policies have been implemented in the United States to facilities producing Ready-to-Eat meat and poultry products after the 2002 turkey deli-meat associated outbreak in the United States (47).

Table 2-1. Major Invasive Listeriosis Outbreaks from 1981 to 2010.

Year	Location	Number of cases (deaths)	Suspected Food	Serotype	Reference
1981	Nova Scotia, Canada	41 (18)	Coleslaw	4b	(70)
1983	Massachusetts, USA	49 (14)	Pasteurized milk	4b	(75)
1985	California, USA	142 (48)	Mexican-style cheese	4b	(71)
1983-1987	Switzerland	122 (31)	Soft cheese	4b	(76)
1987-1989	United Kingdom	355 (94)	Pâté	4bx	(77)
1989-1990	Denmark	26 (6)	Blue mould cheese	4b	(78)
1992	France	279 (85)	Jellied pork tongue	4b	(79)
1993	France	39 (12)	Pork rilletes	4b	(73)
1998-1999	Multistate, USA	108 (14)	Hot dogs	4b	(80)
1995	France	37 (11)	Raw milk soft cheese	4b	(81)
1999	Finland	25 (6)	Butter	3a	(74)
1999-2000	France	10 (3)	Rillettes	4b	(82)

1999-2000	France	32 (10)	Jellied pork tongue	4b	(82)
2000	Multistate, USA	30 (7)	Deli turkey meat	1/2a	(63)
2002	Multistate, USA	54 (8)	Silceable turkey deli-meat	4b	(83)
2002	Quebec, Canada	17	Cheese made from raw milk		(84)
2008	Nationwide, Canada	57 (23)	RTE deli-meats	1/2a	(85)
2009-2010	Austria, Germany and Czech Republic	34 (8)	Acid curd cheese (Quargel)	1/2a	(86)

The majority of previous invasive listeriosis outbreaks were associated with serotype 4b isolates (Table 2-1). However, in recent years, Canadian listeriosis cases and outbreaks were attributed to serotype 1/2a. A recent MLST analysis of 71 important isolates from the National Microbiology Laboratory (NML) and Listeria Reference Service (LRS) collections which consist of 10 most common PFGE types spanning 30 years revealed a predominant clone. This clone belongs to lineage II and is predominantly serotype 1/2a (87). In addition, analysis of 41 listeriosis case clusters between 1995 and 2010 revealed that 75.6% were caused by serotype 1/2a isolates (87). A similar report of surveillance of Canadian listeriosis cases and outbreaks between 1995 and 2004 also revealed serotype 1/2a as the predominant serotype from patients (88).

2.1.8 Cell Envelope

The *L. monocytogenes* cell envelope consists of a cell membrane underneath a peptidoglycan layer. The peptidoglycan is a polymer of altering units of N-acetylmuramic acid and N-acetylglucosamine (89). These chains are cross-linked by peptidic bridges. Two polyanionic polymers are associated with the cell envelope: teichoic acids (TAs) and lipoteichoic acids (LTAs). TAs are covalently associated with the peptidoglycan while LTAs are embedded into the cell membrane by a diacyglycerolipid (89). Both polymers form part of the basis of serotype specificity in *L. monocytogenes* (89). Specifically, TAs are polymers of ribitol-phosphates substituted with alanine residues and various sugars depending on the serotype. LTAs are polymers of glycerophosphate substituted with alanine, galactose and lipids (89). Some *Listeria* surface proteins are associated to the cell wall via LTAs (89).

2.1.9 Surface Proteins

The *L. monocytogenes* strain EGD-e has been studied extensively. At least 4.7% of the coding genes (133/2853 genes) of the *L. monocytogenes* strain EGD-e genome are dedicated to surface proteins (32). The high proportion of surface and secreted protein genes reflect the capacity of *L. monocytogene*s to interact with a variety of surfaces and to colonize many host cell types (39).

2.1.9.1 Anchoring Mechanisms of Surface Proteins

Surface proteins can be covalently or non-covalently linked to the peptidoglycan or are membrane-bound. Surface proteins can be classified by the domains or motifs that allow their association to the cell envelope.

Surface proteins covalently linked to the peptidoglycan contain a C-terminal sorting composed of a LPXTG (leucine-proline-any amino acid-threonine-glycine) motif followed by about 20 amino acids and a stretch of positively charged residues (89). The LPXTG motif is the substrate of sortase A, a membrane-bound transpeptidase, which cleaves the LPXTG motif between threonine and glycine and catalyzes the formation of an amide bond between the carboxyl group of threoine and an amide group in the peptide bridge of the peptidoglycan (89). Sortase B mediates covalent linkage of proteins with a C-terminal NPQTN motif to peptidoglycan (90). In *L. monocytogenes*, two proteins, lmo2185 and lmo2186 have the NPQTN motif. Evidence exists only for lmo2185 as substrate of sortase B (90).

Non-covalent surface protein association with the cell wall is mediated by repeated domains such as GW (glycine-tryptophan) modules, WxL (tryptophan-any amino acid-leucine) and LsyM (lysine motif) domains. GW modules are approximately 80 amino acids in length and contain the dipeptide glycine and tryptophan. GW modules are usually arranged in tandem. The first characterized GW protein was Internalin B (InlB), a virulence factor involved in *L. monocytogenes* entry into mammalian cells. The three GW modules on InlB were sufficient and necessary for its association with the cells (89). GW modules bind to LTAs. Increasing the number of GW modules increased the affinity of GW protein binding to the cell wall (89). WxL domains consist of 160 to 190 amino acids and contain a tryptophan-any amino acid-leucine signature. WxL domains have been observed to be associated with the peptidoglycan in

Enterococcus faecalis (91). LysM domains consist of approximately 40 amino acids and bind non-specifically to the cell wall peptidoglycan (89).

Surface proteins can also associate with the cell membrane. There are three means for this interaction. Firstly, surface proteins can be retained in the membrane via their C-terminal hydrophobic tail of 22 amino acids (32). Secondly, surface proteins can be anchored to the membrane by the N-terminal signal peptide itself if uncleaved (89). Thirdly, lipoproteins are anchored to the membrane by covalent N-terminal lipidation. The N-terminal signal peptide consists of a lipobox followed by a conserved cysteine. After translocation of a lipoprotein across the membrane, prolipoprotein diacylglycerol transferase (Lgt) catalyzes the transfer of a diacylglycerol group to the conserved cysteine. Subsequently, the N-terminal signal peptide is cleaved from the protein by signal peptidase II (SPII), leaving the cysteine residue at the N-terminus of the anchored protein (89).

2.1.9.2 Functions of Surface Proteins

Surface proteins play several roles in *L. monocytogenes,* including cell wall remodelling, protein processing and folding, adhesion to host cells and motility.

Cell wall remodelling is important for cellular processes such as cell growth and division. Since cell wall derivatives are stimulators of host immunity, enzymes promoting the release of cell wall components are important modulators of host immune response (89). There are several surface proteins involved in cell wall remodelling known as autolysins. Certain surface proteins, known as hydrolases, cleave the sugar backbone of the peptidoglycan between alternating units of N-acetylmuramic acid and N-acetylglucosamine that make up a glycan chain. Hydrolases Auto and IspC, anchored by GW modules, have been demonstrated to have N-

acetylglucosaminidase activity (92, 93). MurA is another hydrolase of *L. monocytogenes* anchored by a LysM domain (94). The surface protein Ami is a putative amidase (95). Amidases cleave the bond between the glycan chain and the cross-linking peptide chain of the peptidoglycan. Peptidoglycan assembly requires several enzymes. The family of penicillin binding proteins (PBPs) include β-lactamases as well as transglycosylases, transpeptidases, and D-alanyl-D-alanine carboxypeptidases which are involved in peptidoglycan assembly. Transglycosylases catalyze the insertion of peptidoglycan precursors to the growing end of the glycan chain. D-alanyl-D-alanine carboxypeptidases remove the terminal D-alanine residue from peptidoglycan pentapeptides. Transpeptidases then join the peptide of a precursor to the pre-existing peptidoglycan to form the peptidic link between glycan chains (89). Finally, PgdA, which deacetylates N-acetylglucosamine residues of the glycan chain, was shown to confer resistance to lysozyme, an innate defence mechanism in humans against bacteria (89).

After translocation across the cell membrane, proteins with signal peptides must be cleaved by a signal peptidase (SPase). SipX, SipY and SipZ are three type I SPases and are predicted to cleave signal peptides of preproteins exported by the general Sec pathway (89). SipZ is the major SPase of virulence factors (89). Another class of SPase are the type II SPases which have prolipoproteins as substrates. *Listeria* SPases II consist of LspA and the uncharacterized LspB. YidC/Oxa1/Alb3 proteins serve as chaperones for proteins that remain inserted in the cell membrane (89). Other proteins act as folding catalysts and proteases after proteins have translocation across the cell membrane to assist in protein refolding and remove aberrant proteins from the translocase (89). Prs-A (Prs-A1) and Prs-B (Prs-A2) may be involved in folding of exported proteins (89). Prs-B (Prs-A2) is involved in bacterial adaption in host cells (96). The *Listeria* HtrA-like serine protease is involved in stress response and pathogenesis (89).

Since *L. monocytogenes* is a facultative intracellular pathogen, certain surface proteins are involved in entry into host cells. There are two major proteins that facilitate host cell invasion: InlA and InlB. InlA is sufficient for entry into epithelial cells of intestinal and placenta barriers. This interaction involves interaction between InlA and host cell receptor E-cadherin at the junctions of epithelial cells (97). InlB mediates entry into a variety of host cells by interacting with the Met receptor (hepatocyte growth factor receptor) (98). Vip is another virulence factor that interacts with the Hsp protein Gp96 to trigger cell signal resulting in cell invasion (99).

Surface proteins also mediate adhesion to host cells by binding to specific host cell receptors such as components of the extracellular matrix which surround mammalian cells. Components of the extracellular matrix include structural proteins such as collagen, specialized proteins such as fibronectin and high molecular weight proteins such as proteoglycans (89). Several conserved domains found in *Listeria* surface proteins are involved in the attachment to components of the extracellular matrix. The fibronectin binding protein, FbpA, is required for intestinal and liver colonization by *L. monocytogenes* after oral infection (100). In addition, GW domains of InlB not only bind to LTAs but also to components of mammalian proteoglycan (89). This may enhance interaction of InlB to its cognate host cell receptor, Met. Several *Listeria* surface proteins with the LPXTG motif have Ig-like fold domains. These domains may play a role in adhesion or facilitate bacterial interactions with host cells by projecting a ligand binding region (89).

Listeria surface proteins are involved in motility. *Listeria* movement is propelled by the movement of the flagellum that consists of a long filament anchored to the cell envelope by a flexible hook and basal body (89). The flagellin protein of *Listeria* is produced and assembled on

the cell surface at 20-25 ^{o}C with reduced expression at 37 ^{o}C (89). ActA is known to mediate polymerization of host cell actin for movement in the host cytoplasm (89).

2.1.10 Protein Secretion System

Surface proteins and proteins secreted to the extracellular milieu rely on protein secretion systems to cross the cytoplasmic membrane (101). Six proteins secretion systems were identified in *Listeria* genomes: secretion apparatus (Sec), fimbrilin-protein exporter (FPE), twin-argine translocation (Tat), flagella export apparatus (FEA), WXG100 secretion system (Wss) and the Holins (101).

The *L. monocytogenes* genome encodes all putative components of the Sec system. Although there is currently no direct evidence of a functional Sec system in *Listeria*, proteomic analysis have identified the presence of a large number of proteins, bearing putative Sec-dependent N-terminal signal peptide, in the extracellular supernatant or cell envelope (101). The Sec system is the major translocation system in bacteria (102). The process of protein secretion involves: protein targeting, translocation and protein release (103). Proteins must contain an N-terminal signal peptide to be targeted for translocation to the Sec system. This signal peptide, which can be identified by computer algorithms such as Signal P, consists of three parts: a positively charged amino terminus, a central hydrophobic domain and C-terminal peptidase cleavage site (104).

There are several main components of the Sec system. The first is translocase which functions as a protein conducting channel through the cell membrane. It is a heterotrimeric complex of proteins SecYEG. *E. coli* serves as the paradigm protein secretion mechanism in bacteria (101). In *E. coli,* preprotein can be targeted to the translocase during translation by

signal recognition particle (SRP) or post-translationally by SecB (104). SRP is a complex of 4.5S RNA and a GTPase called fifty-four homolog (FfH) that interacts with the signal sequence or hydrophobic transmembrane segments of nascent membrane proteins (104). FfH interacts with its receptor FfY at the cytoplasmic membrane which interacts directly with the translocase (105). While SecB is not found in Gram-positive bacteria, all components of the SRP complex are found in *Listeria* species (101). In addition, other chaperones involved in protein translocation through the translocase may exist (101). SecA is an ATPase that provides energy for the translocation of preproteins through the SecYEG channel (102). The mechanism of SecA function is unclear, however SecA interacts with the preprotein and the translocase (106).

2.1.11 The PrfA Virulence Regulon

PrfA is a transcription factor that regulates key virulence factors involved in intracellular life cycle of *L. monocytogenes* (31). PrfA can activate transcription directly by binding as a dimer to its palindromic recognition elements known as the PrfA box or tTAACanntGTtAa (31). PrfA integrates several environmental signals to ensure maximal induction of its regulon in the host cell cytosol and repression in environmental habitats (31).

2.2 *Listeria* Diagnostics

2.2.1 Standard *Listeria* Isolation and Detection Methods

Health Canada's Compendium of Analytical Methods (CAM) contains standard methods used to assess foods for microbiological content and to determine compliance to safety standards of foods sold in Canada according to the Food and Drug Act. Health Canada's CAM outlines two methods (MFHPB-30 and MFHPB-07) for *L. monocytogenes* and other *Listeria* species isolation and identification. These culture methods are reference methods that must be performed

to confirm all presumptive positive results obtained from other validated methods (MFLP methods) in Health Canada's CAM.

MFHPB-30 can be used for isolation and identification of *L. monocytogenes* and other *Listeria* species from all foods and environmental surfaces (107). This method involves a primary culture in University of Vermont Media 1 (*Listeria* Enrichment Broth) or UVM1 (LEB), followed by secondary enrichment culture in modified Fraser broth (MFB). These enrichment steps take two to three days to complete. The incorporation of esculin and ferric ammonium citrate in all selective enrichment media of MFHPB-07 and MFHPB-30 allows for presumptive detection of *Listeria* when the medium darkens. All *Listeria* species can hydrolyse esculin to esculetin which reacts with ferric ions in the medium resulting in medium blackening (108).

For *Listeria* isolation and identification, a presumptive *Listeria*-positive MFB culture is streaked on Oxford agar (OXA) and one additional selective plating media. At least five typical colonies picked from plates are subjected to identification and confirmation. Confirmatory tests include hemolysis on blood agar, motility test (umbrella migration in stab agar or tumbling rods in wet-mount) and carbohydrate utilization producing acid without gas. In addition, a selection of optional tests can be used which includes rapid identification kits, catalase test, Gram stain, CAMP test, PCR and serotyping.

Method MFHPB-07 can be used for detection and isolation of *L. monocytogenes* and other *Listeria* species from all food samples, except Ready-to-Eat meat and poultry, smoked fish, kefir and fermented dairy drinks, and environmental samples (107). This method requires the use of Palcam and University of Vermont Media 2 (UVM2) as first and second enrichment culture respectively and takes two to three days to complete. Presumptive *Listeria*-positive UVM2 culture is streaked on OXA agar and one additional selective plating media. In MFHPB-07, the

same protocol as MFHPB-30 is followed for *L. monocytogenes* and other *Listeria* species identification.

2.2.2 Selective Agents, Selective Enrichment Cultures and Plating Medium

Listeria in food, environmental and clinical samples are usually found in trace amounts among background microflora (7). Therefore it is necessary to increase the numbers of *Listeria* with respect to other background microflora for *Listeria* detection and this is achieved through selective enrichment culture. Due to the non-specific nutrient requirements of *Listeria,* the selective enrichment effect in enrichment media is attributed to the use of antibiotics to inhibit background microflora. Inhibitory substances such as lithium chloride, nalidixic acid, acriflavine, polymyxin B, moxalactam and ceftazidime have been used in selective enrichment medium (both liquid and solid medium) for the isolation of *Listeria*.

Selective enrichment media UVM1 (LEB) and UVM2 contain selective agents: nalidixic acid and acriflavine. Nalidixic acid is a widely-used selective agent and one of the most important selective agents to isolate *Listeria* from food and clinical samples (109). Although, nalidixic acid inhibits the growth of Gram-negative bacteria, some Gram-positive cocci and Gram-negative rods still persist in the presence of nalidixic acid (109). Since acriflavine inhibits the growth of Gram-positive cocci (7), nalidixic acid is often used in combination with acriflavine.

Palcam consists of selective agent polymyxin-B in combination with ceftazidime, acriflavine and lithium chloride. Polymyxin B inhibits the growth of Gram-negative rods and enterococci. A combination of polymyxin B and nalidixic acid was useful in isolating *L. monocytogenes* from feces which contain *Enterococcus faecalis* (109). Whereas some

investigators found little added value of polymyxin B in media which already contains nalidixic acid and acriflavine (110), others found that combination of polymyxin B, nalidixic acid and acriflavine to be useful for the isolation of *L. monocytogenes* from certain fermented dairy products (111). Ceftazidime is a broad-spectrum cephalosporin antibiotic, and when used in combination with acriflavine inhibited all background microflora including yeasts and moulds (109). Both Palcam and MFB media include lithium chloride which inhibits the growth of enterococci (108).

Solid media have been developed to isolate colonies of *Listeria* after selective enrichment cultures. McBride Listeria agar is one of the first widely used plating medium (109). The use of lithium chloride/phenylethanol in McBride Listeria agar (MLA) can be used for the selective growth of *Listeria* in the presence of Gram negative bacteria (109). Addition of moxalactam to MLA resulted in lithum chloride phenylethanol maxalactam (LPM) agar. LPM was suitable for isolating *Listeria* from raw meat and poultry and was the plating medium of choice in the earlier version of U.S. Department of Agriculture (USDA) procedure (109). Both Oxford agar and modified Oxford agar containing moxalactam have esculin and ferric ammonium citrate which serve as indicators for *Listeria* species. Both MFHPB-30 and MFHPB-07 utilize Oxford and LPM agars.

Since primary and secondary selective enrichment cultures do not selectively enrich for *L. monocytogenes* among other *Listeria* species, the development of chromogenic agar capable of differentiating *L. monocytogenes* from other *Listeria* species improved the efficiency for isolation of *L. monocytogenes*. In both MFHPB-30 and MFHPB-07 methods, *L. monocytogenes* is detected from a selection of at least five typical *Listeria* colonies on a plating media. Since the conventional plating medium does not discriminate between *L. monocytogenes* and other *Listeria*

species, it is possible that no *L. monocytogenes* colonies are picked even when *L. monocytogenes* colonies are present (7). In addition to Oxford agar, at least one selective solid medium such as Agar Listeria according to Ottaviani and Agosti (ALOA) is recommended in MFHPB-30 and MFHPB-07 methods. The chromogenic agar, ALOA, consists of substrates of phosphotidyl-inositol-specific phospholipase C (PI-PLC) produced only by *L. monocytogenes*. PI-PLC activity results in water-insoluble fatty acids which manifests as opaque halo around *L. monocytogenes* colonies (112). In addition, ALOA contains a substrate of beta-glucosidase found in all *Listeria* species for the detection of all *Listeria* species (112). The addition of lithium chloride, nalixic acid, and/or cycloheximide to ALOA allows for *Listeria* species selection. Other commercially available chromogenic agar include CHROM agar and Rapid' L.mono ® (7).

2.2.3 Rapid *Listeria* Isolation and Detection Methods

Ideally, rapid methods should yield comparable results to standard culture methods but in less time (7). The benefits of rapid detection include reducing the risk of releasing contaminated products for sale by increasing detection efficiency and increasing the shelf-life of products cleared for sale (7). Timely detection of *L. monocytogenes* at critical control points such as food processing environments will also allow for better control of the pathogen in these environments (8).

PCR-based methods are the most frequently reported rapid detection methods. Common PCR targets for *L. monocytogenes* detection include virulence genes: *hly* for listeriolysin O (113-117) and *iap* for invasion-associated protein p60 (113, 118). Other genes such as 23S rRNA (117) have also been used as targets for *L. monocytogenes* detection. Commercial PCR kits,

which include pre-packaged reagents and associated equipment that automates PCR product detection, allow for the adoption of PCR for routine food testing (119, 120).

Although PCR targets DNA which is less influenced by environmental conditions than proteins, PCR inhibitors in food, clinical and environmental samples can confound results (13). In addition, PCR cannot distinguish between live and dead cells. Only the detection of live pathogen provides evidence of associated health risks. Real-time PCR (RT-PCR) which targets mRNA may provide a better indication for the presence of live pathogen. mRNA which has a half-time of a few minutes (121) is quickly degraded after cell death. RT-PCR has many advantages such as elimination of post-amplification sample handling and its quantitative nature. The drawbacks of RT-PCR are difficulties in RNA extraction and DNA contamination, which leads to false positive results (10).

Antibodies can be used in ELISA for pathogen detection or conjugated on solid matrices for pathogen isolation. Antibodies targeting epitopes on surface antigens of intact pathogens can be used to detect live cells (10). The pitfalls of current antibodies includes variable expression in of antigens in selective enrichment cultures (26-28), weak association with the cell surface (122) and lack of expression in some serotypes (19, 28). Research is needed to discover new surface protein biomarkers for *L. monocytogenes* isolation and detection.

2.2.4 Immunological Isolation and Detection Methods

2.2.4.1 Antibodies against *L. monocytogenes* and *Listeria* species

Development of monoclonal antibodies (MAbs) and polyclonal antibodies (PAbs) for use in *L. monocytogenes* isolation and identification is challenging. Ideal diagnostic antibodies should be reactive to all serotypes of *L. monocytogenes* (or at least serotypes implicated in

outbreaks and sporadic cases of listeriosis). In addition, the targets recognized by diagnostic antibodies need to be present in selective enrichment culture conditions and be tightly associated with the cell surface for pathogen capture and detection. Research is still needed to identify potential targets for the development of diagnostic antibodies for *L. monocytogenes.*

Most published reports describe MAbs which have been developed against a complex antigen such as formalin-killed or heat-killed *L. monocytogenes* cells or surface extracts of *L. monocytogenes.* The specific protein target is often undefined. Early MAbs developed were reactive to *L. monocytogenes*, other *Listeria* species and non-*Listeria* bacteria. Siragusa *et al.* developed a series of MAbs against heat-killed, formalin-killed *L. monocytogenes* and cell wall extract of *L. monocytogenes* (23). All but one MAb, P5C9, was specific to *Listeria* and was reactive to *L. monocytogenes*, *L. welshimeri* and *L. innocua* while the other MAbs were reactive to bacteria other than *Listeria* (23). Subsequent studies were successful in developing *Listeria* genus-specific antibodies. Using a crude cell surface protein extract, Bhunia *et al.* developed a MAb, C11E9 that was specific to *L. monocytogenes* and *L. innocua* (20). The target of C11E9 that was extracted from the *L. monocytogenes* cell surface using choatropic agents suggested that the target was non-covalently associated with the cell surface (20). In addition, MAb C11E9 was reactive to several bands at 76, 66, 56, 52 kDa in a denaturing electrophoresis gel and one band in a native electrophoresis gel suggesting that it was reactive to a protein complex (20). Torensma *et al.* used live *L. innocua* and antibiotic attenuated live *L. monocytogenes* to develop seven MAbs (24). Reactivity of most of these MAbs did not correlate with serotype (24). In additions, most MAbs were reactive to one or more *Listeria* species other than *L. monocytogenes.* MAb 55-37 was specific to *L. monocytogenes* isolates tested however the MAb did not react to majority of *L. monocytogenes* isolates tested (24). MAb 55-23 reacted with most

Listeria isolates tested and all *Listeria* species tested (24). Using heat-killed cells as immunogen, Heo *et al*. developed two MAbs 22D10 and 24F6 that react strongly to several serotypes of live *L. monocytogenes* (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab,4b, 4d and 7) except serotypes 4c and 4e cultured in LEB (22). These MAbs were also strongly reactive to several isolates of live *L. innocua* and weakly reactive to *L. ivanovii*, *L. murrayi* and *L. welshimeri* (22). The reactivity of MAb 24F6 was significantly reduced when tested with heat-killed *Listeria* cells (22).

Efforts were made to generate *L. monocytogenes* specific MAbs. As previously mentioned, Bhunia *et al.* developed MAb that was specific to *L. monocytogenes* and *L. innocua* (20). Partial chymotrypsin digestion of crude surface protein extracts from *L. monocytogenes* and *L. innocua* followed by western blotting using MAb C11E9 revealed differential digestion patterns (123). Prior to digestion, MAb C11E9 detected a predominant 66 kDa protein in the surface protein extracts of both *L. monocytogenes* and *L. innocua*. However after digestion of crude surface protein extracts, multiple bands were detected by MAb C11E9 for *L. monocytogenes* while only one band was observed for *L. innocua* (123). It was hypothesized that although the protein targeted by C11E9 is present on both *L. monocytogenes* and *L. innocua*, difference in spatial arrangement of the C119 epitope exist between the two *Listeria* species (123). Since C11E9 was not specific to *L. monocytogenes,* it is possible to mask epitopes targeted by C11E9 to develop *L. monocytogenes* specific MAbs using C11E9. Hence, the *L. monocytogenes* specific MAb EM-7G1 was developed using an immunogen of heat-killed *L. monocytogenes* incubated with MAb C11E9 (123). It was later found that C11E9 and EM-7G1 MAbs both bind to an autolysin, *lmo2691* but at different epitopes (25). As previously mentioned, C11E9 also bound to several proteins while EM-7G1 targeted only protein, lmo2691. Corroborating electron microscopy results revealed more C11E9 foci than EM-7G1 (124).

Certain MAbs specific to *L. monocytogenes* are also serotype specific. MAbs that are specific for 4b serotype isolates of *L. monocytogenes* have been developed from immunization with formalin-killed cells (18, 125).

Selective enrichment culture is often an integral step in *L. monocytogenes* detection. To determine whether certain MAbs are applicable for *L. monocytogenes* detection, studies have been conducted to analyse the reactivity of MAbs to *L. monocytogenes* cultured under various selective enrichment culture conditions. Many MAbs previously reported showed reduced or no reactivity to *L. monocytogenes* in selective enrichment cultures and stress conditions thereby reducing their utility in *L. monocytogenes* detection. Nannapaneni *et al.* tested the reactivity of the *L. monocytogenes* specific MAb, EM-7G1, to *L. monocytogenes* grown in various selective enrichment media. EM-7G1 was reactive to *L. monocytogenes* isolates grown in BHI (Brain Heart Infusion), LEB and LRB (*Listeria* Recovery Broth) but not in UVM or FRB (Fraser broth with ferric ammonium citrate) (27). Moreover, MAb EM-7G1 could not detect *L. monocytogenes* serotypes 4ab, 3b, 4a and 4c isolates (27). Geng *et al.* tested the reactivity of MAbs EM-7G1 and C11E9 with *L. monocytogenes* cells grown in BHI with addition of various stressors such as low pH, high salt and at various temperatures (4, 15, 25, 37, 45 ^{o}C) (124). Brief (3 hours) and prolonged (16-72 hours) exposure to high salt (>1.5% w/v) and temperature (45 ^{o}C) reduced reactivity of both MAbs (124). Prolonged acid and cold exposure had no effect (124). Geng *et al.* also observed differential MAb C11E9 reactivity under various stress conditions in conjunctions with enrichment medium culture (25). Exposure of *L. monocytogenes* to stressors such as acid, cold, heat and salt prior to culture in UVM and FB resulted in minimal reactivity (25). Other MAb targets also showed variable expression in enrichment cultures. A single-chain variable fragment (scFv) screened from a phage display library targets an undefined protein with

differential expression in various enrichment cultures (28). Lack of detection of this protein was observed in many *L. monocytogenes* strains grown in FB, UVM and LEB (28). The differential reactivity of InlB and ActA PAb against *L. monocytogenes* grown in various medium was also observed (26). While InlB PAb was reactive to *L. monocytogenes* isolates in non-selective medium such as BHI and LB, minimal reactivity was observed in BLEB, UVM and FB (26). In contrast, ActA PAb was reactive to *L. monocytogenes* isolates in BLEB, UVM and FB and minimal reactivity was observed in BHI and LB (26). Certain virulence factors such as InlB and ActA may not be ideal targets for pathogen detection as their expression is attuned to *in vivo* conditions (29, 30).

Some MAbs have limited ability to discriminate between live and dead *L. monocytogenes* (27, 126). The ability to detect live cells can provide direct evidence of the health risk associated with *L. monocytogenes* (126). Solve *et al.* developed a MAb that could not detect heat-killed cells, however the MAb's reactivity to cells killed by other methods besides heat-treatment is ambiguous (126). Likewise, Nannapaneni *et al.* developed a MAb that was capable of detection live but not heat-killed *L. monocytogenes* (27). Nonetheless, ability to detect heat-killed cells is still a desirable trait in diagnostic MAbs. Many manufacturers of diagnostic test kits uses a heat-kill step in protocols to reduce the dangers of working with live pathogens to workers (22).

MAbs have also been developed against secreted proteins. These MAbs could be potentially used in testing formats such as ELISA. Erdenlig *et al.* developed MAbs against the listeriolysin O (LLO) toxin and phosphatidylcholine-specific phospholipase C (PC-PLC) from secreted protein extracts of *L. monocytogenes* (21). These MAbs were specific to *L. monocytogenes* and *L. ivanovii*. Nato *et al*. also developed MAbs against native LLO extracted from *L. monocytogenes* culture as well as a conserved peptide found in all thiol-activated toxins

(127). Yu *et al.* developed two MAbs p6007 and p6017 against p60 (122). Although p60 can be found weakly associated with the cell surface, the author tested the culture supernatant for p60.

Research is still needed to develop diagnostic MAbs that target epitopes expressed in standard enrichment culture conditions and are specific for a wide range of *L. monocytogenes* serotypes. In addition, for isolation of pathogen to facilitate downstream detection, protein antigens also need to be tightly associated with the *L. monocytogenes* cell envelope.

2.2.4.2 Immunomagnetic Separation

Immunomagnetic separation (IMS) offers a means of purifying and concentrating live pathogens from sample matrices and background microflora. Sample is usually homogenized in standard selective enrichment media followed by incubation to allow growth of target bacteria (10). Large food particles are removed by low speed centrifugation. A sample of supernatant is combined with antibody-coated magnetic beads and incubated to allow antibodies to bind to cell surface antigens of *L. monocytogenes* (10).

Either direct or indirect IMS can be used for pathogen recovery. In the direct approach, beads, coated with antibodies specific for the target organism, are incubated with samples followed by recovery of the beads by application of a magnetic field. In the indirect approach, specific primary antibodies are added to the sample for attachment of the target organism, followed by addition of beads coated with secondary antibodies to interact with the antibody-bacterium complex. Beads coated with target organism are recovered by application of a magnetic field (128). Subsequently, bacteria captured on beads could be added to liquid medium for additional growth, plated for enumeration or processed for molecular analysis.

Inhibitors in food or other matrices (13) and background microflora (15) can confound results of rapid molecular detection methods. In some reports, IMS is required for the detection of target cells from the food matrix (116). In addition, Hsih *et al.* observed that more than a 2 log difference between two different cell types caused one cell type to be undetected (15). IMS prior to PCR detection circumvented this problem (15). Effective isolation of *L. monocytogenes* by IMS can shorten enrichment culture time and facilitate downstream detection by molecular methods.

Previous reports described the challenges and successes of IMS application in isolation of *L. monocytogenes* from foods. Challenges of IMS include weak antibody-target interactions and non-specific binding of other foodborne pathogens besides *Listeria* species. Hudson *et al.* observed significant loss of *L. monocytogenes* due to the wash steps in IMS (116). Commercial anti-*Listeria* Dynal™ beads were assessed in IMS for *L. monocytogenes* (129). Twenty-four hour enrichment culture was required for detection of low number of *L. monocytogenes* (<10 CFU/g of cheese). Although good limit of detection at 40-100 CFU/ml was achieved, the beads did not discriminate *L. monocytogenes* from non-*Listeria* microflora. Hence no specific enrichment of *L. monocytogenes* was obtained from commercial anti-*Listeria* Dynal™ beads (129).

Many antibodies used for IMS are *Listeria* genus specific but not specific for *L. monocytogenes* (112, 129-131). Recently *L. monocytogenes* specific IMS has been reported. Paoli et al. developed biotinylated single-chain antibody fragments coupled with streptavidin coated beads for *L. monocytogenes* capture, which yielded enrichment of *L. monocytogenes* in a mixture of either *L. monocytogenes* and *L. innocua* or *L. monocytogenes* and *L. ivanovii* grown in non-selective culture (132). Further work should assess the ability of the single-chain antibody fragments to capture *L. monocytogenes* grown in selective enrichment cultures, given that a

previous report described the minimal reactivity of these single-chain antibody fragments with *L. monocytogenes* isolates grown in selective enrichment cultures (28). Mendonca *et al.* also reported the application of IMS to the capture of *L. monocytogenes* and *L. ivanovii*. Although other *Listeria* species were captured at lower capture efficiencies (2.0-2.4%) than that for *L. monocytogenes* (49.2%) and *L. ivannovii* (32.2%), significant numbers of cells (400-480 CFUs) from other *Listeria* species were captured given the amount of cells added. Interestingly, the size of beads used for capture offers different outcomes for pathogen isolation. Mendonca *et al.* reported better capture with smaller 1 μm MyOne beads in comparison to 2.8 μm beads.

2.2.4.3 Antibody-Based Detection of *L. monocytogenes* and *Listeria* species

Enzyme-linked immunosorbent assay (ELISA) is an antibody-based method that involves the immobilization of an antibody onto a solid substrate for bacterial capture. Detection can be achieved with a second antibody covalently linked to an enzyme or the use of a secondary antibody covalently linked to an enzyme that is specific to second antibody. ELISA is relatively robust to sample matrices and therefore is suited for food testing (11). Since typical detection limit for ELISA is approximately ~ 10^4 to 10^6 CFU/ml (10, 11), which is much higher than usual *L. monocytogenes* contamination, selective enrichment culture of test samples is usually required prior to ELISA to amplify *L. monocytogenes* to detectable numbers (16).

There are two ELISA methods using commercial kits outlined in Health Canada's Compendium of Analytical Methods. The first method (MFLP-71) uses the Clearview kit (133). Antibody against *Listeria* flagella proteins are immobilized on a membrane strip. Additional anti-*Listeria* antibodies conjugated to coloured latex are added along with the sample. Presence of antigen results in the antigen sandwiched between immunobilized and coloured latex antibodies which appears as a blue line. Methods MFLP-33 and MFLP-77 of Health Canada's

Compendium of Analytical Methods both uses the VIDAS system (134, 135). MAbs specific for either *L. monocytogenes* or *Listeria* species are immobilized onto the interior of pipette tips which are used in automated sample processing and signal acquisition. While, some studies showed that the VIDAS system for *L. monocytogenes* or *Listeria* species detection is comparable to culture methods for raw foods (136, 137) and certain Ready-to-Eat foods (138), other studies have reported weak non-specific reaction to other bacteria (139) and discrepancies with culture method (140). Other studies have reported ELISA methods with specificity for *Listeria* species but lacking specificity for *L. monocytogenes* (141-143).

2.2.5 Current Methods for *Listeria* Detection Employed by CFIA

Listeria and *L. monocytogenes* detection follows the methods described in Health Canada's Compendium of Analytical Methods. A schematic of *L. monocytogenes* and *Listeria* detection is outlined in Figure 2-1. MFLP-28 and MFLP-15 methods are used to screen for *L. monocytogenes* and *Listeria* contamination in food and environmental samples (119, 120). MFLP-28 is a PCR-based method for the detection of *L. monocytogenes* in food samples. After 24-48 hours of primary selective enrichment culture and 18-24 hours of secondary selective enrichment culture, cultured cells are lysed for DNA template preparation. For bacterial cells cultured from Ready-to-Eat foods, cells are first washed and then lysed. PCR tubes are packaged with all necessary reagents including internal control. The automated BAX system presents "yes", "no", "indeterminate" or "error" results after PCR reactions are complete. MFLP-15 uses the BAX system, similar to MFLP-28, but screens for *Listeria* species from environmental surfaces. After 48 hours of primary enrichment culture, cells are washed, lysed and analyzed by PCR in the BAX PCR system. Acquired results are presented similarly as MFLP-28.

Presumptive positive samples are confirmed by the MFHPB-07 and MFHPB-30 culture methods as previously described. No further action is required with negative results.

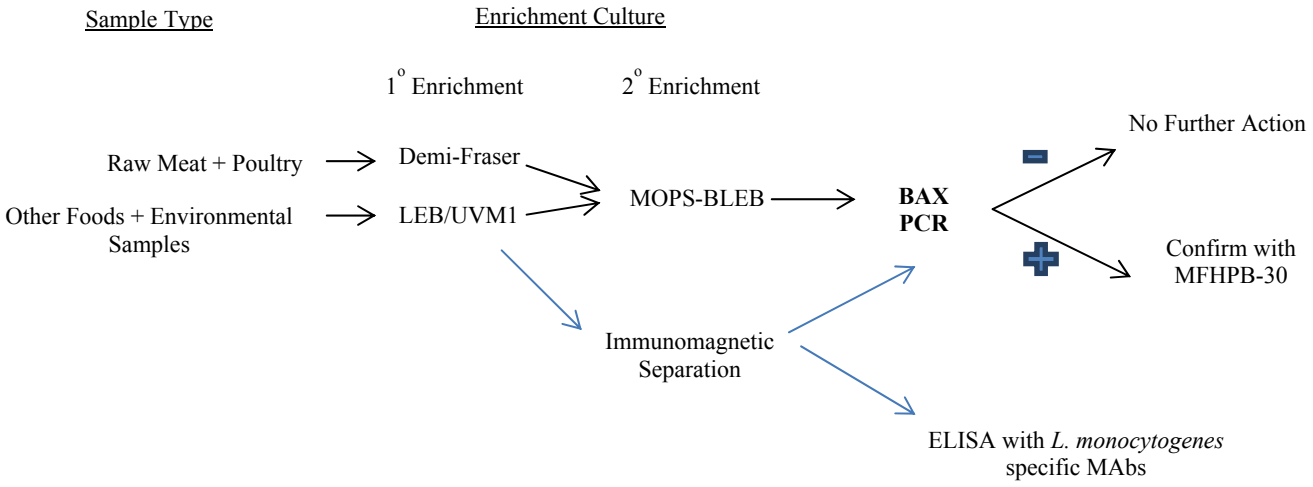


Figure 2-1. Schematic of current *Listeria* detection methods employed by CFIA. The steps in the standard methods are presented in black. Steps which can be improved by *L. monocytogenes* specific MAbs are presented in blue. Immunomagnetic separation can be applied after primary selective enrichment culture to isolate *L. monocytogenes* for *L. monocytogenes* detection by PCR or ELISA.

The development of MAbs specific for *L. monocytogenes* can improve the current detection procedure for *L. monocytogenes* at several steps (Figure 2-1). Specific MAbs capable of capturing *L. monocytogenes* may concentrate the pathogen thereby reducing the selective enrichment culture time needed to yield sufficient *L. monocytogenes* cells for downstream detection. *L. monocytogenes* isolation can also facilitate downstream detection by providing a relatively pure cell population. Since standard selective enrichment culture does not discriminate *L. monocytogenes* from other *Listeria* species, *L. monocytogenes* may go undetected due to other *Listeria* species out-competing the target (15, 144, 145). In addition, PCR inhibitors in both foods samples and the selective enrichment culture broth can affect PCR results negatively. Isolation of *L. monocytogenes* using antibody-based methods produces relatively pure *L. monocytogenes* cells in which substances inhibiting PCR reactions have been removed and thus improves the PCR results. Finally, specific MAbs can be used to detect *L. monocytogenes* grown in selective enrichment culture conditions.

Chapter 3: Materials and Methods

3.1 Bacterial Strains, Plasmids and Growth Conditions

Listeria isolates were incubated overnight on Brain Heart Infusion (BHI) agar or in BHI broth (BD Biosciences, Mississauga, ON, Canada) at 37°C. For assessment of antigen expression in standard enrichment culture, selected *Listeria* isolates were grown according to methods MFHPB-07 (107) and MFHPB-30 (146) published in the Health Canada's Compendium of Analytical Methods. In the MFHPB-07 method, *Listeria* isolates were cultured in 10 ml Palcam broth (medium base and selective supplement from Oxoid, Basingstoke, England) at 35^{o}C for 26-28 h followed by a 1 in 10 fold dilution culture in UVM2 broth (medium base and selective supplement from Oxoid) at 30^{o}C for 26-28 h. In the MFHPB-30 method, bacterial isolates were cultured in 10 ml LEB (UVM1, base and supplement from Oxoid) at 30^{o}C for 48 h followed by a 1 in 10 fold dilution culture in MFB (medium base from EMD, Gibbstown, NJ, USA; supplement from Oxoid) at 35^{o}C for 24 h. Cell concentrations were estimated by measuring the optical density (OD) of the culture at 620 nm and confirmed by plating. *Escherichia coli* stains (DH5α and Rosetta DE3/(pLysS)) used for cloning and recombinant protein expression respectively were cultured in Luria-Bertani (LB) broth (BD Biosciences, Sparks, MD, USA) supplemented with 50 µg/ml kanamycin to maintain the pLIC plasmid derivative. For immunomagnetic separation of *L. monocytogenes* in selective enrichment cultures, approximately 5 CFUs of *L. monocytogenes* strain LI0521 (serotype 4b) was inoculated in 224 ml of either Palcam broth (medium base and selective supplement from Oxoid, Basingstoke, England) or LEB (UVM1, base and supplement from Oxoid, Basingstoke, England) at 35^{o}C for 20-21 h or at 35^{o}C for 20-21 h respectively. Bead suspension with captured bacteria after immunomegatic separation was plated on BHI agar (BD Biosciences) and incubated overnight

(18 hours) at 37°C. Cell concentrations were estimated by measuring the OD of the culture at 620 nm and confirmed by plating.

3.2 Surface Protein Prediction and Candidate Surface Protein Selection from Sequence Comparison

Surface proteins, encoded by the sequenced genome of *L. monocytogenes* serotype 4b strain F2365 (147), were identified using the surface protein prediction software Augur (148), which recognizes secretion and cell envelope retention signals within protein amino acid sequences, and by referencing an annotated report of *L. monocytogenes* surface proteins in strain EGDe (serotype 1/2a) (89) (Tables S1-S7 in Appendix). The F2365 was selected as the reference strain. The F2365 strain is a fully sequenced, serotype 4b strain similar to strain LI0521 used for molecular cloning. The selection criteria for identification of surface protein biomarkers included: (i) proteins not known to be directly regulated by the PrfA transcriptional activator (31) and (ii) proteins with amino acid sequences unique to *L. monocytogenes*. A pBLAST search was performed for each identified surface protein followed by multiple alignments of pBLAST search outputs. Multiple alignments (using CLUSTAL W algorithm with MegAlign software version 5.08 of DNASTAR) consisted of *L. monocytogenes* homologous proteins and heterologous proteins of other *Listeria* species with high sequence similarity. Proteins with regions well conserved among strains of *L. monocytogenes* but variable among other *Listeria* species were selected as candidate surface protein biomarkers.

3.3 Surface Protein Digestion of *L. monocytogenes* and Peptide Purification for Proteomic Analysis

An overnight culture of *L. monocytogenes* strain LI0521 (serotype 4b) in Brain Heart Infusion (BHI) broth at 37 ^{o}C with agitation at 225 rpm in an incubated shaker (MaxQ 4000, Barnstead) was diluted 1 in 100 in BHI broth and subcultured. Bacterial cells were harvested when OD_{620} reached 0.5. Each sample, with approximately 7x 10^{10} cells, was washed three times in 25 ml PBS (pH 7.2). For surface protein digestion, modified sequencing-grade trypsin (Promega, Madison, WI, USA) was added at 12.5 µg (0.5mg/ml) to approximately 7x 10^{10} cells in 1.5 ml of digestion buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$ and 0.6 M sucrose) and the mixture was incubated for 30 min at 37 ^{o}C with 50 rpm agitation. Cells were removed by centrifugation and the supernatant filtered through 0.22 µm centrifuge filter columns (Corning, Lowell, MA, USA). For the negative control, cells were incubated in digestion buffer only prior to filter sterilization and subsequently digested with 12.5 µg of trypsin. Filtrates of both samples were digested overnight at 37 ^{o}C with an additional 2.5 µg of trypsin. Peptides were concentrated and purified using C18 spin columns (Pierce, Rockford, IL, USA) and lyophilized with a concentrator (Oligo Prep OP120 Concentrator, Savant). Peptides were treated with 1 mM DTT (dithiothreitol) (Biorad, Mississauga, Ontario, Canada) and 1 mM iodoacetamide (Sigma, St. Louis, MO, USA) for 30 min in two consecutive steps using 50 mM ammonium bicarbonate, pH 8.0 (Sigma, St. Louis, MO, USA) as solvent and were subsequently concentrated and purified using C18 spin columns. Peptides were lyophilized prior to mass spectrometry analysis.

3.4 LC-MS/MS and MS/MS Spectra Identification

For trials one and two, mass spectrometry analysis was performed on a Waters Synapt High Definition MS (HDMS) system (Milford, MA, USA). The instrument was calibrated by infusion prior to analysis with Glu-Fibrinopeptide (100 fmol/µl). All lyophilized samples were resuspended in 25 µl injection solvent (3% acetonitrile (ACN), 0.2% formic acid, 0.05% trifluoroacetic acid (TFA)). For each sample, triplicate 2 µl aliquots were analyzed by loading onto a Waters Symmetry C18 trap column (180 µm x 20 mm with 5 µm particles) and desalting with 0.1% formic acid in water (solvent A) for 3 min at 5.0 µl/min before separating on a Waters Nano-Acquity ultra-performance liquid chromatography (UPLC) BEH130 C18 reverse-phase analytical column (100 µm x 100 mm with 1.7 µm particles). Chromatographic separation was achieved at a flow rate of 0.5 µl/min over 120 min in six linear steps as follows (solvent B was 0.1% formic acid in ACN): initial, 3% B; 2 min, 10% B; 80 min, 30% B; 100 min, 95% B; 105 min, 95% B; 106 min, 3% B; end, 3% B. The eluting peptides were analyzed by MS and MS/MS in data dependent acquisition (DDA) mode. MS survey scans were 1 second in duration, and MS/MS data were collected on the top four most abundant peaks until either the total ion count exceeded 4000 or 3 s elapsed. All peaks selected for MS/MS analysis during the first analysis of a particular sample were used to generate an exclusion list for the second analysis, and this was reiterated for the third analysis. These iterative exclusion lists were generated and applied in an automated fashion using a program called AutoCat.exe (Waters Ltd). Data were processed using the Mascot software package (Matrix Science). The raw data were processed using Mascot Distiller (version 2.4.2.0) to create Mascot generic files (MGFs), and the data from the triplicate injections of each sample were automatically combined and submitted for database searching. Combining MGFs and queuing data for searching were carried out using Mascot Daemon

(version 2.3.0), and database searches were performed using Mascot (version 2.3), against the NCBI nr database (downloaded on July 3rd, 2012) specifying bacteria taxonomy. A search against a decoy database was performed to obtain an estimation of false discovery rates. Peptide and MS/MS mass tolerances were 60 ppm and 0.05 Da, respectively, and tryptic peptides having charges from 2+ to 4+ and up to one missed cleavages were considered. Carbamidomethylation was specified as a fixed modification and oxidation of methionine, and deamidation of asparagine and glutamine were specified as variable modifications. The Mascot score threshold of $p<0.05$ was considered significant. Mascot identification criteria used included bold red with MudPit scoring and auto reporting of only significant matches.

For trial three, lyophilized peptides were resuspended in 35 µl injection solvent (3% ACN, 0.2% formic acid, 0.05% TFA in water). For each sample, triplicate 5 µl aliquots were analyzed by loading onto a Waters TRIZAIC UPLC nanoTile (85 µm x 100 mm with 1.7 µm beads) which integrates an analytical channel (85 µm x 100 mm with 1.7 µm beads), trapping channel (180 µm x 20 mm with 5 µm beads) and electrospray emitter. Chromatographic separation was achieved at a flow rate of 0.450 µl/min over 60 min in five linear steps as follows (solvent B was 0.1% formic acid in ACN): initial, 3% B; 30 min, 40% B; 32 min, 85% B; 38 min, 85% B; 40 min, 3% B; end, 3% B. The eluting peptides were analyzed by MS and MS/MS using a Waters Synapt HDMS system operating in DDA mode. MS survey scans were 1 second in duration, and MS/MS data were collected on the top four most abundant peaks until either the total ion count exceeded 4000 or 3 s elapsed with collision energy ramping from 14 to 19 volts at low mass and 50 to 64 volts at high mass. All peaks selected for MS/MS analysis during the first analysis of a particular sample were used to generate an exclusion list for the second analysis, and this was reiterated for the third analysis. These iterative exclusion lists were generated and

executed in an automated fashion using a program called AutoCat.exe developed and distributed by Waters. The raw data were processed using Mascot Distiller (version 2.4.2.0) to create Mascot generic files (MGFs), and the data from the triplicate injections of each sample were automatically combined and submitted for database searching. Combining MGFs and queuing data for searching were carried out using Mascot Daemon (version 2.3.0), and database searches were performed using Mascot (version 2.3), against the NCBI nr database (downloaded on November 21st, 2011) specifying bacteria taxonomy. Mascot was searched with a fragment ion mass tolerance of 0.100 Da and a parent ion tolerance of 100 ppm. Iodoacetamide derivative of cysteine was specified in Mascot as a fixed modification. Amidation of the c-terminus, deamidation of asparagine and glutamine and oxidation of methionine were specified in Mascot as variable modifications. The Mascot score threshold of $p<0.05$ was considered significant. Mascot identification criteria used included bold red with MudPit scoring and auto reporting of only significant matches.

3.5 Optimizing Trypsin Digestion for Surface Protein Identification

Cells were grown to 0.6 OD_{620} and washed 3 times with PBS prior to incubation in 0.15 ml of digest buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$ and 0.6 M sucrose) at 37 ^{o}C with agitation at 50 rpm on an incubated shaker (MaxQ 4000, Barnstead). Cells (7 x 10^9) were incubated for 15, 30, 45 or 60 min. After incubation in digest buffer, the supernatant was obtained, mixed 1:1 with 2x SDS-PAGE loading buffer and boiled for 5 min. 30 µl of the sample mix was loaded for SDS-PAGE and subsequent assessed by silver staining. The cell pellets were resuspended in 1 ml of 15% (v/v) glycerol in BHI broth, plated at appropriate dilutions on BHI agar plates and counted for determination of CFU.

3.6 Extraction of mRNA, RT-PCR Analysis of Gene Expression

RT-PCR analysis was performed to assess the gene expression of candidate surface proteins identified by sequence comparison. *L. monocytogenes* strain LI0521 mRNA was extracted from a 16-18 h culture grown to ~1.5 OD_{620} in BHI broth. Bacterial cells (10^9) were treated with RNAprotect bacterial reagent (Qiagen, Toronto, Ontario, Canada) according to the manufacturer's instructions and then treated with 13,000 U of lysozyme (Sigma, St. Louis, MO, USA) in 0.1 ml of Tris-EDTA (TE) buffer for 30 min at 37°C. Subsequently, cells were lysed by mechanical disruption with Lysing Matrix B (MP Biomedicals, Solon, OH, USA) in the FastPrep FP120 cell disrupter (Thermo Electron) according to the manufacturer's instructions. RNA was purified from the cell lysate using a RNeasy mini kit (Qiagen) according to the manufacturer's instructions. The integrity of extracted RNA was analyzed by 1% agarose gel electrophoresis. After mRNA extraction, reverse transcription reaction was performed according to manufacturer's instructions using an Omniscript RT kit (Qiagen) along with 1.0 µg of mRNA, 0.5 µg of random hexamers and 4 units of RNAse inhibitor (Qiagen). Following purification of the PCR product using a PCR cleanup kit (Qiagen), PCR was performed using Pfu polymerase (Thermo Scientific, Ottawa, Ontario, Canada). The 16S gene of *L. monocytogenes* was used as an internal control. A no template control was performed to ensure reagent purity. A RNA template control PCR was performed to ensure no genome DNA is present. Sequences of primers are listed in Table 3-1.

Table 3-1. Oligonucleotide primers used for RT-PCR.

PCR Product Description	Primer Name	primer sequence
LMOf2365_0581 RT-PCR	P939 for	5'-GAATACGCCTACGTCATTGG
	P940 rev	5'-CCACTCCCATTTATCTTCTT
LMOf2365_0578 RT-PCR	P1258 for	5'-GTTTCACCGCTTGCTGATTT
	P1259 rev	5'-CCTCGTCCCACGAATAGGTA
LMOf2365_2117 RT-PCR	P932 for	5'-GGAGCGGTTTATGCCATTAA
	P934 rev	5'-TCATAGCCAGGTGGTGCTG
LMOf2365_0639 RT-PCR	P935 for	5'-TATGGGGAACAAACCACT
	P936 rev	5'-ACTCGTTACCTTACTACC

3.7 5'RACE

The 5' RACE experiments were carried out to identify the transcriptional start site for genes *LMOf2365_0639* and *LMOf2365_0148* using a 5'/3' RACE kit (Roche, Laval, Quebec, Canada) and enzymes from New England Biolabs (NEB) (Whitby, Ontario, Canada). Sequences of gene specific primers are listed in Table 3-2. Reverse transcription reactions were performed with total *L. monocytogenes* RNA to create cDNA using gene specific primers (GSP1) P1120 and P1123 for genes *LMOf2365_0639* and *LMOf2365_0148*, respectively. Primers, dNTPs and RNA were heated at 75 ºC and placed on ice prior to addition of 4 units of RNase inhibitor (Qiagen) and 200 units of M-MuLV reverse transcriptase (NEB). Reverse transcription was carried out at 42ºC for 60 min and then stopped at 90ºC for 10 min. cDNA was purified with a High Pure PCR Product Purification kit (Roche) according to the supplier's instructions. Addition of a poly-A tail to cDNA was accomplished by incubating 10 units of terminal transferase (NEB) with cDNA (150 ng) along with accompanied buffer and dATP at 37ºC for 30 min followed by heat inactivation of the enzyme at 70ºC for 10 min. Poly-A tailed cDNA was

purified as previously described. PCR amplification of the modified cDNA was performed using nested gene specific primers (GSP2) P1121 and P1124 for genes *LMOf2365_0639* and *LMOf2365_0148*, respectively and the oligo-poly T anchor primer included in the 5'/3' RACE kit. Another PCR were performed using the product from the second nested PCR as template. Gene specific primers (GSP3) P1122 and P1125 for the genes *LMOf2365_0639* and *LMOf2365_0148*, respectively and the PCR anchor primer from the 5'/3' RACE kit (Roche) was used. Two 5' RACE trials were performed for each gene. PCR products were gel purified using a gel purification kit (Qiagen) and sequencing verified (McGill University and Génome Québec Innovation Centre) using GSP3 primers P1122 and P1125 for the genes *LMOf2365_0639* and *LMOf2365_0148*, respectively. Promoter prediction was performed using the Softberry BPROM (Prediction of Bacterial Promoters) using the following website:

http://linux1.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb

Table 3-2. Oligonucleotide primers used in 5'RACE.

PCR Product Description	Primer Name	Primer Sequence
Gene Specific Primer (GSP1) for *LMOf2365_0639*	P1120	5'-GGCATAATCTGTTACCCCTGT
Gene Specific Primer (GSP2) for *LMOf2365_0639*	P1121	5'-ATCACTTGTGCTTGATTGTCC
Gene Specific Primer (GSP3) for *LMOf2365_0639*	P1122	5'-GTTGACAACATCTTGAGCGGC
Gene Specific Primer (GSP1) for *LMOf2365_0148*	P1123	5'-CATGTCGCCCGCTTGAACA
Gene Specific Primer (GSP2) for *LMOf2365_0148*	P1124	5'-GCATTGTCAGTTGTCGCCC
Gene Specific Primer (GSP3) for *LMOf2365_0148*	P1125	5'-CAAAATCTGAATGGGAACGG

3.8 Molecular Cloning

The coding sequences of mature proteins (with putative secretion signal peptide and sortase cleavage site within the LPXTG motif removed) were amplified by PCR from the genomic DNA of *L. monocytogenes* serotype 4b (strain LI0521) using the primers listed in Tables 3-3 and 3-5. PCR products were cloned into pLIC-CHIS (16), a ligation-independent inducible expression plasmid, to generate constructs that expressed target proteins with a C-terminal hexa-histidine tag. GST-tag PCR products were amplified from the pGEX-3x GST vector (GE Healthcare). The GST-tag was either cloned alone into pLIC-CHIS or in combination with a *L. monocytogenes* unique sequence within LMOf2365_0639 (Figure 4-7). The GST-fusion product was generated by separate amplification of GST-tag and the *L. monocytogenes* unique sequence within LMOf2365_0639, using primers listed in Table 3-4, followed by amplification of combined PCR products.

Table 3-3. Oligonucleotide primers for cloning surface protein candidates identified by sequence comparison.

PCR Product Description	Primer Name	primer sequence
LMOf2365_0581 antigen	P972 for	5'-TTTAAGAAGGAGATATAAGTCATGCTCTCTGACAATCAGGCATCT
	P973 rev	5'-AGTGGTGGTGGTGGTGGTGAGTCCCAAGAGCTACTTTATTGGC
LMOf2365_0578 antigen	P993 for	5'-TTTAAGAAGGAGATATAAGTCATGGAGTTACCTAAGAGTCCGGAA
	P994 rev	5'-AGTGGTGGTGGTGGTGGTGAGTCGGTCGAGGCGCATCTTCTAG
LMOf2365_2117 antigen	P907 for	5'-TTTAAGAAGGAGATATAAGTCATGGGAGCGGTTTATGCCATTAA
	P908 rev	5'-AGTGGTGGTGGTGGTGGTGAGTCTCATAGCCAGGTGGTGCTG
LMOf2365_0639 antigen	P970 for	5'-TTTAAGAAGGAGATATAAGTCATGGTCAACATTCCTGACCCAGTT
	P971 rev	5'-AGTGGTGGTGGTGGTGGTGAGTCGTGTTTGGTAAAGCGGCATT

Table 3-4. Oligonucleotide primers for cloning GST-rLMOf2365_0639 peptide fusion.

PCR Product Description	Primer Name	primer sequence
LMOf2365_0639 polypeptide	P1035 for	5'-TTTAAAAGGAGATATAAGTCATGAATAATCCAAATATCAATCCCAACCCA
	P1036 rev	5'-TAGGGGACATATTTACTTCTGAGTTAGACGG
GST	P1037 for	5'-AGAAGTAAATATGTCCCCTATACTAGGTTAT
	P1038 rev	5'-AGTGGTGGTGGTGGTGGTGAGTCCCTTTTGGAGGATGGTCGCC

Table 3-5. Oligonucleotide primers for cloning surface protein candidates identified by proteomics.

PCR Product Description	Primer Name	primer sequence
LMOf2365_0148 antigen	P1039 For	5'-TTTAAGAAGGAGATATAAGTCATGGCAGCGGATACCGTTCCCATT
	P1040 Rev	5'-AGTGGTGGTGGTGGTGGTGAGTCGTTGTTGGTAAGGATGTGTTAGCAGA
LMOf2365_0312 antigen	P1077 For	5'-TTTAAGAAGGAGATATAAGTCATGGACGAGAAAGAAAAGAAT
	P1078 Rev	5'-AGTGGTGGTGGTGGTGGTGAGTCGTGGTGTTTTTGCTGCTT
LMOf2365_0546 antigen	P1081 For	5'-TTTAAGAAGGAGATATAAGTCATGTGCGGTAACAGTACATCT
	P1082 Rev	5'-AGTGGTGGTGGTGGTGGTGAGTCTTAGAACCTTTTTCCACATA
LMOf2365_1883 antigen	P1079 For	5'-TTTAAGAAGGAGATATAAGTCATGAATGCTGATTCCATCGCTAAA
	P1080 Rev	5'-AGTGGTGGTGGTGGTGGTGAGTCTTCGCGGCATCTACTTTCTT
LMOf2365_2111 antigen	P1041 For	5'-TTTAAGAAGGAGATATAAGTCATGGCAGCAAAAACACCACAAGGT
	P1042 Rev	AGTGGTGGTGGTGGTGGTGAGTCGTTCCACTTATATCCATTCCGCC
LMOf2365_2742 antigen	P1083 For	5'-TTTAAGAAGGAGATATAAGTCATGGCGGAAGCACCAAATGTA
	P1084 Rev	5'-AGTGGTGGTGGTGGTGGTGAGTCCCATTAAACCACCCTTTTACACC

3.9 Expression and Purification of Recombinant Proteins

Recombinant proteins were expressed in *E. coli* as previously described (149) with some modifications. Briefly, an overnight culture of *E. coli* Rosetta (DE3)/pLysS cells transformed with recombinant expression pLIC-CHIS was sub-cultured at a 1:100 dilution in LB containing kanamycin (50 μg/ml) until an OD_{600} of 0.6 was reached. IPTG (1 mM) was added to the culture to induce the expression of the recombinant protein at 37°C for 3 h. Soluble recombinant proteins were purified by metal affinity chromatography using Ni-NTA Superflow (Qiagen).

3.10 Production of Rabbit Polyclonal Antibodies (PAbs)

Female New Zealand white rabbits (Charles River Laboratories, Senneville, Quebec, Canada) age two to three months were immunized with purified recombinant proteins from metal affinity chromatography. Pre-immune sera were obtained from rabbits and used as negative control. On day 0, 200 µg of purified protein antigen in 0.5 ml PBS (pH 7.2, 7.75 mM Na_2HPO_4, 2.62 mM NaH_2PO_4 and 145 mM NaCl) was emulsified with 0.5 ml of incomplete Freunds adjuvant (IFA) and injected subcutaneously into each rabbit. On days 14 and 28, booster inoculations (100 µg of protein in 0.5 ml PBS with emulsified 0.5 ml IFA) were given. Immune response was assessed by western blot analysis of sera obtained from the test bleed collected on day 42. Rabbits were exsanguinated on day 46. Blood was collected and the sera used as polyclonal antibodies (PAbs). All experiments involving animals were approved by the local Animal Care Committee under the guidelines of the Canadian Council on Animal Care.

3.11 Production of Mouse Monoclonal Antibodies (MAbs)

Three BALB/c and three six-week-old Swiss Webster female mice (Charles River Laboratories) were immunized with purified recombinant rLMOf2365_0639 or rLMOf2365_0148 protein as immunogen over a 2 month period. Pre-immunization sera were obtained from each mouse. On day 0, each mouse was injected subcutaneously with 200 µl of immunogen (30 µg/ml) emulsified with an equal volume of complete Freund's adjuvant. Booster inoculations with the same amount of immunogen emulsified with an equal volume of incomplete Freund's adjuvant were given intraperitoneally on days 28 and 56. Mice were test bled on day 64. One BALB/c and one Swiss Webster mouse were selected for hybridoma fusions based on best immune response using test bleed sera. Five days prior to splenectomy, each

selected mouse was inoculated intraperitoneally with 0.5 µg epinephrine and 100 µg dehydroepiandrosterone sulfate and intravenously with 5 µg of immunogen in 100 µl PBS. Blood was collected from mice on the day of splenectomy. Mouse spleens were removed and splenocytes fused with sp2 myeloma cells. Hybridoma clones were selected in hypoxanthine-aminopterin-thymidine (HAT) medium and hybridoma tissue culture fluids (TCFs) were screened by indirect enzyme-linked immunosorbent assay (ELISA) using immunogen (1 µg/ml, 100 µl/well), and formalin-killed cells (10^8 cells/ml, 100 µl/well) of *L. monocytogenes* strain LI0521 (serotype 4b) and *L. innocua* CLIP2262. For screening of hybridoma clones against rLMOf2365_0639, GST and GST-rLMOf2365_0639 peptides were used at 1 µg/ml (100 µl/well), in addition to formalin-killed cells and the immunogen. Limiting dilution was performed twice on selected clones. The subclasses of immunoglobulins secreted by the hybridoma clones were determined as described (18). TCFs of stable clones were used as MAbs.

3.12 Whole cell *L. monocytogenes* Protein Extraction

Fifty ml of overnight BHI cultures of *L. monocytogenes* LI0521 were pelleted and resuspended in 0.5 M Tris-HCl pH 6.8, 10% (w/v) sodium dodecyl sulfate (SDS). Cell suspension was added to lysing matrix B (MP Biomedicals) and placed in the FastPrep machine (Thermo Electron) for 40 s at setting 6. 0.4 ml of 2x SDS sample buffer (0.1 M Tris-HCl, pH 6.8, 40% (v/v) glycerol, 20% (v/v) β-mercaptoethanol, 4% (w/v) SDS, 0.002% (w/v) Bromophenol Blue) was added and the mixture boiled for 10 min. The supernatant was collected following centrifugation at 15,700x g for 3 min.

3.13 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting

Proteins were separated by size using electrophoresis with a 4% stacking gel and 10% resolving gel. Separated denatured proteins in the gel were visualized either by Coomassie blue staining or by western blotting after transferring proteins onto a nitrocellulose membrane using a semi-dry transfer apparatus (Bio-Rad, Mississauga, ON, Canada) according to manufacturer's instructions. Successful transfer was verified by staining the nitrocellulose membrane with 0.1% (w/v) Ponceau S in 5% (v/v) acetic acid for 1 min. Primary antibodies of either MAbs at 1:25 dilution of TCF or rabbit antiserum at 1:1000 dilution were prepared in PBS containing 3% (w/v) bovine serum albumin (BSA) and used to probe membrane-bound proteins. Secondary antibodies of Peroxidase-AffiniPure goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA, USA) or Peroxidase-AffiniPure goat anti-rabbit IgG (Jackson ImmunoResearch) were used at a 1:1000 dilution in PBS containing 3% (w/v) BSA. Protein bands were visualized using a horseradish peroxidase (HRP) substrate kit (Bio-Rad) according to manufacturer's instructions.

3.14 Immunofluorescence Microscopy

An overnight (16-18 h) culture of *L. monocytogenes* strain LI0521 was centrifuged for 2 min at 16,100x g to obtain 3x 10^8 cells. Live cell pellets were resuspended in PBS with 5% (w/v) BSA and incubated for 1 h at room temperature on a Barnstead/Thermolyne Labquake rotator (Thermo Fisher Scientific). This was followed by incubation with a MAb (in TCF form) diluted 1:50 in PBS with 5% (w/v) BSA or polyclonal antiserum diluted in1:1000 in PBS with 5% (w/v) BSA for 1 h at room temperature. Cells were washed twice with PBS and then incubated with Dylight 488-conjugated goat anti-mouse IgG (H+L) (Jackson ImmunoResearch) at a 1:250 dilution in PBS containing 5% (w/v) BSA for 1 h at room temperature. After 3 times wash, bacterial cells were resuspended in PBS and viewed with an Olympus BX60 fluorescence

microscope using 100x magnification. Fluorescence images of cells were captured with a charge-coupled device (CCD) camera using the QCapture Pro software (Q Imaging).

3.15 Indirect ELISA Assessment of Reactivity of MAbs to *L. monocytogenes* Isolates

The MAbs raised against rLMOf2365_0639 or rLMOf2365_0148 of *L. monocytogenes* strain LI0521 were analyzed for cross-reactivity with *L. monocytogenes* serotypes, other *Listeria* species and foodborne pathogens (Table 3-6) grown on non-selective media of BHI and LB or in selective enrichment cultures. All bacterial cells were formalin-killed prior to ELISA analysis. For preparation of formalin-killed cells, each isolate was cultured overnight in BHI broth at 37 ^{o}C, washed 3 times with PBS, killed overnight by incubation with 0.3% (v/v) formalin in PBS, washed 3 times with PBS and stored at -20 ^{o}C in 50% (v/v) glycerol in PBS. Three ELISA trials in which each trial consisted of duplicate readings for each MAb were performed for assessment of antibody reactivity to formalin-killed cells. Formalin-killed cells were used to coat NUNC Maxisorp^{TM} microtiter plates (Thermo Scientific), at a concentration of 10^8 cells/ml with 100 µl/well, overnight in PBS. After washing with PBS-T (0.05% (v/v) Tween 20 in PBS, pH 7.2), 100 µl TCF of a MAb to LMOf2365_0639, LMOf2365_0148 or an irrelevant MAb (M3042, M3043 or M3044) were added to the wells and incubated for 1 h at room temperature. MAbs M3042, M3043 and M3044 which recognized the lipopolysaccharides of *Salmonella enterica* serovar Typhimurium DT104 (150) served as negative controls. After washing with PBST, 100 µl of peroxidase-AffiniPure goat anti-mouse IgG (Jackson ImmunoResearch) was diluted 1:1000 in PBST and incubated at room temperature for 1 h. After final wash with PBST, a substrate solution containing 0.1% (w/v) 2,2'-azino-bis (3-ethyl-benzthiazdine-6-sulfonic acid) (ABTS) and 3% (v/v) hydrogen peroxide in citrate buffer (0.02 mM citric acid, 0.03 mM tri-sodium citrate) was added for 15 min with moderate shaking. The absorbance was measured at OD_{414}.

The OD_{414} readings obtained from negative controls ranged from 0.03 to 0.2, and thus OD_{414} readings of <0.25, between 0.25 and 0.3, and > 0.3 were considered as negative, weakly positive and positive respectively.

Table 3-6. Bacterial isolates used in indirect ELISA.
Strains were from the Ottawa Laboratory Fallowfield and Listeria Reference Service collections, except those purchased from ATCC.

Strain	Strain Name	Origin	Serotype
L. monocytogenes	HPB6027	food	1/2b
L. monocytogenes	HPB5328	animal	1/2b
L. monocytogenes	HPB5913	food	1/2b
L. monocytogenes	HPB5330	animal	1/2b
L. monocytogenes	LI0586		1/2b
L. monocytogenes	HPB4857	animal	1/2b
L. monocytogenes	OLF09060		1/2b
L. monocytogenes	OLF090401-1		1/2b
L. monocytogenes	OLF090271		1/2b
L. monocytogenes	HPB1031	food	3b
L. monocytogenes	HPB4909	food	3b
L. monocytogenes	HPB61		3c
L. monocytogenes	HPB1265	clinical	4ab
L. monocytogenes	HPB520	environment	4ab
L. monocytogenes	LI0521	clinical	4b
L. monocytogenes	HPB5058	animal	4b
L. monocytogenes	HPB3449	food	4b
L. monocytogenes	HPB5251	animal	4b
L. monocytogenes	HPB5816	food	4b
L. monocytogenes	HPB6092	food	4b
L. monocytogenes	HPB5906	food	4b
L. monocytogenes	HPB5364	animal	4b
L. monocytogenes	HPB1848	food	4b
L. monocytogenes	HPB4534	clinical	4d
L. monocytogenes	HPB18	animal	4e
L. monocytogenes	HPB1861	food	4e
L. monocytogenes	LI0527		1/2a
L. monocytogenes	HPB4705	animal	1/2a
L. monocytogenes	HPB6036	food	1/2a
L. monocytogenes	HPB5327	animal	1/2a
L. monocytogenes	OLF09049		1/2a

L. monocytogenes	OLF09015		1/2a
L. monocytogenes	OLF09011		1/2a
L. monocytogenes	OLF09033		1/2a
L. monocytogenes	OLF09016		1/2a
L. monocytogenes	HPB 2972		1/2c
L. monocytogenes	OLF09022-1		1/2c
L. monocytogenes	HPB1869	food	1/2c
L. monocytogenes	OLF09013		1/2c
L. monocytogenes	HPB5121	food	1/2c
L. monocytogenes	HPB5665	food	3a
L. monocytogenes	HPB3058	food	3a
L. monocytogenes	HPB2768	food	3a
L. monocytogenes	OLF09005		3a
L. monocytogenes	OLF09039		3a
L. monocytogenes	LI0508		3a
L. monocytogenes	HPB3501	clinical	4a
L. monocytogenes	HPB5041	animal	4a
L. monocytogenes	HPB4497	animal	4c
L. monocytogenes	HPB3540	clinical	4c
L. monocytogenes	HPB4479	clinical	4c
L. monocytogenes	HPB4706	clinical	4c
L. monocytogenes	HPB5248	animal	4c
L. seeligeri	HPB24	clinical	
L. seeligeri	ATCC 35967		
L. welshimeri	HPB92	food	
L. innocua	CFIA		
L. innocua	HPB583	animal	
L. innocua	CLIP2262		
L. innocua	ATCC 33090		
L .invanovii	HPB28	animal	
L .invanovii	ATCC 19119		
L. grayi	HPB 29	animal	
C.jejuni	ADRI 1102 NCTC11168		
E.coli 0157:H7	ATCC 43889		
Salmonella enterica serovar Typhimurium	DT104		
B. cereus	B3-37		

3.16 Bacterial Capture using Anti-LMOf2365_0148 and Anti-LMOf2365_0639 MAbs

A high-throughput screening of the ability of anti-LMOf2365_0639 and anti-LMOf2365_0148 MAbs to capture live *L. monocytogenes* was performed by incubating 100 µl of 10^4 cells with 100 µl of MAb tissue culture fluid (TCF) at room temperature for 1 hour with end-over-end rotation. Binding reactions were performed in 200 µl PCR strip tubes (ABgene, Thermo Scientific, Ottawa, Ontario, Canada). Cell pellets are recovered by centrifugation at 10,000 rpm for 5 min and suspended in 200 µl of 0.5% (w/v) BSA in PBS with 20 µl of M-280 Dynal™ beads conjugated with sheep anti-mouse IgG (Life Technologies, Oslo, Norway). Mixture was incubated for 1 hour at room temperature with end-over-end rotation. Beads were washed three times using a Dynal™ MPC-9600 magnet (Invitrogen Dynal™, Oslo, Norway) and plated on BHI agar plates for enumeration. A positive result was considered to be a capture which yielded more than 5 colonies.

3.17 Determination of Capture Efficiency for MAbs

One ml volume of *L. monocytogenes* strain LI0521 (serotype 4b) at concentrations of 10^4, 10^3 and 10^2 cells/ml in PBS was mixed with 200 µl of MAb TCF diluted 1:100 (for M3686, M3697, M3699 and M3700) in PBS; diluted 1:10 (for M3644) in PBS. As a negative control, a MAb specific for *C. jejuni* M1169 was used at 1:100 dilution. The mixture of cells and a MAb was incubated for 30 min at room temperature with end-over-end rotation using a Barnstead/Thermolyne Labquake rotator. A 20 µl suspension of M280 Dynal™ beads conjugated with sheep anti-mouse IgG was added to each mixture prior to aliquoting into sample tube strips (Life Technologies, Burlington, Ontario, Canada) for Beadretriever™ (Thermo Fisher Scientific, Vantaa, Finland) processing. Bacterial capture was performed using a Beadretriever™ with the factory installed *Listeria* program. Samples were loaded into sample tube strips according to

manufacturer's instructions with the following modifications. Six hundred µl and 400 µl of sample and wash buffer (0.05% (v/v) Tween 20 in PBS) respectively were loaded into the first two tubes of each sample tube strip. Following Beadretriever^{TM} run, bead suspensions (100 µl) were plated on BHI for CFU enumeration. Two replications were performed for each trial. Three trials performed on three different days were performed to generate an average number of cells captured. As a negative control, a MAb specific for *C. jejuni* (M1169) was used in conjunction. A positive result in immunomagnetic separation was considered to be a capture which yielded more than 5 colonies..

3.18 Determination of the Capture Ability of MAbs for various *L. monocytogenes* Serotypes and other *Listeria* species and Bacteria.

One ml of bacterial cells at 10^4cells/ml were mixed with 200 µl of MAbs (M3686, M3697, M3699 and M3700) TCF diluted 1:100 in PBS. Capture was performed as previously described in section 3.17. Two technical replications were performed for each trial. Two trials performed on two different days were performed to generate an average number of cells captured. As a negative control, a MAb specific for *C. jejuni* (M1169) was used in conjunction at 1:100 dilution. Strains of *L. monocytogenes* cells used are listed in Table 3-7. A positive result in immunomagnetic separation was considered to be a capture which yielded more than 5 colonies.

Table 3-7. Bacterial strains used for immunomagnetic separation.

Strain	Strain Name	Origin	Serotype
L. monocytogenes	HPB6036	food	1/2a
L. monocytogenes	HPB4857	animal	1/2b
L. monocytogenes	HPB 2972		1/2c
L. monocytogenes	HPB3058	food	3a
L. monocytogenes	HPB3501	clinical	4a
L. monocytogenes	HPB4534	clinical	4d
L. innocua	CLIP2262	food	
L. seeligeri	HPB24	clinical	
L .invanovii	HPB28	animal	
L. grayi	HPB 29	animal	
L. welshimeri	HPB92	food	
Salmonella enterica serovar Typhimurium	ATCC14028		
E.coli O157:H7	ATCC43888		

3.19 Isolation of *L. monocytogenes* from a mixture of *L. monocytogenes* and *L. innocua* and Colony Blot Immunoassay

Ratios of *L. monocytogenes* to *L. innocua* at 1:10 and 1:100 were used to evaluate the ability of MAbs to isolate *L. monocytogenes* from the cell mixture. One ml mixture of *L. monocytogenes* strain LI0521 ($5x10^3$ cells/ml) and *L. innocua* ($5x10^4$ cells/ml or $5x10^5$ cells/ml) were used for capture as previously described in section 3.17. Colony blot immunoassays were performed on captured cells grown on BHI agar to discriminate *L. monocytogenes* from *L. innocua*. Colony blots were performed as previously described (151) with the following modifications. After colony lift from BHI agar plates using Optitran® nitrocellulose membranes

(Schleicher & Schuell, N.H., USA), the membranes were submerged in chloroform for 10 min and dried for 15 min. The membranes were incubated with 5% (w/v) BSA in PBS for 30 min and probed with *L. monocytogenes* specific PAbs (anti-LMOf2365_0639, rabbit antisera diluted 1:1000 in PBS containing 5% (w/v) BSA) followed by Peroxidase-AffiniPure goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA) for 30 min. The membranes were washed twice with PBST (0.05% (v/v) Tween 20, PBS) for 5 min in between antibody incubations. Reactive colonies were visualized after colour development with the substrate solution (0.06 g of 4-chloro-1-napthol in 10 ml methanol, 0.7 ml of 3% (v/v) hydrogen peroxide and 100 ml 0.02 M Tris, 0.5 M NaCl, pH 7.5). Colour development was stopped by washing with water. BHI agar plates of captured cells were photographed and compared against corresponding colony blots to determine the proportion of *L. monocytogenes* from the total cells captured by MAbs.

3.20 Primary Selective Enrichment Culture for Immunomagnetic Separation

Approximately 5 CFU of *L. monocytogenes* (strain LI0521, serotype 4b) in 1 ml of PBS were inoculated in 224 ml either Palcam or LEB/UVM1 according to MFHPB-07 or MFHPB-30, respectively. For Palcam, the culture was incubated at 35 ^{o}C for 20-21 hours with shaking at 250 rpm. For LEB/UVM1 the culture was incubated at 30 ^{o}C for 20-21 hours at 250 rpm. To account for variations in the number of CFU inoculated, the same preparation of *L. monocytogenes* suspension was used to inoculate two identical selective enrichment cultures. Two trials of immunomagnetic separation were performed for each culture. A positive result in immunomagnetic separation was more than 5 colonies captured.

3.21 Size Exclusion Chromatography and Multi-Angle Light Scattering (MALS)

Size exclusion chromatography was performed to determine the molecular weights of LMOf2365_0639 and LMOf2365_0148 using a Superdex 200 10/300 GL column (Amersham Biosciences) controlled by an ÄKTA Purifier FPLC (GE Healthcare). The molecular weights were determined using protein calibration standards (Amersham Biosciences, Buckinghamshire, UK) for size exclusion chromatography. The column was equilibrated with PBS prior to sample injection. To measure the void volume, blue dextran (1 mg/ml) (Amersham Biosciences) was injected into a 100 µl sample loop with a 0.25 ml empty loop volume. Absorbance was measured at OD_{280}. The column ran at a flow rate of 0.5 ml/min and with 1 column volume for elution. Subsequently, a mixture of ribonuclease A (10 mg/ml) and ovalbumin (7 mg/ml) in PBS was similarly injected to the FPLC system. Lastly, a mixture of chymotrypsinogen A (3 mg/ml), albumin (7 mg/ml) and aldolase (6 mg/ml) injected. LMOf2365_0639 protein or LMOf2365_0148 protein (1 mg/ml) was injected and separated similarly to the protein standards. The molecular weights of LMOf2365_0639 and LMOf2365_0148 were calculated from linear equations derived from the plots of Kav versus Log of molecular weights of protein standards (Figures 7-4 and 7-5). Kav is defined as $(V_e - V_o)/(V_t - V_o)$ where V_e is the elution volume, V_o is the void volume and V_t is the column bed volume. MALS was performed to determine the molecular weights of LMOf2365_0639 and LMOf2365_0148 as described (152).

3.22 Preparation of Antibody Fragments

MAbs (M3686, M3692, M3697, M3699 and M3700) were purified from tissue culture fluid (TCF) by affinity chromatography on a column (1 cm x 1 cm) of NHS-activated Agarose (Pierce, Thermo Scientific, Rockford, Illinois, USA) conjugated with rLMOf2365_0148 according to the manufacturer's instructions. MAb M3644 was purified from a column (1 cm x 1

cm) of protein L Agarose (Pierce, Thermo Scientific, Rockford, Illinois, USA) according to manufacturer's instructions. IgG elution buffer (Thermo Scientific, Rockford, Illinois, USA) was used to elute bound IgG. The eluted IgG fractions (1 ml) were neutralized with 100 µl of 1 M Tris pH 9.0. MAbs M3686, M3692, M3697, M3699 and M3700 were all isotype IgG_1. MAb 3644 was isotype IgG_{2a}. Fab fragments were prepared from purified MAbs by IgG digestion using a Mouse IgG_1 Fab and $F(ab')_2$ preparation kit (Pierce, Thermo Scientific, Rockford, Illinois, USA) according to the manufacturer's instructions. For digestion of IgG molecules into Fab fragments using ficin, 25 mM cysteine was used. The purity of Fab fragments were assessed by western blotting using a Fcγ fragment specific Peroxidase-AffiniPure goat anti-Mouse IgG (Jackson ImmunoResearch, West Grove, PA, USA) to ensure all Fc fragments are removed as well as using a light chain specific Peroxidase-AffiniPure goat anti-Mouse IgG (Jackson ImmunoResearch) to detect the presence of Fab. Purified Fabs were concentrated with Amicon Ultra-15 centrifugal filters (3 kDa MWCO) (Merck Millipore, Cork, Ireland). Immediately prior to surface plasmon resonance (SPR) analysis, Fabs were purified and buffer exchanged into HBS-EP buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, pH 7.4, 0.005% P20) using a Superdex 75 10/30 GL column (GE Healthcare) controlled by an ÄKTA Purifier FPLC (GE Healthcare). Only the peak fraction containing Fabs was used for SPR analysis.

3.23 Surface Plasmon Resonance Analysis

The binding of Fab fragments to immobilized LMOf2365_0148 or LMOf2365_0639 was determined by SPR using the BIACORE 3000 (GE Healthcare). Immobilization was carried out at a flow rate of 5 µl/min at 25 ^{o}C. Approximately 500 resonance units (RU) of LMOf2365_0148 and 250 RU of LMOf2365_0639 were immobilized on a research grade CM5 sensor chip. The CM-dextran surfaces were activated with an injection of 50 mM NHS and 200 mM EDC.

Immobilization using a amine coupling kit (GE Healthcare) was performed with 10 µg/ml of LMOf2365_0148 or LMOf2365_0639 in 10 mM acetate at pH 4.0. The remaining binding sites were blocked with a 7 min injection of 1 M ethanolamine at pH 8.5. The reference surface had no ligand but was similarly activated and blocked as the active surface. The affinity measurements were carried out at 25 ^{o}C in HBS-EP running buffer (10 mM HEPES, pH 7.4 containing 150 mM NaCl, 3 mM EDTA and 0.005% surfactant P20). Flow-rates of 30, 20, 40, 20, 40 and 20 µl/min were used for Fabs of M3686, M3692, M3697, M3699, M3700 and M3644 respectively. Fabs M3686, M3692, M3697, M3699, M3700 and M3644 were injected at sample volumes of 60, 200, 40, 20, 40 and 200 µl respectively. Fabs of M3686, M3692, M3697, M3699, M3700 and M3644 were given an injection time of 2, 10, 1, 2 and 1 min, respectively followed by a 10, 10, 12, 5, 5 and 10 min dissociation time, respectively. Binding kinetics of M3686 Fabs was analyzed using single cycle kinetics which involves 5 subsequent injections of increasing concentrations followed by a dissociation of 10 min. Surfaces were regenerated by washing with either HBS-EP running buffer for M3686, M3697, M3699, and M3700 or 10 mM glycine at pH 2.0 and pH 1.5 for M3692 and M3644. Data were analyzed with BIAevaluation 4.1 software and fitted using a 1:1 binding model.

3.24 Epitope Mapping

Overlapping, similarly sized polypeptides of surface proteins LMOf2365_0639 and LMOf2365_0148 were used for epitope mapping. Desired protein coding regions were cloned from *L. monocytogenes* (strain LI0521) genomic DNA (see Tables 3-8 and 3-9 for primer sequences) into the pLIC-CHIS expression plasmids (153). Following the induction of protein expression (as described in section 3.9), cell pellets were collected by centrifugation and lysed to prepare protein samples by boiling for 5 min in 2x SDS-PAGE sample buffer (0.1 M Tris-HCl, pH 6.8, 40% (v/v) glycerol, 20% (v/v) β-mercaptoethanol, 4% (w/v) SDS, 0.002% (w/v)

Bromophenol Blue). SDS-PAGE and western blotting of the prepared protein samples using each corresponding MAb were performed as described in section 3.13.

Table 3-8. Oligonucleotide primers used for epitope mapping of LMOf2365_0639 MAbs.

PCR Product Description	Primer Name	primer sequence
epitope mapping fragment A	P1313 for	5'-TTTAAGAAGGAGATATAAGTCATGAGAAAAATGGGAGTCAA
	P1314 rev	5'-AGTGGTGGTGGTGGTGGTGAGTCGGATTACTGTCTAAATT
epitope mapping fragment B	P1315 for	5'-TTTAAGAAGGAGATATAAGTCATGTTGAAAAGTTATTTAAAT
	P1316 rev	5'-AGTGGTGGTGGTGGTGGTGAGTCTAGCCATCAAAGTTTAC
epitope mapping fragment C	P1317 for	5'-TTTAAGAAGGAGATATAAGTCATGTTGTTCCCTTTTACAACT
	P1318 rev	5'-AGTGGTGGTGGTGGTGGTGAGTCCCAAATTGCTGGTCTGTCTT
epitope mapping fragment D	P1319 for	5'-TTTAAGAAGGAGATATAAGTCATGACATTAAATGCCGAAAATGCT
	P1320 rev	5'-AGTGGTGGTGGTGGTGGTGAGTCGTGTTTGGTAAAGCGGC

Table 3-9. Oligonucleotide primers used for epitope mapping of LMOf2365_0148 MAbs.

PCR Product Description	Primer Name	primer sequence
epitope mapping fragment 1	P1039 For	5'-TTTAAGAAGGAGATATAAGTCATGGCAGCGGATACCGTTCCCATT
	P1321 Rev	5'-AGTGGTGGTGGTGGTGGTGAGTCTTTGACGCCATCAATTTCTTT
epitope mapping fragment 2	P1322 For	5'-TTTAAGAAGGAGATATAAGTCATGGTTCTCCAAAAAATGAATTTC
	P1323 Rev	5'-AGTGGTGGTGGTGGTGGTGAGTCCCGTTATTTGGGTCTAATTCATT
epitope mapping fragment 3	P1324 For	5'-TTTAAGAAGGAGATATAAGTCATGGGCCAAGGCGTTAACGCAATC
	P1325 Rev	5'-AGTGGTGGTGGTGGTGGTGAGTCCCGTAACGTTGCGCATCTGTAAT
epitope mapping fragment 4	P1326 For	5'-TTTAAGAAGGAGATATAAGTCATGGTCACACCAAATGCCGATATT
	P1327 Rev	5'-AGTGGTGGTGGTGGTGGTGAGTCCCGTCGGCTTTTAAAGGAGTTCC
epitope mapping fragment 5	P1328 For	5'-TTTAAGAAGGAGATATAAGTCATGGATATTTTGGAAGCTCTAAAC
	P1329 Rev	5'-AGTGGTGGTGGTGGTGGTGAGTCCCGTTAGCAGTTGCTTTTTGAAC
epitope mapping fragment 6	P1330 For	5'-TTTAAGAAGGAGATATAAGTCATGGGACGTAAAGTCTACAAATCC
	P1040 Rev	5'-AGTGGTGGTGGTGGTGGTGAGTCGTTGTTGGTAAGGATGTGTTAGCAGA
epitope mapping fragment 7	P1331 For	5'-TTTAAGAAGGAGATATAAGTCATGCTAGTAACTGCAATCGACCCA
	P1332 Rev	5'-AGTGGTGGTGGTGGTGGTGAGTCGTGCCACCGTTATTTCCGTT

Chapter 4: Identification of Surface Protein Biomarkers of *L. monocytogenes* Using Bioinformatics and Antibody-based Protein Detection

4.1 Introduction

Listeria monocytogenes is a Gram-positive bacterium that can cause a rare, but serious, human disease referred to as listeriosis primarily by ingestion of foods contaminated with live bacteria. The occurrence of listeriosis is relatively rare as compared to other foodborne illnesses, but it accounts for approximately 3.8% of hospitalizations and 27.6% of deaths among all foodborne illnesses (3). Susceptible individuals include pregnant women and persons with a weak or compromised immune system such as neonates, the elderly, organ transplant recipients of immunosuppressive therapy, cancer patients and individuals living with HIV. During pregnancy, listeriosis can result in aborted fetuses and neonate stillborns. Late onset neonate listeriosis frequently manifests as meningitis. Invasive listeriosis in nonpregnant adults frequently manifests as meningoencephalitis and bacteremia (4).

L. monocytogenes as well as *L. innocua, L. seeligeri, L. welshimeri, L. grayi* and *L. ivanovii* belong to the *Listeria* genus. *L. monocytogenes* causes the vast majority of human illness while rare human listeriosis due to *L. ivanovii* has been reported (44). There are at least 12 recognized *L. monocytogenes* serotypes based on surface somatic and flagellar antigens (17). Results from studies using various subtyping methods have divided the serotypes into three lineages (49) and members of each lineage share common phenotypic characteristics (49). Serotypes 1/2b, 3b, 4b, 4d and 4e are included in lineage I. Serotype 1/2a and 4b are overrepresented in clinical isolates (1, 88). Serotype 1/2a, 3a, 3c and 1/2c are grouped into lineage II and are commonly found in food and food-related and natural environments. Lineage III consists of serotype 4a and 4c and these serotype isolates are involved in animal listeriosis (49).

L. monocytogenes lives as a saprophyte in the natural environment and can enter the food chain through contaminated fresh produce, milk, and other animal products thus posing a serious risk to public health. Identification of foods contaminated with *L. monocytogenes* is thus imperative to reduce the risk of the foodborne illness linked to this pathogen. The standard method for isolation consists of sequential enrichment cultures followed by plating on selective (and often chromogenic) agars. Presumptive colonies are subjected to biochemical tests for confirmation. The whole process is time-consuming and labour-intensive. It is possible that following an abbreviated period of enrichment culture, antibodies can be applied to expedite pathogen detection and/or isolation. Monoclonal antibodies (MAbs) are specific and have high affinity and so are desirable for immunological-based methods of detection and isolation of foodborne pathogens including *L. monocytogenes*. In addition, antibodies targeting intact or live cells provide a direct evidence of a living organism and therefore of an associated health risk.

Research is still needed to identify appropriate surface proteins useful for *L. monocytogenes* detection and isolation. The ideal surface protein should be expressed in standard enrichment culture conditions. For *Listeria* detection, enrichment culture is necessary to increase the density of the target pathogen, usually present in a trace amount in contaminated food and environmental samples, to detectable levels of ~10^4 to 10^6 CFU/ml for ELISA (10, 11) or ~10^1 to 10^3 CFU/ml for PCR (9). In addition, the ideal surface protein should be strongly associated to the cell envelope for use in *L. monocytogenes* isolation. Finally, the surface protein should be expressed in all *L. monocytogenes* serotypes under standard enrichment culture conditions. Certain surface antigens previously examined may not be suitable for *L. monocytogenes* detection due to the lack of expression in enrichment culture conditions (26-28), weak association with the cell surface (122) and lack of conservation in some serotypes (19, 28). Most

surface proteins used as targets for antibody-based methods for *L. monocytogenes* detection are virulence factors regulated by PrfA (154). Although virulence factors are specific to *L. monocytogenes*, expression of these factors in *in vitro* culture is highly variable (26, 155, 156) most likely due to their adapted expression for intra-cellular condition (29, 30). Antibody-based assays with specificity for *L. monocytogenes* have been developed (138-140), however, the antigen targets recognized by these antibodies are unknown and may be shared with other *Listeria* species including other foodborne bacteria (139, 140).

This study aimed to identify novel surface proteins that serve as biomarkers for the isolation and detection of a range of *L. monocytogenes* serotypes. A two-step approach was devised to identify a diagnostic biomarker for *L. monocytogenes.* First, surface antigens with epitopes conserved in various *L. monocytogenes* serotypes, but variable among other *Listeria* species, was identified. Second, monoclonal antibodies (MAbs) were developed to assess whether candidate surface proteins were surface exposed and specifically expressed in a range of *L. monocytogenes* serotypes and in standard selective enrichment culture conditions.

4.2 Results

4.2.1 Selection of Candidate Surface Proteins by Bioinformatics

Bioinformatics analysis identified 130 putative or known surface proteins (Tables S1-S7 in Appendix) from the genome of *L. monocytogenes* strain F2365 (serotype 4b) (147). Of the identified surface proteins, four protein candidates, LMOf2365_0578, LMOf2365_0581, LMOf2365_0639 and LMOf2365_2117, met the selection criteria (see section 3.2 in Materials and Methods) as potential surface biomarkers for *L. monocytogenes*. LMOf2365_0578, a homolog of lmo0549 in the *L. monocytogenes* EGD-e strain, contains two WxL domains

possibly associated with the peptidoglycan (89). LMOf2365_0581 is the homolog of lmo0552 and contains a C-terminal hydrophobic tail. Both LMOf2365_0639 and LMOf2365_2117 have a C-terminal LPXTG motif and are predicted to be covalently linked to the peptidoglycan. A draft genome sequence of *L. monocytogenes* strain LI0521 (serotype 4b), a strain routinely used in this study, has been recently reported (157). The four proteins in strain LI0521 were assessed for their potential as *L. monocytogenes* specific biomarkers.

4.2.2 Transcriptional and Translational Expression of Candidate Proteins in *L. monocytogenes*

Since the candidates selected were all uncharacterized proteins, a preliminary assessment of the gene expression of the candidate proteins in BHI was performed. Reverse-transcriptase PCR revealed mRNA expression of four identified protein candidates (LMOf2365_0578, LMOf2365_0581, LMOf2365_0639 and LMOf2365_2117) in strain LI0521 at the stationary growth phase (Figure 4-1). No PCR product was observed when the extracted RNA was used as template for PCR. This indicated that the PCR product from cDNA template was not due to contamination of genomic DNA.

4.2.3 Production of Recombinant Candidate Proteins

Candidate proteins LMOf2365_0578, LMOf2365_0581, LMOf2365_0639 and LMOf2365_2117 were cloned in the pLIC expression plasmid (Figure 4-2). To assess protein expression, three purified full-length recombinant proteins (rLMOf2365_0578, rLMOf2365_0581 and rLMOf2365_0639) and a truncated C-terminal region (a.a. 340-477) of LMOf2365_2117 were used as antigens to raise rabbit polyclonal antibodies. Full-length LMOf2365_2117 could not be used as antigen because it degraded in the *E. coli* expression host

(data not shown). His-tag western blot and Comassie blue stain of purified recombinant proteins are identical except for LMOf2365_0581. His-tag western blot of purified rLMOf2365_0581 revealed a band of less than 37 kDa in addition to 15 kDa band as compared to the Coomassie blue stained gel (Figure 4-3). The larger band may be dimers of the 15 kDa polypeptide found in trace amounts.

Western blots using polyclonal antibodies revealed single protein bands corresponding to each of the four candidate proteins in *L. monocytogenes* whole cell extract (Figure 4-4). The LMOf2365_0578 (75 kDa) and LMOf2365_0639 (63 kDa) native proteins of *L. monocytogenes* were similar in size to the full-length recombinant proteins produced in *E.coli* (Figure 4-3). The *L. monocytogenes* native protein LMOf2365_2117 was similar to predicted size of 72 kDa. *L. monocytogenes* native protein LMOf2365_0581 was larger than the recombinant protein produced in *E.coli* (Figure 4-3) with a predicted size of 30 kDa.

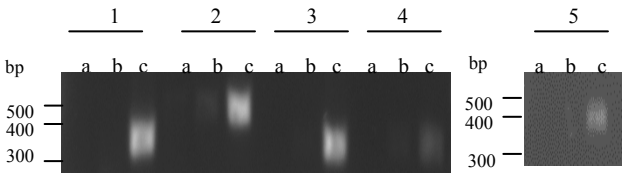


Figure 4-1. RT-PCR detection of candidate genes: LMOf2365_2117, LMOf2365_0639, LMOf2365_0581 and LMOf2365_0578. *L. monocytogenes* strain LI0521 cells were grown in BHI to OD_{620} of ~1.5 (stationary phase). The total RNA extracted from 5 x 10^9 cells was used to prepare cDNA in a reverse transcriptase reaction with random hexamers. The 16S rRNA gene was used as a positive control. PCR was performed using gene specific primers with no template control (lane a), RNA template (lane b) and cDNA template (lane c). Genes: (1) 16s RNA, (2) LMOf2365_2117, (3) LMOf2365_0639, (4) LMOf2365_0581 and (5) LMOf2365_0578 were tested.

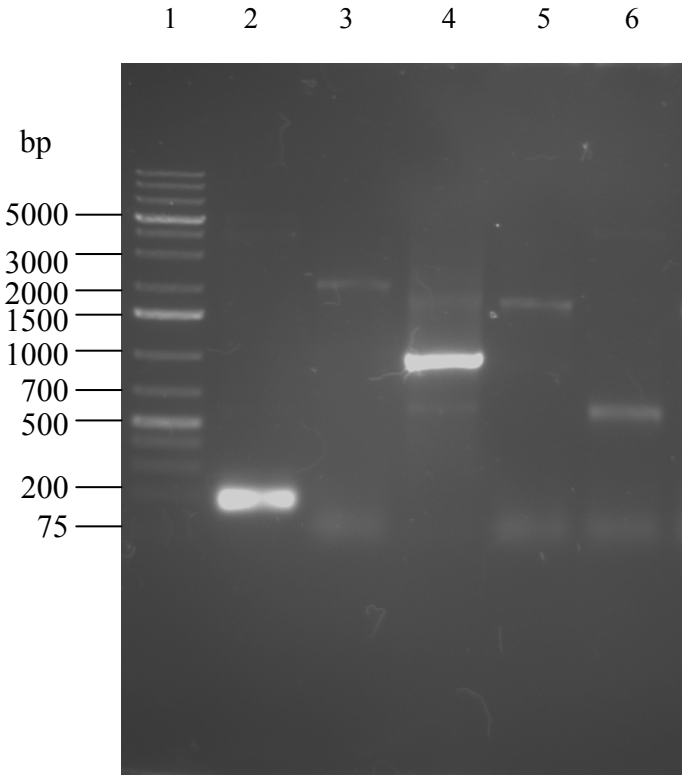


Figure 4-2. PCR Screening of recombinant inducible plasmids for LMOf2365_0578, LMOf2365_0581, LMOf2365_0639 and LMOf2365_2117 expression. Recombinant pLIC-CHIS (lanes 3-6) or empty pLIC-CHIS (lane 2) plasmids were used as template for PCR. T7 promoter and terminator primers that annealed to the plasmid template were used in PCR. Full length genes: *LMOf2365_0578* (lane 3), *LMOf2365_0581* (lane 4) and *LMOf2365_0639* (lane 5) were cloned for each surface protein. The C-terminal region *LMOf2365_2117* (lane 6) was cloned. See Table 3-3 in Materials and Methods for cloning primer sequences. Lane 1 consists of the molecular marker.

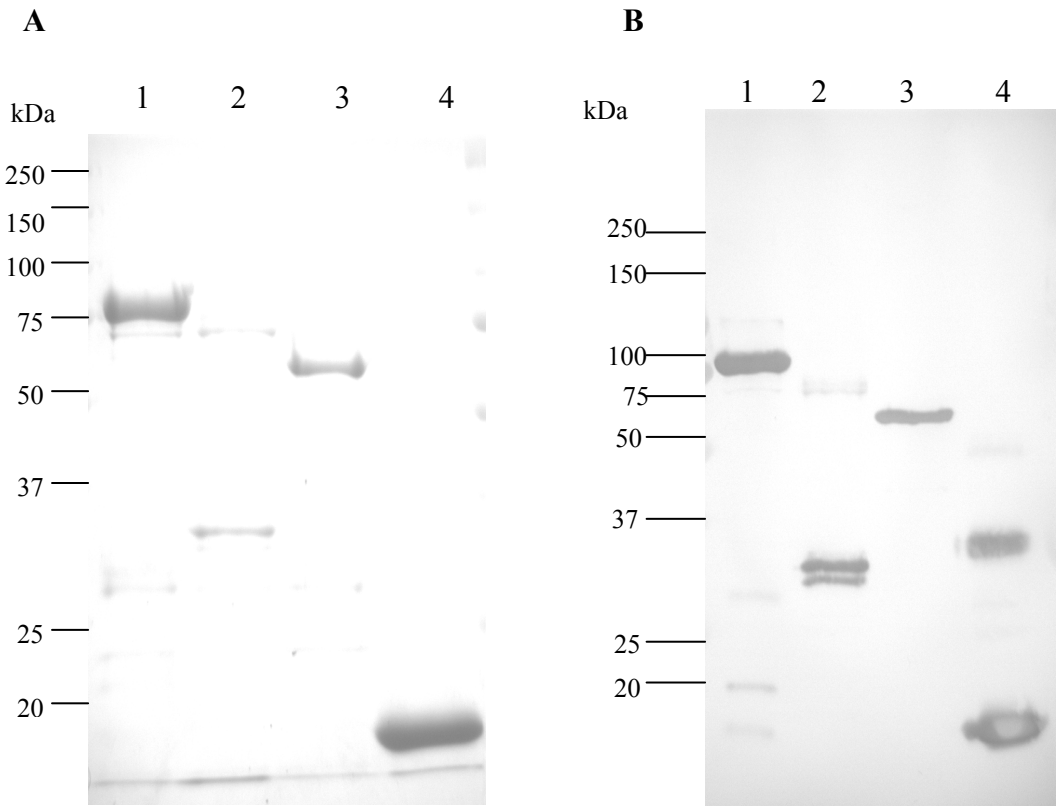


Figure 4-3. Coomassie blue and western blot of purified recombinant surface protein candidates. Panel A: Coomassie blue stain of purified antigens for polyclonal antibody production. Immobilized metal affinity chromatography was performed to purify the His-tagged antigen. Full-length antigens: LMOf2365_0578 (lane 1), LMOf2365_0581 (lane 2) and LMOf2365_0639 (lane 3) were made. The C-terminal region (a.a. 340-477) of LMOf2365_2117 was made (lane 4). Panel B: His-tag western blot of purified antigens for polyclonal antibody production. Purified antigens were assessed for presence of His-tag using (anti-His) MAbs (Qiagen).

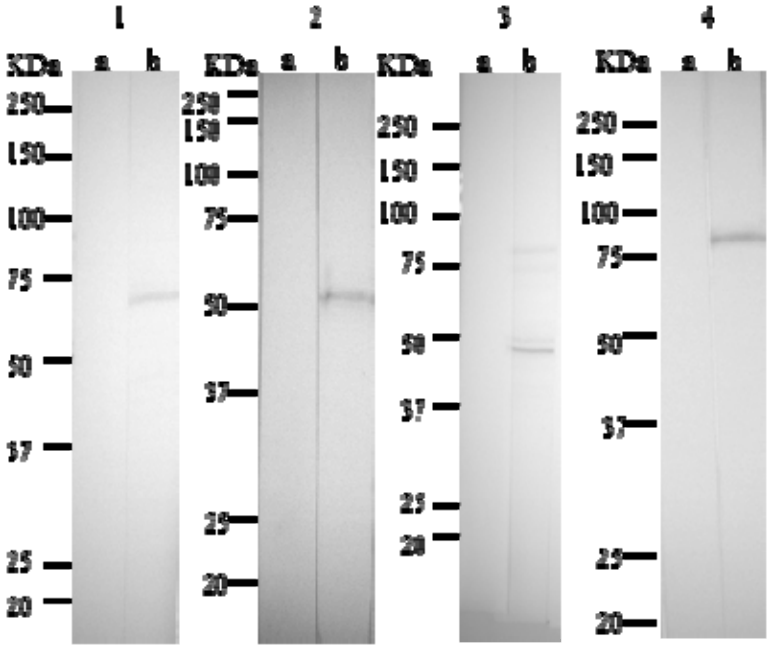


Figure 4-4. Western blot analysis of LMOf2365_0578, LMOf2365_0581, LMOf2365_0639 and LMOf2365_2117 proteins in whole cell extracts of *L. monocytogenes* strain LI0521. An overnight culture (~1.5 OD_{620}, 50 ml) in BHI broth was used to make 0.8 ml of whole cell extract (see section 3.12 in Materials and Methods for details). A 30 µl of whole cell extract sample was loaded onto each lane. Separate proteins on nitrocellulose membrane were probed with the rabbit pre-immune serum (lane a) and specific rabbit anti-serum (lane b). Antibodies against: LMOf2365_2117 (group 1), LMOf2365_0639 (group 2), LMOf2365_0581 (group 3) and LMOf2365_0578 (group 4) were used to probe whole cell extracts.

The surface localization of candidate proteins on *L. monocytogenes* strain LI0521 was examined by immunofluorescence microscopy (IFM) using PAbs. A strong signal was only observed with the PAb to LMOf2365_0639 (Figure 4-5). This indicated that LMOf2365_0639 contained surface exposed epitopes recognized by antibodies. No fluorescence signal was observed with anti-LMOf2365_0639 PAb on *L. innocua*, *L. seeligeri*, *L. grayi* and *L. welshimeri* (Figure 4-6). The LMOf2365_0639 surface protein was thus targeted for the development of monoclonal antibodies (MAbs).

4.2.4 Screening of Monoclonal Antibodies to LMOf2365_0639

Mouse TCFs from hybridomas against rLMOf2365_0639 were screened by ELISA using several antigens. Thirty-five stable clones that reacted with both rLMOf2365_0639 and formalin-killed strain LI0521 whole cells but not with formalin-killed *L. innocua* CLIP11262 whole cells (data not shown) were selected. In addition, a peptide specific to *L. monocytogenes* was used to screen hybridoma clones. A multiple sequence alignment of the proteins similar to LMOf2365_0639, obtained from a BLAST search, revealed a sequence region (a.a. 526-554) specific to *L. monocytogenes* (Figure 4-7). A peptide representing this unique sequence was fused with a GST-tag. Both purified GST-tagged peptide and GST tag (negative control) were used for MAb screening (Figure 4-8). Cloned sequences were confirmed by sequencing (data not shown). TCF from one clone during screening reacted with the GST-tagged peptide but not with the GST alone. MAb secreted from this clone did not react to formalin-killed whole cells of *L. monocytogenes* strain LI0521 and therefore was not selected.

Figure 4-5. Surface localization assessment of LMOf2365_0639, LMOf2365_0578, LMOf2365_0581 and LMOf2365_2117 proteins on live *L. monocytogenes* strain LI0521 by immunofluorescence microscopy. Bacterial cells (2.5 x 10^8) from overnight culture (~1.5 OD_{620}) grown in BHI broth were probed with specific rabbit PAbs (1:1000 dilution) against purified recombinant proteins LMOf2365_0639 (A), LMOf2365_0578 (E), LMOf2365_0581 (I) and LMOf2365_2117 (M). No signal was observed when pre-immune sera were used as a negative control (C, G, K and O). Fluorescence images (A, C, E, G, I, K, M and O) and phase contrast images (B, D, F, H, J, L, N and P) in the same field are shown (magnification 100x).

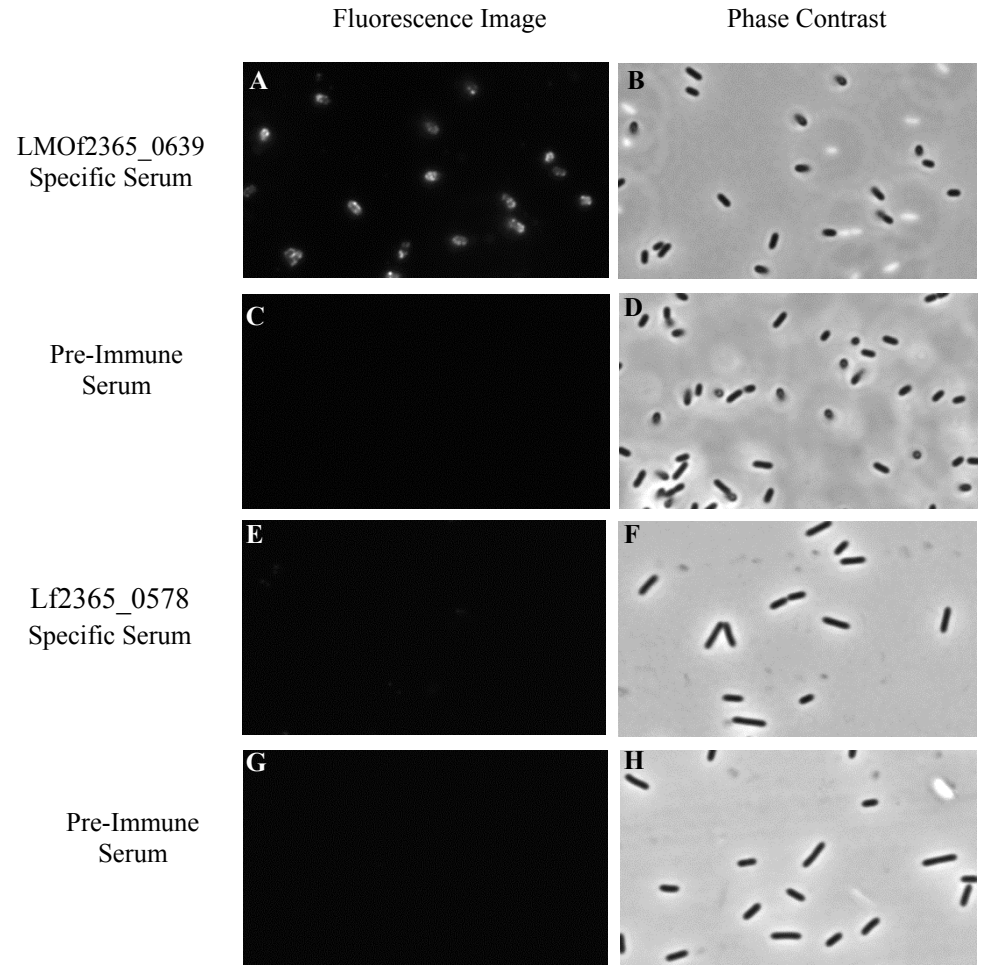

Fluorescence Image
Phase Contrast
LMOf2365_0639 Specific Serum
Pre-Immune Serum
Lf2365_0578 Specific Serum
Pre-Immune Serum
A
B
C
D
E
F
G
H

Figure 4-5.

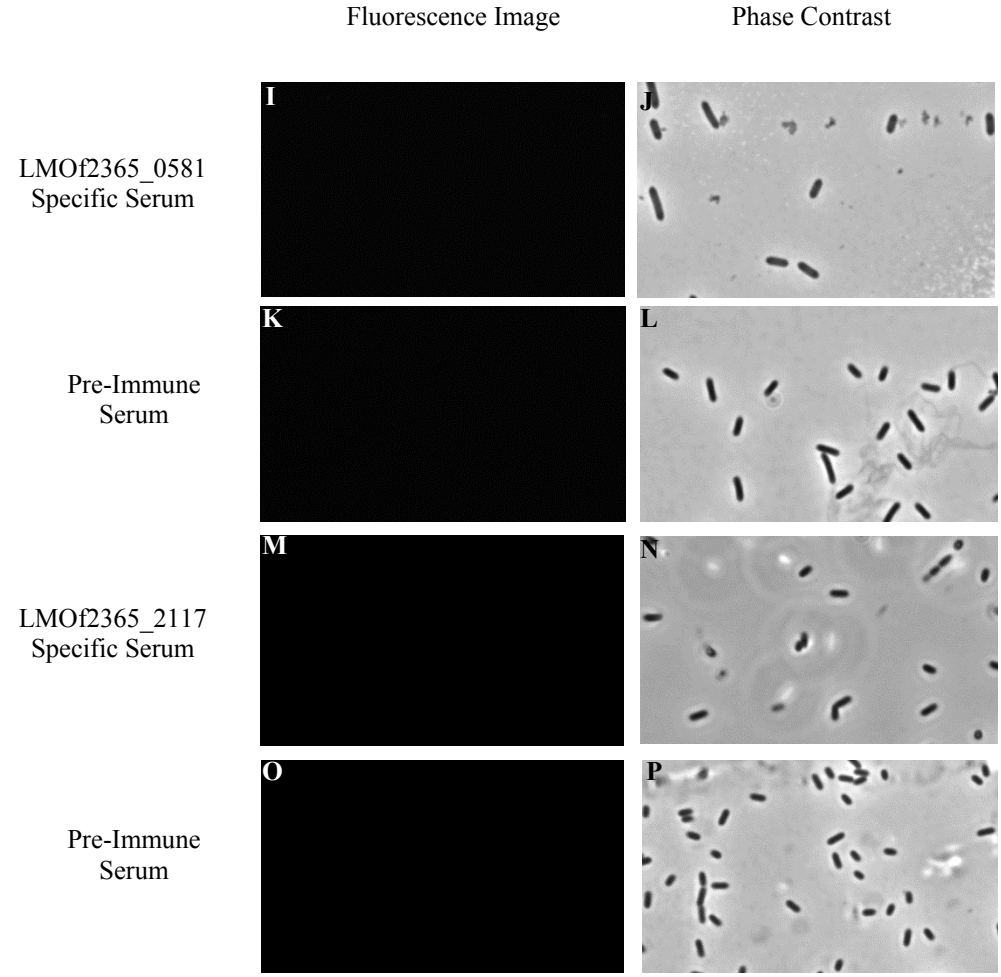


Figure 4-5. Continued.

Figure 4-6. Analysis of LMOf2365_0639 expression on the cell surface of *L. ivanovii*, *L. seeligeri, L. welshimeri* and *L. innocua* by immunofluorescence microscopy. Bacterial cells (2.5×10^8) cells from overnight BHI culture (~1.5 OD_{620}) for each species (*L. ivanovii* (A), *L. seeligeri* (E), *L. welshimeri* (I) and *L. innocua* (M)) were probed with LMOf2365_0639 polyclonal rabbit antisera (1:1000 dilution) (A, E, I and M). Pre-immune sera were used as a negative control (C,G, K and O). Fluorescence images (A, C, E, G, I, K, M and O) and phase contrast images (B, D, F, H, J, L, N and P) in the same field are shown (magnification 100x).

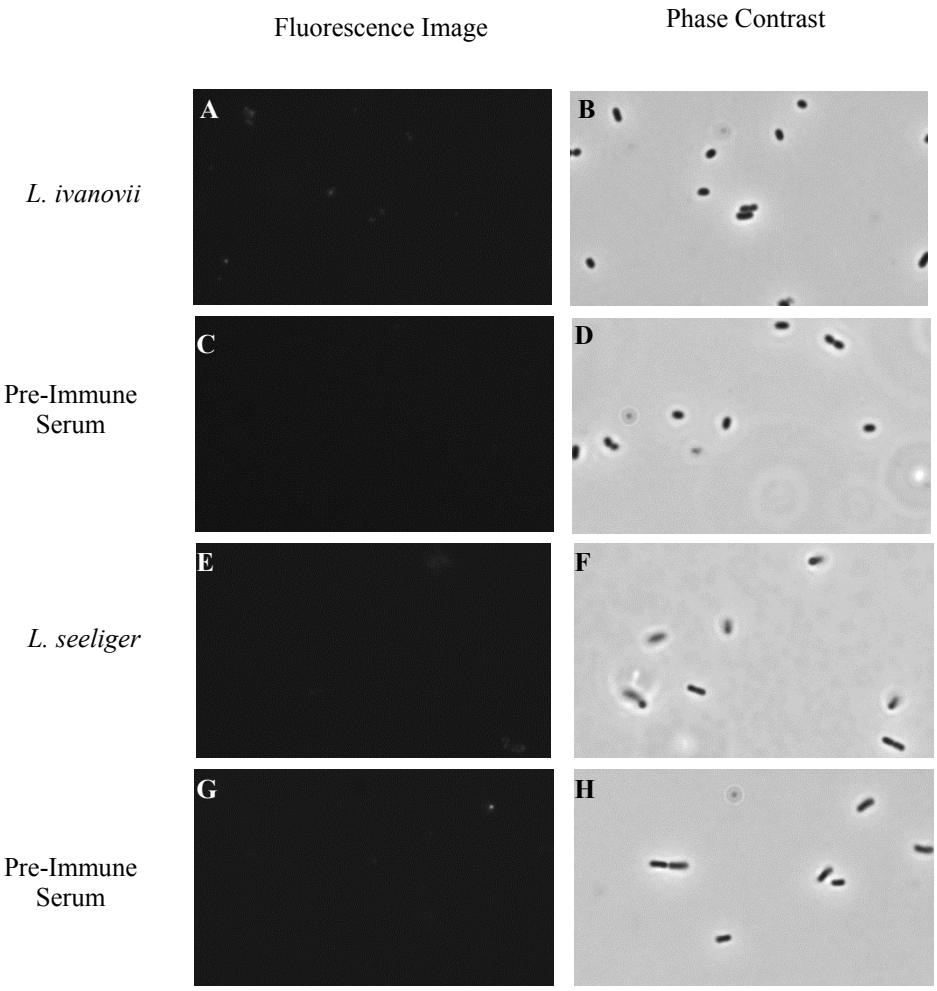

Fluorescence Image
Phase Contrast
L. ivanovii
Pre-Immune Serum
L. seeliger
Pre-Immune Serum
A
B
C
D
E
F
G
H

Figure 4-6.

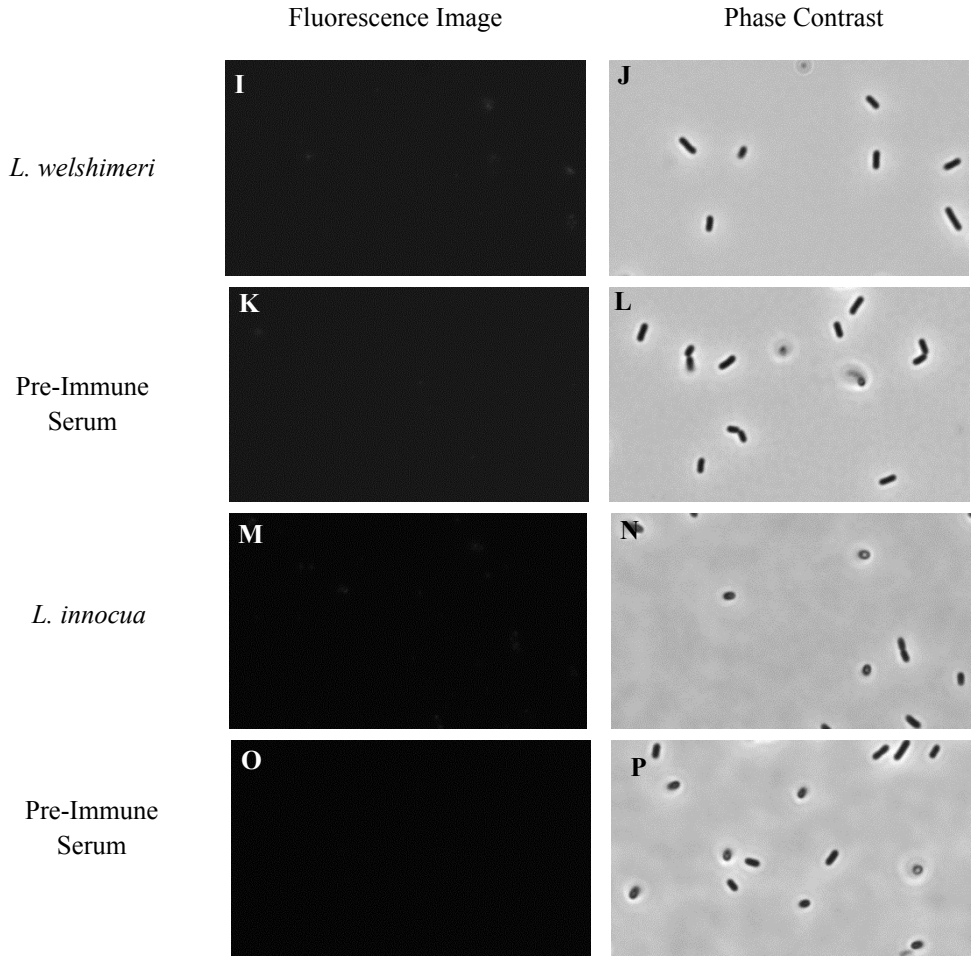
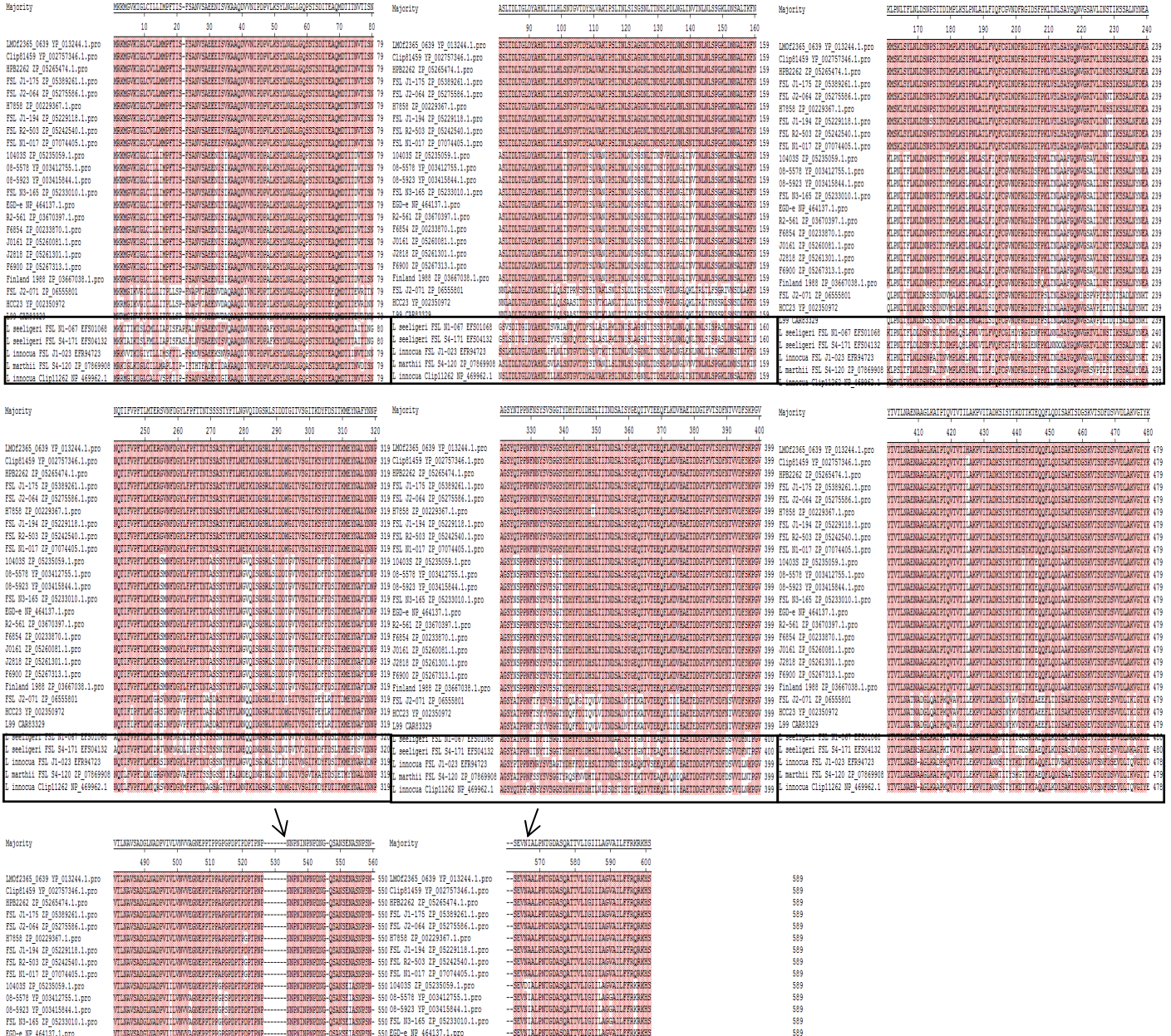


Figure 4-6. Continued.

Figure 4-7. Sequence comparison of sequenced *Listeria* strains. Alignment completed by Clustal W algorithm. Sequences retrieved from NCBI database on September 27, 2010 from pBLAST search. Boxed sequences consist of sequenced *Listeria* strains other than *L. monocytogenes*. Sequence flanked by arrows consists of unique *L. monocytogenes* sequence used for monoclonal antibody screening. Residues highlighted in red are identical to residues of LMOf2365_0639 listed in the first row.



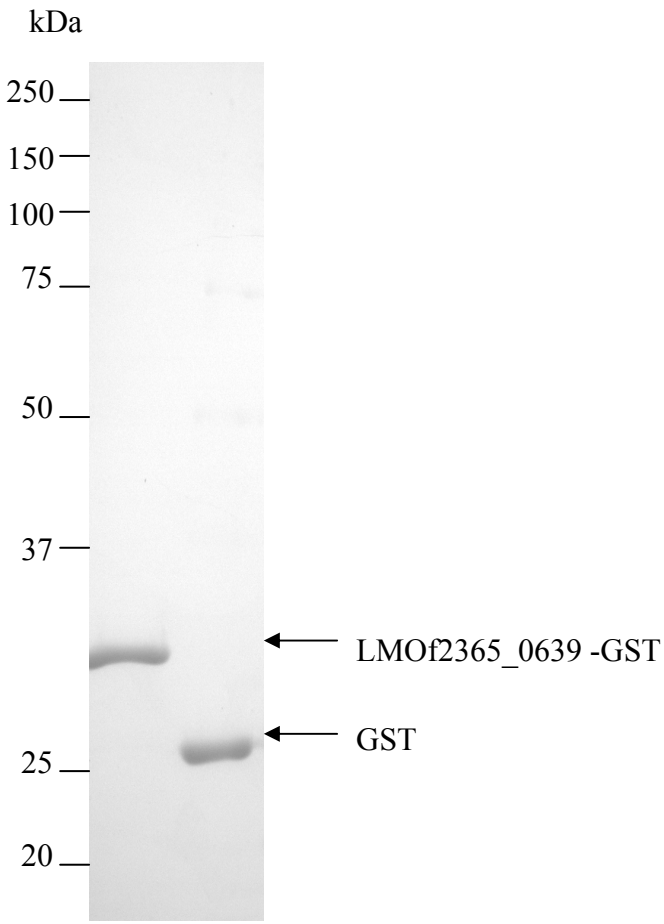


Figure 4-8. SDS-PAGE Coomassie Blue stained gel of purified His-GST and His-GST-peptide. GST from the pGEX-3x GST expression plasmid was cloned into pLIC-CHIS plasmid (153). Peptide that span 526-554 a.a. of LMOf2365_0639 (see Table 3-4 in Materials and Methods for primer sequences) was fused with the GST-tag and cloned into pLIC-CHIS plasmid (153). Immobilized metal affinity chromatography was performed to purify His-tagged proteins.

4.2.5 Identification of MAbs Reactive with Various *L. monocytogenes* Strains Grown in BHI

TCF from 15 clones with the highest OD_{414} values (0.4-1), when analyzed by indirect ELISA with formalin-killed *L. monocytogenes* strain LI0521 grown in BHI, were selected for further characterization. TCF from these clones were further assessed for their reactivity to formalin-killed whole cells of 53 *L. monocytogenes* isolates, 10 isolates from five *Listeria* species and four foodborne pathogens (*C. jejuni*, *Salmonella enterica* serovar Typhimurium, *E. coli* O157:H7 and *B. cereus*) by indirect ELISA (data not shown). Three MAbs, M3643, M3644 and M3651 reacted with 62, 55 and 81% of *L. monocytogenes* isolates tested, respectively. These MAbs detected LMOf2365_0639 on the surface of live *L. monocytogenes* (Figure 4-9) and purified rLMOf2365_0639 antigen by western blot (Figure 4-9).

Reactivity of the three MAbs (M3643, M3644 and M3651) was different between *L. monocytogenes* isolates of different lineages. The strongest ELISA signals were observed with lineage I *L. monocytogenes* isolates of serotype 1/2b, 4b, 4ab and 4d (Figure 4-10). Weaker reactions were observed with lineage II isolates of serotype 1/2a, 1/2c and 3a (Figure 4-10). Weak or no reaction was observed with lineage III isolates of serotype 4a and 4c (Figure 4-10). While MAb M3644 was specific to *L. monocytogenes* isolates (Figure 4-10), M3651 and M3643 were weakly reactive to *L. innocua* isolate HPB583 (Figure 4-10) with an average OD_{414} of 0.27 and 0.35, respectively.

Figure 4-9. MAbs are reactive to live *L. monocytogenes* and purified recombinant LMOf2365_0639. Panel A: Immunofluorescence images of live *L. monocytogenes* strain LI0521 cells probed with each of the three MAbs: M3643 (A and B), M3644 (C and D) and M3651 (E and F) (magnification 100x). Bacterial cells (2.5×10^8) from an overnight BHI culture (~1.5 OD_{620}) were stained with purified IgG (1 ng/µl) and viewed with a fluorescence microscope. TCF of M1169 to *C. jejuni* at a dilution of 1:50 was used as negative control (G and H). Fluorescence images (A, C, E and G) and phase contrast images (B, D, F and H) in the same field are shown. Panel B: Detection of recombinant LMOf2365_0639 by western blot using each of the three MAbs (M3643, M3644 and M3651). Each MAb in TCF was used at 1:25 dilution.

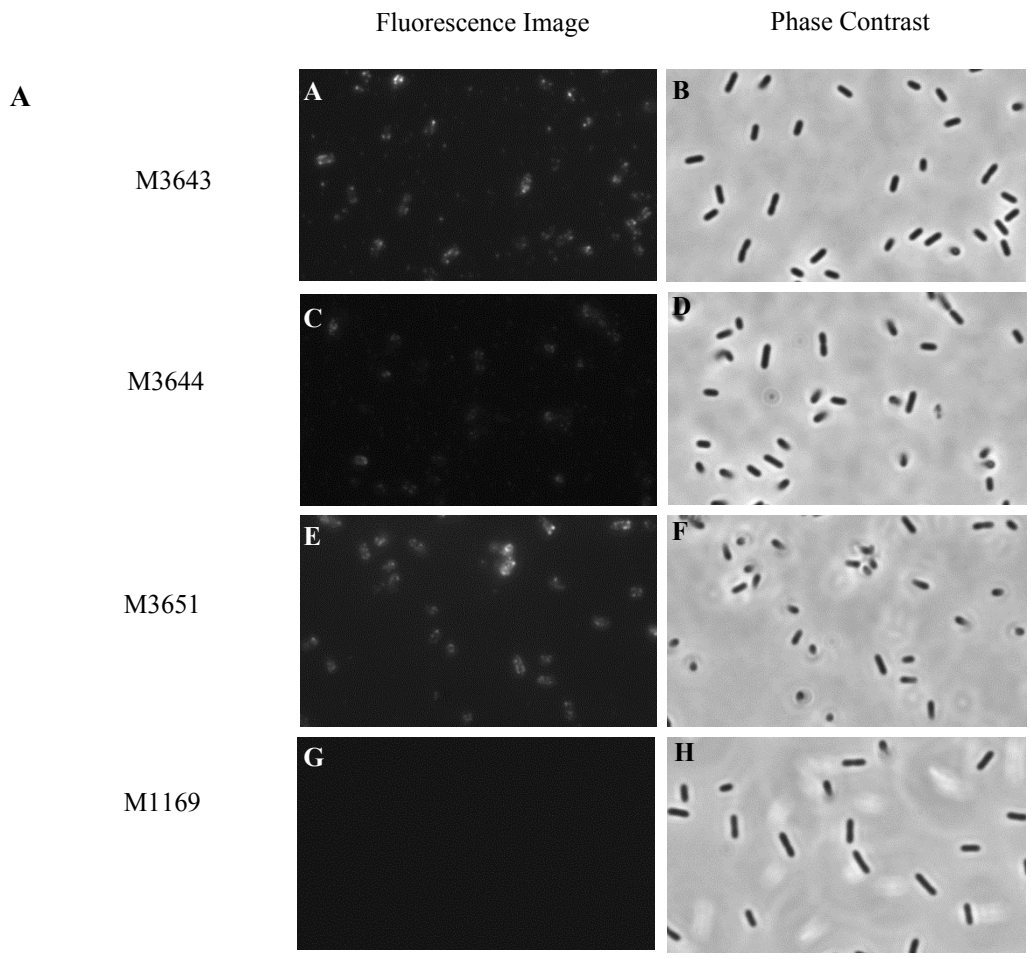
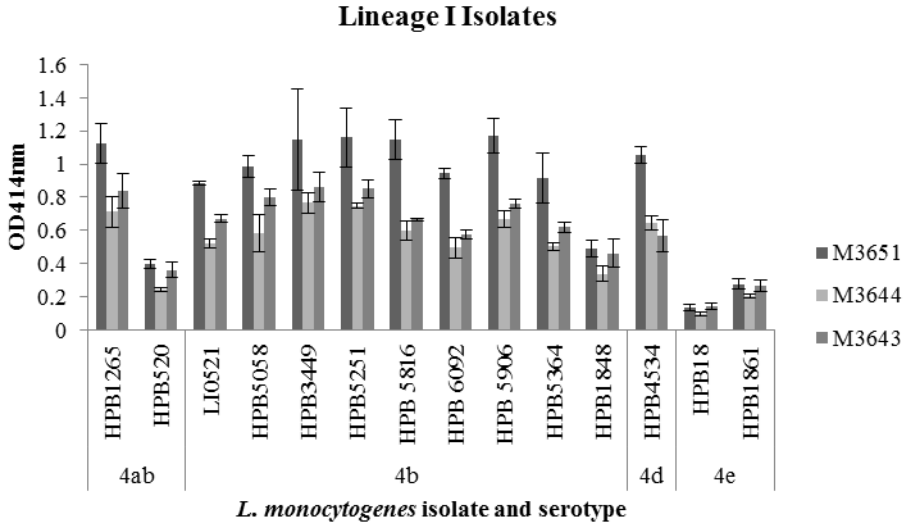
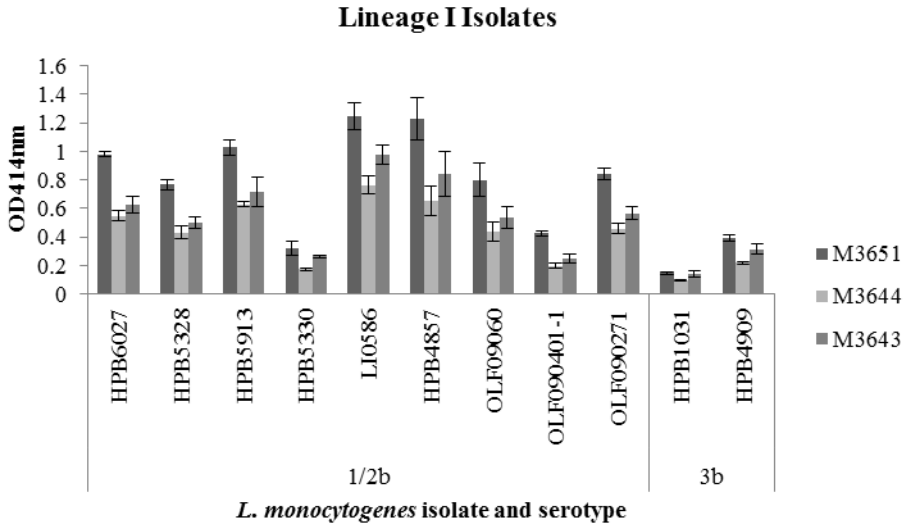
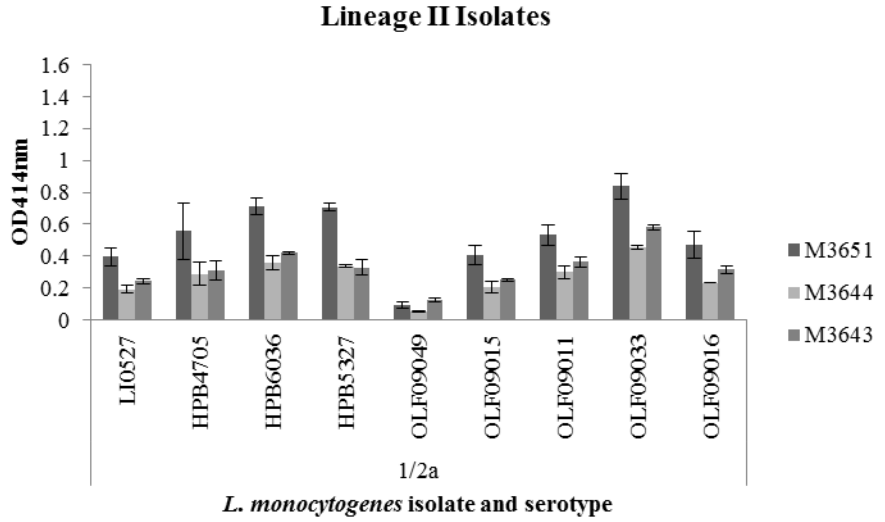
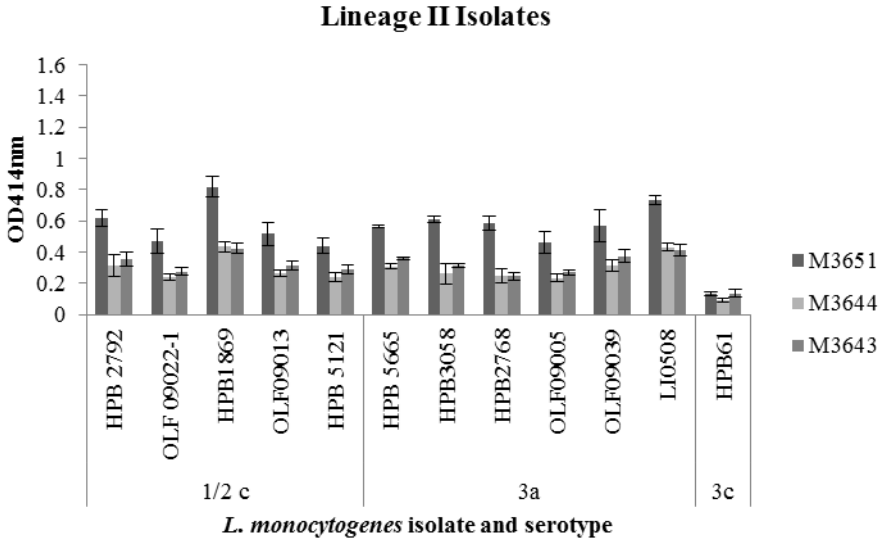

A
Fluorescence Image
Phase Contrast
M3643
M3644
M3651
M1169
A
B
C
D
E
F
G
H
B
M3643 M3644 M3651
KDa
75
50

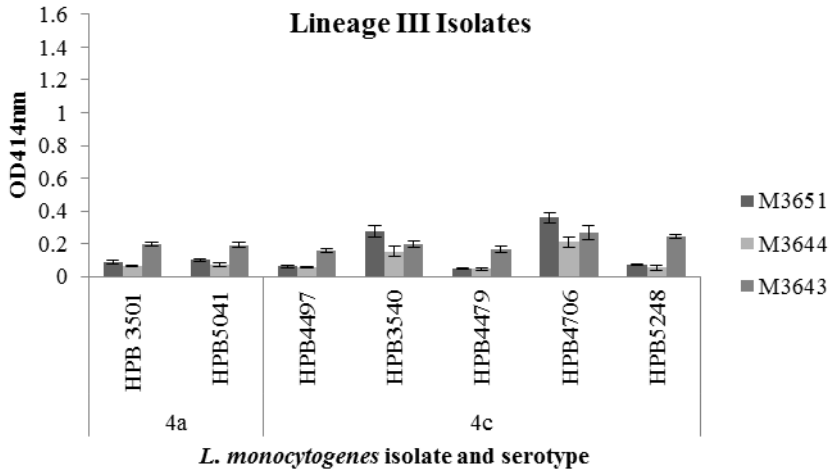
Figure 4-10. Detection of lineage I, II and III isolates of *L. monocytogenes* and other *Listeria* species and bacteria cultured in BHI by indirect ELISA. Overnight *L. monocytogenes* culture was formalin killed and stored in 50% glycerol. A 100 µl formalin-killed bacterial suspension (10^8 cells/ml) was used to coat each well of Maxisorp™ 96-well microtiter plates. Each of the three MAbs (M3651, M3644 and M3643) in undiluted tissue culture fluid was used to detect bacterial cells. Error bars are one standard deviation from the mean of three independent experiments. Two replicates were performed in each experiment. The OD_{414} readings obtained from negative controls ranged from 0.03 to 0.2, and thus OD_{414} readings of <0.25, between 0.25 and 0.3, and > 0.3 were considered as negative, weakly positive and positive, respectively. See section 3.15 in Materials and Methods for details.

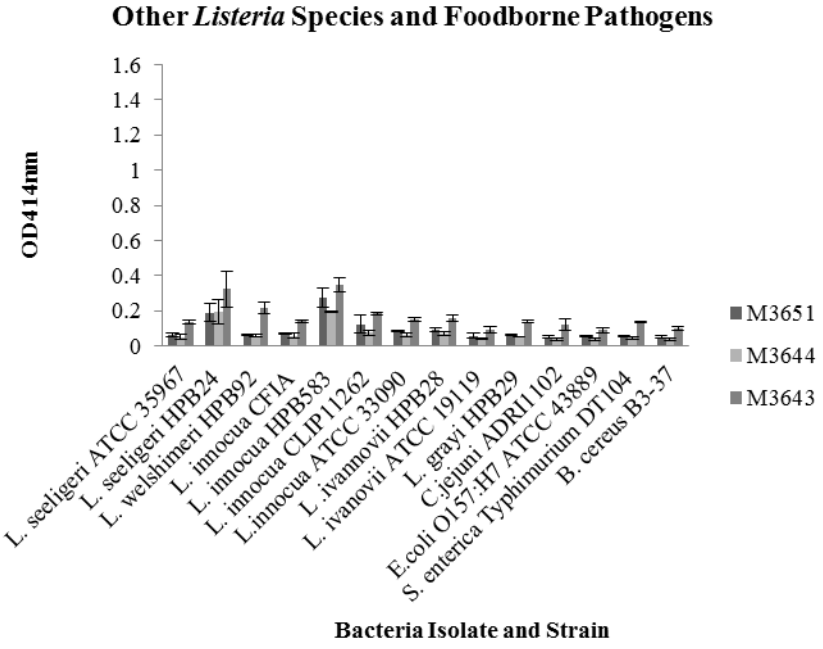

Lineage I Isolates
OD414nm
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
HPB1265
HPB520
LI0521
HPB5058
HPB3449
HPB5251
HPB 5816
HPB 6092
HPB 5906
HPB5364
HPB1848
HPB4534
HPB18
HPB1861
4ab
4b
4d
4e
M3651
M3644
M3643
L. monocytogenes isolate and serotype


Lineage I Isolates
OD414nm
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
HPB6027
HPB5328
HPB5913
HPB5330
LI0586
HPB4857
OLF090060
OLF090401-1
OLF090271
HPB1031
HPB4909
1/2b
3b
M3651
M3644
M3643
L. monocytogenes isolate and serotype


Lineage II Isolates
OD414nm
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
LI0527
HPB4705
HPB6036
HPB5327
OLF09049
OLF09015
OLF09011
OLF09033
OLF09016
1/2a
M3651
M3644
M3643
L. monocytogenes isolate and serotype


Lineage II Isolates
OD414nm
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
HPB 2792
OLF 09022-1
HPB1869
OLF09013
HPB 5121
HPB 5665
HPB3058
HPB2768
OLF09005
OLF09039
LI0508
HPB61
1/2 c
3a
3c
M3651
M3644
M3643
L. monocytogenes isolate and serotype

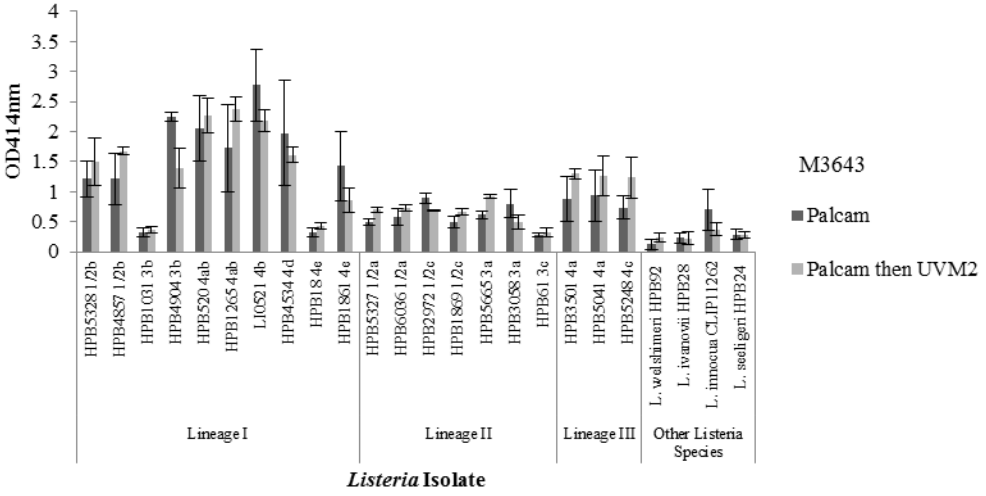

Lineage III Isolates
OD414nm
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
M3651
M3644
M3643
HPB 3501
HPB5041
HPB4497
HPB3540
HPB4479
HPB4706
HPB5248
4a
4c
L. monocytogenes isolate and serotype


Other Listeria Species and Foodborne Pathogens
OD414nm
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
M3651
M3644
M3643
L. seeligeri ATCC 35967
L. seeligeri HPB24
L. welshimeri HPB92
L. innocua CFIA
L. innocua HPB583
L. innocua CLIP11262
L.innocua ATCC 33090
L .ivanovii HPB28
L. ivanovii ATCC 19119
L. grayi HPB29
C.jejuni ADRI1102
E.coli O157:H7 ATCC 43889
S. enterica Typhimurium DT104
B. cereus B3-37
Bacteria Isolate and Strain

4.2.6 Expression of the LMOf2365_0639 Protein during Enrichment Culture

Enrichment culture is necessary to detect target pathogen, usually present in trace amounts, in contaminated food and environmental samples. Hence potential application of any of these MAbs in bacterial detection requires the expression of the LMOf2365_0639 antigen under selective enrichment culture conditions. The ability of the three MAbs (M3643, M3644 and M3651) to detect 20 *L. monocytogenes* isolates and other *Listeria* species isolates grown in standard enrichment cultures according to MFHPB-07 (146) and MFHPB-30 (107), described in Health Canada's Compendium of Analytical, was assessed by indirect ELISA Methods (Figures 4-10 and 4-11). The MAbs were reactive to eight out of eleven lineage I isolates and all six lineage II isolates tested. This result demonstrated the potential usefulness of LMOf2365_0639 as a biomarker for *L. monocytogenes* detection. The MAbs were not reactive to two isolates from lineage I (serotype 3b, 4e) and one isolate (serotype 3c) from lineage II. Of note, M3643 was the only MAb reactive to the 4a and 4c serotype isolates from lineage III (Figure 4-11 and 4-12). In addition, *L. innocua* CLIP11262 was reactive to all three MAbs tested in both primary and secondary enrichment culture of MFHPB-07 and MFHPB-30. The observation that MAbs M3643, M3644 and M3651 reacted similarly to the isolates tested under primary and secondary culture conditions of both MFHPB-07 and MFHPB-30 methods indicated the consistency and reliability of the ELISA results.

Figure 4-11. Detection of *Listeria* isolates cultured according to the MFHPB-07 method by indirect ELISA. Both bacterial cells grown in Palcam at 35 ^{o}C for 26-28 hours and then in UVM2 at 30 ^{o}C for 26-28 hours were used in the experiments. A 100 µl formalin-killed bacterial suspension (10^8 cells/ml) was used to coat each well of Maxisorp^{TM} 96-well microtiter plates. Each of the three MAbs (M3643, M3644 and M3651) in undiluted tissue culture fluid was used to detect bacterial cells. Error bars are one standard deviation from the mean of three independent experiments. Two replicates were performed in each experiment. The OD_{414} readings obtained from negative controls ranged from 0.03 to 0.2, and thus OD_{414} readings of <0.25, between 0.25 and 0.3, and > 0.3 were considered as negative, weakly positive and positive respectively. See section 3.15 in Materials and Methods for details.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3.5
3
2.5
2
1.5
1
0.5
0
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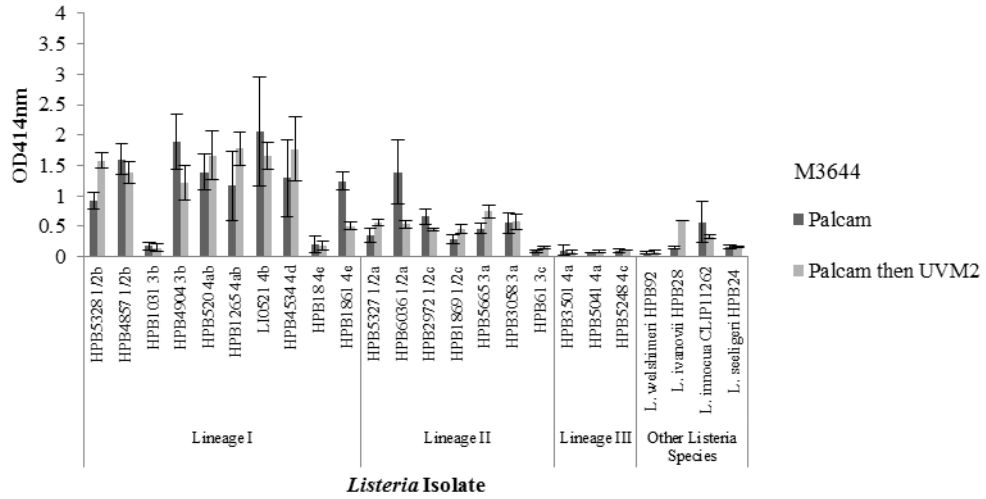
HPB4857 1 2b
HPB1031 3b
HPB4904 3b
HPB5204ab
HPB1265 4ab
LI0521 4b
HPB4534 4 d
HPB18 4e
HPB1861 4e
HPB5327 1 2a
HPB6036 1 2a
HPB2972 1 2c
HPB1869 1 2c
HPB5665 3a
HPB3088 3a
HPB61 3c
HPB3501 4a
HPB5041 4a
HPB5248 4c
L. welshimeri HPB92
L. ivanovii HPB28
L. innocua CLIP11262
L. seeligeri HPB24
Lineage I
Lineage II
Lineage III
Other Listeria Species
Listeria Isolate
M3643
Palcam
Palcam then UVM2


OD414nm
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HPB5328 1 2b
HPB4857 1 2b
HPB1031 3b
HPB4904 3b
HPB5204ab
HPB1265 4ab
LI0521 4b
HPB4534 4 d
HPB18 4e
HPB1861 4e
HPB5327 1 2a
HPB6036 1 2a
HPB2972 1 2c
HPB1869 1 2c
HPB5665 3a
HPB3088 3a
HPB61 3c
HPB3501 4a
HPB5041 4a
HPB5248 4c
L. welshimeri HPB92
L. ivanovii HPB28
L. innocua CLIP11262
L. seeligeri HPB24
Lineage I
Lineage II
Lineage III
Other Listeria Species
Listeria Isolate
M3644
Palcam
Palcam then UVM2

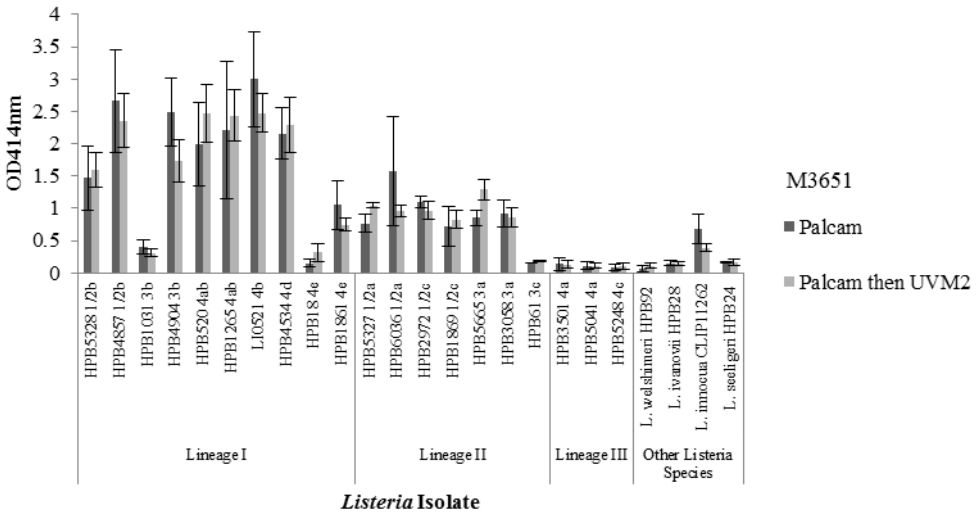
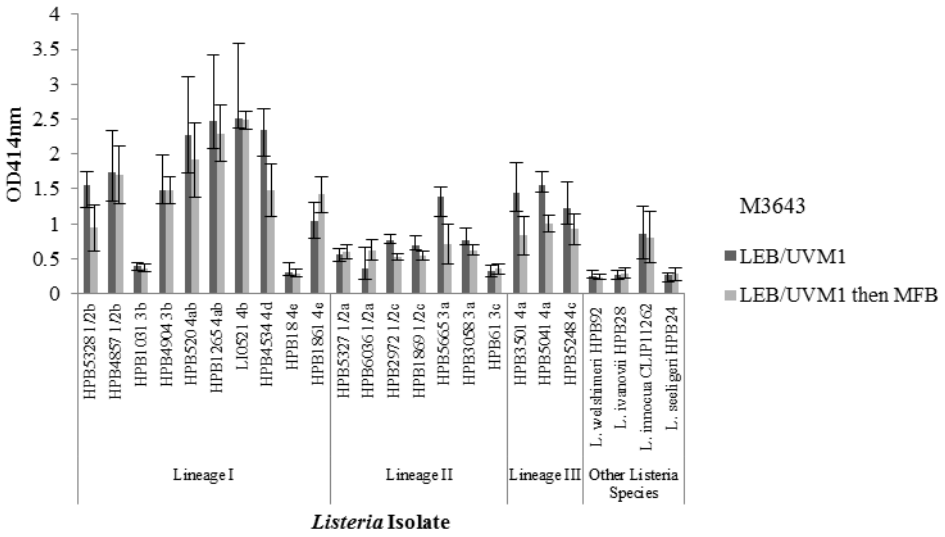
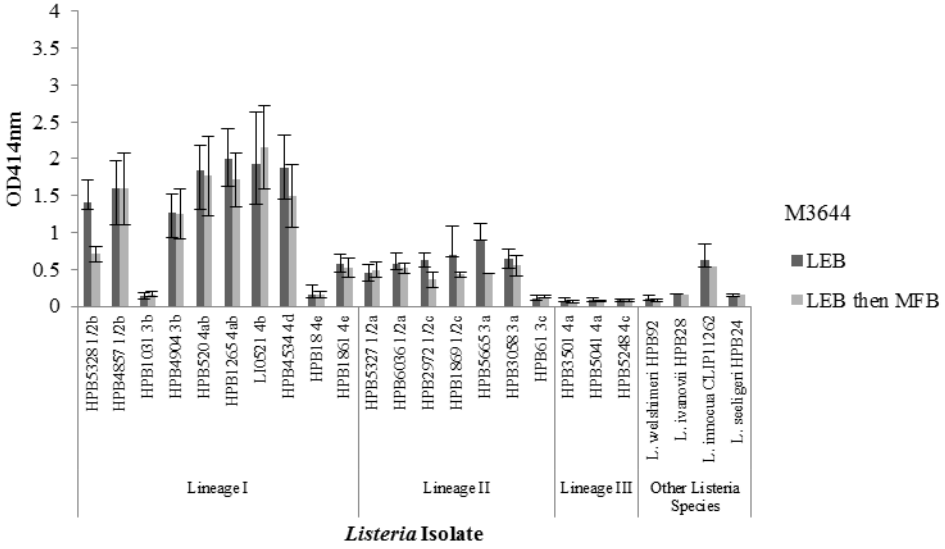

OD414nm
4
3.5
3
2.5
2
1.5
1
0.5
0
M3651
Palcam
Palcam then UVM2
HPB5328 1 2b
HPB4857 1 2b
HPB1031 3b
HPB4904 3b
HPB5204 ab
HPB1265 4ab
L10521 4b
HPB4534 4d
HPB18 4e
HPB1861 4e
HPB5327 1 2a
HPB6036 1 2a
HPB2972 1 2c
HPB1869 1 2c
HPB5665 3a
HPB3058 3a
HPB61 3c
HPB3501 4a
HPB5041 4a
HPB5248 4e
L. welshimeri HPB92
L. ivanovii HPB28
L. innocua CLIP11262
L. seeligeri HPB24
Lineage I
Lineage II
Lineage III
Other Listeria Species
Listeria Isolate

Figure 4-12. Detection of *Listeria* isolates cultured according to the MFHPB-30 method by indirect ELISA. Both bacterial cells grown in Listeria Enrichment Broth (LEB) at 30 ^{o}C for 48 hours and then in Modified Fraser Broth (MFB) at 35 ^{o}C for 24 hours were used in the experiments. A 100 µl formalin-killed bacterial suspension (10^8 cells/ml) was used to coat each well of Maxisorp^{TM} 96-well microtiter plates. Each of the three MAbs (M3643, M3644 and M3651) in undiluted tissue culture fluid was used to detect bacterial cells. Error bars are one standard deviation from the mean of three independent experiments. Two replicates were performed in each experiment. The OD_{414} readings obtained from negative controls ranged from 0.03 to 0.2, and thus OD_{414} readings of <0.25, between 0.25 and 0.3, and > 0.3 were considered as negative, weakly positive and positive respectively. See section 3.15 in Materials and Methods for details.


OD414nm
4
3.5
3
2.5
2
1.5
1
0.5
0
M3643
LEB/UVM1
LEB/UVM1 then MFB
HPB5328 1/2b
HPB4857 1/2b
HPB1031 3b
HPB4904 3b
HPB520 4ab
HPB1265 4ab
LI0521 4b
HPB4534 4d
HPB18 4e
HPB1861 4e
HPB5327 1/2a
HPB6036 1/2a
HPB2972 1/2c
HPB1869 1/2c
HPB5665 3a
HPB3058 3a
HPB61 3c
HPB3501 4a
HPB5041 4a
HPB5248 4c
L. welshimeri HPB92
L. ivanovii HPB28
L. innocua CLIP11262
L. seeligeri HPB24
Lineage I
Lineage II
Lineage III
Other Listeria Species
Listeria Isolate


OD414nm
4
3.5
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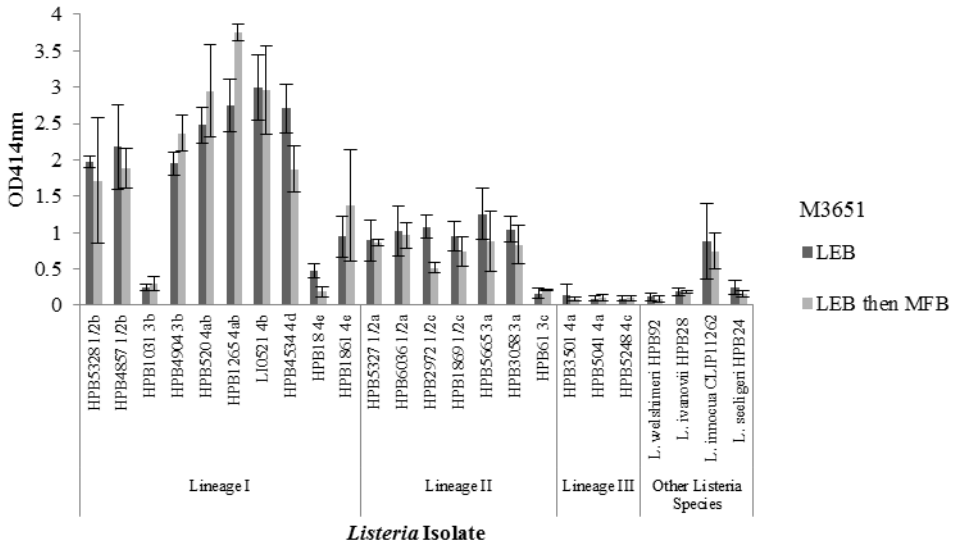
2
1.5
1
0.5
0
M3644
LEB
LEB then MFB
HPB5328 1/2b
HPB4857 1/2b
HPB1031 3b
HPB4904 3b
HPB520 4ab
HPB1265 4ab
LI0521 4b
HPB4534 4d
HPB18 4e
HPB1861 4e
HPB5327 1/2a
HPB6036 1/2a
HPB2972 1/2c
HPB1869 1/2c
HPB5665 3a
HPB3058 3a
HPB61 3c
HPB3501 4a
HPB5041 4a
HPB5248 4c
L. welshimeri HPB92
L. ivanovii HPB28
L. innocua CLIP11262
L. seeligeri HPB24
Lineage I
Lineage II
Lineage III
Other Listeria Species
Listeria Isolate


OD414nm
4
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HPB5328 I/2b
HPB4857 I/2b
HPB1031 3b
HPB4904 3b
HPB5204 4ab
HPB1265 4ab
L10521 4b
HPB4534 4d
HPB184 4e
HPB1861 4e
HPB5327 I/2a
HPB6036 I/2a
HPB2972 I/2c
HPB1869 I/2c
HPB5665 3a
HPB3058 3a
HPB61 3c
HPB3501 4a
HPB5041 4a
HPB5248 4c
L. welshimeri HPB92
L. ivanovii HPB28
L. innocua CLIP11262
L. seeligeri HPB24
Lineage I
Lineage II
Lineage III
Other Listeria Species
Listeria Isolate
M3651
LEB
LEB then MFB

4.2.7 Epitope Mapping for MAbs M3651, M3644 and M3643

Four recombinant protein fragments (A a.a. 1-174, B a.a. 48-260, C a.a. 261-448 and D a.a. 421-560) spanning LMOf2365_0639 (Figure 4-13) were produced. MAbs M3651 and M3644 recognized a region within fragment A (a.a.1-47). Since the N-terminus of the cloned antigen used for MAb production started from a.a. 41, the epitope of M3651 and M3644 appeared to encompass the sequence VNIPDPV (a.a. 41-47). Since M3643 recognized both fragments A and B, M3643 likely recognized the sequence LKSYLNGLLGQ common to both protein fragments immediately adjacent to VNIPDPV (Figure 4-14).

Multiple alignment of LMOf2365_0639 with its homologous proteins revealed similarities among *L. monocytogenes* strains of the same lineage. Where the epitope of MAbs M3651 and M3644 was mapped, a single amino acid change at the second valine residue (VNIPDPV) was observed between lineage I and lineages II/III strains and *L. Seeligeri* (Figure 4-14). Sequenced strains of *L. innocua* and *L. marthii* also contained the VNIPDPV sequence found in lineage I strains (Figure 4-14). The epitope recognized by M3643, LKSYLNGLLGQ, was identical among *L. monocytogenes* lineage I, II and III strains as well as strains of other *Listeria* species (Figure 4-14).

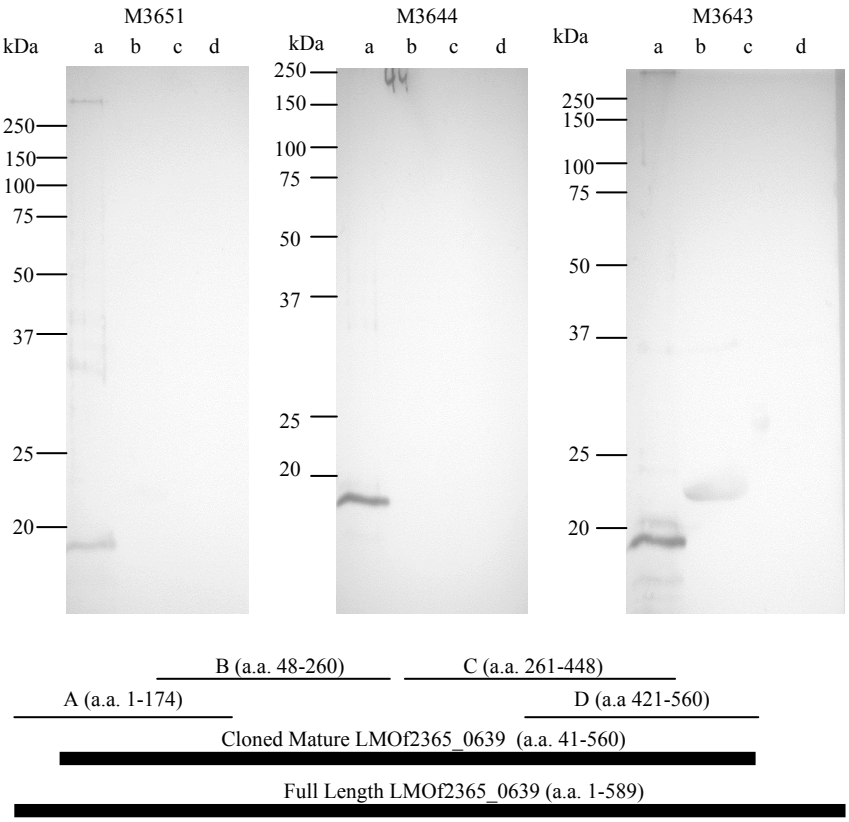


Figure 4-13. Epitope mapping for M3651, M3644 and M3643 using four overlapping protein fragments (A, B, C and D) that span the full-length protein of LMOf2365_0639 protein. Each of the three MAbs (M3651, M3644 and M3643) in TCF was used at a dilution of 1:25 to probe the protein fragments transferred onto nitrocellulose membranes.

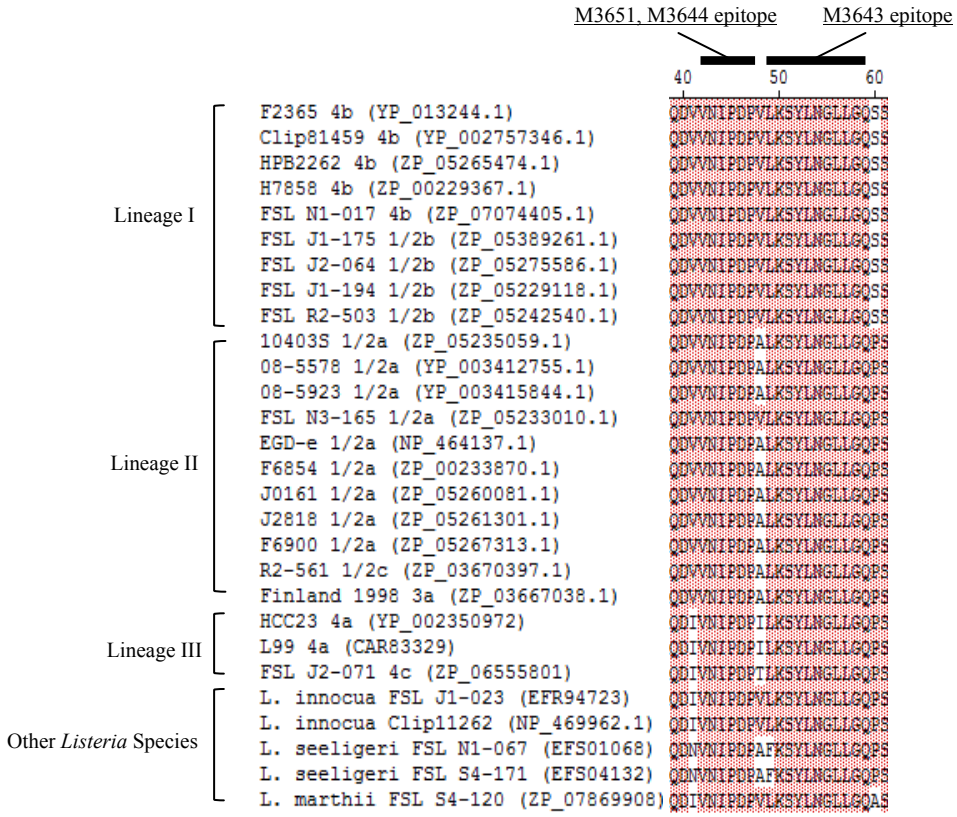


Figure 4-14. Comparison of N-terminal residues (40 to 60) of the LMO2365_0639 protein with the corresponding region in *L. monocytogenes* and other *Listeria* species by multiple alignment. Strain name with NCBI protein accession numbers in brackets are listed for each sequence. Sequences retrieved from NCBI database on September 27, 2010 to January 6, 2011.

4.2.8 Transcription Start Site Determination of *LMOf2365_0639*

The transcription start site of the gene, *LMOf2365_0639* was determined to investigate possible mechanisms of how the gene is regulated. The identification of possible transcriptional regulation elements upstream of the gene may provide insight into *LMOf2365_0639* expression. Using 5' RACE (see section 3.7 in Materials and Methods for details), the transcription start site was mapped to nucleotide "G" which was 48 base pairs from the first base of the start codon (Figure 4-15). Using the bacterial promoter prediction program BPROM (Softberry Inc.), sigma B promoter sequences were observed directly upstream of the transcription start site.

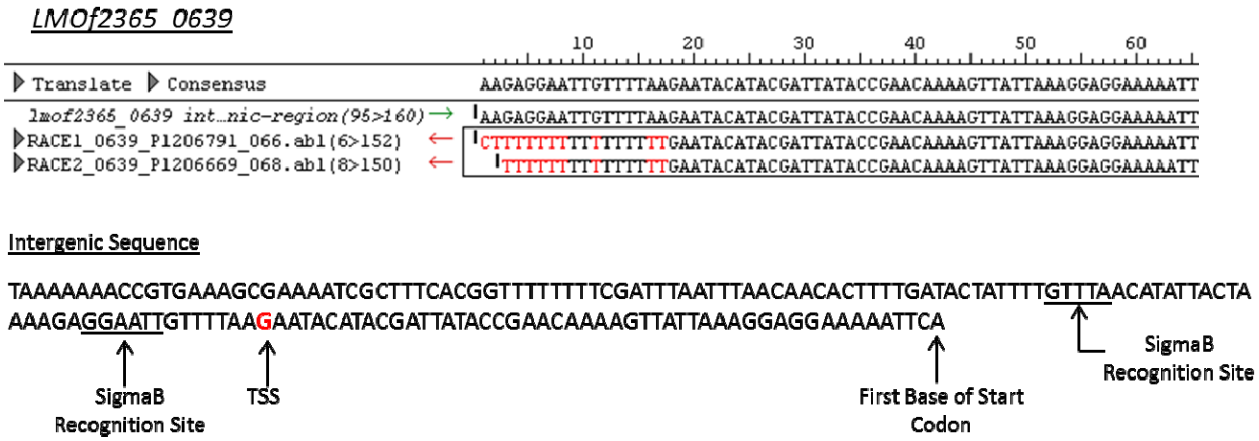


Figure 4-15. Transcription start site of *LMOf2365_0639*. Two independent 5' RACE experiments were performed to determine the transcription start site (TSS). PCR products (RACE1_0639 and RACE2_0639) from 5' RACE were sequenced and aligned to *LMOf2365_0639* intergenic region to determine the TSS. See section 3.7 in Materials and Methods for details of 5' RACE.

4.3 Discussion

The aim of this work was to identify candidate surface proteins for detection of *L. monocytogenes*. To this end, a two-step approach was performed. This approach involved candidate selection based on sequence comparisons of surface proteins followed by assessment of surface exposure and expression in enrichment culture conditions pertinent to *L. monocytogenes* detection. Of the four candidates initially identified, only LMOf2365_0639 was readily detected on the surface of live *L. monocytogenes* as revealed by IFM. To evaluate if LMOf2365_0639 can act as a potential biomarker for *L. monocytogenes* detection, MAbs were raised against this protein and assessed extensively for their reactivity with formalin-killed *L. monocytogenes* isolates and isolates of other *Listeria* species and other foodborne bacterial pathogens. Of the 35 MAbs generated against rLMOf2365_0639, three MAbs (M3651, M3644 and M3643) recognized a majority of 53 *L. monocytogenes* isolates grown in non-selective media. MAbs (M3643, M3644 and M3651) against LMOf2365_0639 revealed specific LMOf2365_0639 expression in *L. monocytogenes* lineage I and II isolates in non-selective and selective enrichment media. MAb M3643 was reactive to *L. monocytogenes* lineage I, II and III isolates in selective enrichment media. Expression of LMOf2365_0639 in selective enrichment in a range of *L. monocytogenes* serotypes makes it a potential biomarker for *L. monocytogenes* isolation and detection from environmental and foods samples.

All four candidate surface proteins were detected by western blot of the whole cell extract however, only LMOf2365_0639 was readily detected on live *L. monocytogenes* using PAb in immunofluorescence microscopy. The lack of signal for the other candidate proteins may be due to a lack of surface exposure. Certain *L. monocytogenes* surface proteins such as InlB require peptidoglycan cleavage for surface exposure (158). Sequential washes prior to

immunofluorescence microscope imaging may also have prevented detection of candidate proteins by immunofluorescence microscopy. Surface proteins such as LMOf2365_0578 and LMOf2365_0581 are non-covalently associated with the cell envelope therefore may be washed away prior to immunofluorescence microscope imaging. Finally, although expression is observed in the whole cell extract, LMOf2365_0578, LMOf2365_0581 and LMOf2365_2117 may be not readily secreted.

Immunofluorescence microscopy of live *L. monocytogenes* using anti-LMOf2365_0639 PAbs revealed that surface exposed epitopes exist within LMOf2365_0639. Hence, MAbs were developed to identify such surface exposed epitopes. During sequence comparison, a region of LMOf2365_0639, near the C-terminus, that is conserved among *L. monocytogenes* strains and highly variable among other *Listeria* species was identified. To assess whether this region was surface exposed, this region along with formalin-killed whole *L. monocytogenes* was used to screen hybridoma clones specific to LMOf2365_0639. MAbs specific to this region were not reactive to formalin-killed whole *L. monocytogenes* cells. Conceivably, since the region is close to the anchoring LPXTG motif, it is likely sequestered in *L. monocytogenes* cells. Therefore this region may not be an ideal target for a diagnostic antibody targeting whole *L. monocytogenes*. MAbs M3643, M3644 and M3651 were all reactive to live *L. monocytogenes* and mapped to the N-terminal region of LMOf2365_0639. These results suggest that the N-terminal region of LMOf2365_0639 is surface exposed.

The MAbs reported here were more reactive to lineage I isolates than to isolates belonging to other lineages (Figures 4-10, 4-11 and 4-12). Differences in reactivity could be epitope-related. A single amino acid difference (VNIPDPV to VNIPDPA) exists between lineage I and lineage II/III strains at the location of the M3651 and M3644 epitope. The

rLMOf2365_0639 used to immunize mice for MAb production contained the VNIPDPV sequence. The strong reactivity of MAbs M3651 and M3644 to lineage I isolates may be due to lineage I specific epitopes on the immunogen cloned from the lineage I isolate LI0521. The difference in reactivity observed between lineages may also be attributed to differential expression of LMOf2365_0639 between lineages. A proteomic study of several *L. monocytogenes* isolates of various serotypes reported that total surface protein patterns of isolates can be grouped by lineage classification (159). In addition, while the epitope recognized by M3643 is identical between sequenced *L. monocytogenes* isolates and other *Listeria* species isolates, M3643 was highly specific to *L. monocytogenes* in BHI. Other *L. monocytogenes* serotype specific MAbs have targeted antigens that are not serotype specific (19). A panel of MAbs against the GW modules of *L .monocytogenes* autolysin, IspC reacted specifically to 4b and 4ab isolates even though the targeted GW modules are encoded in the genomic sequences of other *L. monocytogenes* serotypes and other *Listeria* species (19).

Selected *L. monocytogenes* isolates were grown in enrichment culture according to methods described in Health Canada's Compendium of Analytical Methods. ELISA results indicated that the three selected MAbs were more reactive to lineage I isolates than lineage II isolates grown in enrichment cultures. Unlike previous studies of potential diagnostic surface proteins which reported reduced expression under specific conditions (25-27, 124), the stable expression of LMOf2365_0639 in enrichment culture makes it a candidate biomarker.

A previous study developed PAb against four *L. monocytogenes* proteins one of which was lmo0610, the homolog of LMOf2365_0639 (25). The study reported weak expression of lmo0610 in *L. monocytogenes* V7 (serotype 1/2a) cultured in Fraser, UVM and BHI broths by western blot. In addition, SDS-PAGE analysis of total surface proteins revealed less protein

contents for bacteria grown in Fraser and BHI broths than those cultured in BLEB, LRB and UVM broths (25). Although western blot revealed minimal levels of lmo0610 expression by Geng *et al.* (25), using MAbs M3643, M3644 and M3651, serotype 1/2a *L. monocytogenes* isolates was detected by indirect ELISA grown in UVM1 and MFB and most serotype 1/2a isolates grown in BHI was detected using M3651 in this study.

While M3651 and M3644 were not reactive to lineage III isolates of serotype 4a and 4c, M3643 was able to detect these serotypes during enrichment culture. This finding may be explained by the fact that the epitope recognized by M3643 was different from that recognized by M3651 and M3644. Conceivably, the LMOf2365_0639 protein was expressed in these 4a and 4c isolates during enrichment culture.

Although the three MAbs specifically recognize *L. monocytogenes* cultured in BHI, they detected the *L. innocua* CLIP11262 in both primary and secondary enrichment cultures according to MFHPB-07 and MFHPB-30 methods. An ortholog of LMOf2365_0639 exists in *L. innocua* CLIP11262. By sequence comparison, sequence identity is observed between *L. monocytogenes* lineage I isolates and *L. innocua* CLIP11262 where epitopes of M3643, M3644 and M3651 are mapped (Figure 4-14). Therefore the observed reactivity to *L. innocua* CLIP11262 can be explained by the presence of M3643, M3644 and M3651 epitopes on *L. innocua* CLIP11262. Differential protein expression between non-selective and selective media has been observed in other studies. PAbs against the protein ActA were reactive to *L. monocytogenes* isolates in BLEB, UVM and FB and minimal reactivity was observed in BHI and LB (26). Stress response to selective agents within enrichment culture may induce the expression of LMOf2365_0639 in *L. innocua* which is otherwise absent in non-selective culture such as BHI (Figure 4-10). The MAbs are potentially applicable for *L. monocytogenes* detection and, to

lesser extent, *L. innocua* detection. The application of these MAbs can provide specific information about the presence of the two *Listeria* species for subsequent conformational tests (107, 146).

The presence of sigma B promoter sequences upstream of the transcription start of *LMOf2365_0639* was observed. *LMOf2365*_0639 expression may be regulated by the stress responsive alternative sigma factor, sigma B. The homolog of *LMOf2365_0639*, *lmo0610* which has identical sigma B promoter sequences was regulated by sigma B (160). The expression of *lmo0610* was at a significantly lower level in sigma B null mutant compared to wild type *L. monocytogenes* (160) in high salt in which sigma B activity is stimulated. Moreover, *lmo0610* expressed at various temperatures correlate with the sigma B activity (161, 162). Possible regulation of *LMOf2365*_0639 by sigma B may allow *LMOf2365*_0639 to be expressed under the conditions relevant to *L. monocytogenes* detection. While no sigma B activity was observed at the log phase growth of *L. monocytogenes* in BHI, the presence of high salt concentration in the culture induced the sigma B activity (161). High salt content in selective enrichment culture media such as Fraser broth (modified Fraser broth), UVM1 (LEB), UVM2 and certain foods may stimulate the activity of sigma B, thereby the expression of *LMOf2365_0639*. In addition, commonly used food preservatives such as nisin have been demonstrated to induce sigma B activity (163). Hence, *LMOf2365*_0639 is expected to be expressed in foods and selective enrichment cultures.

This study provides evidence that antigen LMOf2365_0639 is a useful surface biomarker that can be explored for the development of antibody- or capture-based diagnostic methods for *L. monocytogene*s. It is expressed in lineage I and II isolates of *L. monocytogenes* which includes serotype 1/2a, 1/2b and 4b that are associated with 95% of human listeriosis outbreaks and

sporadic cases (164). The three MAbs (M3651, M3644 and M3643) were shown to be specific for *L. monocytogenes* and showed no reaction to other *Listeria* species with the exception of weak reaction with one *L. innocua* isolate (HPB583) in BHI culture and reaction to *L. innocua* CLIP11262 in enrichment cultures. In addition, these MAbs showed binding to epitopes in the N-terminal region of LMOf2365_0639 that is accessible on the surface of live cells and can therefore be readily applied to the diagnostic isolation and detection of *L. monocytogenes* from foods and environmental samples following culture enrichment.

Chapter 5: Identification of Surface Protein Biomarkers using Proteomic Analysis

5.1 Introduction

L. monocytogenes infection can result in the rare but serious food-borne illness listeriosis (4). The average fatality rate of listeriosis is 20-30% (1). Given the ubiquity of *L. monocytogenes* and its ability to survive a range of pH, osmolarity and temperature conditions (165, 166), *L. monocytogenes* poses a serious health risk to susceptible individuals (4). Conventional identification of *L. monocytogenes* involves enrichment cultures, isolation by plating followed by biochemical tests which can take more than a week to complete (13). Although molecular methods can expedite *L. monocytogenes* detection, inhibitory substances in the food and environmental samples can confound test results. *L. monocytogenes* is therefore separated and concentrated from the original sample matrix prior to molecular detection.

Several proteomic studies have identified numerous *L. monocytogenes* surface proteins through the extraction of various components of the cell envelope (167-171). These studies indicate the expression of certain surface proteins, but their surface exposure on the external side of the cell wall is unknown. For instance, the surface protein Internalin B is buried in the peptidoglycan and requires peptidoglycan cleavage for its exposure (158). The information of whether a protein is surface-exposed is required for the development of diagnostic reagents capable of live cell isolation and detection.

The study of surface exposed proteins of *L. monocytogenes* may results in identification of diagnostic targets for the isolation and detection of viable *L. monocytogenes* in test samples. To this end, tryspin digest was performed to bacterial cells to release surface-exposed peptides for mass spectrometry analysis. This approach allowed the recovery of peptides from the

exposed regions of surface proteins while minimizing those from regions that are not readily accessible to trypsin for the identification of surface exposed proteins. One hundred and seventy-four proteins were identified in at least two of three trials in either the negative control or during cell digest (Table 5-1). Nineteen surface, 21 extracellularly secreted, 132 cytoplasmic and two phage proteins were identified. Seventeen surface proteins out of a total of 76 proteins were identified in one of three trials (Table 5-2).

5.2 Results and Discussion

5.2.1 Optimizing Digestion for Surface Protein Identification

Several optimization studies were performed to minimize cell lysis and the contamination of cytoplasmic protein. First, cell lysis was monitored by examining the protein content of supernatants by SDS-PAGE with silver staining and by CFU counting. Cells were incubated in digest buffer and the supernatant and cell pellets were collected at 15, 30, 45 and 60 minutes. At 45 minutes, protein content within the supernatant increased (Figure 5-1) while CFUs decreased (Figure 5-2). The maximum incubation time prior to cell lysis was determined to be 30 minutes. Second, the potential for trypsin to induce cell lysis at 30 minutes of incubation was assessed by comparing CFUs of trypsin-treated and untreated bacterial samples. No statistically significant difference between medians of the two sample groups was observed using the Mann-Whitney U test (SYSTAT version 10) (Figure 5-3).

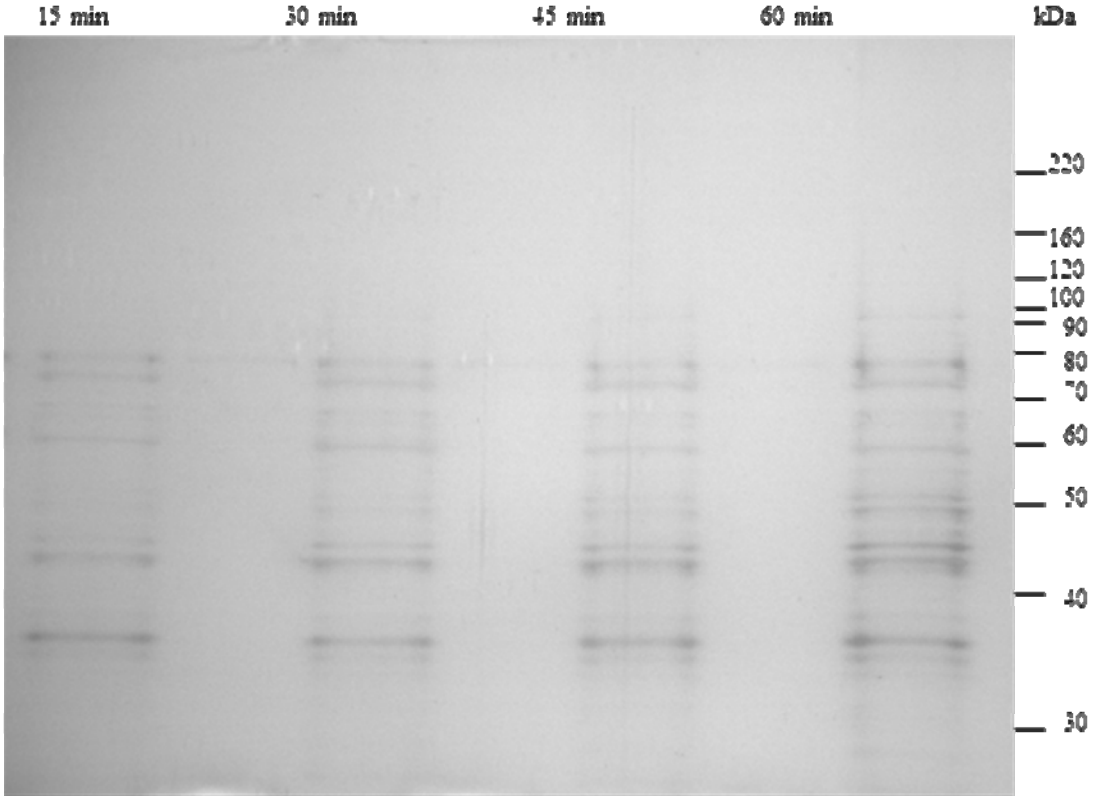


Figure 5-1. SDS-PAGE and silver-staining of supernatant after incubation in digestion buffer. *L. monocytogenes* cells were incubated in digest buffer for 15, 30, 45 and 60 minutes and the supernatant analyzed by SDS-PAGE and silver staining.

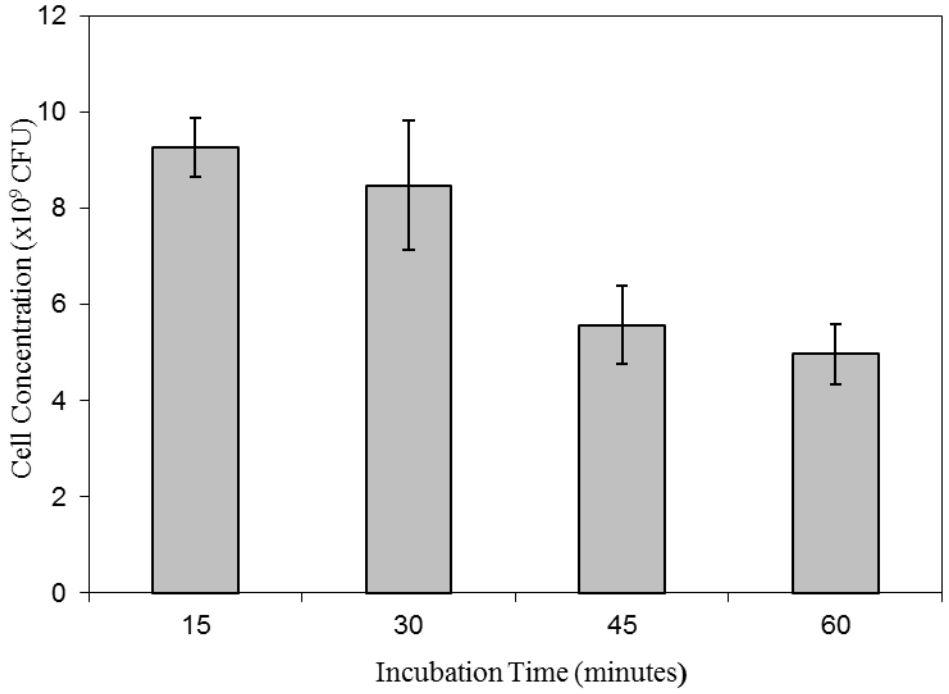


Figure 5-2. Colony-forming units (CFUs) of *L. monocytogenes* cells after incubation in digestion buffer. Cells (7×10^9) were incubated in digest buffer for 15, 30, 45 and 60 minutes. Cells were subsequently pelleted, resuspended in 1 ml of 15% glycerol in Brain Heart Infusion (BHI) broth and plated at appropriate concentrations for CFU counting. For each time point, at least triplicate CFU counts were made. Error bars represent standard deviation. The graph shows cell concentration (y-axis) versus incubation time (x-axis). Cells numbers were reduced after incubation in digest buffer at 45 and 60 minutes.

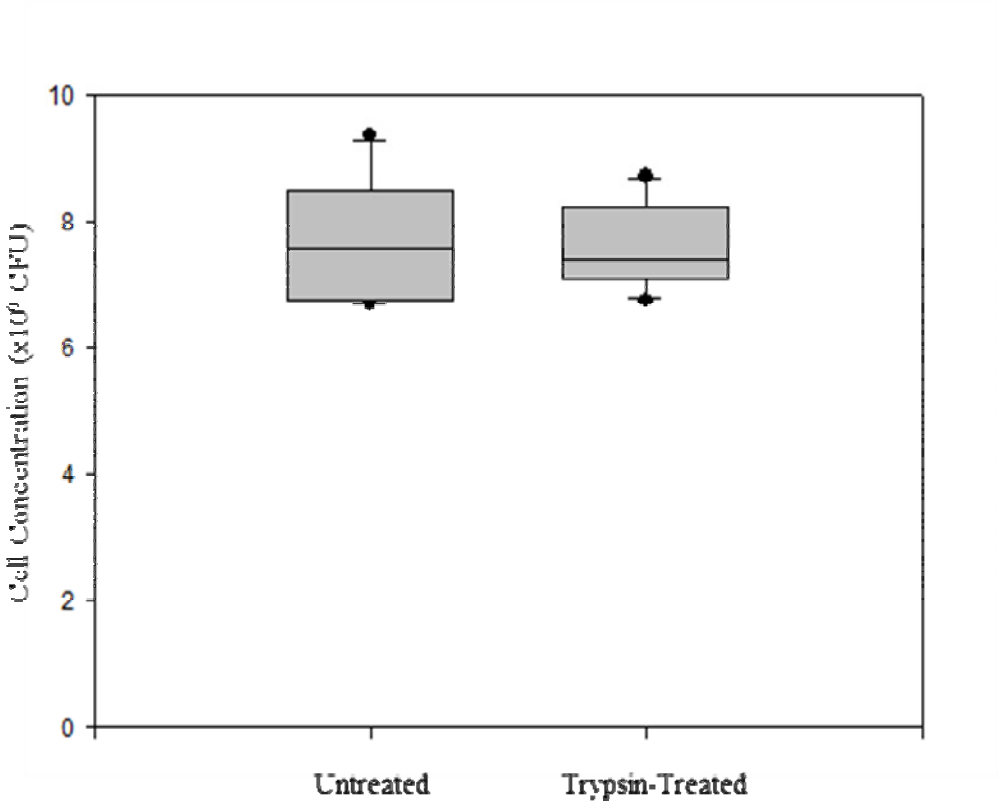


Figure 5-3. Colony-forming units (CFUs) of trypsin treated and untreated *L. monocytogenes* cells. Cells (7 x 10^9) were taken from a culture grown to 0.6 OD_{620} and washed 3 times with PBS prior to incubation in 0.15 ml of digest buffer. For the trypsin treated sample, 1.25 µg of trypsin was added for 30 min at 37 ^oC with 50 rpm agitation. The untreated sample was incubated for 30 min in digest buffer only. Cell pellets were recovered and re-suspended in 1 ml of 15% glycerol in PBS and stored at -20 ^oC. Cell suspensions were thawed once for serial dilution and CFU count. Six independent experiments were performed for each condition. Mann-Whitney U test was performed to assess possible difference between untreated and trypsin-treated sample groups. Mann-Whitney U test statistic is 18 with a two-tailed probability of 1.0000. The two sample groups are not significantly different ($P \geq 0.05$, two-tailed test).

Identified proteins were classified into three groups: cell surface (gene ontology (GO): 0009986) proteins, proteins secreted to the extracellular region (GO: 0005576) and cytoplasmic proteins.

5.2.2 Cell Surface Proteins Identified

Identified proteins that were unique to trypsin-treated cells and contained a secretion signal were classified as cell surface (GO: 0009986) proteins (Table 5-1). Among the 19 surface proteins identified, two autolysins, p45 (LMOf2365_2478) and Ami (LMOf2365_2530), were detected. The N-acetylglucosamine deacetylase PgdA (LMOf2365_0434) was identified. Previous evidence suggests that PgdA is surface associated. PgdA has a N-terminal Sec mediated secretion signal (172). The absence of deacetylated peptidoglycan precursors indicates that deacetylation, mainly mediated by PgdA (173), occurs on mature extracellular peptidoglycan (174). Two proteases: FtsH (LMOf2365_0231) and HtrA (LMOf2365_0312) were identified. FtsH consists of multi-transmembrane domains and *B. substilis* FstH is localized to the cell surface (175). HtrA found in *L. monocytogenes* is present in the extracellular milieu (170). Two penicillin binding proteins: PBPD1 (LMOf2365_2742) and PBPD3 (LMOf2365_1883) was also observed both of which have been identified within *L. monocytogenes* cell wall extract (176). Lipoprotein PrsA-2 (LMOf2365_2252) was identified. Proteomic analysis of lipoproteins indicated that a significant amount of PrsA-2 is anchored to the cytoplasmic membrane (177). LMOf2365_2694 which consists of a LPXTG motif that confers covalent attachment to the peptidoglycan was identified. An ATP-binding cassette (ABC) transporter permease with a multi-transmembrane domain (LMOf2365_2148), a putative transcriptional regulator LMOf2365_2491, a putative lipase (LMOf2365_2121), MreC (LMOf2365_1566) as well as

LMOf2365_0620, LMOf2365_0621, LMOf2365_1085, LMOf2365_1514, LMOf2365_2087 and LMOf2365_2330 were identified.

Table 5-1. Proteins of *L. monocytogenes* strain LI0521 (serotype 4b) identified in untreated and trypsin-treated cells.

F2365 Locus Tag[a]	EGD-e Locus Tag[b]	Gene Name	Function	Peptide Hits[c] (Mascot Score[d])						Predicted Localization[g]	kDa
				Un-treated 1[e]	Trypsin Treated 1[f]	Un-treated 2[e]	Trypsin Treated 2[f]	Un-treated 3[e]	Trypsin Treated 3[f]		
LMOf2365_0053	lmo0044	*rpsF*	30s ribosomal protein S6	1 (53)	2 (149)	0	1 (55)	0	1 (57)	Cytoplasmic	11
LMOf2365_0055	lmo0046	*rpsR*	30S ribosomal protein S18	0	1 (33)	0	1 (25)	0	0	Cytoplasmic	9
LMOf2365_0153*	lmo0135	*LMOf2365_0153*	oligopeptide ABC transporter substrate-binding protein	34 (809)	32 (830)	39 (767)	39 (519)	53 (2131)	63 (3467)	Lipid Anchor, Sec Secreted, SP-II[i] Cleaved	58
LMOf2365_0188	lmo0177	*metG*	methionyl-tRNA synthetase	0	5 (46)	0	5 (44)	0	0	Cytoplasmic	76
LMOf2365_0208	lmo0197	*spoVG-2*	-	0	3 (80)	2 (57)	0	3 (251)	2 (194)	Cytoplasmic	11
LMOf2365_0210	lmo0199	*prs-1*	ribose-phosphate pyrophosphokinase	0	1 (27)	0	1 (51)	0	0	Cytoplasmic	35
LMOf2365_0213*	lmo0202	*hly*	Listeriolysin O	8 (71)	15 (191)	10 (69)	13 (153)	0	0	Sec Secreted, SP-I[h] Cleaved	59
LMOf2365_0215*	lmo0204	*actA*	actin-assembly-inducing protein	3 (61)	6 (78)	5 (67)	11 (104)	0	9 (299)	Transmembrane Anchor, Sec Secreted, SP-I[h] Cleaved	66
LMOf2365_0231	lmo0220	*ftsH*	ATP-dependent metalloprotease	0	5 (69)	0	4 (93)	0	0	Multi-transmembrane Anchor, Sec Secreted, SP-I[h] Cleaved	76
LMOf2365_0240	lmo0228	*lysS*	lysyl-tRNA synthetase	0	4 (37)	0	4 (52)	0	0	Cytoplasmic	57
LMOf2365_0249*	lmo0237	*gltX*	glutamyl-tRNA synthetase	0	5 (78)	0	6 (109)	2 (38)	0	Cytoplasmic	56
LMOf2365_0260*	lmo0248	*rplK*	50S ribosomal protein L11	0	4 (194)	0	4 (166)	6 (158)	6 (305)	Cytoplasmic	15
LMOf2365_0262*	lmo0250	*rplJ*	50S ribosomal protein L10	0	5 (130)	0	3 (93)	0	2 (199)	Cytoplasmic	18

LMOf2365_0263*	lmo0251	*rplL*	50s ribosomal protein L7/L12	2 (61)	5 (85)	2 (65)	17 (92)	0	12 (188)	Cytoplasmic	12
LMOf2365_0274*	lmo0258	*rpoB*	DNA-directed RNA polymerase	0	11 (139)	0	12 (164)	0	4 (76)	Cytoplasmic	133
LMOf2365_0275*	lmo0259	*rpoC*	DNA-directed RNA polymerase	0	7 (54)	0	9 (114)	6 (149)	12 (441)	Cytoplasmic	135
LMOf2365_0312*	lmo0292	*htrA*	serine protease	0	10 (158)	0	11 (250)	0	30 (1963)	Possibly Sec Secreted	53
LMOf2365_0376*	lmo0355	*LMOf2365_0376*	fumarate reductase flavoprotein subunit	3 (54)	5 (100)	5 (90)	5 (136)	1 (80)	16 (351)	Sec Secreted, SP-II^{i} Cleaved	54
LMOf2365_0434*	lmo0415	*pgdA*	N-acetylglucosamine deacetylase	0	6 (160)	0	7 (198)	6 (120)	12 (696)	N-terminal Membrane Anchor (No Signal Cleavage Site), Sec Secreted	52
LMOf2365_0480	lmo0443	*LMOf2365_0480*	transcriptional regulator	0	1 (68)	0	1 (60)	0	3 (212)	Cytoplasmic	34
LMOf2365_0568	lmo0539	*LMOf2365_0568*	tagatose 1,6-diphosphate aldolase	0	2 (65)	3 (31)	0	0	5 (91)	Cytoplasmic	38
LMOf2365_0570*	lmo0541	*LMOf2365_0570*	ABC transporter substrate-binding protein	15 (316)	14 (228)	14 (348)	10 (172)	5 (435)	3 (561)	Lipid Anchor, Sec Secreted, SP-II^{i} Cleaved	34
LMOf2365_0582	lmo0553	*LMOf2365_0582*	-	0	0	0	0	3 (153)	3 (424)	Cytoplasmic	24
LMOf2365_0620	lmo0601	*LMOf2365_0620*	-	0	2 (21)	0	2 (47)	0	0	Sec Secreted, SP-I^{h} Cleaved	39
LMOf2365_0621	lmo0592	*LMOf2365_0621*	-	0	0	0	1 (37)	0	3 (200)	Possibly Sec Secreted	20
LMOf2365_0762*	lmo0727	*glmS*	glucosamine-fructose-6-phosphate aminotransferase	2 (32)	7 (184)	2 (32)	5 (123)	0	2 (140)	Cytoplasmic	66
LMOf2365_0808	lmo0791	*LMOf2365_0808*	lipoprotein	1 (81)	2 (77)	4 (81)	2 (57)	7 (152)	0	Sec Secreted, SP-II^{i} Cleaved	24

LMOf2365_0990*	lmo0970	*fabI*	enoyl-ACP reductase	0	2 (30)	0	3 (84)	0	3 (95)	Cytoplasmic	28
LMOf2365_0992	lmo0972	*dltC*	D-alanyl carrier protein	0	4 (133)	0	2 (60)	7 (34)	10 (565)	Cytoplasmic	9
LMOf2365_1024*	lmo1003	*ptsI*	phosphoenolpyruvate-protein phosphotransferase	2 (47)	7 (100)	0	8 (105)	8 (110)	11 (421)	Cytoplasmic	56
LMOf2365_1062	lmo1041	*modA*	molybdenum ABC transporter substrating binding protein	3 (82)	0	6 (39)	0	0	0	Lipid Anchor, Sec Secreted, SP-II[i] Cleaved	28
LMOf2365_1073*	lmo1052	*pdhA*	pyruvate dehydrogenase E1 alpha subunit	0	13 (158)	0	12 (164)	4 (70)	0	Cytoplasmic	41
LMOf2365_1074*	lmo1053	*pdhB*	pyruvate dehydrogenase E1 beta subunit	0	14 (115)	5 (65)	12 (190)	7 (203)	10 (499)	Cytoplasmic	35
LMOf2365_1075*	lmo1054	*LMOf2365_1075*	dihydrolipoamide acetyltransferase	3 (38)	9 (290)	4 (74)	11 (302)	3 (98)	4 (355)	Cytoplasmic	58
LMOf2365_1084	lmo1067	*typA*	GTP-binding protein	0	2 (31)	0	5 (78)	0	0	Cytoplasmic	69
LMOf2365_1085	-	*LMOf2365_1085*	-	0	7 (170)	0	7 (61)	0	0	Sec Secreted, SP-I[h] Cleaved	31
LMOf2365_1089	lmo1072	*pyc*	pyruvate carboxylase	0	4 (54)	0	4 (38)	0	0	Cytoplasmic	128
LMOf2365_1090*	lmo1073	*LMOf2365_1090*	ABC transporter substrate-binding protein	7 (183)	5 (129)	10 (240)	7 (145)	0	10 (276)	Lipid Anchor, Sec Secreted, SP-II[i] Cleaved	33
LMOf2365_1099	lmo1078	*galU*	UTP-glucose-1-phosphate uridylyltransferase	0	3 (66)	0	5 (68)	0	0	Cytoplasmic	33
LMOf2365_1110	lmo1096	*guaA*	GMP synthase	0	2 (34)	0	6 (71)	0	0	Cytoplasmic	58
LMOf2365_1146	lmo1138	*clpP-1*	ATP-dependent Clp protease proteolytic subunit	0	0	0	1 (36)	3 (62)	0	Cytoplasmic	21

LMOf2365_1284*	lmo1267	*tig*	trigger factor	4 (59)	10 (296)	2 (41)	9 (116)	0	12 (264)	Cytoplasmic	48
LMOf2365_1286	lmo1268	*clpX*	ATP-dependent protease ATP-binding subunit	0	1 (58)	0	3 (59)	0	6 (274)	Cytoplasmic	47
LMOf2365_1298	lmo1280	*codY*	transcriptional repressor CodY	0	1 (45)	0	0	0	3 (266)	Cytoplasmic	29
LMOf2365_1317	lmo1299	*glnA*	glutamine synthetase, type I	0	3 (47)	0	4 (42)	0	0	Cytoplasmic	51
LMOf2365_1323	lmo1305	*tkt-2*	transketolase	0	6 (115)	0	4 (82)	0	0	Cytoplasmic	72
LMOf2365_1331	lmo1314	*frr*	ribosome recycling factor	0	2 (30)	0	2 (35)	0	0	Cytoplasmic	21
LMOf2365_1336*	lmo1319	*proS*	prolyl-tRNA synthetase	0	3 (65)	0	6 (97)	0	1 (70)	Cytoplasmic	63
LMOf2365_1339*	lmo1322	*nusA*	transcription elongation factor	0	0	0	6 (107)	3 (67)	0	Cytoplasmic	42
LMOf2365_1347	lmo1330	*rpsO*	30S ribosomal protein S15	0	1 (121)	2 (48)	2 (83)	0	0	Cytoplasmic	11
LMOf2365_1348	lmo1331	*pnp*	polynucleotide phosphorylase/ polyadenylase	0	2 (41)	0	0	0	5 (218)	Cytoplasmic	80
LMOf2365_1374	lmo1357	*accC*	acetyl-CoA carboxylase biotin carboxylase subunit	0	3 (52)	0	2 (56)	0	0	Cytoplasmic	50
LMOf2365_1381*	lmo1364	*cspL*	major cold-shock protein	2 (47)	4 (99)	2 (31)	6 (64)	4 (198)	6 (325)	Cytoplasmic	5
LMOf2365_1395*	lmo1376	*gnd*	6-phosphogluconate dehydrogenase	6 (35)	6 (107)	4 (80)	8 (116)	14 (280)	8 (539)	Cytoplasmic	52
LMOf2365_1407*	lmo1388	*tcsA*	CD4+ T-cell-stimulating antigen	27 (629)	22 (437)	25 (395)	19 (211)	27 (920)	21 (989)	Lipid Anchor, Sec Secreted, SP-II[i] Cleaved	38
LMOf2365_1414	lmo1395	*LMOf2365_1414*	DNA-binding domain containing protein	0	2 (66)	0	2 (59)	0	0	Cytoplasmic	34

LMOf2365_1417*	lmo1398	*recA*	recombination protein RecA	0	0	0	3 (125)	2 (52)	6 (422)	Cytoplasmic	38
LMOf2365_1467	lmo1448	*ppaC*	inorganic pyrophosphatase, manganese-dependent	0	1 (61)	0	3 (74)	0	0	Cytoplasmic	34
LMOf2365_1492*	lmo1473	*dnaK*	heat shock protein DnaK	10 (57)	20 (399)	9 (40)	20 (187)	8 (113)	10 (300)	Cytoplasmic	66
LMOf2365_1493	lmo1474	*grpE*	heat shock protein grpE	0	3 (57)	0	2 (62)	0	0	Cytoplasmic	22
LMOf2365_1514	lmo1495	*LMOf2365_1514*	-	0	2 (30)	0	2 (73)	0	5 (243)	N-terminal Membrane Anchor (No Signal Cleavage Site), Sec Secreted	23
LMOf2365_1515	lmo1496	*greA*	transcription elongation factor	0	4 (36)	0	2 (38)	0	0	Cytoplasmic	17
LMOf2365_1523	lmo1504	*alaS*	alanyl-tRNA synthetase	0	3 (53)	0	6 (46)	0	0	Cytoplasmic	57
LMOf2365_1550	lmo1531	*queA*	S-adenosylmethionine-tRNA ribosyltransferase-isomerase	0	3 (28)	0	3 (59)	0	0	Cytoplasmic	38
LMOf2365_1561	lmo1542	*rplU*	50s ribosomal protein L21	2 (61)	0	2 (37)	1 (62)	0	0	Cytoplasmic	11
LMOf2365_1566	lmo1547	*mreC*	rod shape-determining protein MreC	0	1 (42)	0	2 (31)	0	0	N-terminal Membrane Anchor (No Signal Cleavage Site), Sec Secreted	32
LMOf2365_1567	lmo1548	*mreB*	rod shape-determining protein MreB	0	3 (53)	0	2 (52)	0	0	Cytoplasmic	36
LMOf2365_1580	lmo1559	*thrS*	threonyl-tRNA synthetase	0	2 (60)	0	3 (29)	0	2 (38)	Cytoplasmic	73
LMOf2365_1592*	lmo1570	*pykA*	pyruvate kinase	0	22 (165)	0	13 (236)	7 (89)	10 (545)	Cytoplasmic	63

LMOf2365_1600	lmo1578	*pepQ*	proline dipeptidase	0	3 (33)	0	1 (74)	0	6 (212)	Cytoplasmic	40
LMOf2365_1601	lmo1579	*ald*	alanine dehydrogenase	0	0	0	0	2 (42)	2 (103)	Cytoplasmic	37
LMOf2365_1603*	lmo1581	*ackA*	acetase kinase	0	6 (61)	2 (40)	4 (81)	0	0	Cytoplasmic	45
LMOf2365_1605	lmo1583	*tpx*	thiol peroxidase	0	1 (46)	0	2 (56)	0	2 (181)	Cytoplasmic	18
LMOf2365_1618*	lmo1596	*rpsD*	30S ribosomal protein S4	0	6 (249)	3 (26)	6 (190)	0	6 (277)	Cytoplasmic	23
LMOf2365_1620	lmo1599	*ccpA*	catabolite control protein A	0	2 (48)	0	3 (82)	0	3 (293)	Cytoplasmic	37
LMOf2365_1621	lmo1600	*aroA*	bifunctional 3-deoxy-7-phosphoheptulonate synthase/chorismate mutase	0	0	0	2 (32)	0	3 (82)	Cytoplasmic	40
LMOf2365_1678*	lmo1657	*tsf*	elongation factor Ts	5 (120)	19 (288)	7 (79)	14 (218)	6 (88)	3 (145)	Cytoplasmic	33
LMOf2365_1679*	lmo1658	*rpsB*	30S ribosomal protein S2	0	8 (228)	2 (42)	9 (140)	2 (32)	6 (368)	Cytoplasmic	28
LMOf2365_1695	lmo1671	*LMOf2365_1695*	laminin-binding surface protein	5 (38)	0	6 (32)	0	0	0	Lipid Anchor, Sec Secreted, SP-II^{i} Cleaved	37
LMOf2365_1733	lmo1709	*map*	methionine aminopeptidase	0	2 (86)	0	3 (90)	0	0	Cytoplasmic	28
LMOf2365_1762*	lmo1738	*LMOf2365_1762*	amino acid ABC transporter substrate-binding protein	7 (133)	6 (95)	9 (190)	6 (91)	7 (316)	0	Lipid Anchor, Sec Secreted, SP-II^{i} Cleaved	31
LMOf2365_1779*	lmo1754	*gatB*	aspartyl/glutamyl-tRNA amidotransferase subunit B	0	8 (123)	0	7 (81)	0	0	Cytoplasmic	53
LMOf2365_1780	lmo1755	*gatA*	aspartyl/glutamyl-tRNA amidotransferase subunit A	0	4 (26)	0	4 (75)	0	1 (66)	Cytoplasmic	52

LMOf2365_1782*	lmo1757	*LMOf2365_1782*	similar to sex pheromone staph-cAM373	11 (224)	6 (157)	9 (251)	8 (132)	2 (82)	3 (265)	Lipid Anchor, Sec Secreted, SP-II[i] Cleaved	41
LMOf2365_1808	lmo1783	*rplT*	50s ribosomal protein L20	3 (45)	5 (176)	2 (135)	3 (121)	0	0	Cytoplasmic	14
LMOf2365_1810	lmo1785	*infC*	translation initiation factor IF-3	0	2 (93)	0	2 (60)	0	0	Cytoplasmic	16
LMOf2365_1814	lmo1787	*rplS*	50S ribosomal protein L19	0	2 (53)	0	2 (46)	3 (54)	0	Cytoplasmic	13
LMOf2365_1824	lmo1797	*rpsP*	30S ribosomal protein S16	1 (70)	2 (82)	1 (54)	1 (49)	0	0	Cytoplasmic	10
LMOf2365_1835*	lmo1807	*fabG*	3-oxoacyl-ACP reductase	0	0	0	3 (116)	1 (33)	4 (247)	Cytoplasmic	26
LMOf2365_1875*	lmo1847	*LMOf2365_1875*	manganese binding lipoprotein	6 (74)	4 (54)	4 (92)	0	0	5	Lipid Anchor, Sec Secreted, SP-II[i] Cleaved	35
LMOf2365_1879*	lmo1851	*LMOf2365_1879*	endopeptidase	0	4 (57)	0	7 (116)	2 (55)	6 (282)	N-terminal Membrane Anchor (No Signal Peptide Cleavage Site), Sec Secreted	54
LMOf2365_1883*	lmo1855	*LMOf2365_1883*	D-alanyl-D-alanine carboxypeptidase PBPD3	0	6 (57)	0	5 (90)	0	12 (379)	N-terminal Membrane Anchor (No Signal Peptide Cleavage Site), Sec Secreted	31
LMOf2365_1906	lmo1877	*fhs*	formate-tetrahydrofolate ligase	0	1 (38)	0	2 (53)	3 (121)	3 (299)	Cytoplasmic	46
LMOf2365_1925	lmo1896	*asnC*	asparaginyl-tRNA synthetase	0	2 (47)	0	2 (25)	0	3	Cytoplasmic	49
LMOf2365_1963*	lmo1934	*hup*	histone-like bacterial DNA-binding protein HU	4 (163)	5 (159)	4 (151)	6 (74)	6 (312)	4 (198)	Cytoplasmic	10
LMOf2365_1967*	lmo1938	*rpsA*	30S ribosomal protein S1	0	5 (80)	0	6 (85)	0	0	Cytoplasmic	41

LMOf2365_1989*	lmo1959	*LMOf2365_1989*	Iron compound ABC transporter substrate-binding protein	4 (76)	3 (112)	0	1 (36)	0	2 (83)	Sec Secreted, SP-II[i] Cleaved	34
LMOf2365_2002	lmo1978	*zwf*	glucose-6-phosphate 1-dehydrogenase	0	1 (36)	0	1 (57)	0	0	Cytoplasmic	56
LMOf2365_2041	lmo2016	*LMOf2365_2041*	cold shock protein	1 (60)	5 (113)	1 (61)	4 (54)	6 (303)	7 (377)	Cytoplasmic	7
LMOf2365_2044	lmo2019	*ileS*	isoleucyl-tRNA synthesis	1 (55)	0	1 (45)	5 (43)	0	0	Cytoplasmic	104
LMOf2365_2045	lmo2020	*divIVA*	cell division protein	0	1 (51)	0	1 (50)	0	0	Cytoplasmic	20
LMOf2365_2064	lmo2032	*ftsZ*	cell division protein	0	3 (74)	0	0	0	1 (94)	Cytoplasmic	41
LMOf2365_2065	lmo2033	*ftsA*	cell division protein FtsA	0	2 (39)	0	5 (47)	0	0	Cytoplasmic	46
LMOf2365_2087	lmo2056	*LMOf2365_2087*	-	0	0	0	1 (34)	0	7 (246)	N-terminal Membrane Anchor (No Signal Peptide Cleavage Site), Sec Secreted	40
LMOf2365_2099*	lmo2068	*groEL*	chaperonin GroEL	0	11 (122)	0	10 (98)	0	0	Cytoplasmic	57
LMOf2365_2100*	lmo2069	*groES*	co-chaperonin GroES	0	5 (163)	0	3 (78)	3 (71)	7 (198)	Cytoplasmic	10
LMOf2365_2111*	lmo2079	*LMOf2365_2111*	-	0	0	0	5 (142)	5 (291)	4 (158)	Lipid Anchor, Sec Secreted, SP-II[i] Cleaved	41
LMOf2365_2121	lmo2089	*LMOf2365_2121*	lipase	0	0	0	1 (34)	0	3 (195)	N-terminal Membrane Anchored (No Signal Peptide Cleavage Site), Sec Secreted	38
LMOf2365_2148	lmo2115	*LMOf2365_2148*	ABC transporter permease	0	3 (88)	0	3 (72)	0	0	Multi-transmembrane Anchor, Sec Secreted, SP-I[h] Cleaved	72

LMOf2365_2186	lmo2154	*nrdF*	ribonucleotide-diphosphate reductase subunit beta	0	1 (65)	0	1 (47)	0	0	Cytoplasmic	40
LMOf2365_2217*	lmo2184	*LMOf2365_2217*	ferrichrome binding lipoprotein	4 (98)	3 (91)	3 (104)	3 (81)	2 (62)	0	Lipid Anchor, Sec Secreted, SP-II[i] Cleaved	32
LMOf2365_2229*	lmo2196	*oppA*	oligopeptide binding lipoprotein	9 (185)	12 (345)	10 (181)	17 (160)	0	10 (435)	Lipid Anchor, Sec Secreted, SP-II[i] Cleaved	63
LMOf2365_2231	lmo2198	*trpS*	tryptophanyl-tRNA synthetase	0	0	0	2 (61)	0	1 (64)	Cytoplasmic	37
LMOf2365_2234	lmo2201	*fabF*	3-oxoacyl-ACP (acyl carrier protein) synthase	0	6 (57)	0	6 (39)	1 (30)	0	Cytoplasmic	44
LMOf2365_2235	lmo2202	*fabH*	3-oxoacyl-ACP (acyl carrier protein) synthase	0	2 (67)	0	2 (85)	0	0	Cytoplasmic	34
LMOf2365_2252*	lmo2219	*prsA-2*	foldase	0	7 (210)	0	8 (126)	0	3 (152)	Lipid Anchor, Sec Secreted, SP-II[i] Cleaved	33
LMOf2365_2281	lmo2248	*LMOf2365_2281*	-	0	3 (38)	0	4 (88)	0	0	Cytoplasmic	24
LMOf2365_2330	lmo2360	*LMOf2365_2330*	transmembrane protein	0	1 (32)	0	2 (47)	0	3 (261)	Multi-transmembrane Anchor, Sec Secreted, SP-I[h] Cleaved	97
LMOf2365_2338*	lmo2367	*pgi*	glucose-6-phosphate isomerase	7 (176)	16 (336)	8 (75)	16 (253)	2 (150)	3 (263)	Cytoplasmic	50
LMOf2365_2382	lmo2411	*sufB*	FeS assembly protein	0	2 (33)	0	3 (43)	0	0	Cytoplasmic	53
LMOf2365_2385*	lmo2414	*sufD*	FeS assembly protein	0	4 (59)	0	6 (122)	4 (80)	6 (245)	Cytoplasmic	48
LMOf2365_2386*	lmo2415	*LMOf2365_2386*	Fe-S cluster assembly ABC-type	0	3 (95)	0	4 (90)	0	0	Cytoplasmic	30

LMOf2365_2396	lmo2425	*gcvH*	glycine cleavage system protein H	0	2 (43)	0	2 (57)	0	0	Cytoplasmic	14
LMOf2365_2402*	lmo2431	*LMOf2365_2402*	Iron compound ABC transporter substrate-binding protein	17 (218)	9 (101)	13 (319)	12 (182)	13 (720)	7 (221)	Lipid Anchor, Sec Secreted, SP-II^{i} Cleaved	34
LMOf2365_2428*	lmo2455	*eno*	enolase	10 (118)	21 (507)	13 (184)	16 (162)	4 (110)	3 (152)	Cytoplasmic	46
LMOf2365_2429	lmo2456	*gpmA*	phosphoglyceromutase	0	6 (82)	0	8 (99)	0	0	Cytoplasmic	56
LMOf2365_2430*	lmo2457	*tpiA*	triosephophate isomerase	4 (67)	7 (118)	3 (67)	5 (119)	6 (298)	10 (547)	Cytoplasmic	27
LMOf2365_2431	lmo2458	*pgk*	sphoglycerate kinase	0	0	0	5 (55)	0	7 (221)	Cytoplasmic	42
LMOf2365_2432	lmo2459	*gap*	glyceraldehyde-3-phosphate dehydrogenase	0	3 (37)	0	3 (57)	0	1 (34)	Cytoplasmic	36
LMOf2365_2441	lmo2468	*clpP*	ATP-dependent Clp protease proteolytic subunit	0	3 (63)	0	3 (60)	0	4 (218)	Cytoplasmic	22
LMOf2365_2448	lmo2475	*LMOf2365_2448*	phosphoglucomutase/ phosphomannomutase	0	3 (57)	0	2 (54)	3 (92)	1 (40)	Cytoplasmic	64
LMOf2365_2478*	lmo2505	*spl*	peptidoglycan lytic protein	0	4 (90)	0	4 (56)	0	5 (315)	N-terminal Membrane Anchor (No Signal Peptide Cleavage Site), Sec Secreted	43
LMOf2365_2483	lmo2510	*secA*	preprotein translocase subunit	0	1 (80)	0	1 (62)	0	0	Cytoplasmic	94
LMOf2365_2491*	lmo2518	*LMOf2365_2491*	transcriptional regulator, LytR Family	0	6 (203)	0	6 (142)	0	4 (278)	N-terminal Membrane Anchor (No Signal Peptide Cleavage Site), Sec Secreted	39

LMOf2365_2511	lmo2538	*upp*	uracil phosphoribosyltransferase	0	5 (55)	0	7 (40)	0	0	Cytoplasmic	23
LMOf2365_2528*	lmo2556	*fbaA*	fructose-1,6-bisphosphate aldolase type II	6 (50)	9 (204)	3 (70)	9 (82)	7 (316)	7 (519)	Cytoplasmic	30
LMOf2365_2530	lmo2558	*ami*	autolysin	0	1 (61)	0	1 (31)	0	0	GW Domain Cell Wall Anchored, Sec Secreted, SP-I[h] Cleaved	102
LMOf2365_2531	lmo2559	*pyrG*	CTP synthetase	0	4 (26)	0	6 (49)	0	3 (175)	Cytoplasmic	60
LMOf2365_2533	lmo25615	*argS*	arginyl-tRNA synthetase	0	2 (52)	0	3 (29)	0	0	Cytoplasmic	63
LMOf2365_2550	lmo2578	-	lipoprotein	0	0	0	2 (45)	2 (91)	0	Lipid Anchor, Sec-secreted, SP-II[i] Cleaved	32
LMOf2365_2569	lmo2596	*rpsI*	30S ribosomal protein S9	0	2 (44)	0	1 (39)	0	3 (183)	Cytoplasmic	14
LMOf2365_2570	lmo2597	*rplM*	50S ribosomal protein L13	0	4 (58)	0	3 (81)	0	0	Cytoplasmic	16
LMOf2365_2578*	lmo2605	*rplQ*	50S ribosomal protein L17	2 (70)	5 (86)	2 (62)	4 (87)	0	3 (45)	Cytoplasmic	15
LMOf2365_2579*	lmo2606	*rpoA*	DNA-directed RNA polymerase	3 (22)	6 (192)	3 (39)	5 (212)	0	0	Cytoplasmic	35
LMOf2365_2580	lmo2607	*rpsK*	30S ribosomal protein S11	0	1 (71)	0	2 (47)	0	0	Cytoplasmic	14
LMOf2365_2586*	lmo2613	*rplO*	50S ribosomal protein L15	4 (79)	3 (123)	4 (89)	3 (105)	0	0	Cytoplasmic	16
LMOf2365_2588	lmo25615	*rpsE*	30S ribosomal protein S5	0	1 (60)	0	2 (41)	2 (71)	3 (94)	Cytoplasmic	17
LMOf2365_2589	lmo2616	*rplR*	50S ribosomal protein L18	0	3 (150)	0	2 (211)	5 (242)	6 (407)	Cytoplasmic	13
LMOf2365_2590	lmo2617	*rplF*	50S ribosomal protein L6	3 (33)	6 (106)	0	0	0	1 (53)	Cytoplasmic	19
LMOf2365_2591*	lmo2618	*rpsH*	30S riboosomal protein S8	0	4 (124)	0	3 (90)	0	0	Cytoplasmic	15
LMOf2365_2593*	lmo2620	*rplE*	50S ribosomal protein L5	4 (98)	6 (181)	4 (82)	6 (133)	4 (145)	4 (271)	Cytoplasmic	20
LMOf2365_2595*	lmo2622	*rplN*	50S ribosomal protein L14	0	3 (77)	0	2 (75)	0	0	Cytoplasmic	13

LMOf2365_2597	lmo2624	*rpmC*	50S ribosomal protein L29	0	2 (64)	0	3 (55)	0	0	Cytoplasmic	7
LMOf2365_2598	lmo2625	*rplP*	50S ribosomal protein L16	2 (38)	3 (36)	0	3 (129)	0	0	Cytoplasmic	16
LMOf2365_2599	lmo2626	*rpsC*	30S ribosomal protein S3	0	2 (79)	0	4 (48)	0	0	Cytoplasmic	25
LMOf2365_2600*	lmo2627	*rplV*	50S ribosomal protein L22	0	4 (96)	0	4 (74)	0	3 (244)	Cytoplasmic	13
LMOf2365_2601*	lmo2628	*rpsS*	30S ribosomal protein S19	2 (72)	3 (51)	2 (45)	2 (74)	0	0	Cytoplasmic	10
LMOf2365_2602*	lmo2629	*rplB*	50S ribosomal protein L2	5 (70)	6 (95)	5 (42)	9 (78)	0	4 (137)	Cytoplasmic	31
LMOf2365_2603	lmo2630	*rplW*	50S ribosomal protein L23	0	3 (54)	0	3 (76)	0	0	Cytoplasmic	11
LMOf2365_2604*	lmo2631	*rplD*	50S ribosomal protein L4	6 (63)	7 (232)	6 (91)	8 (133)	0	0	Cytoplasmic	23
LMOf2365_2605*	lmo2632	*rplC*	50S ribosomal protein L3	5 (77)	8 (269)	6 (77)	9 (145)	3 (96)	3 (206)	Cytoplasmic	23
LMOf2365_2606	lmo2633	*rpsJ*	30S ribosomal protein S10	0	4 (79)	0	3 (38)	0	0	Cytoplasmic	12
LMOf2365_2610*	lmo2637	*LMOf2365_2610*	similar to sex pheromone cAD1	7 (42)	6 (97)	7 (98)	5 (74)	14 (599)	18 (831)	Sec Secreted, SP-II^{i} Cleaved	33
LMOf2365_2632*	lmo2653	*tuf*	elongation factor Tu	9 (98)	46 (488)	8 (74)	38 (403)	23 (420)	39 (1482)	Cytoplasmic	43
LMOf2365_2633*	lmo2654	*fus*	elongation factor G	9 (61)	19 (225)	14 (137)	25 (316)	9 (212)	14 (542)	Cytoplasmic	77
LMOf2365_2634*	lmo2655	*rpsG*	30S ribosomal protein S7	0	4 (64)	0	5 (72)	3 (177)	3 (426)	Cytoplasmic	13
LMOf2365_2635	lmo2656	*rpsL*	30S ribosomal protein S12	0	1 (87)	0	1 (53)	0	0	Cytoplasmic	15
LMOf2365_2683	lmo2703	*LMOf2365_2683*	-	0	2 (26)	0	1 (21)	0	0	Cytoplasmic	11
LMOf2365_2694	lmo2714	*LMOf2365_2694*	cell wall surface anchor family protein	0	1 (26)	0	2 (71)	0	0	LPXTG Cell Wall Anchor, Sec Secreted, SP-I^{h} Cleaved	35
LMOf2365_2742*	lmo2754	*LMOf2365_2742*	D-alanyl-D-alanine carboxypeptidase PBPD1	0	6 (95)	0	9 (103)	0	9 (255)	Sec Secreted, SP-I^{h} Cleaved	48

LMOf2365_2746	lmo2758	*guaB*	inosine-5'-monophosphate dehydrogenase	0	0	0	1 (51)	4 (51)	0	Cytoplasmic	53
LMOf2365_2770	lmo2779	*ychF*	GTP--binding protein	0	2 (64)	0	1 (27)	0	0	Cytoplasmic	41
-	-	*gp25*	-	0	0	0	0	3 (177)	2 (73)	-	16
-	-	*gp32*	-	5 (148)	3 (118)	3 (85)	2 (65)	3 (63)	5 (284)	-	27

a) Locus tags of the annotated genome from L. monocytogenes F2365 (147).
b) Locus tags of the annotated genome from L. monocytogenes EGD-e (178).
c) Number of peptides identified in three trials. Trials are designated as 1, 2, or 3.
d) Mascot score associated with each peptide hit.
e) Peptides derived from proteins found in extracellular region or cell lysis. Cells were incubated with digest buffer only. Peptides released into solution were separated from cells by filtration and subsequently digested with trypsin (see section 3.3 in Materials and Methods).
f) Peptides derived from surface associated cells. Cells were digested with trypsin. Peptides released into solution were separated from cells by filtration (see section 3.3 in Materials and Methods).
g) Location information is from LocateP annotation (179).
*) Scaffold (version Scaffold_3.3.1, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide and protein identifications were accepted at greater than 95% confidence as specified by Peptide Prophet (180) and Protein Prophet (181) respectively. Protein was observed in at least two trials.
h) Signal Peptidase Type I.
i) Signal Peptidase Type II.

Selected surface protein candidates were presented in bold red.

Table 5-2. Proteins identifications observed in only one trial.

F2365 Locus tag[a]	EGD-e Locus tag[b]	Gene Name	Function	Peptide Hits[c] (Mascot Score[d])						Predicted Localization[g]	kDa
				Un-treated 1[e]	Trypsin Treated 1[f]	Un-treated 2[e]	Trypsin Treated 2[f]	Un-treated 3[e]	Trypsin Treated 3[f]		
LMOf2365_0002	lmo0002	*dnaN*	DNA polymerase III subunit beta	0	0	0	3 (66)	0	0	Cytoplasmic	42
LMOf2365_0006	lmo0006	*gyrB*	DNA gyrase subunit B	0	0	0	1 (79)	0	0	Cytoplasmic	73
LMOf2365_0016	lmo0013	*qoxA*	quinol oxidase AA3, subunit II	0	0	0	1 (37)	0	0	Multi-transmembrane Anchor, Sec Secreted, SP-II[i] Cleaved	42
LMOf2365_0167	lmo0152	*LMOf2365_0167*	solute-binding family 5 protein	0	0	0	3 (54)	0	0	Lipid Anchor, Sec Secreted, SP-II[i] Cleaved	62
LMOf2365_0202	lmo0191	*LMOf2365_0202*	-	0	0	0	1 (28)	0	0	Cytoplasmic	27
LMOf2365_0206	lmo0195	*LMOf2365_0206*	ABC transporter permease	0	0	0	2 (55)	0	0	Multi-transmembrane Anchor, Sec Secreted, SP-I[h] Cleaved	43
LMOf2365_0221	lmo0210	*idH*	L-lactate dehydrogenase	0	1 (40)	0	0	0	0	Cytoplasmic	34
LMOf2365_0230	lmo0219	*LMOf2365_0230*	hypoxanthine phosphoribosyltransferase	0	1 (39)	0	0	0	0	Cytoplasmic	75
LMOf2365_0237	lmo0225	*folB*	dihydroneopterin aldolase	0	1 (57)	0	0	0	0	Cytoplasmic	14
LMOf2365_0244	lmo0232	*LMOf2365_0244*	ClpC ATPase	0	1 (40)	0	0	0	0	Cytoplasmic	91
LMOf2365_0253	lmo0241	*LMOf2365_0253*	RNA methyltransferase	0	0	0	0	0	3 (153)	Cytoplasmic	28

LMOf2365_0261	lmo0249	*rplA*	50s ribosomal protein L1	3 (56)	0	0	0	0	0	Cytoplasmic	25
LMOf2376_0337	lmo0319	*bglH-1*	beta-glucosidase	0	0	0	0	0	2 (40)	Cytoplasmic	54
LMOf2365_0471	lmo0433	*inlA*	internalin A	0	0	0	0	0	2 (42)	LPXTG Cell Wall Anchor, Sec-secreted, SP-I^{h} Cleaved	80
-	lmo0434	*inlB*	internalin B	0	0	0	2 (23)	0	0	GW Domain Cell Wall Anchored, Sec Secreted, SP-I^{h} Cleaved	29
LMOf2365_0546	lmo0517	*LMOf2365_0546*	phosphoglycerate mutase	0	0	0	0	0	7 (478)	Lipid Anchor, Sec Secreted, SP-II^{i} Cleaved	29
LMOf2365_0583	lmo0554	*LMOf2365_0583*	alcohol dehydrogenase, iron-dependent	0	0	0	0	0	2 (38)	Cytoplasmic	43
LMOf2365_0638	lmo0609	*LMOf2365_0638*	rhodanase-like domain containing protein	0	1 (59)	0	0	0	0	Cytoplasmic	11
LMOf2365_0802	lmo0786	*LMOf2365_0802*	ACP phosphodiesterase	0	0	0	0	0	5 (132)	Cytoplasmic	23
LMOf2365_0827	lmo0811	*cah*	carbonic anhydrase	0	1 (38)	0	0	0	0	Cytoplasmic	27
LMOf2365_0872	lmo0855	*ddl*	D-alanyl-alanine synthetase A	0	0	0	2 (79)	0	0	Cytoplasmic	41
LMOf2365_0912	lmo0893	*LMOf2365_0912*	anti-sigma factor antagonist	0	0	0	1 (30)	0	0	Cytoplasmic	13
LMOf2365_0928	lmo0906	*gor*	glutathione reductase	0	0	0	0	0	3 (225)	Cytoplasmic	49
LMOf2365_0950	lmo0929	*LMOf2365_0950*	sortase	0	0	0	4 (49)	0	0	N-terminal Membrane Anchor (No Signal Peptide Cleavage Site), Sec Secreted	25

LMOf2365_0970	lmo0950	*LMOf2365_0970*	-	0	0	0	0	0	2 (130)	N-terminal Membrane Anchor (No Signal Peptide Cleavage Site), Sec Secreted	32
LMOf2365_1023	lmo1002	*ptsH*	phosphocarrier protein HPr	0	0	0	6 (46)	0	0	Cytoplasmic	9
LMOf2365_1231	lmo1222	*pheT*	phenylalanyl-tRNA synthetase subunit beta	0	0	0	3 (55)	0	0	Cytoplasmic	88
LMOf2365_1235	lmo1226	*MmpL*	MmpL family membrane protein	0	0	0	0	0	2 (68)	Multi-transmembrane Anchor, Sec Secreted, SP-I^{h} Cleaved	115
LMOf2365_1308	lmo1291	*LMOf2365_1308*	acyltransferase	0	0	0	0	0	2 (71)	Multi-transmembrane Anchor, Sec Secreted, SP-I^{h} Cleaved	71
LMOf2365_1377	lmo1360	*folD*	bifunctional 5,10-methylene-tetrahydrofolate dehydrogenase/tetrahydrofolate cyclohydrolase	0	0	0	4 (42)	0	0	Cytoplasmic	31
LMOf2365_1425	lmo1406	*pfl-1*	formate acetyltransferase	0	0	0	6 (59)	0	0	Cytoplasmic	84
LMOf2365_1433	lmo1414	*LMOf2365_1433*	acetyl-CoA acetyltransferase	0	0	0	2 (45)	0	0	cytoplasmic	41
LMOf2365_1453	lmo1434	*LMOf2365_1453*	metallo-beta-lactamase	0	1 (37)	0	0	0	0	cytoplasmic	62
LMOf2365_1456	lmo1437	*asd*	aspartate-semialdehyde dehydrogenase	0	2 (69)	0	0	0	0	Cytoplasmic	38
LMOf2365_1457	lmo1438	*LMOf2365_1457*	penicillin binding protein PBPB1	0	0	1 (39)	0	0	0	N-terminal Membrane Anchor (No Signal Peptide Cleavage Site), Sec Secreted	80
LMOf2365_1491	lmo1472	*dnaJ*	chaperone protein dnaJ	0	0	0	1 (49)	0	0	Cytoplasmic	41

LMOf2365_1518	lmo1499	*LMOf2365_1518*	-	0	3 (41)	0	0	0	0	N-terminal Membrane Anchor (No Signal Peptide Cleavage Site), Sec Secreted	40
LMOf2365_1520	lmo1501	*LMOf2365_1520*	-	0	1 (33)	0	0	0	0	Cytoplasmic	12
LMOf2365_1538	lmo1519	*aspS*	aspartyl-tRNA synthetase	0	0	0	6 (49)	0	0	Cytoplasmic	66
LMOf2365_1549	lmo1530	*tgt*	queuine tRNA-ribosyltransferase	0	1 (36)	0	0	0	0	Cytoplasmic	43
LMOf2365_1556	lmo1537	*obgE*	GTPase	0	1 (30)	0	0	0	0	Cytoplasmic	47
LMOf2365_1588	lmo1566	*icd*	isocitrate dehydrogenase	0	0	0	0	0	4 (76)	Cytoplasmic	46
LMOf2365_1594	lmo1572	*accA*	acetyl-CoA carboxylase carboxyltransferase subunit alpha	0	0	0	1 (44)	0	0	Cytoplasmic	35
LMOf2365_1602	lmo1580	*LMOf2365_1602*	universal stress protein	0	0	0	2 (26)	0	0	Cytoplasmic	17
LMOf2365_1624	lmo1603	*LMOf2365_1624*	aminopeptidase	0	0	0	0	0	3 (30)	Cytoplasmic	41
LMOf2365_1629	lmo1607	*LMOf2365_1629*	tRNA-binding domain-containing protein	0	0	0	4 (47)	0	0	Cytoplasmic	23
LMOf2365_1662	lmo1641	*acnA*	aconitate hydratase	0	0	0	4 (30)	0	0	Cytoplasmic	98
LMOf2365_1684	lmo1660	*leuS*	leucyl-tRNA synthetase	0	0	0	4 (30)	0	0	cytoplasmic	92
LMOf2365_1688	lmo1664	*LMOf2365_1688*	-	0	0	0	2 (37)	0	0	Cytoplasmic	44

LMOf2365_1735	lmo1711	*LMOf2365_1735*	aminopeptidase	0	0	0	2 (26)	0	0	LPXTG Cell Wall Anchor, Sec Secreted, SP-I[h] Cleaved	45
LMOf2365_1781	lmo1756	*gatC*	aspartyl/glutamyl-tRNA amidotransferase subunit C	0	0	0	1 (47)	0	0	Cytoplasmic	11
LMOf2365_1830	lmo1803	*ftsY*	cell division ABC transporter substrate-binding protein	0	1 (31)	0	0	0	0	Cytoplasmic	36
LMOf2365_1863	lmo1835	*carB*	carbamoyl phosphate synthase large subunit	0	0	0	0	0	3 (262)	Cytoplasmic	118
LMOf2365_1921	lmo1892	*LMOf2365_1921*	penicillin-binding protein PBPA1	0	0	0	2 (41)	0	0	N-terminal Membrane Anchor (No Signal Peptide Cleavage Site), Sec Secreted	33
LMOf2365_1957	lmo1928	*aroA*	chorismate synthase	0	0	0	1 (27)	0	0	Cytoplasmic	42
LMOf2365_2135	lmo2103	*eutD*	phosphotransacetylase	0	0	0	5 (64)	0	0	Cytoplasmic	34
LMOf2365_2151	lmo2118	*glmM*	phosphoglucosamine mutas	0	1 (78)	0	0	0	0	Cytoplasmic	49
LMOf2365_2159	lmo2125	*LMOf2365_2159*	maltose/maltodextrin ABC transporter maltose/maltodextrin-binding protein	0	0	0	0	0	1 (68)	Lipid Anchor, Sec Secreted, SP-II[i] Cleaved	46
LMOf2365_2187	lmo2155	*LMOf2365_2187*	-	0	0	0	3 (78)	0	0	Cytoplasmic	87
LMOf2365_2218	lmo2185	*LMOf2365_2218*	-	0	0	0	1 (46)	0	0	LPXTG Cell Wall Anchor, Sec-secreted, SP-I[h] Cleaved	63
LMOf2365_2221	lmo2188	*pepF*	oendopeptidase F	0	0	0	4 (40)	0	0	Cytoplasmic	69

LMOf2365_2238	lmo2205	*gpm*	phosphoglycerate mutase	0	0	0	1 (21)	0	0	Cytoplasmic	26
LMOf2365_2239	lmo2206	*clpB*	-	0	0	0	0	0	5 (88)	Cytoplasmic	98
LMOf2365_2256	lmo2223	*LMOf2365_2256*	-	0	2 (75)	0	0	0	0	Cytoplasmic	13
LMOf2365_2262	lmo2229	*LMOf2365_2262*	penicillin-binding protein, PBPA2	0	0	0	0	0	8 (556)	N-terminal Membrane Anchor (No Signal Peptide Cleavage Site), Sec Secreted	78
LMOf2365_2328	lmo2358	*LMOf2365_2328*	glucosamine-6-phosphate isomerase	0	1 (35)	0	0	0	0	Cytoplasmic	27
LMOf2365_2461	lmo2488	*uvrA*	excinuclease ABC subunit A	0	0	0	0	0	1 (29)	Cytoplasmic	107
LMOf2365_2484	lmo2511	*yfiA*	ribosomal subunit interface protein	0	1 (37)	0	0	0	0	Cytoplasmic	22
LMOf2365_2499	lmo2526	*murA-1*	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	0	0	0	2 (33)	0	0	Cytoplasmic	46
LMOf2365_2502	lmo2529	*atpD-2*	F0F1 ATP synthase subunit beta	0	0	0	2 (37)	0	0	Cytoplasmic	52
LMOf2365_2583	lmo2610	*infA*	translation initiation factor IF-1	0	0	0	1 (24)	0	0	Cytoplasmic	8
LMOf2365_2594	lmo2621	*rplX*	50S ribosomal protein L24	0	0	0	1 (54)	0	0	Cytoplasmic	11
LMOf2365_2670	lmo2691	*murA*	autolysin	0	0	0	2 (55)	0	0	N-terminal Membrane Anchor (No Signal Peptide Cleavage Site), Sec Secreted	64
-	-	*gp31*	putative repressor protein [Listeria phage A118]	0	0	0	1 (32)	0	0	-	12

-	-	*gp33*		0	0	0	0	0	6 (235)	-	28
-	-	*gp59*	Listeria phage 2389	0	0	0	0	3 (97)	0	-	18

a) Locus tags of annotated *L. monocytogenes* F2365 genome (147).
b) Locus tags of annotated *L. monocytogenes* EGD-e genome (178).
c) Number of peptides identified in three trials. Trials are designated as 1, 2, or 3.
d) Protein Mascot score associated with each set of peptides identified.
e) Peptides derived from secretion or cell lysis. Cells were incubated with digest buffer only. Peptides released into solution were separated from cells by filtration and subsequently digested with trypsin (see section 3.3 in Materials and Methods).
f) Peptides derived from surface associated cells. Cells were digested with trypsin. Peptides released into solution were separated from cells by filtration (see section 3.3 in Materials and Methods).
g) Location information is from LocateP annotation (179).
h) Signal Peptidase Type I.
i) Signal Peptidase Type II.

Selected surface protein candidate was presented in bold red.

5.2.3 Secreted Proteins Identified

Proteins that contained a secretion signal and had similar numbers of peptide hits in the negative control and trypsin treatment of *L. monocytogenes* cells were classified as proteins "secreted to the extracellular region (GO: 0005576)" (Table 5-1). Twenty-one proteins from this group were identified. The majority of proteins in this group such as LMOf2365_0153, Hly (LMOf2365_0213), ActA (LMOf2365_0215), TcsA (LMOf2365_1407), LMOf2365_1762, LMOf2365_1875, LMOf2365_1879, LMOf2365_1989, LMOf2365_2111, OppA (LMOf2365_2229) and LMOf2365_2610 were previously identified as secreted proteins (170). Other proteins such as LMOf2365_0376, LMOf3265_0570, LMOf2365_0808, ModA (LMOf2365_1062), LMOf2365_1090, LMOf2365_1695, LMOf2365_1782, LMOf2365_2217, LMOf2365_2402 and LMOf2365_2550 were also identified along with two phage proteins: gp25 and gp32.

5.2.4 Cytoplasmic Proteins Identified

One hundred and thirty-two cytoplasmic proteins were identified (Table 5-1), 59 of which were identified in previous proteomic studies of *L. monocytogenes* (169, 170, 182, 183). Although the identification of cytoplasmic proteins such as ribosomal proteins and chaperones suggested some degree of cell lysis, the lack of a statistically significant difference between the CFU count of trypsin-treated and undigested bacteria suggested that the conditions under which the trypsin digest were performed did not lead to significant cell lysis. Although some cytoplasmic proteins have been demonstrated to be surface localized (169, 184, 185), further work is needed to establish their roles on the cell surface.

Chapter 6: Assessment of *L. monocytogenes* Surface Proteins Identified from Proteomics Analysis for Use as Diagnostic Biomarkers

6.1 Introduction

Several proteins associated with the *L. monocytogenes* cell envelope were identified in the proteomic analysis described in chapter 5 (186). This proteomic screen provided evidence for the expression and surface exposure of identified proteins on *L. monocytogenes*. Surface proteins from this proteomic screen were assessed for their potential as diagnostic biomarkers for *L. monocytogenes.*

Surface proteins expressed in a range of *L. monocytogenes* serotypes in selective enrichment cultures are of particular interest since *L. monocytogenes* in environment and food samples is often found in trace amounts and requires selective enrichment culture to reach sufficient cell numbers for *L. monocytogenes* detection (9-11). The objective of this study was to assess the expression of candidate surface associated proteins, identified in the previous proteomic screen, on a range of *Listeria* isolates grown under selective enrichment culture conditions according to Health Canada's MFHPB-07 and MFHPB-07 methods.

6.2 Results

6.2.1 Surface Proteins Candidates from Proteomic Analysis

Proteins identified in the proteomic screen of *L. monocytogenes* surface proteins were assessed for their potential as diagnostic biomarkers. Six proteins: LMOf2365_0148, LMOf2365_0312, LMOf2365_0546, LMOf2365_1883, LMOf2365_2111 and LMOf2365_2742 were selected. Selection criteria included proteins with a secretion signal, high Mascot scores with multiple peptides identified and specific identification in experimental samples (with the exception of protein LMOf2365_2111). In addition, selected proteins also contained regions that were conserved among *L. monocytogenes* strains but variable among other *Listeria* species

which were identified using a pBLAST search followed by multiple-alignment of identified surface proteins.

Peptides of protein LMOf2365_2111 were identified in the experimental sample in trial 2 and in both the experimental and negative control samples in trial 3 (Table 5-1). Proteins LMOf2365_0312, LMOf2365_1883 and LMOf2365_2742 were identified in at least two of three trials and only in experimental samples (Table 5-1). Protein LMOf2365_0546 was identified once in the experimental sample of trial 3 (Table 5-2). Protein LMOf2365_0148 was identified in a preliminary proteomic screen. Twenty-one peptides of LMOf2365_0148 were detected only in the experimental sample generating a Mascot score of 658.

6.2.2 Production of Recombinant Candidate Proteins

Gene sequences encoding candidate proteins LMOf2365_0148, LMOf2365_0312, LMOf2365_0546, LMOf2365_1883, LMOf2365_2111 and LMOf2365_2742 were cloned into the pLIC-CHIS expression plasmid for the production of respective recombinant proteins (Figure 6-1). To assess the expression of candidate proteins in *L. monocytogenes*, purified, full-length, recombinant proteins (rLMOf2365_0148, rLMOf2365_0312, rLMOf2365_0546, rLMOf2365_1883, rLMOf2365_2111 and rLMOf2365_2742) were used as antigens to raise rabbit polyclonal antibodies. Purified, His-tagged proteins were detected by His-tag western blot and by SDS-PAGE with Coomassie blue staining. Western blot (panel A, Figure 6-2) and Coomassie blue stained gel (panel B, Figure 6-2) showed identical sizes of each purified recombinant proteins were identical except for rLMOf2365_2742. A band of approximately 45 kDa (indicated by arrows in Figure 6-2) were present in both His-tag western blot and Coomassie blue stained gel for the purified rLMOf2365_2742 preparation however four other

protein bands above and below the 45 kDa were also present in the Comassie blue stained gel (panel A, Figure 6-2). The theoretical size of LMOf2365_2742 is 48 kDa. The 45 kDa band is considered to be LMOf2365_2742 while the other bands may be contaminating *E. coli* proteins.

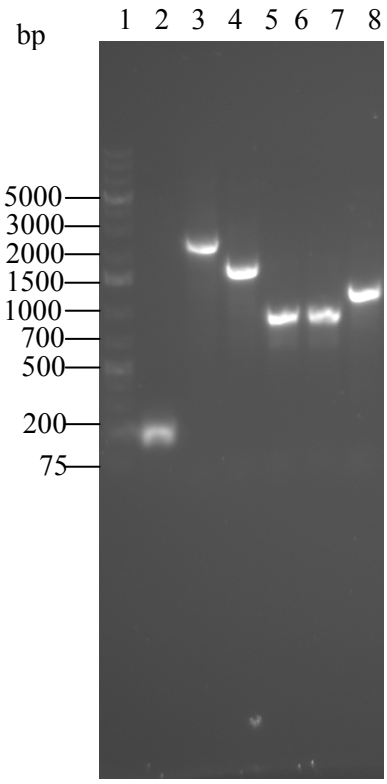


Figure 6-1. PCR screen of recombinant plasmids of LMOf2365_0148, LMOf2365_0312, LMOf2365_0546, LMOf2365_2111 and LMOf2365_2742. Recombinant pLIC-CHIS (lanes 3-8) or empty pLIC-CHIS (lane 2) plasmids were used as templates for PCR. T7 promoter and terminator primers that annealed to the plasmid template were used in PCR. Full length genes: *LMOf2365_0148* (lane 3), *LMOf2365_0312* (lane 4), *LMOf2365_0546* (lane 5), *LMOf2365_1883* (lane 6), *LMOf2365_2111* (lane 7) and *LMO2f2365_2742* (lane 8) were cloned into pLIC-CHIS plasmids. See Table 3-5 in Materials and Methods for primer sequences used in PCR cloning. Lane 1 consists of DNA markers.

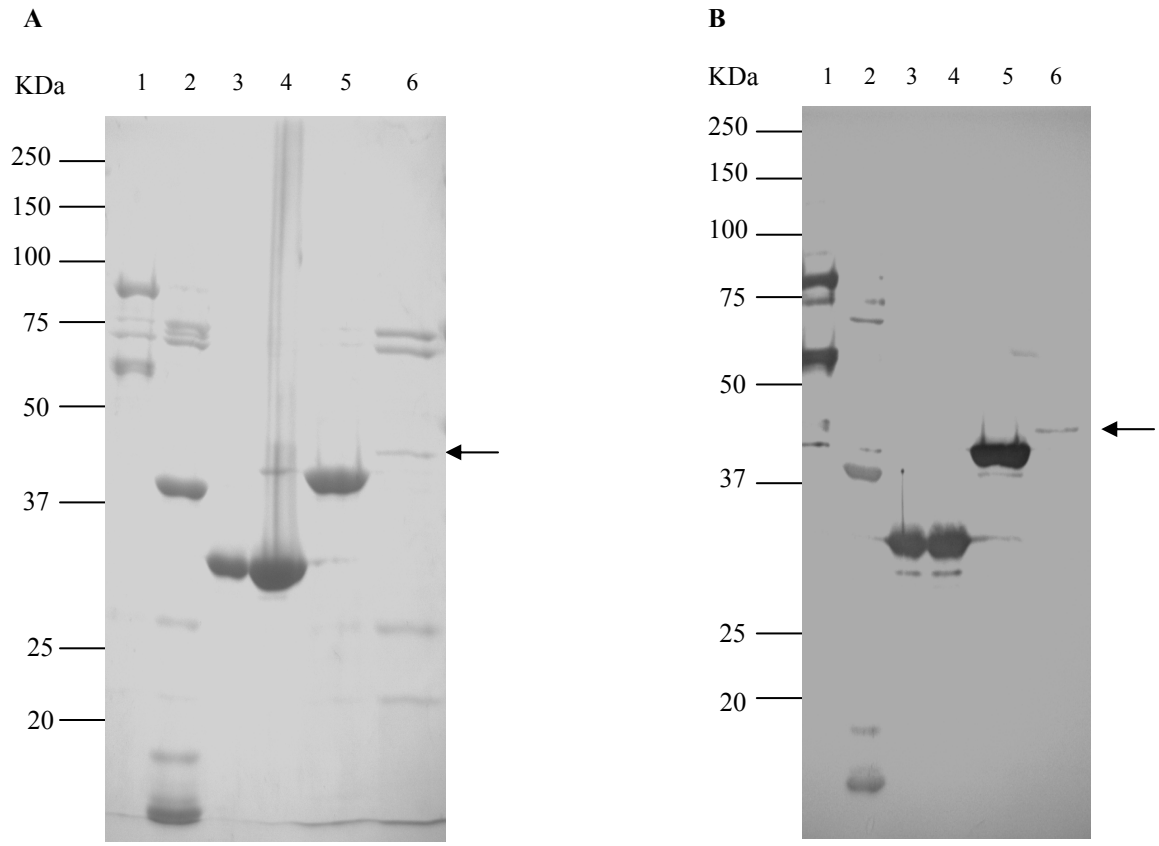


Figure 6-2. Coomassie blue stained SDS-PAGE gel and western blot analysis of purified recombinant surface protein candidates. Panel A: Coomassie blue stained gel of purified recombinant proteins used for polyclonal antibody production. Immobilized metal affinity chromatography was performed to purify the His-tagged proteins. Full-length recombinant proteins: LMOf2365_0148 (lane 1), LMOf2365_0312 (lane 2), LMOf2365_0546 (lane 3), LMOf2365_1883 (lane 4), LMOf2365_2111 (lane 5) and LMOf2365_2742 (lane 6) were purified. Panel B: His-tag western blot of purified recombinant proteins. Purified proteins were assessed for the presence of a His-tag using (anti-His) MAbs (Qiagen). Arrows indicate the LMOf2365_2742 protein.

6.2.3 Expression and Surface Localization of Candidate Proteins in *L. monocytogenes*

LMOf2365_0148, LMOf2365_0312, LMOf2365_0546, LMOf2365_1883, LMOf2365_2111 and LMOf2365_2742 were detected using polyclonal antibodies (PAbs) in the whole cell extract of *L. monocytogenes* strain LI0521 by western blots (Figure 6-3). A predominant band of approximately 75 kDa recognized by the anti-LMOf2365_0148 PAb was similar in size to the theoretical size of 78 kDa. Anti-LMOf2365_0312 PAbs recognized two protein bands of around 75 kDa which were larger than the predicted size of LMOf2365_0312 (~50 kDa). Although this discrepancy existed, the recombinant LMOf2365_0312 preparation also contained two protein bands around 75 kDa (Figure 6-2) similar in size to the native proteins observed in the *L. monocytogenes* whole cell extract (Figure 6-3). The sizes of LMOf2365_0546, LMOf2365_1883, LMOf2365_2111 and LMOf2365_2742 detected by PAbs in both *L. monocytogenes* whole cell extracts (Figure 6-3) and recombinant protein preparations (Figure 6-2) were close to their respective theoretical sizes of 29, 31, 41 and 48 kDa.

Immunofluorescence microscopy (IFM) of live *L. monocytogenes* cells using PAbs was performed to probe the surface localization of each candidate protein. While LMOf2365_0546 and LMOf2365_2742 anti-sera revealed weak IFM signals, a strong IFM signal was observed with PAb to LMOf2365_0148 (Figure 6-4). LMOf2365_0312, LMOf2365_1883 and LMOf2365_2111 could not be detected using PAbs against LMOf2365_0312, LMOf2365_1883 and LMOf2365_2111 respectively (Figure 6-4). The LMOf2365_0148 antigen was thus selected for the development of MAbs which were then assessed for their potential as diagnostic MAbs.

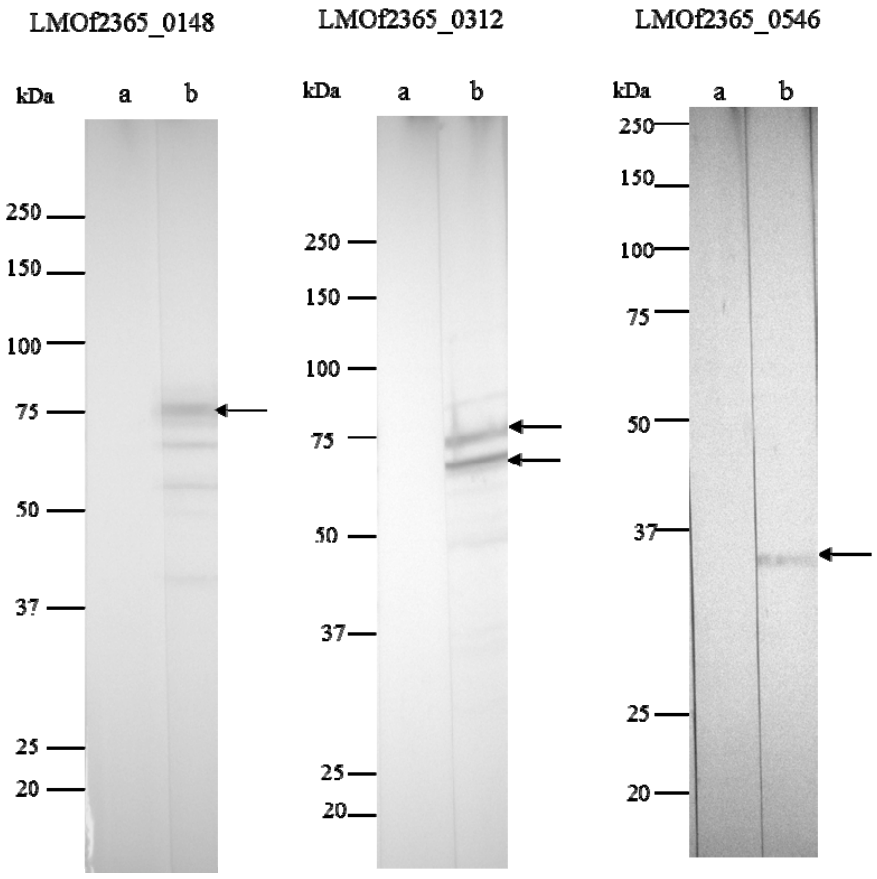


Figure 6-3. Western blot analysis of LMOf2365_0148, LMOf2365_0312, LMOf2365_0546, LMOf2365_1883, LMOf2365_2111 and LMOf2365_2742 proteins in whole cell extracts of *L. monocytogenes* strain LI0521. An overnight culture (~1.5 OD_{620}, 50ml) in BHI broth was used to make 0.8 ml of whole cell extract (see section 3.12 in Materials and Methods for details). A 30 µl of whole cell extract sample was loaded onto each lane of the SDS-PAGE gel. Separated whole cell extract components were transferred to nitrocellulose membrane which was probed with specific rabbit anti-serum (lane b) and with rabbit pre-immune serum (lane a). Arrows indicate surface protein bands recognized by PAbs.

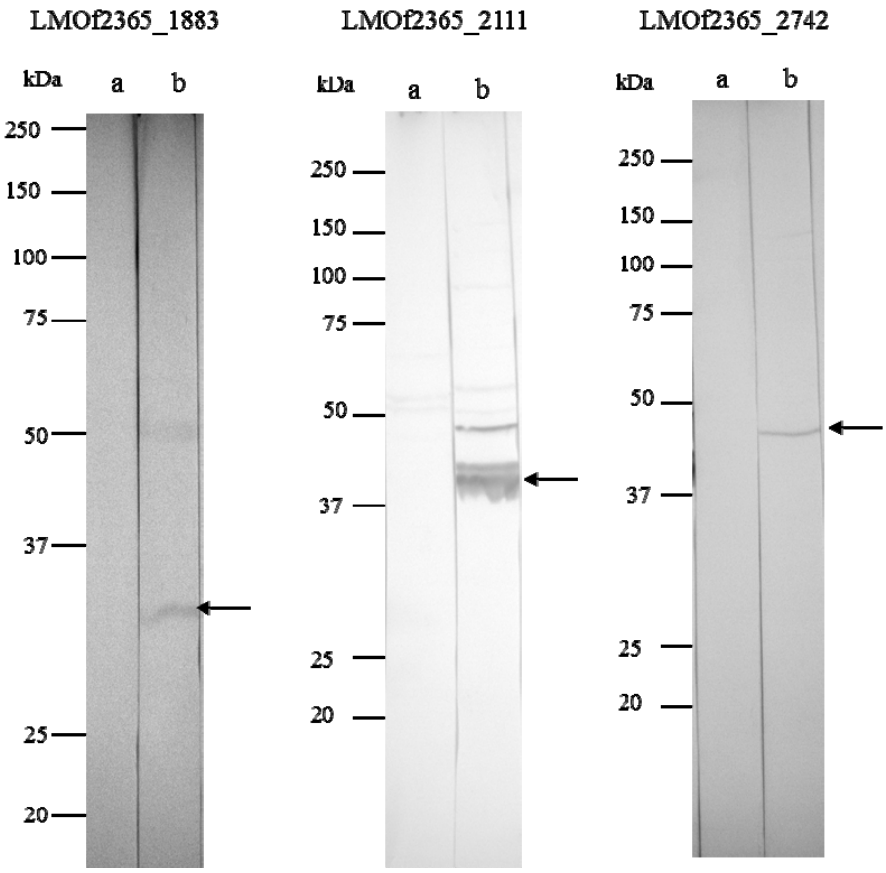
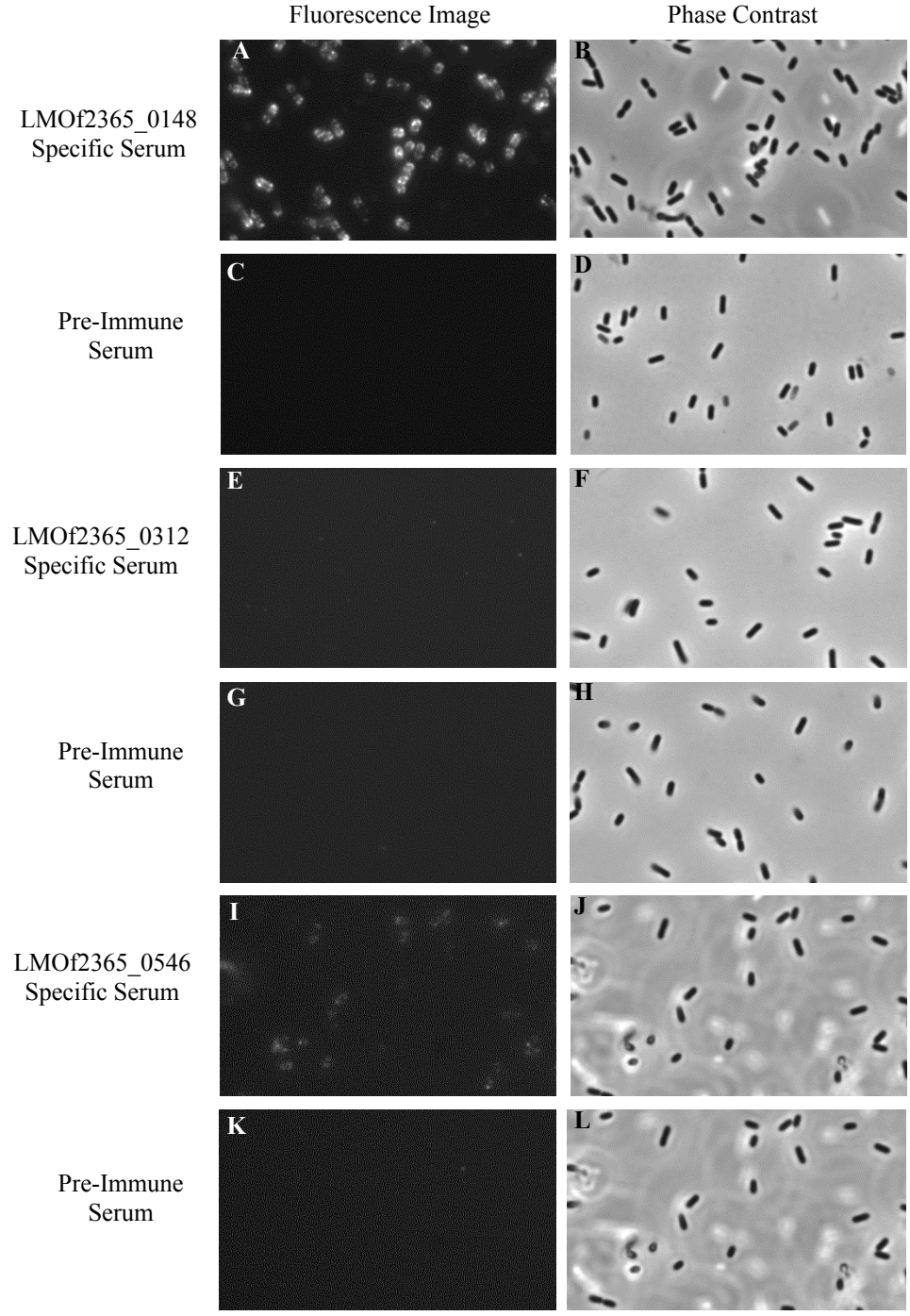


Figure 6-3 Continued.

Figure 6-4. Surface localization assessment of LMOf2365_0148, LMOf2365_0312, LMOf2365_0546, LMOf2365_1883, LMOf2365_2111 and LMOf2365_2742 proteins on live *L. monocytogenes* strain LI0521 cells by immunofluorescence microscopy. Bacterial cells (2.5×10^8) from overnight culture (~1.5 OD_{620}) grown in BHI broth were probed with specific rabbit PAbs (1:1000 dilution) against purified recombinant proteins LMOf2365_0148 (A), LMOf2365_0312 (E), LMOf2365_0546 (I), LMOf2365_1883 (M), LMOf2365_2111 (Q) and LMOf2365_2742 (U). No signal was observed when pre-immune sera were used as a negative control (C,G, K, O, S and W). Fluorescence images (A, C, E, G, I, K, M, O, Q, S, U and W) and phase contrast images (B, D, F, H, J, L, N, P, R, T, V and X) in the same field are shown (magnification 100x).


Fluorescence Image
Phase Contrast
LMOf2365_0148 Specific Serum
Pre-Immune Serum
LMOf2365_0312 Specific Serum
Pre-Immune Serum
LMOf2365_0546 Specific Serum
Pre-Immune Serum
A
B
C
D
E
F
G
H
I
J
K
L

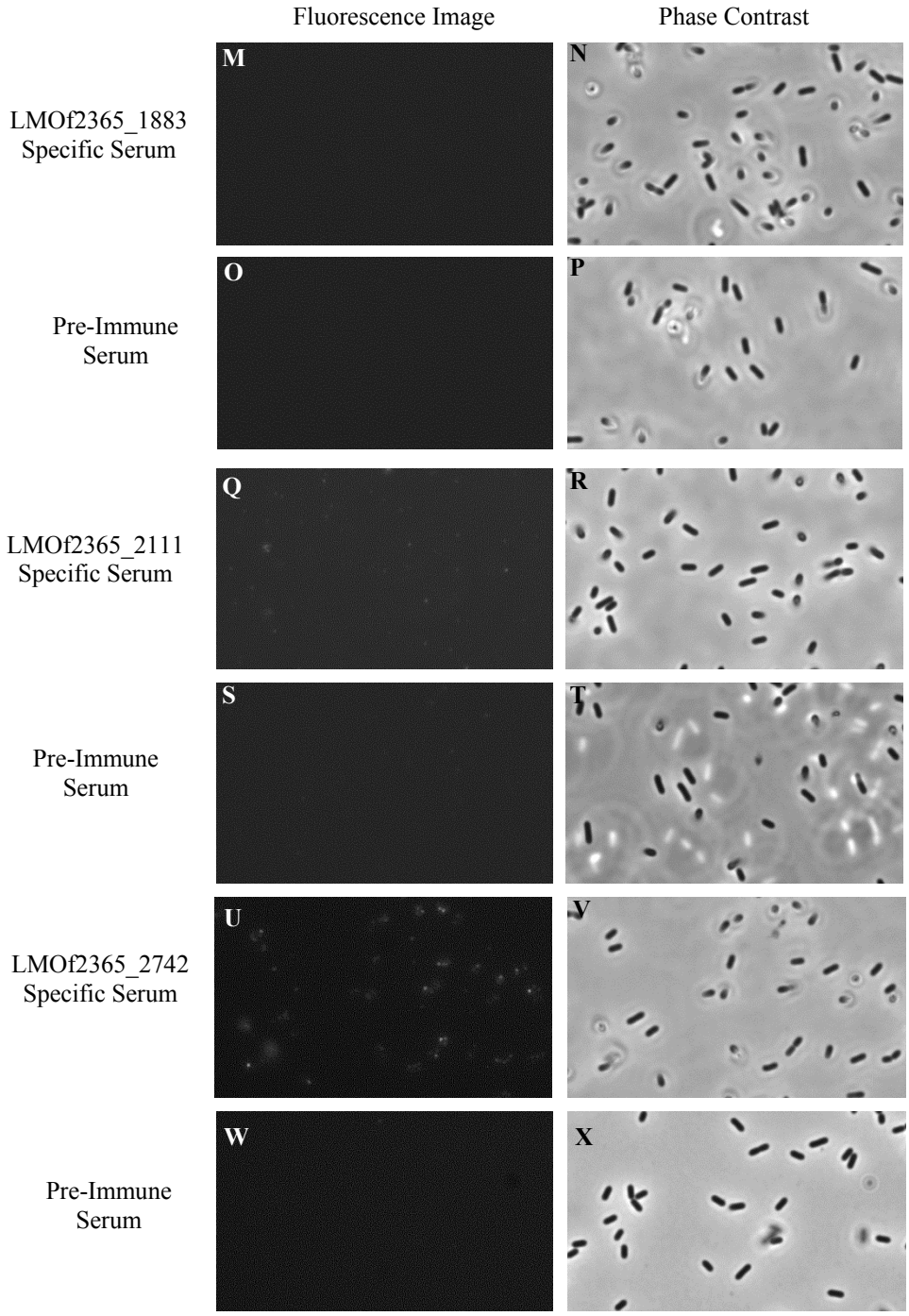
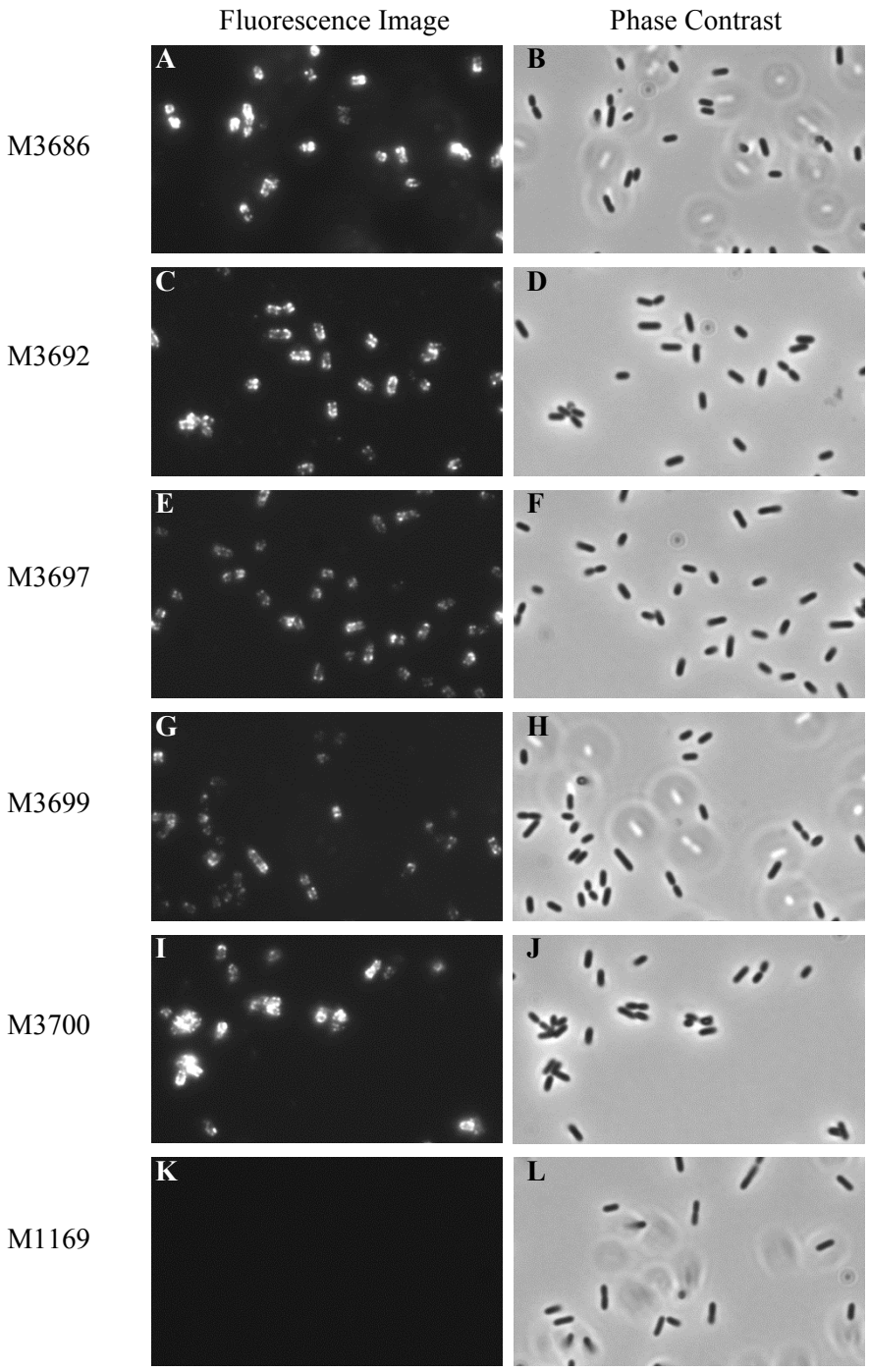


Figure 6-4 Continued.

6.2.4 Development of LMOf2365_0148 Monoclonal Antibodies

Hybridoma clones secreting MAbs to LMOf2365_0148 were identified by indirect ELISA screening with several antigen preparations. Twenty-four stable hybridoma clones were found to secrete MAbs that reacted with both rLMOf2365_0148 and formalin-killed *L. monocytogenes* strain LI0521 cells but not with formalin-killed *L. innocua* CLIP11262 cells (data not shown). Of 24 MAbs, 10 reacted with the fewest number of other *Listeria* species isolates tested. Indirect ELISA assessment with formalin-killed whole cells of 53 *L. monocytogenes* isolates revealed that five (M3686, M3692, M3697, M3699 and M3700) out of the ten MAbs tested reacted to the highest number of *L. monocytogenes* isolates. These MAbs also detected LMOf2365_0148 on the surface of live *L. monocytogenes* strain LI0521 (Figure 6-5).

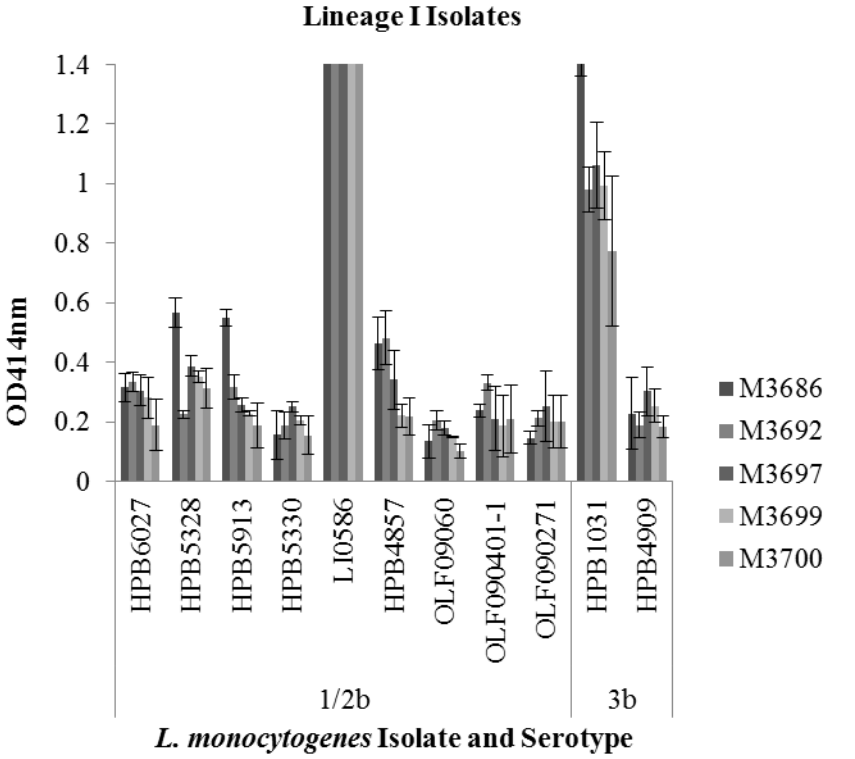
Figure 6-5. Immunofluorescence images of live *L. monocytogenes* strain LI0521 cells probed with MAbs raised against rLMOf2365_0148. Bacterial cells (2.5 x 10^8) from an overnight BHI culture (~1.5 OD_{620}) were stained with M3686 (A), M3692 (C), M3697 (E), M3699 (G), M3700 (I) and irrelevant M1169 (K). Purified IgG (1vng/µl) was used except TCF of M1169 to *C. jejuni* at a dilution of 1:50 was used as negative control (K and L). Fluorescence images (A, C, E, G, I and K) and phase contrast images (B, D, F, H, J and L) in the same field are shown (magnification 100x).

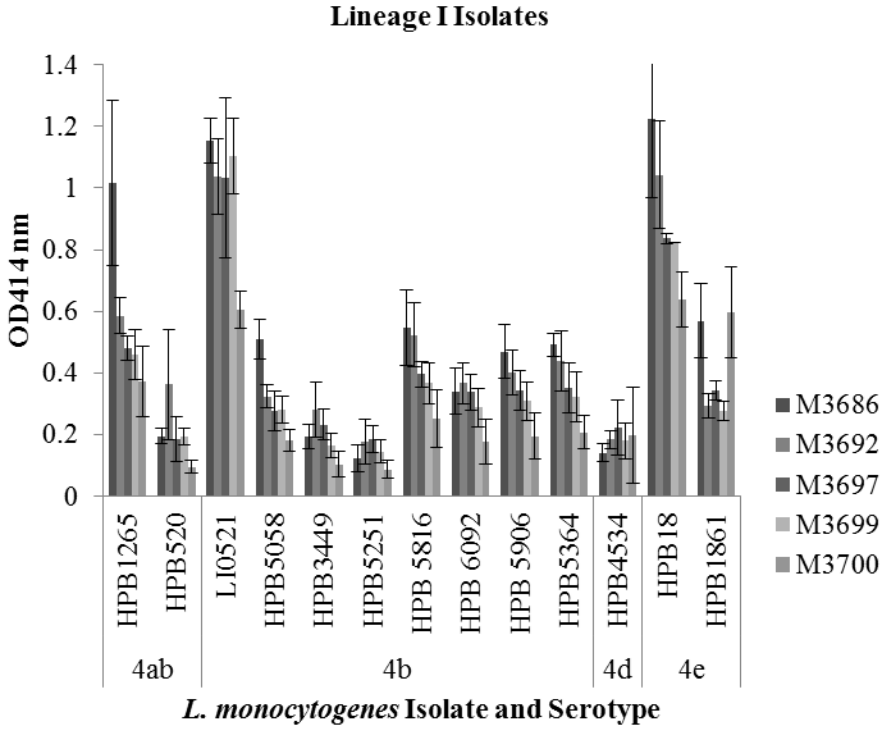

Fluorescence Image
Phase Contrast
M3686
M3692
M3697
M3699
M3700
M1169
A
B
C
D
E
F
G
H
I
J
K
L

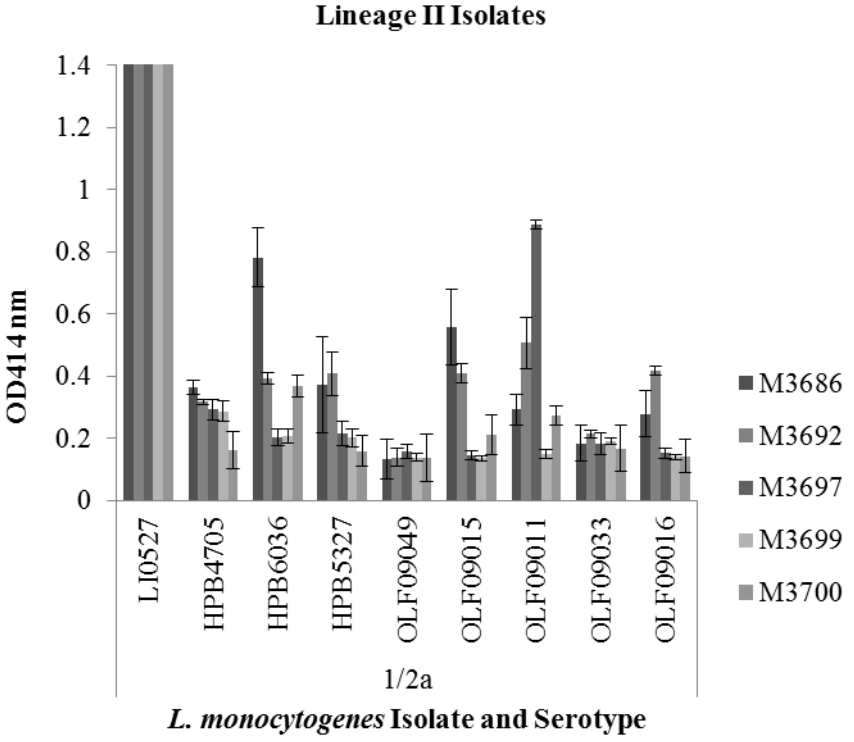
6.2.5 Reactivity of LMOf2365_0148 MAbs to *Listeria* and Other Bacterial Isolates

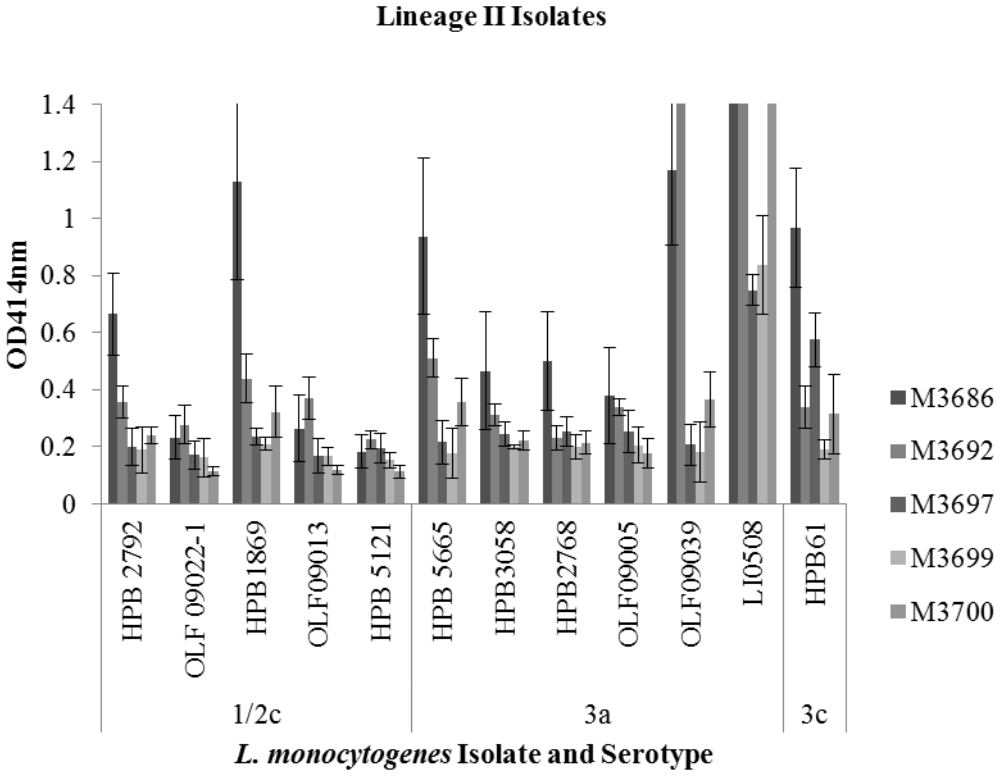
MAbs M3686, M3692, M3697, M3699 and M3700 were not reactive with the majority of the *L. monocytogenes* isolates grown in non-selective BHI medium (Figure 6-6). However, high reactivity was observed in *L. monocytogenes* isolates LI0586 (serotype 1/2b), HPB1031 (serotype 3b), LI0521 (serotype 4b), HPB18 (serotype 4e), LI0527 (serotype 1/2a) and LI0508 (serotype 3a) (Figure 6-6). These isolates belong to different serotypes with no apparent relationship between them. Of the five MAbs, M3686 reacted to the highest number of *L. monocytogenes* isolates. M3686 was reactive to 3b, 3c, 1/2a, 1/2c and 3a serotype isolates that were not detected by other MAbs. The MAbs were not reactive to other *Listeria* species and foodborne bacteria except M3686 which was reactive to *L. innocua* ATCC 33090 and *L. seeligeri* ATCC 35967 and M3692 which was reactive to *L. seeligeri* HPB24 (Figure 6-6).

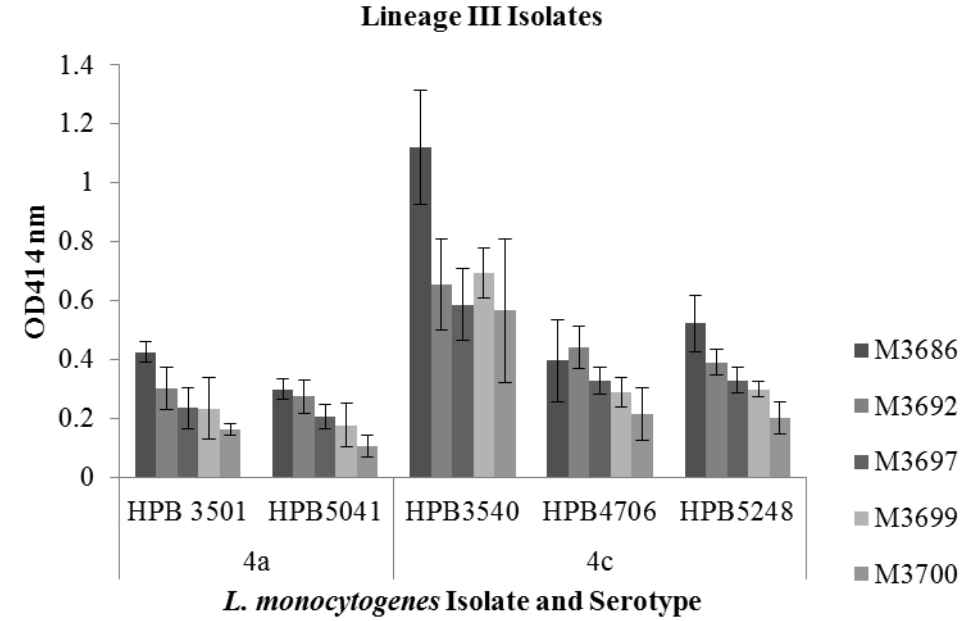
Figure 6-6. Detection of lineage I, II and III isolates of *L. monocytogenes* and other *Listeria* species and bacteria cultured in BHI by indirect ELISA. Cells from an overnight culture were formalin-killed and stored in 50% glycerol. A 100 µl of formalin-killed bacterial suspension (10^8 cells/ml) was used to coat each well of Maxisorp™ 96-well microtiter plates. Each of the five MAbs (M3686, M3692, M3697, M3699 and M36700) in undiluted tissue culture fluid was used to detect bacterial cells. Error bars are one standard deviation from the mean of three independent experiments. Two replicates were performed in each experiment. The OD_{414} readings obtained from negative controls ranged from 0.03 to 0.2, and thus OD_{414} readings of <0.25, between 0.25 and 0.3, and > 0.3 were considered as negative, weakly positive and positive respectively. See section 3.15 in Materials and Methods for details. OD_{414} readings of *L. monocytogenes* isolates which exceeded the maximum value on the y-axis and cannot be represented in the figure were reported in Table 6-1.


Lineage I Isolates
OD414nm
1.4
1.2
1
0.8
0.6
0.4
0.2
0
HPB6027
HPB5328
HPB5913
HPB5330
LI0586
HPB4857
OLF090401
OLF090401-1
OLF090271
HPB1031
HPB4909
1/2b
3b
M3686
M3692
M3697
M3699
M3700
L. monocytogenes Isolate and Serotype


Lineage I Isolates
OD414nm
1.4
1.2
1
0.8
0.6
0.4
0.2
0
HPB1265
HPB520
LI0521
HPB5058
HPB3449
HPB5251
HPB 5816
HPB 6092
HPB 5906
HPB5364
HPB4534
HPB18
HPB1861
4ab
4b
4d
4e
M3686
M3692
M3697
M3699
M3700
L. monocytogenes Isolate and Serotype


Lineage II Isolates
OD414nm
1.4
1.2
1
0.8
0.6
0.4
0.2
0
LI0527
HPB4705
HPB6036
HPB5327
OLF09049
OLF09015
OLF09011
OLF09033
OLF09016
1/2a
M3686
M3692
M3697
M3699
M3700
L. monocytogenes Isolate and Serotype


Lineage II Isolates
OD414nm
1.4
1.2
1
0.8
0.6
0.4
0.2
0
HPB 2792
OLF 09022-1
HPB1869
OLF09013
HPB 5121
HPB 5665
HPB3058
HPB2768
OLF09005
OLF09039
LI0508
HPB61
1/2c
3a
3c
M3686
M3692
M3697
M3699
M3700
L. monocytogenes Isolate and Serotype


Lineage III Isolates
OD414 nm
1.4
1.2
1
0.8
0.6
0.4
0.2
0
HPB 3501
HPB5041
HPB3540
HPB4706
HPB5248
4a
4c
L. monocytogenes Isolate and Serotype
M3686
M3692
M3697
M3699
M3700

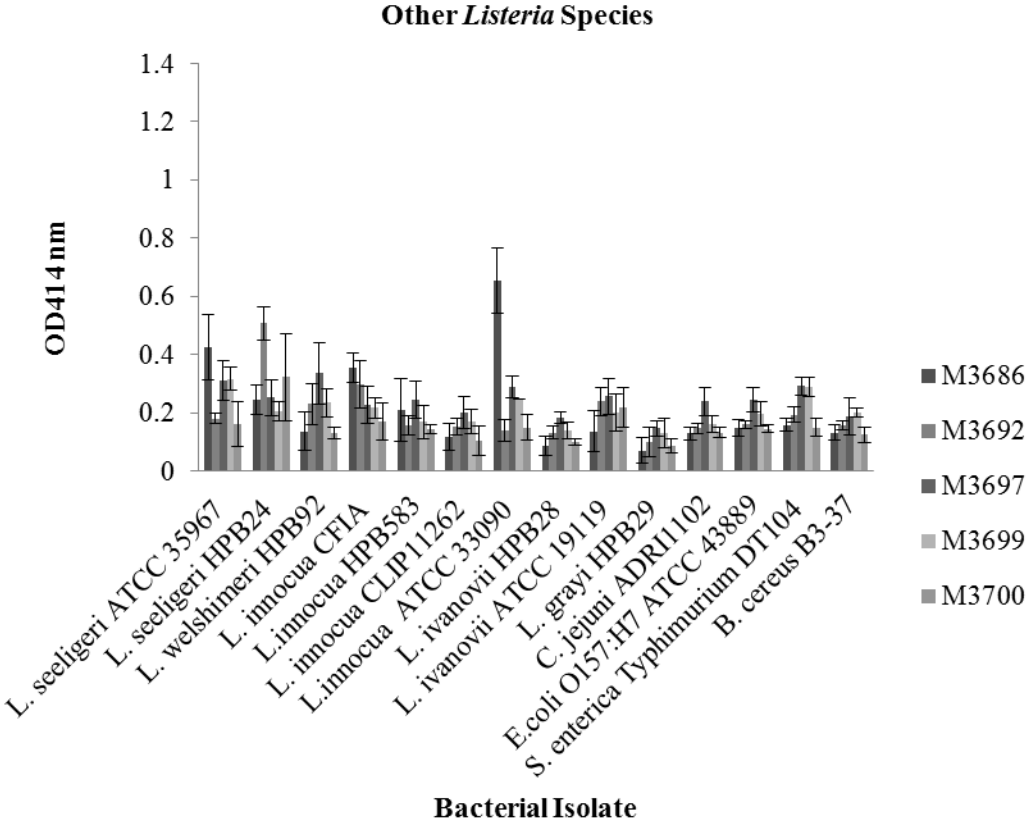

Other Listeria Species
OD414 nm
1.4
1.2
1
0.8
0.6
0.4
0.2
0
L. seeligeri ATCC 35967
L. seeligeri HPB24
L. welshimeri HPB92
L. innocua CFIA
L.innocua HPB583
L. innocua CLIP11262
L.innocua ATCC 33090
L. ivanovii HPB28
L. ivanovii ATCC 19119
L. grayi HPB29
C. jejuni ADRI1102
E.coli O157:H7 ATCC 43889
S. enterica Typhimurium DT104
B. cereus B3-37
Bacterial Isolate
M3686
M3692
M3697
M3699
M3700

Table 6-1. OD_{414} readings of highly reactive *L. monocytogenes* isolates. OD_{414} readings of *L. monocytogenes* isolates which exceeded the maximum value on the y-axis in Figure 6-6.

MAbs	Average OD_{414nm} Reading of *L. monocytogenes* Isolates						
	LI0586	HPB1031	HPB18	LI0527	HPB1869	OLF09039	LI0508
M3686	3.90±0.11	1.77±0.41	1.23±0.26	3.64±0.24	1.13±0.34	1.17±0.26	3.53±0.24
M3692	3.77±0.13			3.76±0.32		1.59±0.12	2.05±0.24
M3697	3.72±0.20			3.88±0.11			
M3699	3.50±0.15			3.56±0.15			
M3700	3.82±0.23			3.51±0.58			2.22±0.55

6.2.6 Expression of the LMOf2365_0148 Protein during Enrichment Culture

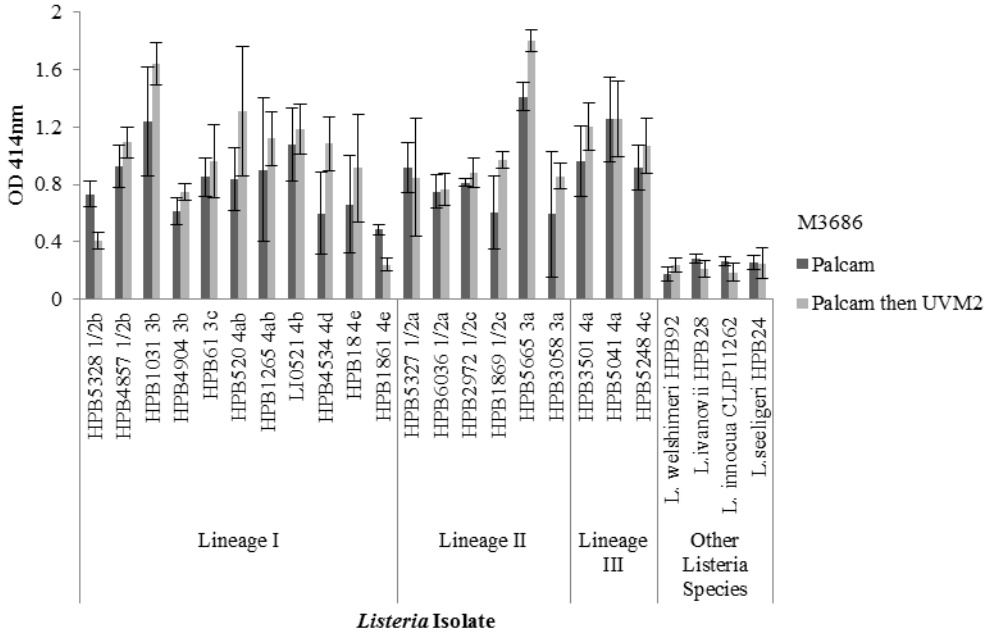
Selective enrichment culture is a necessary step to increase the number of the target pathogen, often present in trace amounts in foods, to a detectable level of ~10^4 to 10^6 CFU/ml (10, 11) for ELISA or to ~10^1 to 10^3 CFU/ml for PCR (9). Therefore, to act as a potential diagnostic protein biomarker for *L. monocytogenes*, the LMOf2365_0148 protein must be expressed under selective enrichment culture conditions. To this end, the ability of the five MAbs (M3686, M3692, M3697, M3699 and M3700) to detect 20 *L. monocytogenes* isolates and four other *Listeria* species isolates grown in standard enrichment cultures according to MFHPB-07 (146) and MFHPB-30 (107) described in Health Canada's Compendium of Analytical Methods was assessed using indirect ELISA.

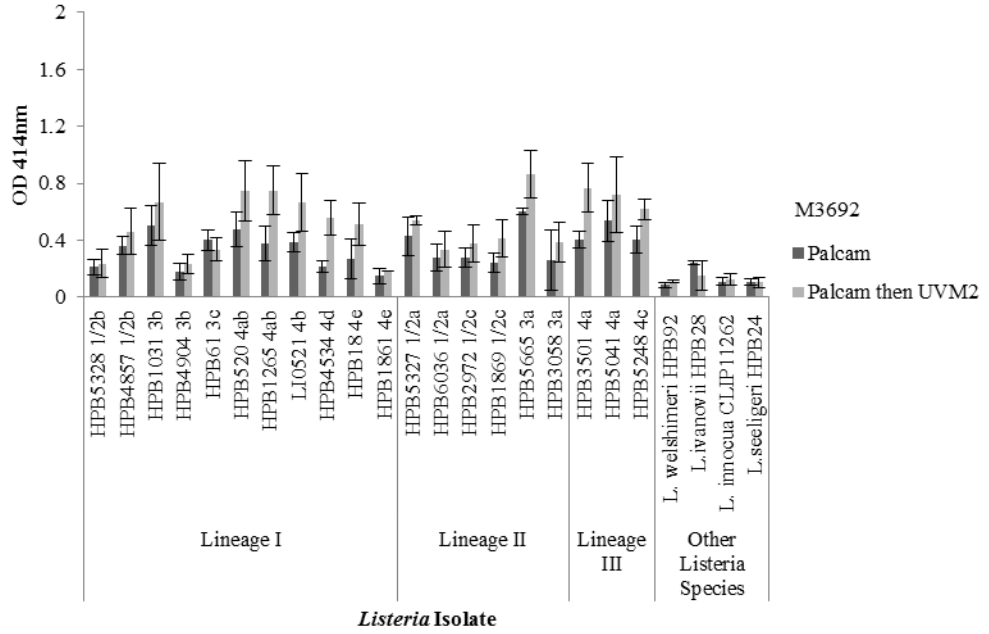
In primary (Palcam) and secondary (UVM2) selective enrichment culture conditions of MFHPB-07, M3686 detected all *L. monocytogenes* isolates except one 4e isolate in UVM2 (Figure 6-7). The reactivity profiles of M3692 and M3700 to *Listeria* isolates were similar to that of M3686 but with reduced OD_{414} readings. M3697 and M3699 reacted to lineage I and III *L. monocytogenes* isolates but were either weakly or not reactive to most lineage II *L.*

monocytogenes isolates. M3697, M3699 and M3700 were reactive to *L. welshimeri* in UVM2 (Figure 6-7).

In primary (LEB/UVM1) and secondary (MFB) selective enrichment culture conditions of MFHPB-30, M3686 reacted to all *L. monocytogenes* isolates, *L. seeligeri* and *L. innocua* in MFB (Figure 6-8). M3692 reacted to lineage III isolates but weakly reacted with many lineage I and II *L. monocytogenes* isolates. M3692 also reacted to *L. ivanovii* in LEB/UVM1 (Figure 6-8).M3697 and M3699 reacted to lineage I and III isolates of *L. monocytogenes* and *L. welshimeri* but were weakly reactive to lineage II *L. monocytogenes* isolates. M3700 was reactive to all *L. monocytogenes* isolates, *L. welshimeri* isolates and *L. ivanovii* isolates.

Figure 6-7. Detection of *Listeria* isolates cultured according to the MFHPB-07 method by indirect ELISA. Bacterial cells grown in Palcam at 35 ^{o}C for 26-28 hours and then in UVM2 at 30 ^{o}C for 26-28 hours were used in indirect ELISA. A 100 µl formalin-killed bacterial suspension (10^8 cells/ml) was used to coat each well of Maxisorp™ 96-well microtiter plates. Each of the five MAbs (M3686, M3692, M3697, M3699 and M3700) in undiluted tissue culture fluid was used to detect bacterial cells. Error bars are one standard deviation from the mean of three independent experiments. Two replicates were performed in each experiment. The OD_{414} readings obtained from negative controls ranged from 0.03 to 0.2, and thus OD_{414} readings of <0.25, between 0.25 and 0.3, and > 0.3 were considered as negative, weakly positive and positive respectively. See section 3.15 in Materials and Methods for details.


OD 414nm
2
1.6
1.2
0.8
0.4
0
M3686
Palcam
Palcam then UVM2
HPB5328 1/2b
HPB4857 1/2b
HPB1031 3b
HPB4904 3b
HPB61 3c
HPB520 4ab
HPB1265 4ab
LI0521 4b
HPB4534 4d
HPB18 4e
HPB1861 4e
HPB5327 1/2a
HPB6036 1/2a
HPB2972 1/2c
HPB1869 1/2c
HPB5665 3a
HPB3058 3a
HPB3501 4a
HPB5041 4a
HPB5248 4c
L. welshimeri HPB92
L.ivanovii HPB28
L. innocua CLIP11262
L.seeligeri HPB24
Lineage I
Lineage II
Lineage III
Other Listeria Species
Listeria Isolate


OD 414nm
2
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1.2
0.8
0.4
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M3692
Palcam
Palcam then UVM2
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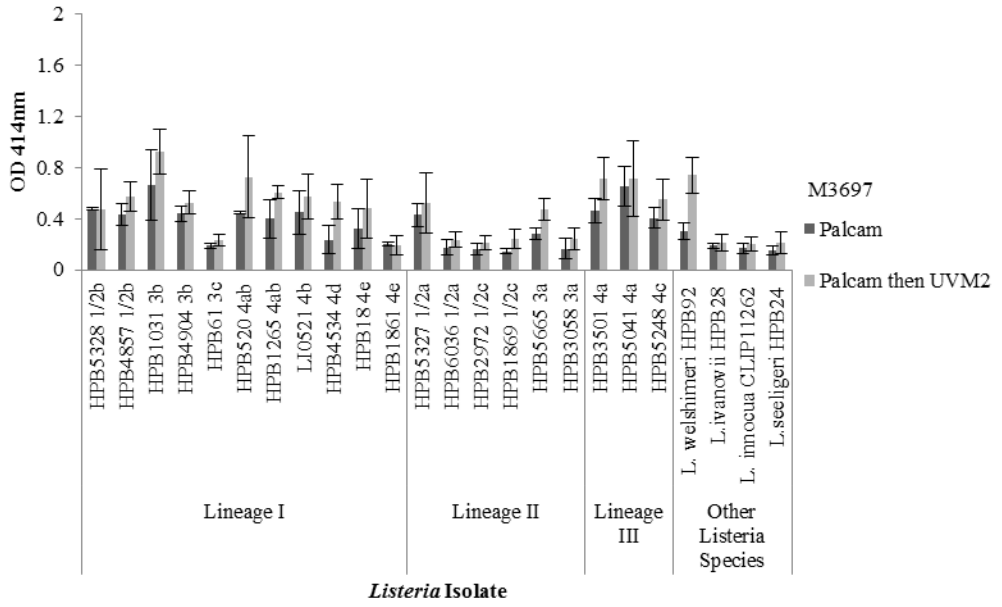
HPB4904 3b
HPB61 3c
HPB520 4ab
HPB1265 4ab
LI0521 4b
HPB4534 4d
HPB18 4e
HPB1861 4e
HPB5327 1/2a
HPB6036 1/2a
HPB2972 1/2c
HPB1869 1/2c
HPB5665 3a
HPB3058 3a
HPB3501 4a
HPB5041 4a
HPB5248 4c
L. welshimeri HPB92
L.ivanovii HPB28
L. innocua CLIP11262
L.seeligeri HPB24
Lineage I
Lineage II
Lineage III
Other Listeria Species
Listeria Isolate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1.2
0.8
0.4
0
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HPB4904 3b
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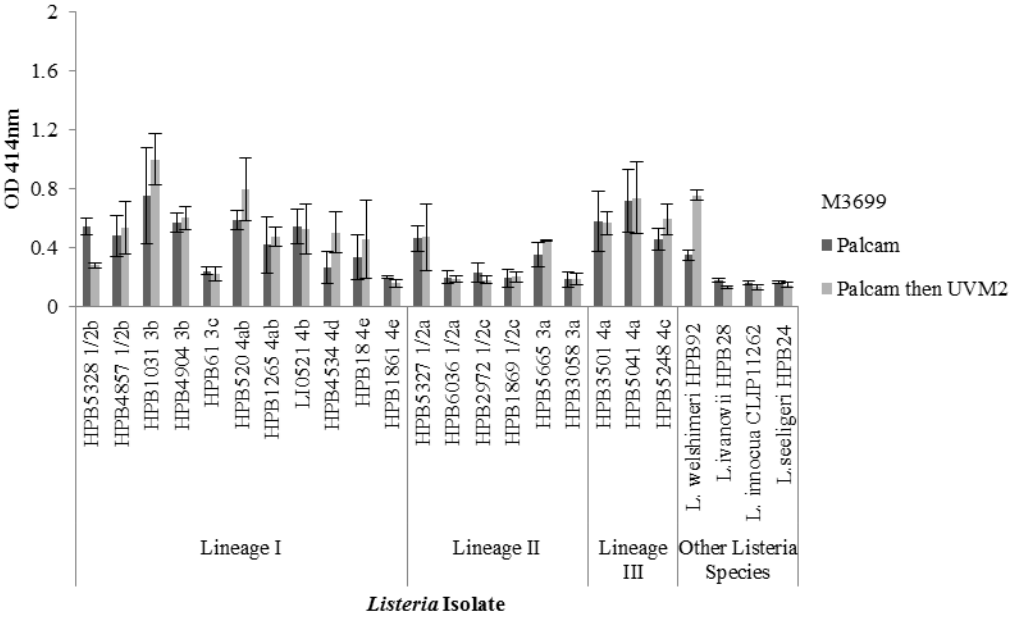
HPB1265 4ab
LI0521 4b
HPB4534 4d
HPB18 4e
HPB1861 4e
HPB5327 1/2a
HPB6036 1/2a
HPB2972 1/2c
HPB1869 1/2c
HPB5665 3a
HPB3058 3a
HPB3501 4a
HPB5041 4a
HPB5248 4c
L. welshimeri HPB92
L.ivanowii HPB28
L. innocua CLIP11262
L.seeligeri HPB24
Lineage I
Lineage II
Lineage III
Other Listeria Species
Listeria Isolate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0.4
0
M3699
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HPB61 3c
HPB520 4ab
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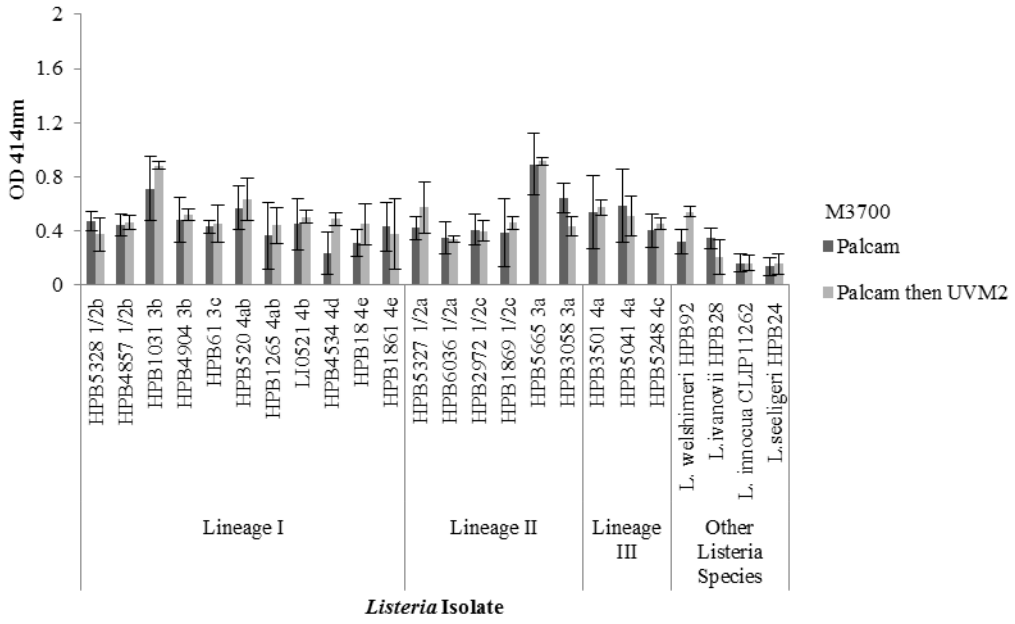
LI0521 4b
HPB4534 4d
HPB18 4e
HPB1861 4e
HPB5327 1/2a
HPB6036 1/2a
HPB2972 1/2c
HPB1869 1/2c
HPB5665 3a
HPB3058 3a
HPB3501 4a
HPB5041 4a
HPB5248 4c
L. welshimeri HPB92
L.ivanowii HPB28
L. innocua CLIP11262
L.seeligeri HPB24
Lineage I
Lineage II
Lineage III
Other Listeria Species
Listeria Isolate


OD 414nm
2
1.6
1.2
0.8
0.4
0
M3700
Palcam
Palcam then UVM2
HPB5328 1/2b
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HPB4904 3b
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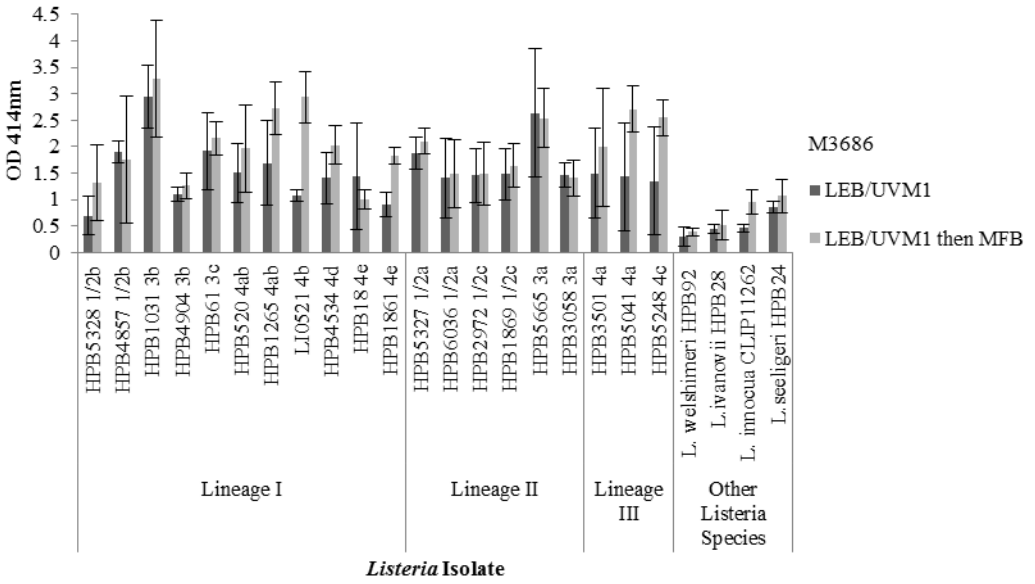
LI0521 4b
HPB4534 4d
HPB18 4e
HPB1861 4e
HPB5327 1/2a
HPB6036 1/2a
HPB2972 1/2c
HPB1869 1/2c
HPB5665 3a
HPB3058 3a
HPB3501 4a
HPB5041 4a
HPB5248 4c
L. welshimeri HPB92
L.ivanowii HPB28
L. innocua CLIP11262
L.seeligeri HPB24
Lineage I
Lineage II
Lineage III
Other Listeria Species
Listeria Isolate

Figure 6-8. Detection of *Listeria* isolates cultured according to the MFHPB-30 method by indirect ELISA. Bacterial cells grown in Listeria Enrichment Broth (LEB) at 30 ^{o}C for 48 hours and then in Modified Fraser Broth (MFB) at 35 ^{o}C for 24 hours were used in indirect ELISA. A 100 µl formalin-killed bacterial suspension (10^8 cells/ml) was used to coat each well of Maxisorp^{TM} 96-well microtiter plates. Each of the five MAbs (M3686, M3692, M3697, M3699 and M3700) in undiluted tissue culture fluid was used to detect bacterial cells. Error bars are one standard deviation from the mean of three independent experiments. Two replicates were performed in each experiment. The OD_{414} readings obtained from negative controls ranged from 0.03 to 0.2, and thus OD_{414} readings of <0.25, between 0.25 and 0.3, and > 0.3 were considered as negative, weakly positive and positive, respectively. See section 3.15 in Materials and Methods for details.


OD 414nm
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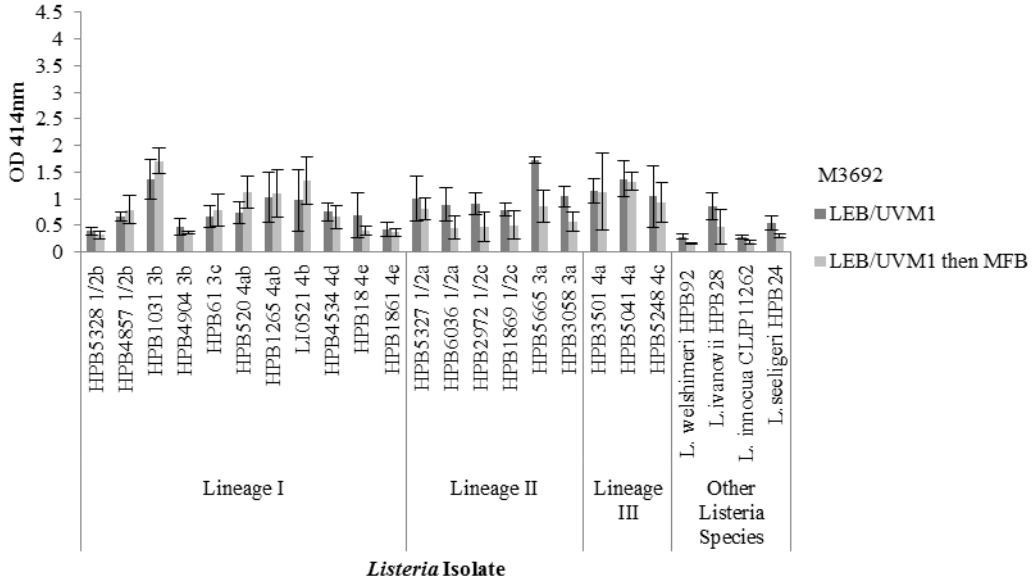
4
3.5
3
2.5
2
1.5
1
0.5
0
M3686
LEB/UVM1
LEB/UVM1 then MFB
HPB5328 1/2b
HPB4857 1/2b
HPB1031 3b
HPB4904 3b
HPB61 3c
HPB520 4ab
HPB1265 4ab
LI0521 4b
HPB4534 4d
HPB18 4e
HPB1861 4e
HPB5327 1/2a
HPB6036 1/2a
HPB2972 1/2c
HPB1869 1/2c
HPB5665 3a
HPB3058 3a
HPB3501 4a
HPB5041 4a
HPB5248 4c
L. welshimeri HPB92
L.ivanowii HPB28
L. innocua CLIP11262
L.seeligeri HPB24
Lineage I
Lineage II
Lineage III
Other Listeria Species
Listeria Isolate


OD 414nm
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4
3.5
3
2.5
2
1.5
1
0.5
0
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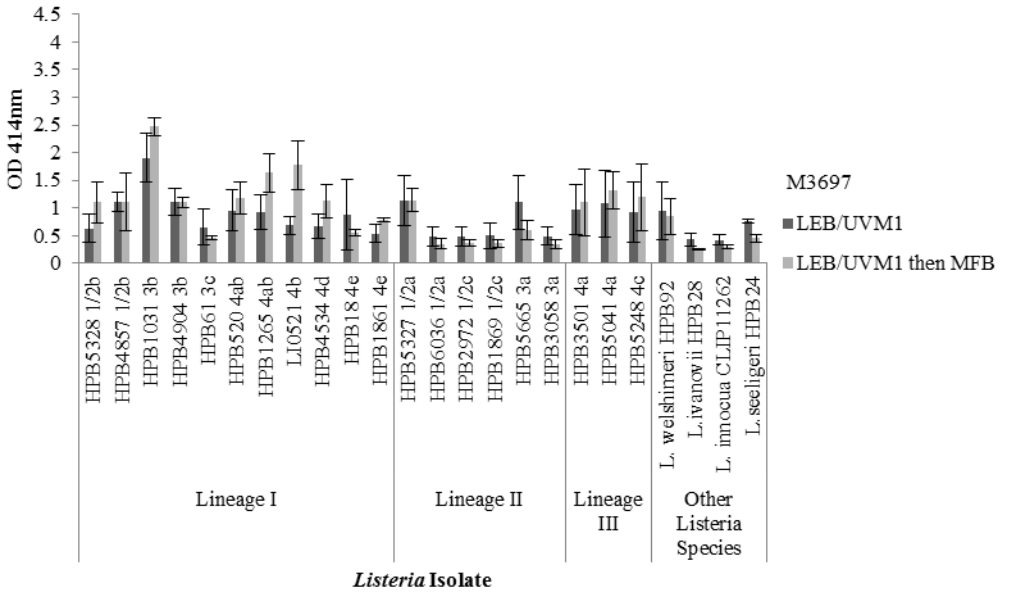
LEB/UVM1
LEB/UVM1 then MFB
HPB5328 1/2b
HPB4857 1/2b
HPB1031 3b
HPB4904 3b
HPB61 3c
HPB520 4ab
HPB1265 4ab
LI0521 4b
HPB4534 4d
HPB18 4e
HPB1861 4e
HPB5327 1/2a
HPB6036 1/2a
HPB2972 1/2c
HPB1869 1/2c
HPB5665 3a
HPB3058 3a
HPB3501 4a
HPB5041 4a
HPB5248 4c
L. welshimeri HPB92
L.ivanowii HPB28
L. innocua CLIP11262
L.seeligeri HPB24
Lineage I
Lineage II
Lineage III
Other Listeria Species
Listeria Isolate


OD 414nm
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4
3.5
3
2.5
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1.5
1
0.5
0
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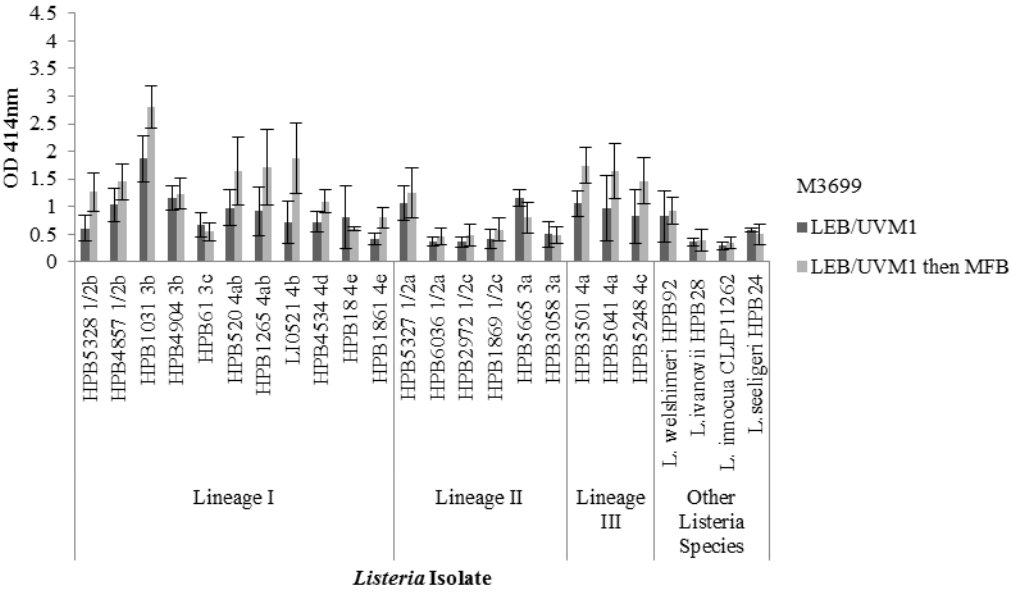
LEB/UVM1 then MFB
HPB5328 1/2b
HPB4857 1/2b
HPB1031 3b
HPB4904 3b
HPB61 3c
HPB520 4ab
HPB1265 4ab
LI0521 4b
HPB4534 4d
HPB18 4e
HPB1861 4e
HPB5327 1/2a
HPB6036 1/2a
HPB2972 1/2c
HPB1869 1/2c
HPB5665 3a
HPB3058 3a
HPB3501 4a
HPB5041 4a
HPB5248 4c
L. welshimeri HPB92
L.ivanowii HPB28
L. innocua CLIP11262
L.seeligeri HPB24
Lineage I
Lineage II
Lineage III
Other Listeria Species
Listeria Isolate


OD 414nm
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4
3.5
3
2.5
2
1.5
1
0.5
0
M3699
LEB/UVM1
LEB/UVM1 then MFB
HPB5328 1/2b
HPB4857 1/2b
HPB1031 3b
HPB4904 3b
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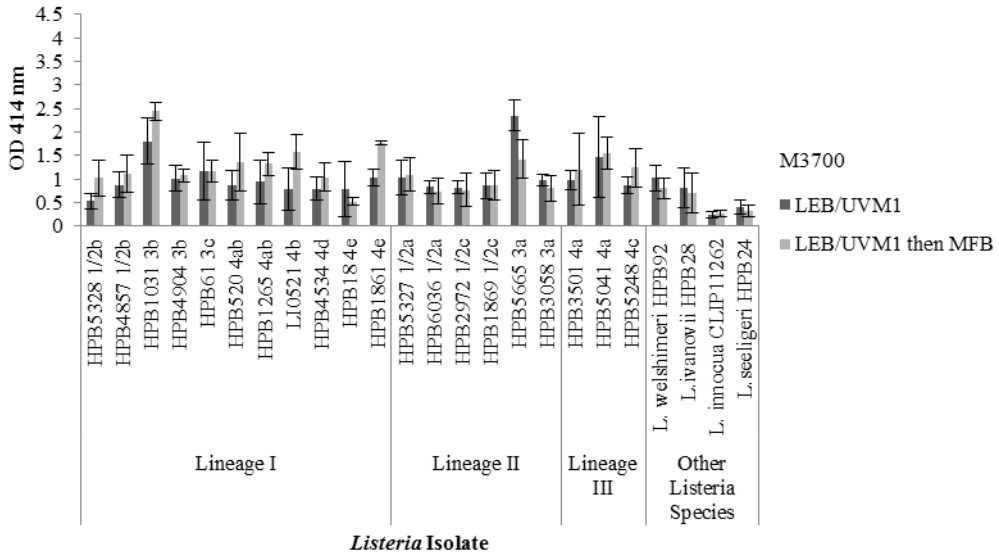
HPB520 4ab
HPB1265 4ab
LI0521 4b
HPB4534 4d
HPB18 4e
HPB1861 4e
HPB5327 1/2a
HPB6036 1/2a
HPB2972 1/2c
HPB1869 1/2c
HPB5665 3a
HPB3058 3a
HPB3501 4a
HPB5041 4a
HPB5248 4c
L. welshimeri HPB92
L.ivanowii HPB28
L. innocua CLIP11262
L.seeligeri HPB24
Lineage I
Lineage II
Lineage III
Other Listeria Species
Listeria Isolate


OD 414 nm
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
M3700
LEB/UVM1
LEB/UVM1 then MFB
HPB5328 1/2b
HPB4857 1/2b
HPB1031 3b
HPB4904 3b
HPB61 3c
HPB520 4ab
HPB1265 4ab
LI0521 4b
HPB4534 4d
HPB18 4e
HPB1861 4e
HPB5327 1/2a
HPB6036 1/2a
HPB2972 1/2c
HPB1869 1/2c
HPB5665 3a
HPB3058 3a
HPB3501 4a
HPB5041 4a
HPB5248 4c
L. welshimeri HPB92
L. ivanovii HPB28
L. innocua CLIP11262
L. seeligeri HPB24
Lineage I
Lineage II
Lineage III
Other Listeria Species
Listeria Isolate

6.2.7 Determination of Transcription Start Site for the *LMOf2365_0148* Gene

The transcription start site of the *LMOf2365_0148* gene was determined to investigate the possible mechanisms by which the gene is regulated. Possible transcriptional regulation elements upstream of the gene that may provide insight into the control of *LMOf2365_0148* expression. Using 5' RACE (see section 3.7 in Materials and Methods for details), the transcription start site (TSS) was mapped to nucleotide "T" which was 60 base pairs from the first base of the start codon. The TSS was determined from two independent experiments (Figure 6-9). Sigma 70 (the primary sigma factor) promoter sequences were found directly upstream of the transcription start site by using the bacterial promoter prediction program BPROM (Softberry Inc.).

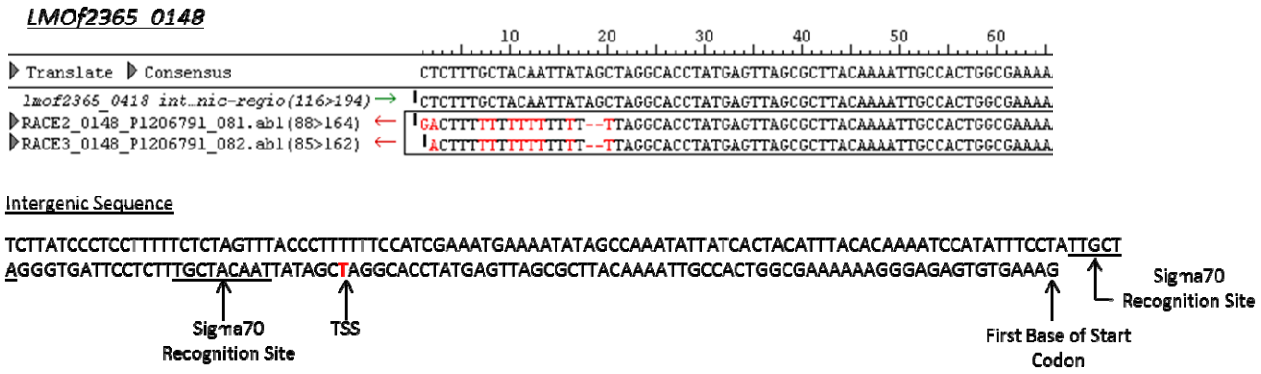


Figure 6-9. Transcription start site of the *LMOf2365_0148* gene. Two independent 5' RACE experiments were performed to determine the transcription start site (TSS). PCR products (RACE2_0148 and RACE3_0148) from 5' RACE were sequenced and aligned to intergenic region upstream of the *LMOf2365_0148* TSS. See section 3.7 in Materials and Methods for details of the 5' RACE experiment.

6.3 Discussion

The purpose of this work was to assess the surface proteins identified in Chapter 5 as potential biomarkers for the isolation and detection of *L. monocytogenes*. Among the various surface proteins identified, six candidates (LMOf2365_0148, LMOf2365_0312, LMOf2365_0546, LMOf2365_1883, LMOf2365_2111 and LMOf2365_2742) were selected. These candidates contained regions which were conserved among *L. monocytogenes* but variable in other *Listeria* species. They were found solely or predominantly in the samples from trypsin-treated *L. monocytogenes* cells and had high Mascot scores with multiple peptides identified. An uncharacterized protein with a LPXTG motif for covalent association to the peptidoglycan, annotated as LMOf2365_0148, was selected due to strong signal of anti-LMOf2365_0148 PAbs binding to the cell surface as observed by immunofluorescence microscopy. MAbs against the LMOf2365_0148 homolog from *L. monocytogenes* strain LI0521 (serotype 4b) (157) reacted to various *L. monocytogenes* isolates grown under standard enrichment culture conditions (MFHPB-07 and MFHPB-30) (107, 146). Of note, MAb M3686 reacted to *L. monocytogenes* isolates from all three lineages and has the potential for use in *L. monocytogenes* isolation and detection from foods and food processing environments.

Of the six candidate proteins selected, LMOf2365_0148, LMOf2365_0546 and LMOf2365_2742 had exposed epitopes on the surface of live *L. monocytogenes* cells as revealed by immunofluorescence microscopy using specific PAbs. The homolog of LMOf2365_0148, lmo0130 was identified in two proteomic screens of proteins associated with the peptidoglycan (168, 187) as well as in the culture supernatant in the *L. monocytogenes* EGD-e strain (170). The homolog of LMOf2365_0546, lmo0517 is a lipoprotein with a putative phosphoglycerate mutase domain that has not previously identified in any proteomic studies. The homolog of

LMOf2365_2742, lmo2754 or PBPD1 has been isolated from the cell membrane (188). Immunofluorescence microscopy with anti-LMOf2365_0148 PAbs revealed significantly stronger signals in comparison to those of anti-LMOf2365_0546 and LMOf2365_2742 PAbs. There are several possible explanations for this observation. One explanation is the differential protein expression of these proteins on *L. monocytogenes*. In addition, the putative covalent linkage of LMOf2365_0148 to the peptidoglycan may contribute to its strong association with the peptidoglycan and allow for its detection after the multiple washing steps involved in immunofluorescence microscopy. Although all six candidates were detected in the whole cell extract, candidate proteins LMOf2365_0312, LMOf2365_1883 and LMOf2365_2111 were not observed on the cell surface by immunofluorescence microscopy of live *L. monocytogenes.* Both LMOf2365_0312 and LMOf2365_2111 have been previously identified in cell culture supernatant (170), suggesting that their association to the cell envelope may be weak. It is also possible that these proteins do not contain surface epitopes readily accessible to antibodies (158). In addition, trypsin is a smaller molecule than antibodies. Surface proteins that are accessible to trypsin may not be accessible to larger proteins such as antibodies. Hence, surface proteins identified by trypsin digest and proteomics may not necessarily be detected by immunofluorescence microscopy using antibodies raised against the recombinant proteins. Trypsin conjugated to agarose have been used by other researchers (189) and may be applied in future proteomic studies of surface proteins on live cells to identify surface proteins that are accessible to larger molecular such as antibodies.

Identification of a transcription start site of *LMOf2365_0148* and two sigma 70 (a general sigma factor) promoter sequences in the intergenic region immediately upstream

LMOf2365_0148 indicated that *LMOf2365_0148* expression is under the direction of its own promoter.

ELISA analysis of MAbs to rLMOf2365_0148 (M3686, M3692, M3697, M3699 and M3700) using 53 *L. monocytogenes* isolates, 10 isolates from five other *Listeria* species and four other foodborne pathogens (*C. jejuni*, *Salmonella enterica* serovar Typhimurium, *E. coli* O157:H7 and *B. cereus*) revealed that *L. monocytogenes* isolates such as LI0586 (serotype 1/2b), HPB1031 (serotype 3b), LI0521 (serotype 4b), HPB18 (serotype 4e), LI0527 (serotype 1/2a) and LI0508 (serotype 3a) were the most reactive to all MAbs tested. No apparent relationship was observed between these reactive isolates. In previous studies, certain MAbs were found to be specific for a serotype (19) or lineages (MAbs to rLMOf2365_0639) while other MAbs showed sporadic reactivity (24). Further research is needed to decipher possible commonalities between isolates that were strongly reactive to the MAbs developed here.

The reactivity of anti-LMOf2365_0148 MAbs to *L. monocytogenes* and *Listeria* species isolates cultured in primary and secondary selective enrichment conditions of MFHPB-07 and MFHPB-30 was tested. MAbs M3686, M3692 and M3700 reacted to *L. monocytogenes* isolates belonging to all three lineages with M3686 exhibiting higher OD_{414} readings than other MAbs. MAbs M3697, M3699 were reactive to lineage I and III isolates but were weakly or not reactive to lineage II isolates. Similarly, M3686 reacted to the most lineage II isolates in the non-selective culture, BHI, while M3692, M3697, M3699 and M3700 were weakly or not reactive to the same isolates. The reactivity of M3686 with more *L. monocytogenes* isolates may be due to its higher affinity than the other MAbs examined and/or surface exposure of its epitope. In addition, the tissue culture fluid (TCF) of M3686 may also have a higher concentration of IgG than other TCF preparations.

Since selective enrichment cultures are necessary prior to detection by ELISA, the expression of LMOf2365_0148 in standard selective enrichment cultures makes LMOf2365_0148 a good biomarker for *L. monocytogenes* detection using ELISA. With the exception of one serotype 4e isolate, M3686 was reactive to all *L. monocytogenes* isolates grown in both primary and secondary selective enrichment cultures of MFHPB-07. Moreover, M3686 was specific to *L. monocytogenes* and did not react with the four other *Listeria* species tested. These results suggest that M3686 may be applied to *L. monocytogenes* detection by ELISA after selective enrichments cultures according to the MFHPB-07 method. In selective enrichments cultures of MFHPB-30, M3686 was less specific to *L. monocytogenes* as the MAb was reactive to *L. innocua* in secondary selective enrichment culture and *L. seeligeri* in primary and secondary selective enrichment cultures. For specific detection of *L. monocytogenes* by ELISA using culture prepared according to the MFHPB-30 procedure, M3686 may need to be used in conjunction with a different MAb. A MAb such as M3643 against LMOf2365_0639 (described in Chapter 4) may be used in combination with M3686. M3643 reacted to all three lineages of *L. monocytogenes* and had reduced reactivity to *L. innocua.* M3643 and M3686 used together in a sandwich ELISA may allow for specific *L. monocytogenes* detection. Nonetheless, MAbs reactive to *Listeria* species can be applicable to surveillance of food processing environments since the detection of *Listeria* species other than *L. monocytogenes* warrants sanitation of the food processing plant (8).

The ability of the MAbs to detect *L. monocytogenes* grown in standard selective enrichment cultures is a desirable quality. No or limited reactivity of previously developed MAbs to *L. monocytogenes* cultured in UVM (equivalent to UVM1 in MFHPB-07) and FB (equivalent to MFB in MFHPB-30) broths (26-28) was observed. In contrast, the MAbs developed in this

study reacted similarly with isolates grown in primary and secondary selective enrichment cultures. Specifically, MAb M3686 reacted to isolates belonging to all three lineages of *L. monocytogenes*. This suggests that M3686 can be applied to *L. monocytogenes* detection by ELISA following selective enrichment culture.

Chapter 7: *L. monocytogenes* Capture Using Immunomagnetic Separation with Monoclonal Antibodies

7.1 Introduction

L. monocytogenes infection in susceptive individuals often results in a fatality rate of about 30% (1). This pathogen is widely distributed in the environment (5, 6) and can easily contaminate food processing environments and foods (7). Ready-to-Eat foods that can sustain the growth of *L. monocytogenes* pose a serious health hazard because these foods require no heating and *L. monocytogenes* can multiply in these foods at refrigeration temperatures. Due to the serious consequences of *L. monocytogenes* infection and the fact that *L. monocytogenes* cannot be eliminated in the environment, a continuous effort is needed to prevent *L. monocytogenes* from contaminating in foods and to identify the presence of this organism in foods.

Although advances in molecular methods such as PCR have been made to faciliate *L. monocytogenes* detection, these methods still rely on the purity of DNA or RNA templates. PCR inhibitors from the food matrix (13) and selective enrichment cultures (14) as well as genetic materials from non-target bacteria (15) in samples can confound test results of molecular methods. Hence sample preparation is the bottleneck of rapid *L. monocytogenes* detection by molecular detection methods. Antibody-based isolation of foodborne pathogens such as monoclonal antibodies coated on magnetic beads offers a means of capturing *Listeria* bacteria from complex food matrices. Advantages of using immunomagnetic separation (IMS) include: concentration of targeted viable bacteria thereby reducing culture time prior to detection, and removal of inhibitory factors and background microflora that may confound molecular test results.

Reports have documented the development of MAbs against surface proteins for *L. monocytogenes* isolation (131, 132). Nonetheless, there are currently no *L. monocytogenes* specific MAb that recognize various serotypes for pathogen isolation.

The aim of this work was to identify and characterize MAbs capable of catpuring *L. monocytogenes* specifically. This involved screening MAbs developed against surface proteins LMOf2365_0639 and LMOf2365_0148 for *L. monocytogenes* capture ability. The MAbs with the best capture ability were further evaluated for their ability to capture seven serotypes of *L. monocytogenes*, five other *Listeria* species and two other foodborne pathogens. The capture ability of the MAbs was also tested with *L. monocytogenes* grown in selective enrichment cultures according to MFHPB-07 and MFHPB-30 methods. In addition, epitope mapping and kinetic characterization of several selected MAbs were performed.

7.2 Results

7.2.1 Determining of the Limit of Detection and Capture Efficiency of the Best MAbs for *L. monocytogenes* Isolation

A preliminary screen was performed for the capture ability of all MAbs against both rLMOf2365_0639 and rLMOf236_0418 proteins (method described in Materials and Methods section 3.16). Of the 35 hybridoma clones against rLMOf2365_0639, MAb M3644 exhibited capture ability. Of the 24 hybridoma clones against rLMOf2365_0148, 16 MAbs exhibited capture ability. Four of 16 MAbs to rLMOf2365_0148 that captured the most cells and MAb M3644 to rLMOf2365_0639 were selected and assessed for their limits of detection and capture efficiencies. To this end, indirect IMS in which cells are incubated with antibodies prior to addition of beads coated with anti-mouse secondary antibodies was performed. MAbs in the

form of tissue culture fluid (TCF) were used. To avoid cell loss due to washing, the antibody solution was not removed once added to the cells. Theoretically, both insufficient and excessive amounts of MAb can reduce capture capacity. An excess of free MAb can compete with MAbs in complex with the pathogen for binding to secondary antibodies coated on beads. Hence a series of different TCF dilutions (1:200, 1:100, 1:50, 1:10, 1:5 and 1:2) were assessed to find the dilution that resulted in the most cells captured. A dilution of 1:10 for M3644 and a dilution of 1:100 for M3686, M3697, M3699 and M3700 were found to be the optimal dilutions for IMS.

Using live *L. monocytogenes* strain LI0521 (serotype 4b) cells for IMS, MAb M3644 against rLMOf2365_0639 had a capture limit of 10^4 cells with a capture efficiency of 0.2%, at a concentration of 10^4 cells/ml (Table 7-1). MAbs M3686, M3697, M3699 and M3700 against rLMOf2365_0148 had a capture limit of 10^3 cells (Table 7-1). Capture efficiencies for M3686, M3697, M3699 and M3700 were 3.8, 2.7, 1.7 and 2.3% respectively at 10^3 cells/ml (Table 7-1). With a concentration of 10^4 cells/ml, similar capture efficiencies were observed for M3686, M3697, M3699 and M3700 at 3.7, 2.6, 2.0 and 2.8 % respectively (Table 7-1). MAbs M3686, M3697, M3699 and M3700 had the highest capture efficiencies and the lowest capture limit and were selected for further evaluation.

Table 7-1. Capture Efficiency at Various Input Cell Numbers for five selected MAbs.

MAb	Input Cell Number[a]	Average Number of Colonies Captured	Capture Efficiency (%)
M1169	10^4	0.3	0.003
	10^3	0.3	0.03
	10^2	0.0	0.00
M3644	10^4	21.0	0.2
	10^3	1.3	0.1
	10^2	0.3	0.3
M3686	10^4	372.5	3.7
	10^3	37.5	3.8
	10^2	2.8	2.8
M3697	10^4	260.3	2.6
	10^3	26.5	2.7
	10^2	0.8	0.8
M3699	10^4	195.3	2.0
	10^3	16.8	1.7
	10^2	2.8	2.8
M3700	10^4	282.8	2.8
	10^3	22.8	2.3
	10^2	3.5	3.5

[a] Capture was performed using *L. monocytogenes* strain LI0521 (serotype 4b) at three different cell numbers in 1 ml PBS (see section 3.17 in Materials and Methods for details) with M3644 against rLMOf2365_0639 and MAbs M3686, M3697, M3699 and M3700 against rLMOf2365_0148. The irrelevant MAb M1169 against *C. jejuni* was used as a negative control. Three independent experiments with two replicate each time were performed to obtain the average number of colonies captured. A positive result in IMS is >5 CFUs captured.

7.2.2 Determination of the Ability of Selected MAbs to Capture *L. monocytogenes* Serotype: 1/2a, 1/2b, 1/2c, 3a, 4a and 4d cells

Since other *L. monocytogenes* serotypes in addition to serotype 4b (lineage I) have been involved in listeriosis, the ability of the selected MAbs to capture other serotypes from three lineages (I, II and III) of *L. monocytogenes* was investigated. Specifically, serotype 1/2b (lineage I), serotypes 1/2a, 1/2b and 3a (lineage II) and serotypes 4a and 4d (lineage III) were tested in the capture experiments. MAbs 3686 and M3700 captured cells of all *L. monocytogenes* serotypes tested (Table 7-2). M3697 captured *L. monocytogenes* serotype 4a and 1/2b cells (Table 7-2). M3699 only captured *L. monocytogenes* serotype 4a cells (Table 7-2).

Table 7-2. Capture of various *L monocytogenes* serotypes by selected MAbs.

MAb	*L. monocytogenes* Serotype[a]	Average Number of Colonies Captured	Capture Efficiency (%)
M1169	1/2a	0	0
	1/2b	0	0
	1/2c	0.2	0.002
	3a	0.3	0.003
	4a	0	0
	4d	0	0
M3686	1/2a	24.3	0.2
	1/2b	31.3	0.3
	1/2c	14.8	0.1
	3a	10.3	0.1
	4a	71.3	0.7
	4d	15.8	0.2
M3697	1/2a	0.3	0.003
	1/2b	5.8	0.06
	1/2c	0	0
	3a	0	0
	4a	32.5	0.3
	4d	3.0	0.03
M3699	1/2a	0.3	0.003
	1/2b	0.3	0.003
	1/2c	0.3	0.003
	3a	0.5	0.005
	4a	34.3	0.3
	4d	1.3	0.01
M3700	1/2a	24.8	0.2
	1/2b	30.0	0.3
	1/2c	16.5	0.2
	3a	6.75	0.07
	4a	66.5	0.7
	4d	16.5	0.2

[a] Six additional serotypes (1/2a, 1/2b, 1/2c, 3a, 4a and 4d) besides serotype 4b of *L. monocytogenes* were used to assess the capture ability of M3686, M3697, M3699 and M3700. Capture was performed using 10^4cell in 1ml PBS (see section 3.18 in Materials and Methods for details). The irrelevant MAb M1169 against *C. jejuni* was used as a negative control. Two independent experiments with two replicate each time were performed to obtain the average

number colonies captured. See Table 3-7 in Materials and Methods for isolate names. A positive result in IMS is >5 CFUs captured.

7.2.3 Determination of the Capture Specificity of Selected MAbs

Since the selective enrichment culture routinely used in *L. monocytogenes* detection indiscriminately enriches all members of the *Listeria* genus, MAbs that are specific to *L. monocytogenes* would facilitate downstream detection by concentrating the pathogen from selective enrichment culture. The capture specificity of MAbs M3686, M3697, M3699 and M3700 was evaluated with other *Listeria* species (*L. seeligeri, L.innocua, L. ivanovii, L. grayi* and *L. welshimeri*) and two other Gram-negative foodborne pathogens (*Salmonella enterica* serovar Typhimurium and *E. coli* O157:H7). MAbs M3686 and M3699 were unable to capture *L. seeligeri, L.innocua, L. ivanovii, L. grayi* and *L. welshimeri* as well as *Salmonella enterica* serovar Typhimurium and *E. coli* O157:H7 (Tables 7-3 and 7-4). MAbs M3697 and M3700 were unable to capture *L. seeligeri, L.innocua, L. ivanovii, L. grayi, Salmonella enterica* serovar Typhimurium and *E. coli* O157:H7 but were able to capture *L. welshimeri* (Tables 7-3 and 7-4).

Table 7-3. Capture ability of MAbs M3686, M3697, M3699 and M3700 to other *Listeria* species.

Listeria Species	MAb	Average Number of Colonies Captured[a]	Capture Efficiency (%)
L. seeligeri	M1169	0	0
	M3686	0	0
	M3697	2.8	0.03
	M3699	0	0
	M3700	0	0
L. innocua	M1169	0	0
	M3686	0	0
	M3697	0	0
	M3699	0	0
	M3700	0	0
L. ivanovii	M1169	0	0
	M3686	0	0
	M3697	0	0
	M3699	0	0
	M3700	0	0
L. grayi	M1169	0.3	0.003
	M3686	0	0
	M3697	0.3	0.003
	M3699	0.3	0.003
	M3700	0	0
L. welshimeri	M1169	0	0
	M3686	0	0
	M3697	22.8	0.2
	M3699	0.5	0.005
	M3700	25	0.3

[a] Capture was performed using 10^4 cell in 1 ml PBS (see section 3.18 in Materials and Methods for details). The irrelevant MAb M1169 against *C. jejuni* was used as a negative control. Two independent experiments with two replicate each time were performed to obtain the average number colonies captured. See Table 3-6 in Materials and Methods for isolate names. A positive result in IMS is >5 CFUs captured.

Table 7-4. Capture ability of MAbs M3686, M3697, M3699 and M3700 to *Salmonella enterica* serovar Typhimurium and *E.coli* O157:H7.

Bacteria	MAb	Average Number of Colonies Captured[a]	Capture Efficiency (%)
Salmonella enterica serovar Typhimurium	M1169	3.25	0.0325
	M3686	2	0.02
	M3697	2	0.02
	M3699	2.5	0.025
	M3700	1.25	0.0125
E.coli O157:H7	M1169	0	0
	M3686	0	0
	M3697	0.5	0.005
	M3699	0.25	0.0025
	M3700	0.25	0.0025

[a] Capture was performed using 10^4cell in 1ml PBS (see section 3.18 in Materials and Methods for details). The irrelevant MAb M1169 against *C. jejuni* was used as a negative control. Two independent experiments with two replicate each time were performed to obtain the average number colonies captured. A positive result in IMS is >5 CFUs captured.

7.2.4 Isolation of *L. monocytogenes* from a Mixture Containing *L. innocua* and *L. monocytogenes* cells

One purpose of IMS is to isolate target cells in the presence of other non-target cells. Hence, the ability of MAbs M3686, M3697, M3699 and M3700 to separate *L. monocytogenes* from a mixture which contained excessive non-target *L. innocua* was studied. *L. monocytogenes* cells were mixed with *L. innocua* cells at ratios of 1:10 and 1:100. Colony blot immunoassays using *L. monocytogenes* specific antibody (PAbs to rLMOf2365_0639) were performed to assess the proportion of *L. monocytogenes* cells recovered from a mixture of *L. monocytogenes* and *L. innocua* cells (Figure 7-1). If the MAbs were not specific to *L. monocytogenes* cells, then the proportion of captured cells for each *Listeria* species would be similar as the initial proportion. Following IMS and plating of captured cells, a colony blot immunoassay of resulting colonies using PAbs (against rLMOf2365_0639) was performed to determine the percentage of *L. monocytogenes* out of the total cells captured. As a negative control for the specificity of colony blot immunoassay for *L. monocytogenes*, approximately 100 CFUs of *L. innocua* cells were plated and detected by colony blot immunoassay using *L. monocytogenes* specific PAbs. Almost no *L. innocua* colonies were detected by after colony blot immunoassay (Figure 7-1).

At a ratio of 1:10 (*L. monocytogenes*: *L. innocua*), 75.0, 73.3, 54.4 and 82.8% of the cells captured by MAbs M3686, M3697, M3699 and M3700 were *L. monocytogenes*, respectively; at a ratio of 1:100 (*L. monocytogenes*: *L. innocua*), 52.3, 72.3, 63.6 and 75.1% of the cells captured were *L. monocytogenes*. Of note, a small proportion of colonies at the edge of plates were not lifted onto the membrane for colony blot immunoassay due to the smaller size of the membrane. Therefore the percentage of *L. monocytogenes* out of the cells captured may be under-estimated.

Nonetheless, percentages of captured *L. monocytogenes* cells were well above 10% and 1% of *L. monocytogenes* present in the mixture containing *L. innocua*.

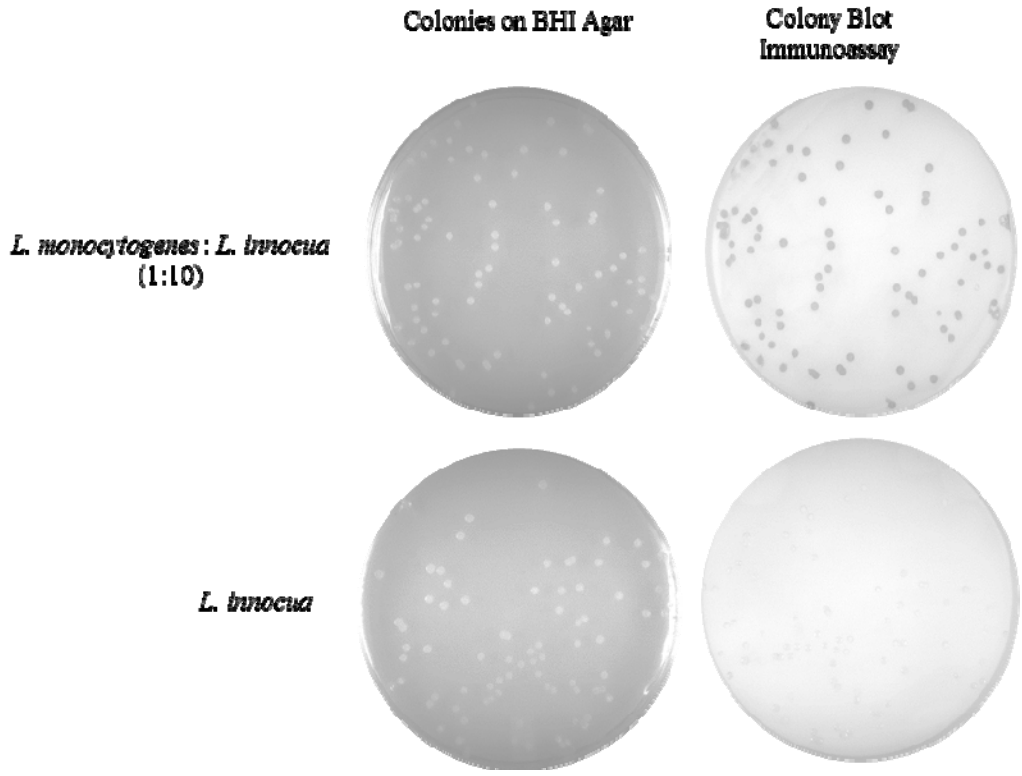


Figure 7-1. Example colony blot immunoassay after immunomagnetic separation of *L. monocytogenes* from a mixture of *L. monocytogenes* and *L. innocua* using MAb M3697. One ml a mixture of *L. monocytogenes* strain LI0521 ($5x10^3$ cells) and *L. innocua* ($5x10^4$ cells) at a ratio of 1:10 respectively was incubated with M3697 for IMS. Captured cells were plated and detected by colony blot immunoassays as described in section 3.19 of Materials and Methods using *L. monocytogenes* specific PAb raised against rLMOf2365_0639. As a negative control, approximately 100 CFUs of *L. innocua* was plated and detected similarly using *L. monocytogenes* specific PAb.

7.2.5 Capture of *L. monocytogenes* cultured in Selective Enrichment Media

The trace quantity of *L. monocytogenes* that may exist in food and environmental samples must be amplified and enriched in selective enrichment cultures to detectable levels prior to *L. monocytogenes* detection. MFHPB-07 (146) and MFHPB-30 (107) are standard Canadian selective enrichment culture methods for *Listeria* isolation and detection. To expedite *L. monocytogenes* isolation and to facilitate downstream pathogen detection by molecular methods, it is possible to capture *L. monocytogenes* in primary selective enrichment culture using IMS. *L. monocytogenes* strain LI0521 (serotype 4b) was grown in primary selective enrichment culture of Palcam or LEB/UVM1 according to MFHPB-07 and MFHPB-30, respectively.

The IMS procedure for selective enrichment culture of *L. monocytogenes* was optimized in preliminary experiments. Due to variability associated with inoculation of one CFU (in 1 ml) of *L. monocytogenes* into 224 ml of Palcam or LEB/UVM1, approximately five CFUs were used for inoculation. At an inoculum of five CFUs in 224 ml of Palcam culture at 35 ^{o}C, a concentration of 10^5 – 10^6 CFU/ml was reached after 20-21 hours. To minimize or eliminate possible inhibitory effects of salts in selective enrichment culture, the culture was diluted with PBS prior to IMS. Two, five, ten and one hundred fold dilutions of the selective enrichment culture were tested. Five fold dilution resulted in the most cells captured therefore this dilution was adopted for subsequent experiments.

Twenty to twenty-one hours was determined as the minimum culture time for the IMS method used here (Table 7-5). At an inoculum of five CFUs in 224 ml of UVM1/LEB medium at 30 ^{o}C, a cell concentration of 10^5 CFU/ml was reached after 30 hours. Similarly, the culture was diluted five times with PBS prior to IMS. Thirty hours in UVM1/LEB was determined as the minimum culture time for the IMS method described here (Table 7-5).

Table 7-5. Capture ability of MAbs M3686, M3697, M3699 and M3700 to *L. monocytogenes* after abbreviated culture in selective enrichment media.

	Average Number of Colonies Captured[a]	
MAb	Palcam (20-21 hours)	LEB/UVM1 (30 hours)
M1169	3.25	0
M3686	114.75	48
M3697	24	9
M3699	87.5	9.25
M3700	107.25	42

[a] Approximately five CFUs of *L. monocytogenes* (strain LI0521 serotype 4b) were inoculated in 224ml of either Palcam (primary selective enrichment broth for MFHPB-07) or LEB/UVM1 (primary selective enrichment broth for MFHPB-30) and incubated for 20-21 hours or 30 hours respectively. Average number of colonies captured was obtained from two immunomagnetic separation experiments from two cultures inoculated together. See section 3.20 in Materials and Methods for details. The irrelevant MAb M1169 against *C. jejuni* was used as a negative control. A positive result in IMS is >5 CFUs captured.

7.2.6 Determination of Dissociation Constants of Monoclonal Antibodies

Since MAb affinity may be important in its capture ability, the equilibrium dissociation constants (K_D) of MAbs M3686, M3697, M3699, M3700, M3644 and M3692 were determined by surface plasmon resonance (SPR). The dissociation constant measured is the reciprocal of the affinity constant. MAbs M3686, M3697, M3699 and M3700 were assessed due to their ability for *L. monocytogenes* capture. MAb M3692 was initially selected because it was among the five characterized MAbs (M3686, M3692, M3697, M3699 and M3700) tested for reactivity against a range of *L. monocytogenes* and other *Listeria* species grown in non-selective and selective enrichment cultures (Chapter 6). MAb M3644 was selected because it was the only MAb against LMOf2365_0639 capable of *L. monocytogenes* capture.

Preliminary size exclusion chromatography of rLMOf2365_0148 and rLMOf2365_0639 revealed that both proteins were not monomers in solution (Figures 7-2 and 7-3). The elution volume of chymotrypsinogen was higher than ribonuclease, although chymotrypsinogen has a larger molecular weight (Figures 7-2 and 7-3). For the calculation of the molecular weights of rLMOf2365_0148 and rLMOf2365_0639 by size exclusion, only albumin, aldolase, ribonuclease A and ovalbumin were used (Figures 7-4 and 7-5). The apparent molecular mass of LMOf2365_0639 is 143.3 kDa while the theoretical molecular mass is 55.7 kDa. The apparent molecular mass of LMOf2365_0148 is 160.3 kDa while the theoretical molecular mass is 76.16 kDa. These results suggested that both LMOf2365_0639 and LMOf2365_0148 are not monomers in solution and cannot be used as the analyte for SPR. Hence rLMOf2365_0148 and rLMOf2365_0639 were immobilized on the dextran matrix of the sensochip while fragment antigen binding (Fabs) fragments, made from purified IgG, served as the monomeric analyte.

With the assistance of Dr. Tanha (NRC), the molecular weights of LMOf2365_0639 and LMOf2365_0148 were more accurately determined using multi-angle light scattering (MALS). The average molecular mass of LMOf2365_0639 in solution was determined to be 53.23 kDa by MALS from two independent experiments. This determined molecular mass was close to the molecular mass of 55.71 kDa deduced from the amino acid sequence. Likewise using MALS, the molecular mass of LMOf2365_0148 was determined as 57.45 kDa which is less than the molecular mass of 76.16 kDa deduced from the amino acid sequence. These more accurate measurements with MALS indicated that both LMOf2365_0639 and LMOf2365_0148 were monomers in solution. Nonetheless, the experimental design of the SPR analysis for dissociation constant determination is sound regardless of whether the purified antigens are monomeric or multimeric.

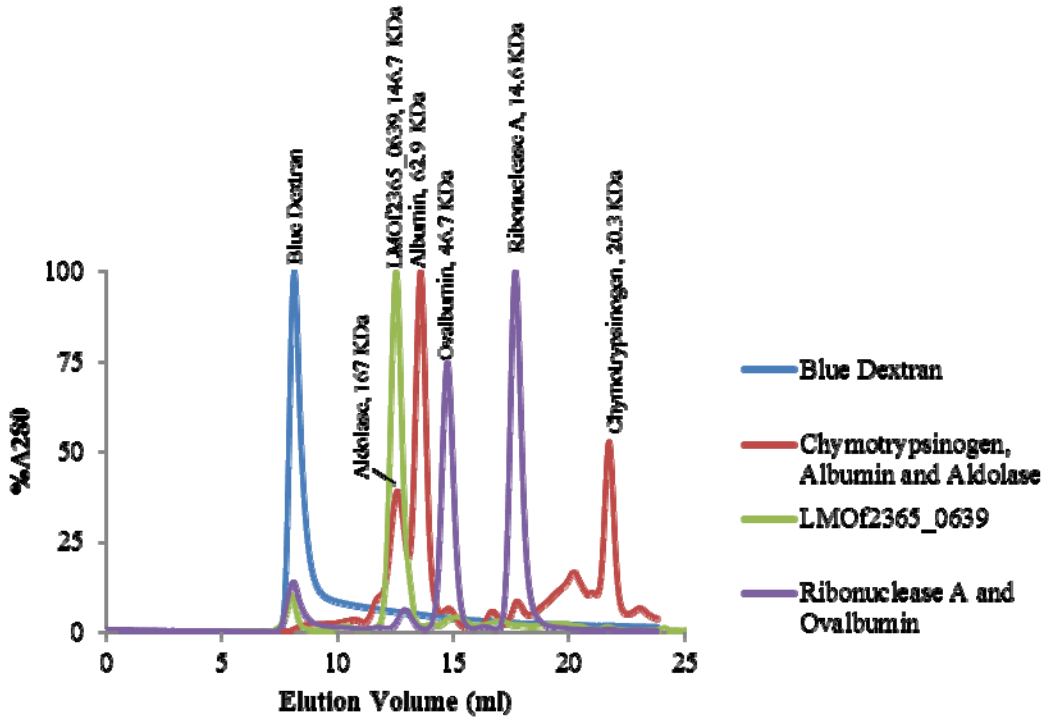


Figure 7-2. Size exclusion chromatography of LMOf2365_0639. LMOf2365_0639 protein (represented by green line) was purified by size exclusion chromatography on a Superdex 200 column (10 x 300 mm). The column was calibrated with commercial globular standards (chymotrypsinogen, albumin, aldolase, ribonuclease A and ovalbumin) to determine the molecular size of LMOf2365_0639 protein in solution. Proteins were separated at a flow rate of 0.5 ml/min in PBS. The elution volume of blue dextran indicates the void volume. The data is plotted as % A280 [(absorbance- minimum absorbance)/maximum absorbance x 100] against the elution volume (ml). Refer to Materials and Methods, section 3.21 for experimental details.

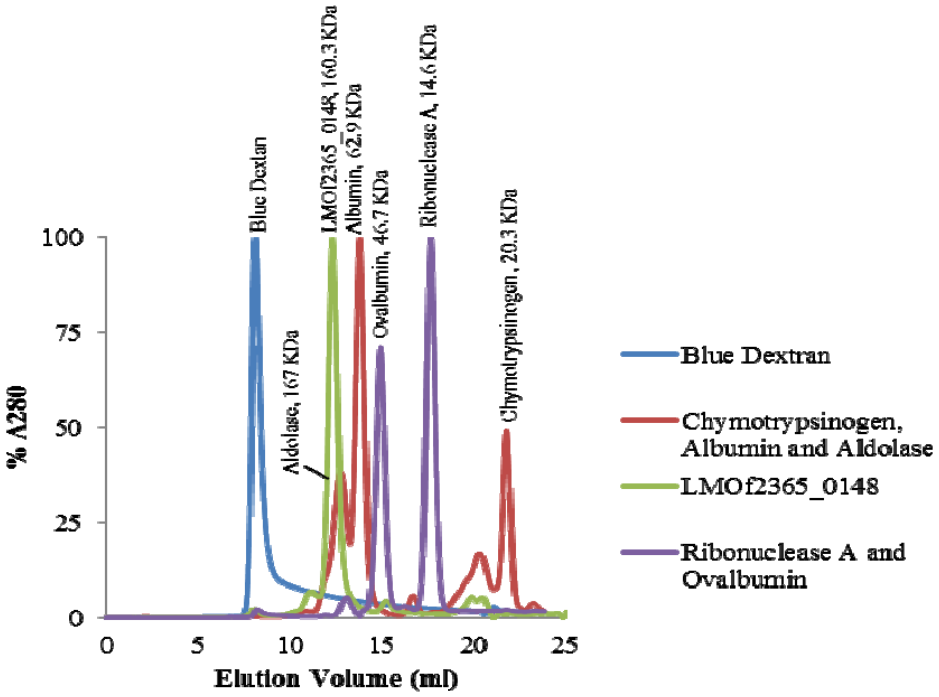


Figure 7-3. Size exclusion chromatography of LMOf2365_0148. LMOf2365_0148 protein (represented by green line) was purified by size exclusion chromatography on a Superdex 200 column (10 x 300 mm). The column was calibrated with commercial globular standards (chymotrypsinogen, albumin, aldolase, ribonuclease A and ovalbumin) to determine the molecular size of LMOf2365_0148 protein in solution. Proteins were separated at a flow rate of 0.5 ml/min in PBS. The elution volume of blue dextran indicates the void volume. The data is plotted as % A280 [(absorbance- minimum absorbance)/maximum absorbance x 100] against the elution volume (ml). Refer to Materials and Methods, section 3.21 for experimental details.

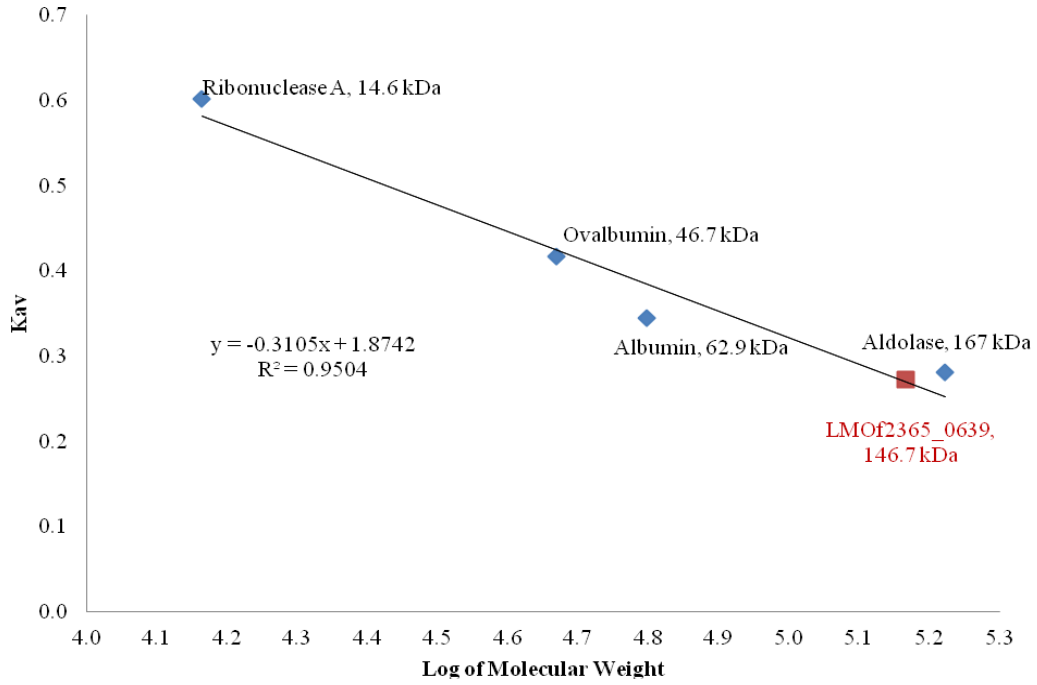


Figure 7-4. Determination of the apparent molecular mass of LMOf2365_0639 using size exclusion chromatography and protein standards. A plot of Kav versus the log of the molecular weights for ribonuclease A, ovalbumin, albumin and aldolase based on which the molecular weight of LMOf2365_0639 is calculated to be 146.7 kDa from a Kav of 0.27.

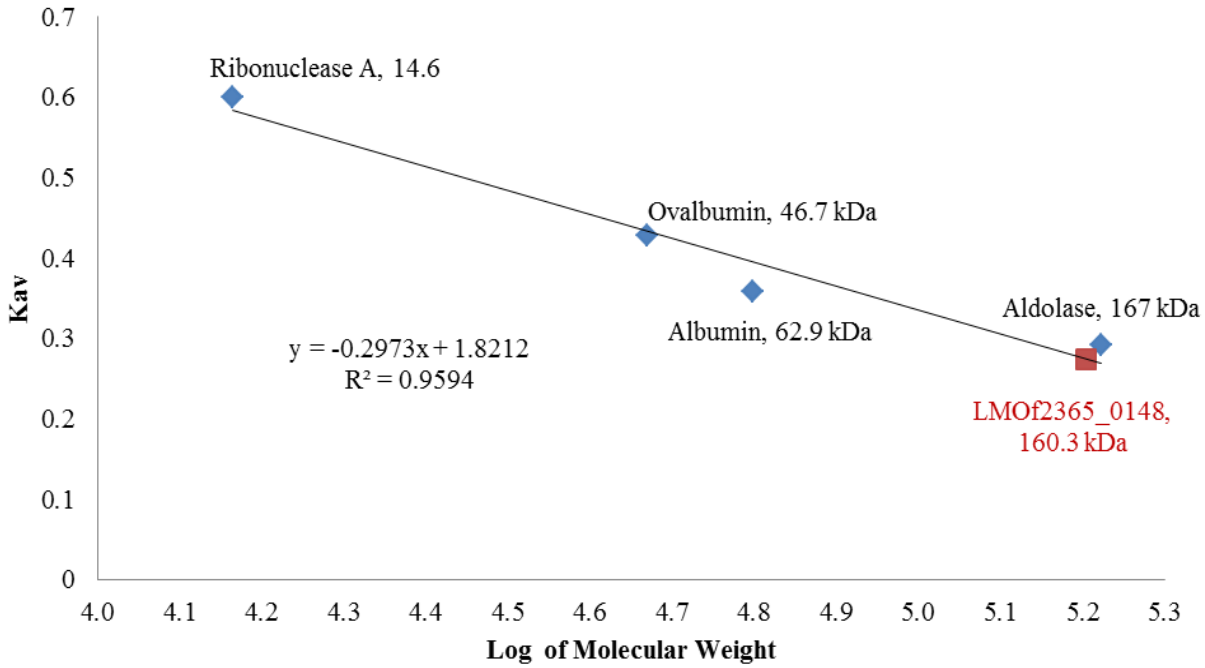


Figure 7-5. Determination of the apparent molecular mass of LMOf2365_0148 using size exclusion chromatography and protein standards. A plot of Kav versus the log of the molecular weights for ribonuclease A, ovalbumin, albumin and aldolase based on which the molecular weight of LMOf2365_0148 is calculated to be 160.3 kDa from a Kav of 0.27.

Since the preliminary results from size exclusion chromatography experiments suggested that LMOf2365_0639 and LMOf2365_0639 are not monomers in solution, rLMOf2365_0148 or rLMOf2365_0639 was therefore immobilized on the dextran matrix of the sensorchip while fragment antigen binding (Fab) fragments, made from purified IgG, were injected as the monomer analyte over the immobilized recombinant protein allowing fitting of SPR data to a 1:1 interaction model. The MAbs IgG was purified from tissue culture fluid (TCF). The TCF contained bovine IgG in addition to antigen specific IgG secreted by the mouse hybridoma clone. To ensure the purity of IgG used for SPR analysis, affinity chromatography was performed to purify M3692, M3697, M3699 and M3700 using a column of LMOf2365_0148 conjugated agarose (Figure 7-6). M644 was purified using a column of protein L conjugated agarose. Protein L binds κ light chain of M3644 but has no affinity for bovine IgG in the TCF. Fabs were generated by digestion of purified IgG with commercial ficin resin and purified using a commercial protein A spin column (Figure 7-7). Undigested IgG or Fc fragments can be removed using protein A column as evident in the protein A column elution (Figure 7-7). The flow-through containing purified Fab fragments from the protein A column was assessed for purity. Western blot using Fc fragment specific antibodies reveal no Fc fragments in the purified Fab preparation (Figure 7-7). Western blot using light chains specific antibody showed the presence of Fab (Figure 7-7). Immediately prior to SPR analysis, size exclusion chromatography was performed on purified Fabs. Only peak fractions were collected for the SPR analysis (Figure 7-8).

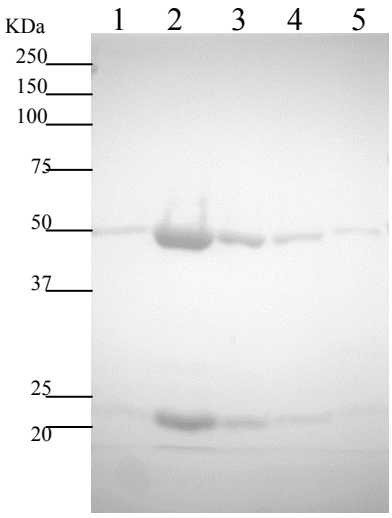


Figure 7-6. SDS-PAGE Analysis of IgG purified from tissue culture fluid. Purification of M3697 IgG from TCF is illustrated here as an example. IgGs were purified from TCF by affinity chromatography on a column of LMOf2365_0148 conjugated agarose. Lanes 1 to 5 show eluted IgG samples from fraction numbers 6 to 10 collected during chromatography, respectively. Refer to section 3.22 in Materials and Methods for experimental details on IgG purification.

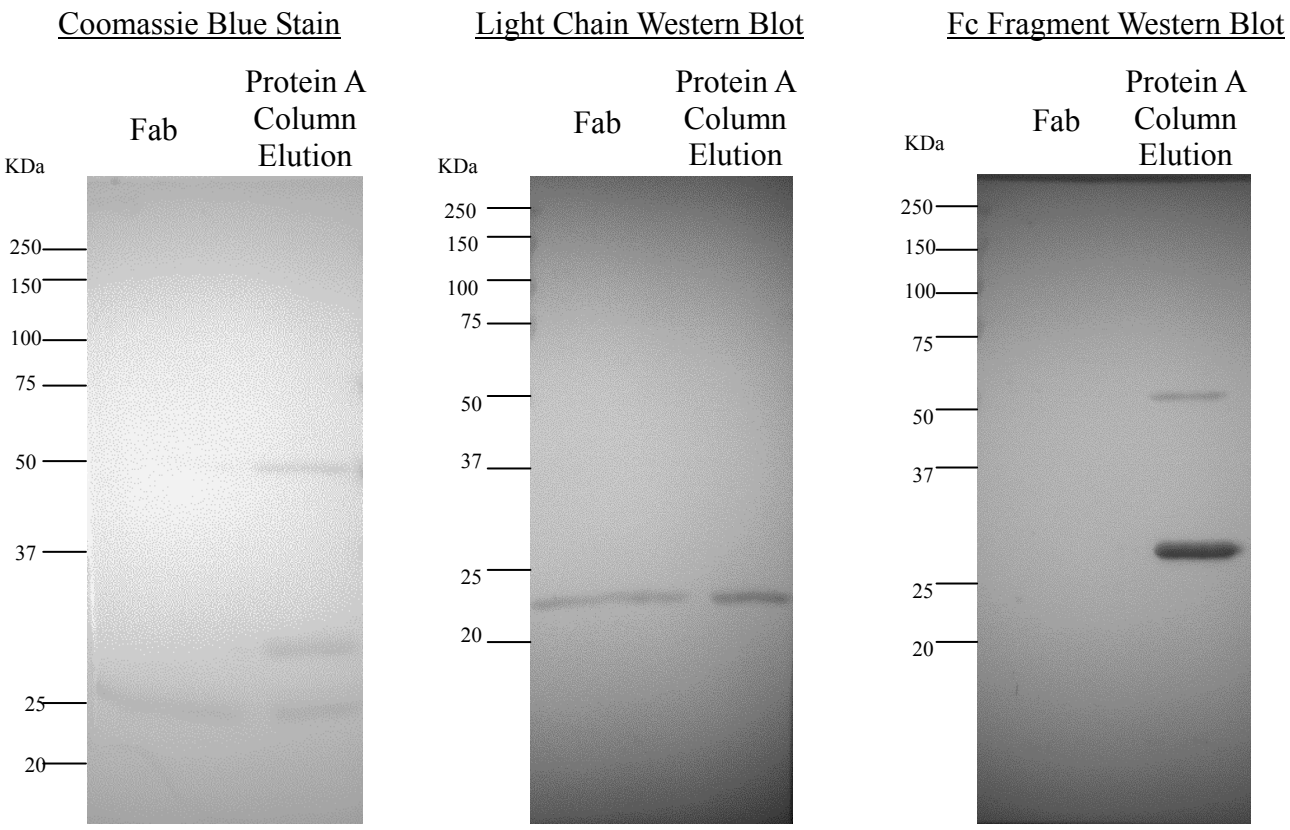


Figure 7-7. Fab of M3697 IgG purified from protein A agarose. After IgG digestion, Fab was purified using protein A. Samples of purified Fab and eluate from protein A, after Fab purification, were analyzed by Coomassie blue stain and by light chain and Fc fragment western blots.

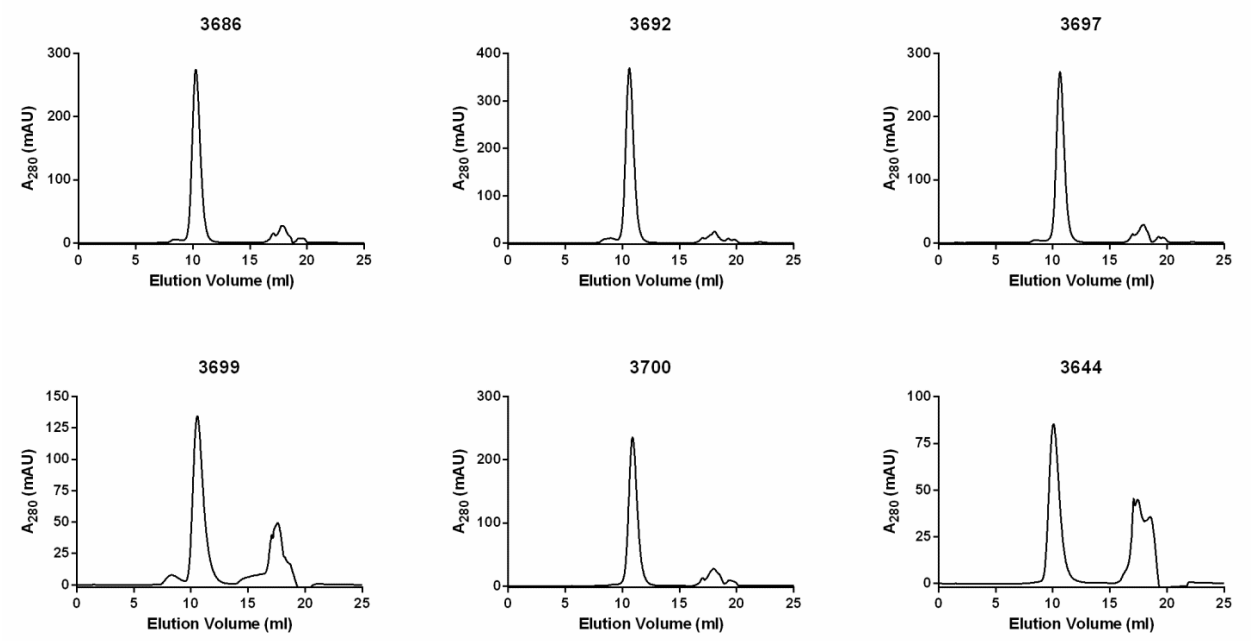


Figure 7-8. Size exclusion chromatography of purified Fab of M3686, M3692, M3697, M3699, M3700 and M3644. Prior to SPR analysis each Fab was purified by size exclusion chromatography. Only peak fractions were used for SPR.

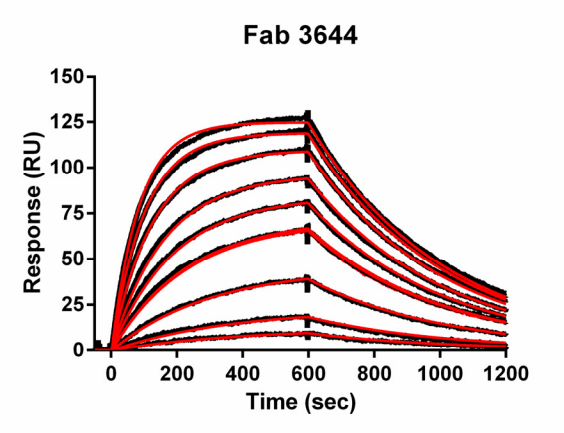
SPR analysis was performed to study the binding affinities of Fabs for their cognate antigen. A range of Fab concentrations were passed over antigen immobilized on the sensorchip (Figure 7-9). Interaction between the Fab and antigen on the sensorchip surface were detected in real time and are represented as sensorgrams which plots resonance units (RUs) with respect to time (Figure 7-9). All Fab data sets show good fitting to the 1:1 binding model (Figure 7-9).

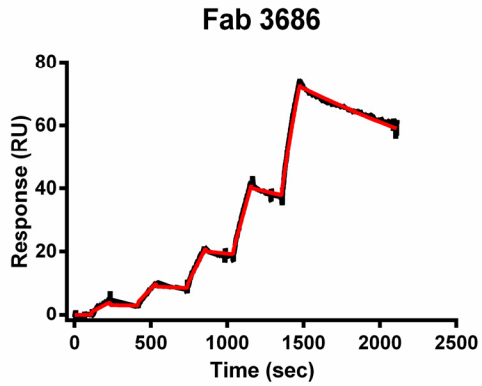
All six MAbs had high affinities as K_D was in the nanomolar range (Table 7-6). Interestingly, MAb M3692 has the lowest dissociation constant at 0.9 nM but was incapable of capturing *L. monocytogenes*.

Table 7-6. Association (ka) and dissociation (kd) rate constants and equilibrium dissociation (K_D) constants for M3644, M3686, M3692, M3697, M3699 and M3700.

MAb	ka (1/Ms)	kd (1/s)	K_D (M)
M3644	3.83×10^{5}	2.41×10^{-3}	6.30×10^{-9}
M3686	3.40×10^{5}	4.81×10^{-4}	1.41×10^{-9}
M3692	2.12×10^{5}	1.88×10^{-4}	8.87×10^{-10}
M3697	1.84×10^{6}	3.84×10^{-2}	2.08×10^{-8}
M3699	1.92×10^{6}	4.95×10^{-2}	2.58×10^{-8}
M3700	1.49×10^{6}	2.08×10^{-2}	1.40×10^{-8}

Figure 7-9. SPR sensogram for Fab 3644, 3686, 3692, 3697, 3699 and 3700. SPR sensorgram showing Fab binding to immobilized antigen at: 0.5, 1, 2.5, 5, 5, 7.5, 10, 15, 20 and 25 nM for M3644, 1.25, 2.5, 5, 10 and 20 nM for M3686, 0.5, 1, 2.5, 5, 7.5, 10 and 10 nM for M3692, 0.5, 1, 2.5, 5, 7.5, 10, 10, 25, 50, 75 and 100 nM for M3697, 1, 2.5, 5, 7.5, 10, 25, 25, 50 and 75 nM for M3699 and 0.5, 1, 2.5, 5, 7.5, 10, 25, 25, 50 and 75 nM for M3700. Red lines represent fitted curve and black line represent raw measurements. Binding kinetics of Fab M3686 was analyzed using single cycle kinetics which involves 5 subsequent injections of increasing concentrations followed by a dissociation of 10 min.


Fab 3644
Response (RU)
Time (sec)


Fab 3686
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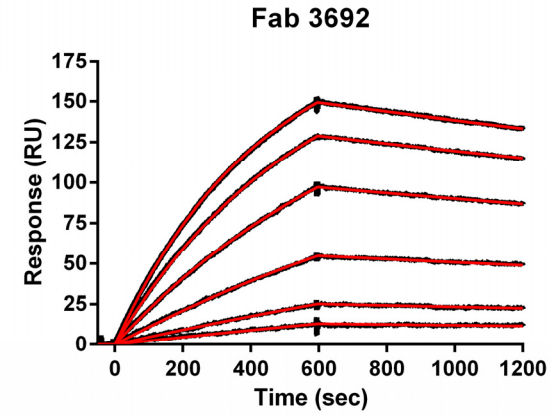
Fab 3692
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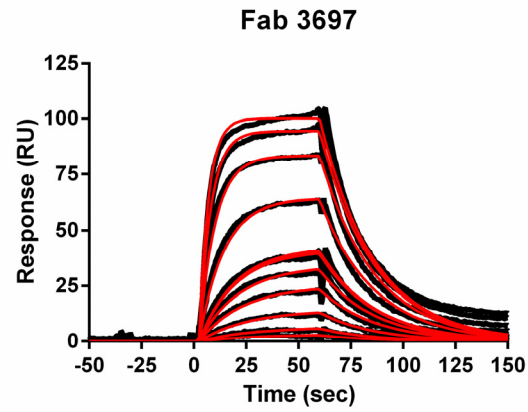
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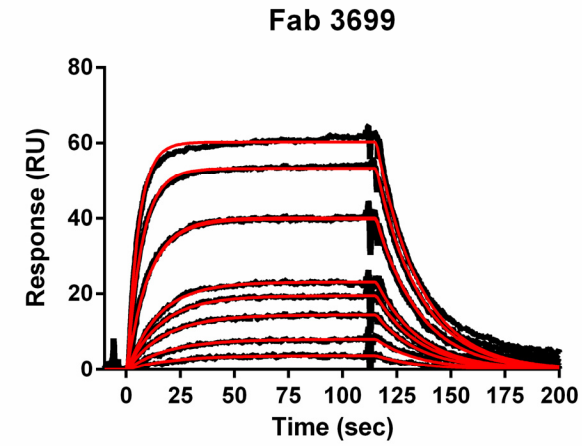
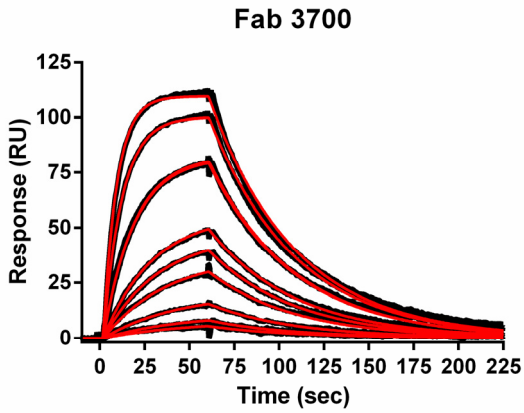

Fab 3699
Response (RU)
Time (sec)

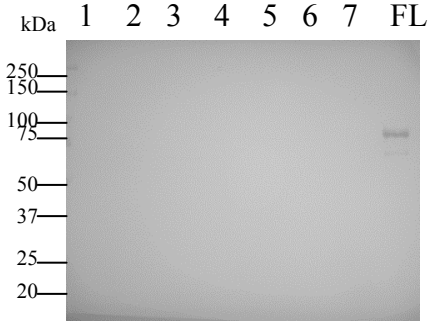

Fab 3700
Response (RU)
Time (sec)

7.2.7 Epitope Mapping for M3686, M3692, M3697, M3699 and M3700

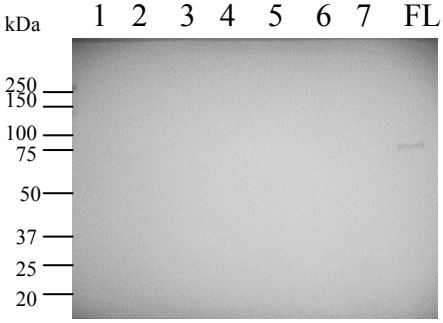
Seven overlapping recombinant polypeptides (Figure 7-10) that covered the length of LMOf2365_0148 protein were used to map the epitopes for MAbs (M3686, M3692, M3697, M3699 and M3700) raised against rLMOf2365_0148. Epitope mapping for the MAbs may predict their specificity to *L. monocytogenes* strains and may also explain why certain MAbs such as M3692 with high affinity for its antigen cannot capture *L. monocytogenes* cells. Of the five MAbs assessed, only the epitope for M3692 was mapped to a region overlapped by peptides 6 and 7 and close at the C-terminal region of the LMOf2365_0148 protein (Figure 7-10). Western blot of the other four MAbs (M3686, M3697, M3699 and M3700) detected the denatured full-length LMOf2365_0148 protein but were not reactive to any polypeptides derived from LMOf2365_0148 (Figure 7-10). PAbs detected the presence of all polypeptides (Figure 7-10). These results showed that although M3686, M3697, M3699 and M3700 were reactive to the denatured full-length protein, the three MAbs (M3697, M3699 and M3700) were not reactive to polypeptide fragments derived from the same protein.

Figure 7-10. Epitope mapping for M3686, M3692, M3697, M3699 and M3700 by western blot analysis of using seven overlapping polypeptide fragments of LMOf2365_0148. Crude extracts of *E. coli* expressing a polypeptide fragment or purified full-length LMOf2365_0148 (1 ng), were analyzed by SDS-PAGE and western blotting using respective MAb in the form of TCF (dilution 1: 25). To assess the presence of LMOf2365_0148 polypeptides, rabbit PAb to LMOf2365_0148 (dilution 1:1000), was used to probe crude extracts of *E. coli* expressing polypeptide fragments. Lanes 1 to 7 correspond to polypeptide 1 to 7 in the schematic diagram. The schematic diagram showed the location of each polypeptides with respect to the cloned mature LMOf2365_0148, used for PAb and MAb development, and the full-length LMOf2365_0148. Lane FL consisted of purified mature LMOf2365_0148.

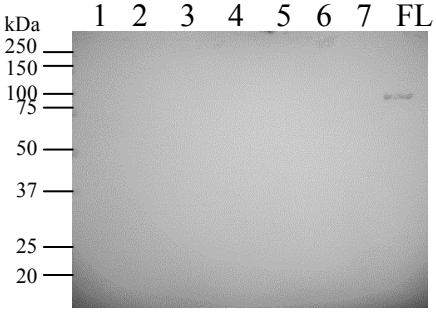
M3686 Western Blot



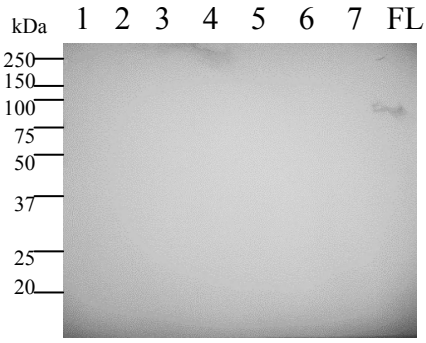
M3700 Western Blot



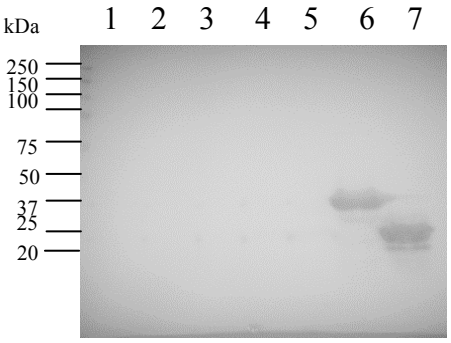
M3697 Western Blot



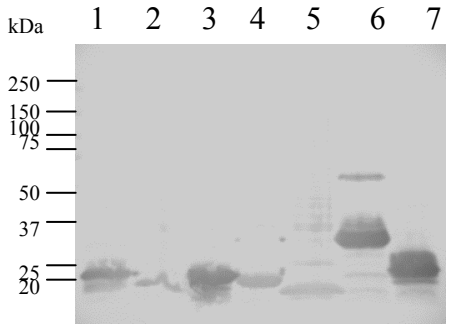
M3699 Western Blot

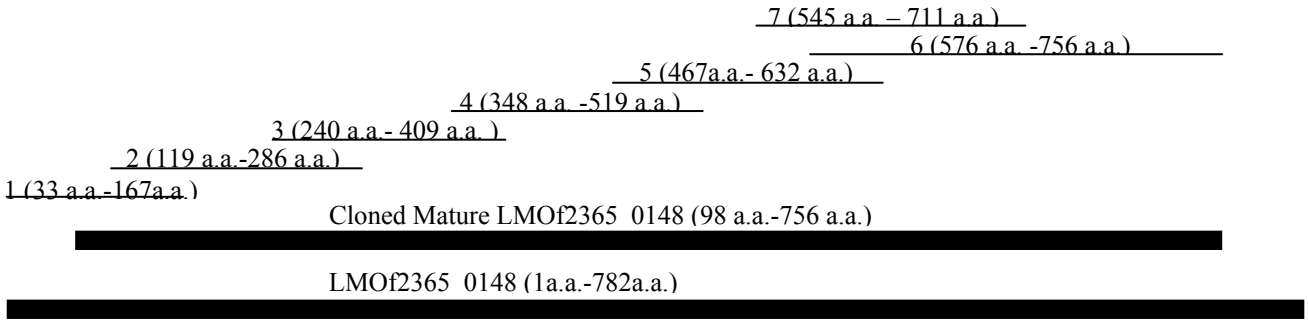


M3692 Western Blot



LMOf2365_0148 Western Blot





7.3 Discussion

In Chapters 4 and 6, MAbs against *L. monocytogenes* surface proteins LMOf2365_0639 and LMOf2365_0148 were developed. The ability of several selected MAbs to capture live *L. monocytogenes* cells was investigated here for practical applications. The affinity of these MAbs for their respective antigens was measured by SPR analysis to provide some possible explanation for the capture ability observed.

Of the 35 MAbs against rLMOf2365_0639, only M3644 exhibited capture ability although many have the potential for *L. monocytogenes* detection by ELISA. Fifteen out of the 24 MAbs against rLMOf2365_0148 were capable of capturing *L. monocytogenes* with M3686, M3697, M3699 and M3700 exhibiting the best capture efficiencies. M3644 against rLMOf2365_0639 captured fewer cells than the four MAbs (M3686, M3697, M3699 and M3700) against rLMOf2365_0148. MAb in the form of TCF was diluted at five dilutions (1:200, 1:100, 1:50, 1:10, 1:5 and 1:2) to select for the most optimal working concentration. The number of cells captured by M3644 was consistently one order of magnitude less than the four MAbs against rLMOf2365_0148 regardless of the dilution used. The reduced capture ability of M3644 appeared not to be due to the affinity of M3644 for its epitope because the dissociation constant of M3644 was comparable to those of M3686, M3697, M3699 and M3700 (Table 7-6).

The reduced or no capture ability of MAbs developed against rLMOf2365_0639 could be due to the properties of the LMOf2365_0639 protein on *L. monocytogenes* cell surface rather than properties of the MAbs. One possible explanation is a lower level of LMOf2365_0639 expression as compared with LMOf2365_0148. In addition, the distribution of the LMOf2365_0639 on the cell surface may contribute to the reduced or no capture of corresponding MAbs. The diameter of the Dynal^{TM} beads used in this study was 2.8 µm while a

L. monocytogenes cell is about 0.5 µm wide and 2 µm long. Conceivably, a maximum of two beads can bind to one *L. monocytogenes* cell. Possible diffused localization of the LMOf2365_0639 proteins on the surface of *L. monocytogenes* may result in decreased avidity and thereby decreased capture.

Epitope mapping for M3686, M3692, M3697, M3699 and M3700 by western blot analysis of overlapping polypeptide fragments of LMOf2365_0148 yielded results only for M3692 (Figure 7- 10). Interestingly the epitope for M3692 is location at the C-terminal region of LMOf2365_0148 where the protein is presumably anchored to peptidoglycan via its LPXTG motif. Although M3692 had the lowest dissociation constant ($8.87x10^{-10}$ M) among all the MAbs analyzed by SPR, M3692 did not capture live *L. monocytogenes* cells. The location of the M3692 epitope at the protein anchor motif may have prevented the MAb from capturing live cells. The ability of M3692 to detect formalin-killed cells as revealed by indirect ELISA (Figures 6-6, 6-7 and 6-8) and live cells as revealed by in immunofluorescence microscopy (Figure 6-5) suggested that its epitope, to some degree, was surface exposed. Peptides of LMOf2365_0148 identified from the proteomics study of surface exposed proteins revealed higher coverage in the N-terminal region of LMOf2365_0148 than its C-terminal region (Figure 7-11). It is conceivable that the C-terminal region of LMOf2365_0148 was less surface-exposed thus less accessible to antibodies. Other researchers have noted the important of epitope surface exposure in pathogen detection. Lack of epitope surface exposure has been reported to limit the binding of MAbs to *Bacillus anthracis* spores (190). Given challenges such as weak association of beads to cells (116) and the inhibitory effects of cell motility on IMS (191), any potential limitations may abrogate the capture ability of a MAb.

MNKFFKKTTHVLLVAGLTIGLTAPFTGTTAQAAADTVPIQILGINDFHGALETASKDASGSPIGGADYLATN
LDNATNSFLQANPGATTDNAIRVQAGDMVGASPAVSGLLQDEPTMKVLQKMNFEVGTLGNHEFDEGLPE
YKRILDGVSTNKFGPIVEAYPRVKSDMKIVAANVVNKGTNTVAEGFLPYYVKEIDGVKVGFIGIVTTEIPNL
VLANHIKDYDFLDEAETIVKYSAELRGQGVNAIVVLSHVPALSTGNPNTGTKQDVAGEAANMITKANELD
PNNSVDLVLAGHNHQYTNGLVGKTRIVQSYNNGKAFSDVTGELDKTTGDFVTPPDAKITYNTRSVTPNADI
TAVTEDAKSRIEGVINETIGLANKDVISRDTNPDNKAIDDKESELGNMITDAQRYMANKAGADVDFAMTN
NGGIRSDLTTRLANGQNEITWGAAQAVQPFGNILQVVEMTGADILEALNQQYLSNQTYFLQISGLKYTFTD
TDDLDHAYKVASVTTEDGTPLKADQKYKVVINDFLFGGGDGFSAFKKANLVTAIDPDTETFINYIKDQKAA
GKVITAQKEGRKVYKSQAEIDKETEDAAIKAIKDATKINKLAEKDKTLTGTTLPGATVSVQKATANARMA
LAAGPNATADANGKFSVDVTSLNLKKGDQITTTITDPNGYSTTFQATVQAAATTPPDNGNGGTDNGNGNG
NNGGTDGNGGTNNGNGSGTNGGTTTTEDPTTTTPNTSTTGTSANTSLPTTGDTAGFATVFGIVLTTTALYV
LRKRS

Figure 7-11. Coverage of LMOf2365_0148 protein from mass spectrometry analysis. Peptide sequences of LMOf2365_0148 identified by mass spectrometry analysis are highlighted in gray while the underlined sequence contained the epitope for MAb M3692.

The reason why M3686, M3697, M3699 and M3700 did not react to overlapping polypeptides (~16 kDa) of LMOf2365_0148 used for epitope mapping is unclear. These MAbs reacted with the SDS-treated full length LMOf2365_0148 protein in western blot suggested that they recognized a linear epitope. Nonetheless, these MAbs did not react with large fragments of the protein under denaturing conditions. It appears that a full-length LMOf2365_0148 is required for recognition by these four MAbs.

The capture experiments revealed the greatest capture efficiency for M3686 in comparison to M3697, M3699 and M3700 (Table 7-1). This finding is consistent with the measured dissociation constants of antibody-antigen interaction. The dissociation constant for M3686 (1.41×10^{-9} M) is one order of magnitude less than those for M3697, M3699 and M3700 (Table 7-6). Antibody affinity may be a contributing factor in capture efficiency as many reports have demonstrated the importance of antibody affinity in immunoassay sensitivities (192-194).

The four MAbs M3686, M3697, M3699 and M3700 was extensively analyzed for their capture ability using isolates of several *L. monocytogenes* serotypes, other *Listeria* species and other non-*Listeria* bacteria. M3686 was specific for capturing *L. monocytogenes* as it was incapable of capturing *L. seeligeri, L. innocua, L. ivanovii, L. grayi*, *L. welshimeri, E.coli* O157:H7 and *Salmonella enterica* serovar Typhimurium. Moreover, MAbs M3686, M3697, M3699 and M3700 were able to enrich for *L. monocytogenes* from a mixture of *L. monocytogenes* and *L. innocua* with 10 or 100 times more *L. innocua* cells than *L. monocytogenes* cells. One important quality of a MAb is its ability to enrichment for the target pathogen. High background microflora can produce false negative results in PCR detection (15). The remarkable specificity of M3686 is encouraging as the use of this MAb in IMS will allow

for isolation of *L. monocytogenes* from enrichment cultures which likely contains a high background of other *Listeria* species (7, 144, 145).

Previous studies have reported the use of MAbs for the IMS of *Listeria* species (15, 116, 129, 130, 195-197). The MAbs that were previously shown to have high capture efficiencies for *L. monocytogenes* also recovered considerable numbers of other *Listeria* species. Paoli *et al.* reported IMS of *L. monocytogenes* grown in BHI. These researchers found high capture efficiencies of 17.34-1.38% for the *L. monocytogenes* strains tested and significant capture of other *Listeria* species (500 – 10 CFU counts with an input of 10^5 cells). Mendonca *et al.* reported a MAb with capture efficiencies of 49.2 and 32.2% for *L. monocytogenes* and *L. ivanovii*, respectively. They observed significant capture of *L. innocua* and *L. marthii* (480 and 400 CFUs, respectively) at an input of $2x10^4$ cells under the same conditions. In this study, the *L. monocytogenes* capture efficiencies for M3686, M3697, M3699 and M3700 were 3.7, 2.6, 2.0 and 2.8 % with an input of 10^4 cells. These numbers are lower than those reported in other studies, 200 to 370 CFUs, nevertheless, were captured (Table 7-1). For the purpose of obtaining isolated colonies for subsequent molecular detection, the capture efficiencies of MAbs generated here was sufficient. Importantly, M3686 is highly specific for *L. monocytogenes* capture. No CFUs were recovered by M3686 using 10^4 cells for each of *L. seeligeri, L. innocua, L. ivanovii, L. grayi* and *L. welshimeri*; less than three CFUs were isolated using 10^4 cells for each of *E. coli* O157:H7 and *Salmonella enterica* serovar Typhimurium. Both specificity and capture efficiency are equally important when developing MAbs for *L. monocytogenes* isolation. Capture efficiencies are not only dependent on the quality of the antibodies used but also on many aspects of the experimental procedure. These include initial cell concentration (131), the size (131) and concentration of the beads as well as the number of wash steps after pathogen capture (116).

Therefore experimental details should be considered when making comparison of the results between studies.

M3686 exhibited the ability to capture seven *L. monocytogenes* serotypes 1/2a, 1/2b, 1/2c, 3a, 4b, 4a and 4d. However, this MAb captured 10 times the number of *L. monocytogenes* serotype 4b cells as other serotypes. This difference in capture efficiency between serotypes has been previously reported (132). The MAbs developed here appear to be suitable for capturing all *L. monocytogenes* serotypes at a cell concentration of 10^4 CFU/ml.

In addition to testing the ability of M3686, M3697, M3699 and M3700 to capture *L. monocytogenes* grown in non-selective BHI medium, this study also tested the ability of the selected MAbs to capture *L. monocytogenes* grown in primary selective enrichment cultures according to MFHPB-30 and MFHPB-07 methods. All MAbs (M3686, M3697, M3699 and M3700) were capable of *L. monocytogenes* capture after abbreviated 20-21 hours and 30 hours in primary selective enrichment culture according to MFHPB-30 and MFHPB-07, respectively. While other MAbs with *L. monocytogenes* capture ability in BHI (132) did not react to *L. monocytogenes* grown in selective enrichment cultures (28), the ability of the MAbs developed here to capture *L. monocytogenes* grown in enrichment culture makes them applicable to practical diagnostic testing. In addition, the IMS method in this study was adapted to the Beadretriever ™ platform to facilitate multiple testing steps, prevent operator fatigue and increase efficiency.

In conclusion, the use of the selected MAbs against the previously discovered surface protein biomarkers for *L. monocytogenes* capture was successful. The results show that MAbs M3686, M3697, M3699 and M3700 were capable of *L. monocytogenes* capture after abbreviated

primary selective enrichment culture according to both MFHB-07 and MFHP-30 methods. Particularly, M3686 specifically captured seven serotypes of *L. monocytogenes.* While these MAbs were capable of capturing significant numbers of *L. monocytogenes* serotype 4b cells, they captured reduced numbers of *L. monocytogenes* cells of other serotypes. Further research is needed to assess capture of *L. monocytogenes* of various serotypes in naturally or artificially contaminated foods samples after selective enrichment culture. Nonetheless, the results reported here are an encouraging step towards the development of an automated IMS method applicable for routine isolation of *L. monocytogenes* from food and environmental samples.

Chapter 8: General Discussion and Conclusions

Problems still exist in current *L. monocytogenes* detection which involves sample preparation followed by detection. In sample preparation, selective enrichment cultures are time-consuming. Moreover, certain *Listeria* species such as *L. innocua* can out-compete the growth of *L. monocytogenes* in selective enrichment cultures to confound subsequent *L. monocytogenes* detection (144, 145). In detection, rapid molecular methods such as PCR are liable to inhibitory substances found in foods and selective enrichment cultures (13, 14, 15). Immunological detection methods such as ELISA are more resistant to sample complexity (16). However, antibodies that have well-defined antigens and are specific to various serotypes of *L. monocytogenes* are still eagerly sought. The goal of this work was to identify surface proteins of *L. monocytogenes* that could be used as diagnostic biomarkers. Antibodies developed against identified biomarkers may be applied to the isolation and detection of *L. monocytogenes* from test samples and thus resolve the problems plaguing *L. monocytogenes* detection.

In this study, extensive work was done to discover and assess *L. monocytogenes* surface proteins as diagnostic biomarkers. Surface protein diagnostic biomarkers were identified for *L. monocytogenes* using two novel approaches: sequence comparison of surface proteins by bioinformatics tools and a non-gel proteomic screen of surface proteins by mass spectrometry. Of the 130 surface protein genes identified from the genome of *L. monocytogenes* F2365 (serotype 4b), four surface proteins were selected based the presence of extensive amino acid sequences conserved among *L. monocytogenes* and variable among other *Listeria* species. Polyclonal antibodies against candidate proteins were used to probe the expression and surface accessibility of candidate surface proteins prior to the development of MAbs against the selected proteins of *L. monocytogenes*. This approach first identified the target for MAb development in

contrast to the majority of previously MAbs raised against *L. monocytogenes* or *Listeria* species which have undefined targets (18, 20-24, 28, 123, 125, 126). The identification of the protein antigen target allowed for a more detailed characterization of antigens and MAbs with diagnostic potential. Studies such as antigen expression in various conditions and in various *Listeria* species and antibody affinity determination and epitope mapping of diagnostic MAbs are important for pathogen detection (131, 190). In addition, previous proteomics studies of *L. monocytogenes* surface proteins, extracted from various components of the cell envelope (167-171), do not provide information on whether certain proteins are surface accessible. This work was the first to identify surface exposed proteins of *L. monocytogenes* for the selection of diagnostic biomarkers.

Application of the two different approaches led to the discovery of two novel surface proteins: LMOf2365_0639 and LMOf2365_0148 as biomarkers of *L. monocytogenes*. MAbs raised against LMOf2365_0639 and LMOf2365_0148, reported in this study, exhibited new properties that have not been described in the literature. Specifically, MAbs against both proteins reacted to several serotypes of *L. monocytogenes* grown in selective enrichment culture. In addition, some MAbs to LMOf2365_0148 were capable of capturing *L. monocytogenes* grown in selective enrichment culture and were highly specific to *L. monocytogenes*. Moreover, this work was the first to characterize the affinity of MAbs capable of *L. monocytogenes* capture thereby providing insight into the development of MAbs capable of *L. monocytogenes* capture.

MAbs M3643 and M3686 were reactive to the majority of *L. monocytogenes* isolates grown in selective enrichment culture. MAb M3643, raised against LMOf2365_0639, reacted to *L. monocytogenes* isolates of all three lineages grown in selective enrichment cultures of MFHPB-07 and MFHPB-30 routinely used in *Listeria* detection at the CFIA. These isolates represented serotypes 1/2b, 4ab, 4b, 4d and 4e from lineage I, 1/2a, 1/2c and 3a from lineage II

and 4a and 4c from lineage III. Likewise, MAb M3686, raised against LMOf2365_0148, reacted to *L. monocytogenes* isolates belonging to all three lineages. These isolates were serotypes 1/2b, 3b, 4ab, 4b, 4d and 4e from lineage I, 1/2a, 1/2c and 3a from lineage II and 4a and 4c from lineage III. Although selective enrichment cultures are often necessary for *L. monocytogenes* detection by PCR (9) or ELISA (10, 11), many previous studies of MAbs against *L. monocytogenes* did not demonstrate the practical utility of the MAbs to detect *L. monocytogenes* grown in selective enrichment culture. MAbs that were investigated in practical application showed lack of reactivity with *L. monocytogenes* cells grown in selective enrichment cultures (25, 26, 28). Moreover, unlike the work presented here, antibody-pathogen reactivity was tested only with a single or a limited number of *L. monocytogenes* serotypes (25, 26, 28) or under one selective enrichment culture condition (22). Although reactivity to *L. innocua* and *L. seeligeri* was observed with M3643 and M3686 respectively, specific detection of *L. monocytogenes* may be obtained if M3643 and M3686 are used together in a sandwich ELISA. Nonetheless, the presence of other *Listeria* species warrants sanitation of the food processing plants (8). MAbs reactive to other *Listeria* species are also useful in surveillance of food processing environments. This work, which extensively evaluated the reactivity of MAbs raised against both surface proteins LMOf2365_0639 and LMOf2365_0148 with various serotypes of *L. monocytogenes*, from all three lineages grown in all standard selective enrichment culture conditions used by the CFIA, provided evidence to support the utility of MAbs developed here for *L. monocytogenes* isolation and detection.

Several MAbs (M3686, M3697, M3699 and M3700) against LMOf2365_0148 were able to capture live *L. monocytogenes* cells. The capture efficiencies of the MAbs tested (3.7-2.0% for an input of 10^4 cells) were not as high as those reported other studies (131, 132), nevertheless,

between 200 to 370 CFUs were captured. For the purpose of generating isolated colonies for subsequent molecular detection, the capture efficiency of MAbs generated here was sufficient.

Although studies have reported high capture efficiencies to *L. monocytogenes*, a significant amount of other *Listeria* species were captured (131, 132). One study reported high capture efficiencies of 17.34-1.38% for the *L. monocytogenes* strains tested. However, significant capture of other *Listeria* species (500 – 10 CFUs) observed with an input of 10^5 cells (132). Likewise, another study reported a MAb that is specific to *L. monocytogenes* and *L. ivanovii* with high respective capture efficiencies of 49.2 and 32.2% (131). However, at $2x10^4$ cells as input, significant capture of *L. innocua* and *L. marthii* (480 and 400 CFUs, respectively) was observed (131).

Of interest, M3686 is highly specific in immunomagnetic separation of *L. monocytogenes*. M3686 did not capture *L. innocua, L. seeligeri, L. welshimeri, L. ivanovii,* and *L. grayi* or *Salmonella enterica* serovar Typhimurium and *E. coli* O157:H7. Moreover, in a mixture of *L. monocytogenes* and *L. innocua* with *L. innocua* cells 10 and 100 fold in excess, all MAbs tested enriched for *L. monocytogenes.* This capture specificity of M3686 may prove to be particularly useful since selective enrichment cultures are not selective only for *L. monocytogenes* but for *Listeria* species*,* and *L. innocua* has been observed to outcompete *L. monocytogenes* in selective enrichment cultures (144, 145).

MAbs M3686, M3697, M3699 and M3700 were also capable of capturing *L. monocytogenes* after relatively abbreviated growth in primary selective enrichment culture conditions of MFHPB-07 and MFHPB-30. The ability of these MAbs to capture and enrich *L.*

monocytogenes cells can shorten the time necessary for selective enrichment culture prior to *L. monocytogenes* detection.

The dissociation constants of MAbs M3686, M3697, M3699, M3700 as well as M3644 and M3692 were determined. Of the four MAbs (M3686, M3697, M3699 and M3700) extensively tested for *L. monocytogenes* capture, M3686 had the highest capture efficiency as well as the strongest affinity for the pathogen (i.e. the lowest dissociation constant). The dissociation constant of M3686 (1.41×10^{-9}M) was one log lower than that of MAbs M3697, M3699 and M3700. Studies have demonstrated the importance of affinity in the sensitivity of immunoassays (192-194). This work reinforced the notion that antibody affinity plays a role in capture ability. Interestingly, although M3692 had a very low dissociation constant of 8.87×10^{-10} M, it was not capable of capturing *L. monocytogenes.* This was surprising since it was expected that MAbs with high affinity for their surface targets should capture the bacteria. The epitope recognized by M3692 was mapped to the C-terminal region of LMOf2365_0148, close to the protein anchor which consisted of the LPXTG motif. The location of the M3692 epitope may play a role in abrogating the live cell capture ability of M3692. Other researchers have noted the importance of targeting surface exposed epitopes for pathogen detection as lack of surface exposure of the epitope was attributed to the minimal binding capacity of a MAb with a very low dissociation constant (3.30×10^{-10} M) (190). Moreover, while the majority of the MAbs against LMOf2365_0148 were capable of capturing *L. monocytogenes,* only one MAb M3644 against LMOf2365_0639 had capture ability at reduced capture efficiency compared to anti-LMOf2365_0148 MAbs. The dissociation constant of M3644 is comparable to that of M3686 although disparity exists in their capture efficiency. This suggests that qualities of the epitope

such as its localization on the intact cell and its level of expression may affect capture efficiency in addition to the affinity of a MAb.

In conclusion, the identification and assessment of surface proteins for *L. monocytogenes* isolation and detection provided insights into the selection of diagnostic biomarkers. This study was successful in identifying two surface protein biomarkers that were expressed in *in vitro* culture. Both LMOf2365_0639 and LMOf2365_0148 are expressed in *in vitro* culture, especially in selective enrichment cultures routinely used for isolation and detection *L. monocytogenes*. MAbs developed against LMOf2365_0639 and LMOf2365_0148 may potentially be used in ELISA for *L. monocytogenes* detection and MAbs developed against LMOf2365_0148 may be used in immunomagnetic separation of *L. monocytogenes* to facilitate and expedite *L. monocytogenes* detection.

Future work is needed to evaluate the efficacy of MAbs developed here for isolation and detection of *L. monocytogenes* in food samples. Foods naturally or artificially contaminated with *L. monocytogenes*, subjected to sample homogenization and selective enrichment cultures, would serve as the ultimate test samples to assess the efficacy of MAbs in *L. monocytogenes* isolation and detection.

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Chapter 10: Contributions of Collaborators

Chapters 4 and 6:

Jen Ronholm, Hanhong Dan, Teela O'Neill, Dr. Min Lin and I prepared the formalin-killed cells cultured in non-selective medium used for indirect ELISA. I prepared the formalin-killed cells cultured in selective enrichment media. I worked with Cathie Elmgren, Jenni Widdison and Kristin Arnold of Monoclonal Antibody Unit at CFIA to develop monoclonal antibodies. I worked with Sally Lloyd and Qi Gao Fu of the Small Animal Colony at CFIA to develop polyclonal antibodies.

Chapter 5:

The proteomic study of surface associated proteins was carried in collaboration Marybeth Creskey and Dr. Terry Cyr at Health Canada. Marybeth performed mass spectrometry analysis of purified peptides that I prepared. Marybeth also outlined mass spectrometry procedures.

Chapter 7:

Hanhong Dan and I performed the immunomagnetic separation experiments.

The dissociation constants of MAbs were determined in collaboration Henk van Faassen and Roger MacKenzie at the National Research Council. I prepared purified Fab fragments and antigens. Henk carried the SPR analysis, interpreted the data and generated Figure 7-9.

Dr. Jamshid Tanha , Kandalaft, Hiba and Dae Yong Kim at the National Research Council, Canada determined the molecular mass of purified LMOf2365_0639 and LMOf2365_0148 using Multi-Angle Light Scattering.

Appendix

Table S1. Proteins of F2365 *L. monocytogenes* containing LysM domain.

Gene Locus	Gene Name	Possible Function	Other Domains	EGDe Homolog	Gene Name
LMOf2365_0611	iap	-	p60	lmo0582 [bcde]	p60/iap
LMOf2365_0899	-	-	-	lmo0880	-
LMOf2365_1970	-	-	-	lmo1941	-
LMOf2365_1321	-	-	-	lmo1303	-
LMOf2365_2495	-	N-acetylmuramidase	-	lmo2522	-
LMOf2365_2670	-	N-acetyl-glucosaminidase	-	lmo2691 [ad]	MurA

Table S2. Proteins of F2365 *L. monocytogenes* containing p60 domain.

Gene Locus	Gene Name	Possible Function	Other Domains	EGDe Homolog	Gene Name
LMOf2365_0406	-	gamma- D-glutamyl-L-mDpm peptidase	-	lmo0394	-
LMOf2365_0611	iap	gamma- D-glutamyl-L-mDpm peptidase	-	lmo0582 [bcde]	iap
LMOf2365_2478	-	gamma- D-glutamyl-L-mDpm peptidase	-	lmo2505 [bcde]	spl

Table S3. Proteins of F2365 *L. monocytogenes* containing GW modules.

Gene Locus	Gene Name	Possible Function	Number of GW Modules	EGDe Homolog
LMOf2365_1093	-	N-acetyl-glucosaminidase	7	-
LMOf2365_1224	-	N-acetylmuramyl-L-alaine amidase	1	lmo1215
LMOf2365_1225	-	N-acetyl-glucosaminidase	1	lmo1216 [d]
LMOf2365_1540	ami	N-acetylmuramyl-L-alaine amidase	2	lmo1521 [d]
LMOf2365_2236	-	N-acetyl-glucosaminidase	2	lmo2203
LMOf2365_2530	-	N-acetylmuramyl-L-alaine amidase	6	lmo2558 [de]
LMOf2365_2564	-	N-acetyl-glucosaminidase	4	lmo2591 [d]
LMOf2365_2693	-	-	1	lmo2713

Table S4. Proteins of F2365 *L. monocytogenes* containing LPXTG sorting motif.

Gene Locus	Gene Name	Other Features	EGDe Homolog	Gene Name
LMOf2365_0148	-	nucleotidase	lmo0130 [b]	-
LMOf2365_0174	-	collagen binding domain and 4 Can B	lmo0159	-
LMOf2365_0175	-	collagen binding domain, 2 Can B repeats	lmo0160 [b]	-
LMOf2365_0186	-	1 PKD repeat and 1 Muc BP repeat	lmo0175	-
LMOf2365_0281	InlC2	-	lmo0263 [ad]	InlH
LMOf2365_0282	InlD	-	lmo0264	C-terminus InlH
LMOf2365_0283	InlE	-	lmo0264	InlE
LMOf2365_0338	-	-	lmo0320	vip
LMOf2365_0345	-	5 LRR repeats, LRR adjacent IR region and	lmo0327 [a]	-
LMOf2365_0347	-	11 LRR domains and 1 PKD repeat	lmo0331	-
LMOf2365_0350	-	28 LRR domains, 8 PKD repeats and 3 Muc	lmo0333	InlI
LMOf2365_0429	lnlF	-	lmo0409	lnlF
LMOf2365_0471	lnA	-	lmo0433 [a]	-
LMOf2365_0498	-	-	lmo0463	-
LMOf2365_0543	-	6 LRR and 2 PKD repeats	lmo0514	-
LMOf2365_0579	-	-	lmo0550	-
LMOf2365_0605	-	-	lmo0576	-
LMOf2365_0639	-	8 LRR and 2 PKD repeats	lmo0610 [a]	-
LMOf2365_0656	-	4 Cna Repeats	lmo0627	-
LMOf2365_0761	-	-	lmo0725	-
LMOf2365_0768	-	8 LRR, 2 Muc BP repeats and 1 Big 3	lmo0732	-

Gene Locus	Gene Name	Other Features	EGDe Homolog	Gene Name
LMOf2365_0852	-	2 separate Muc BP repeats	-	-
LMOf2365_0859	-	7 Big 3 domains	lmo0842 [b]	-
LMOf2365_0899	-	LysM domain	lmo0880 bd	-
LMOf2365_1144	-	11 LRR and 1 adjacent LRR domain	lmo1136	-
LMOf2365_1307	-	8 LRR and 2 PKD repeats	lmo1290	-
LMOf2365_1432	-	3 Muc BP repeats	lmo1413 [a]	-
LMOf2365_1690	-	11 PKD repeats	lmo1666 [ad]	-
LMOf2365_1974	-	7 Big 3 domains	lmo0842	-
LMOf2365_2052	-	-	lmo0463	-
LMOf2365_2117	-	3 Cna B repeats	lmo2085 [a]	-
LMOf2365_2210	-	1 N-terminal and 5 C-terminal Cna B repeats,	lmo2178	-
LMOf2365_2212	-	2 Muc BP repeats	lmo2179	-
LMOf2365_2370	-	10 LRR, 7 Muc BP repeats	lmo2396	-
LMOf2365_2694	-	-	lmo2714 [bd]	-
LMOf2365_2812	-	15 LR, 1 LRR adjacent IR region and 4 Muc BP	lmo2821	InlJ

Table S5. Lipoproteins of F2365 *L. monocytogenes.*

Substrate binding proteins of ABC transporter system.

Gene Locus	Gene Name	Possible Function	EGDe Homolog	Gene Name
LMOf2365_0153	-	similar to oligopeptide binding lipoproteins	lmo0135 [cde]	-
LMOf2365_0167	-	similar to oligopeptide binding lipoproteins	lmo0152 [cd]	-
LMOf2365_0192	-	similar to sugar binding proteins	lmo0181	-
LMOf2365_0168	-	similar to zinc (II) binding lipoprotein	lmo0153 [cd]	-
LMOf2365_0267	-	similar to sugar binding proteins	n/a	-
LMOf2365_0305	-	similar to substrate binding lipoproteins	lmo0285 [ce]	-
LMOf2365_0570	-	similar to iron compound binding lipoproteins	lmo0541 [c]	-
LMOf2365_0876	-	similar to sugar binding proteins	lmo0859	-
LMOf2365_1037	-	similar to glycine betaine binding protein	lmo1016 [c]	gbuC
LMOf2365_1062	ModA	similar to molybdate binding proteins	lmo1041 [ce]	-
LMOf2365_1090	-	similar to metal ion binding proteins	lmo1073 [e]	-
LMOf2365_1695	-	similar to adhesion proteins and Mn/Zn binding proteins	lmo1671 [cde]	-
LMOf2365_1754	-	similar to sugar binding proteins,	lmo1730 [e]	-
LMOf2365_1762	-	similar to amino acid binding proteins	lmo1738 [cde]	-
LMOf2365_1875	-	similar to manganese binding lipoprotein	lmo1847 [cde]	-
LMOf2365_1407	-	similar to substrate binding lipoproteins; CD4+ T-cell-stimulating antigen	lmo1388 [cde]	TcsA
LMOf2365_1989	-	similar to ferrichrome binding lipoprotein	lmo1959 [cd]	-
LMOf2365_2031	-	similar to sugar binding proteins	lmo2007	-
LMOf2365_2159	-	similar to maltose/maltodextrin binding lipoproteins	lmo2125	-
LMOf2365_2217	-	similar to ferrichrome binding lipoproteins	lmo2184	-

Gene Locus	Gene Name	Possible Function	EGDe Homolog	Gene Name
LMOf2365_2217	-	similar to ferrichrome binding lipoproteins	lmo2184	-
LMOf2365_2227	-	-	lmo2194 [c]	OppC
LMOf2365_2229	-	similar to oligopeptide binding lipoprotein	lmo2196 [c]	OppA
LMOf2365_2319	-	similar to amino acid binding lipoproteins	lmo2349 [cd]	-
LMOf2365_2388	-	similar to substrate binding lipoproteins, and to pheromone cOB1	lmo2417 [e]	-
LMOf2365_2402	-	similar to ferrichrome binding lipoproteins	lmo2431 [c]	-
LMOf2365_2472	ModA	similar to phosphate binding lipoproteins	lmo2499	-
LMOf2365_2542	-	similar to dipeptide binding proteins	lmo2569 [c]	-
LMOf2365_2615	-	similar to serine/threonine protein phosphatase	lmo2642	-
LMOf2365_2830	-	similar to sugar binding proteins	lmo2839	-

Enzymatic

Gene Locus	Gene Name	Possible Function	EGDe Homolog	Gene Name
LMOf2365_0016	-	similar to AA3-600 quinol oxidase subunit II	lmo0013[de]	QoxA
LMOf2365_0376	-	similar to flavocytochrome c fumarate reductase chain A	lmo0355	-
LMOf2365_0546	-	similar to phosphoglycerate mutase	lmo0517	-
LMOf2365_0965	-	similar to metallo-beta-lactamase, DNA binding and competence protein (ComEC and ComEA of B. subtilis)	lmo0945	-
LMOf2365_0991	-	involved in synthesis of D-alanyl-LTA	lmo0971 [c]	DltD
LMOf2365_1398	-	similar to membrane insertase OxaA	Lmo1379	OxaA1
LMOf2365_1463	prsA-1	similar to foldase PrsA	lmo1444	PrsA
LMOf2365_1827	-	similar to protein tyrosine-phosphatase	lmo1800	-
LMOf2365_1900	-	serine protease	n/a	-
LMOf2365_1932	-	similar to thioredoxin	lmo1903	-
LMOf2365_2252	prsA-2	similar to foldase PrsA	lmo2219 [e]	PrsB
LMOf2365_2550	ModA	similar to hydrolase	lmo2578	-
LMOf2365_2609	-	similar to thiamine biosynthesis lipoprotein ApbE	lmo2636	-
LMOf2365_2803	-	similar to D-alanyl-D-alanine carboxypeptidase	lmo2812	-
LMOf2365_2844	-	similar to membrane insertase OxaA	lmo2854	OxaB

Other

Gene Locus	Gene Name	Possible Function	EGDe Homolog	Gene Name
LMOf2365_0056	-	unknown	lmo0047 [e]	-
LMOf2365_0218	-	unknown	lmo0207	-
LMOf2365_0329	-	unknown	lmo0303	-
LMOf2365_0342	-	unknown	lmo0324	-
LMOf2365_0539	-	unknown	lmo0510	-
LMOf2365_0646	-	unknown	lmo0617	-
LMOf2365_0808	-	unknown	lmo0791 [e]	-
LMOf2365_0840	-	unknown	lmo0821	-
LMOf2365_1085	-	unknown	lmo1068 [e]	-
LMOf2365_1282	-	unknown	lmo1265	-
LMOf2365_1357	-	unknown	lmo1340	-
LMOf2365_1669	-	unknown	lmo1649	-
LMOf2365_1782	-	similar to sex pheromone staph-cAM373	lmo1757 [e]	-
LMOf2365_2111	-	unknown	lmo2079 [d]	-
LMOf2365_2112	-	unknown	lmo2080	-
LMOf2365_2387	-	unknown	lmo2416 [d]	-
LMOf2365_2568	-	unknown	lmo2595	-
LMOf2365_2610	-	similar to sex pheromone cAD1	lmo2637 [cd]	-

Unspecified

Gene Locus	Gene Name	Possible Function	EGDe Homolog	Gene Name
LMOf2365_0068	-	unknown	lmo0057	-
LMOf2365_0475	-	unknown	n/a	-
LMOf2365_0605	-	unknown	lmo0576	-
LMOf2365_0864	-	Unknown, similar to Glutamine ABC transporter (binding and transport protein)	lmo0847 [c]	-
LMOf2365_1281	-	unknown	lmo1264	-
LMOf2365_1890	-	unknown	lmo1862	-
LMOf2365_2250	-	unknown	lmo2217	-
LMOf2365_2631	-	unknown	n/a	-
LMOf2365_2694	-	unknown	lmo2714	-

Table S6. Proteins of F2365 *L. monocytogenes* containing C-terminal hydrophobic tail.

Gene Locus	Gene Name	Possible Function	Other Domains	EGDe Homolog	Gene Name
LMOf2365_0215	ActA	Actin-assembly-inducing protein	-	lmo0204	ActA
LMOf2365_0557	-	Similar to UDP-glucose 6-dehydrogenase	-	lmo0528	-
LMOf2365_0581	-	unknown	-	lmo0552	-
LMOf2365_0615	-	unknown	-	lmo0586	-

Table S7. Summary of surfaced proteins identified in *L. monocytogenes* strain F2365.

Cell Surface Anchor	Number of Proteins
LsyM	6
p60	3
GW	8
LPXTG Motif	36
NXZTN	2
Lipoprotein	71
C-terminal Hydrophobic Tail	4

a *L. monocytogenes* EGD-e protein identified only within purified peptidoglycan (168).
b *L. monocytogenes* EGD-e protein and its *L. innocua* orthologue both identified within purified peptidoglycan (168).
c Identified membrane protein (171).
d Identified excreted protein (170).
e Identified cell wall protein (169).