

EVIDENCE FOR THE *N*-ACETYLGLUCOSAMINIDASE ACTIVITY OF A CELL
WALL-ASSOCIATED AUTOLYSIN ISPC AND ITS SUITABILITY AS A DIAGNOSTIC
MARKER FOR *LISTERIA MONOCYTOGENES* SEROTYPE 4B

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By

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Abstract

Listeria monocytogenes is the etiological agent of a life-threatening, opportunistic infection caused by the ingestion of contaminated foods. Although *L. monocytogenes* is divided into 13 serotypes, 98% of human illness is caused by serotype 1/2a, 1/2b and 4b strains, with serotype 4b accounting for almost all the major outbreaks of human listeriosis. The principle objective of this work was to develop surface-binding monoclonal antibodies (MAbs) highly specific for serotype 4b, as well as characterize their antigen targets to aid in the detection and isolation of serotype 4b strains using an antibody based procedure. To create such antibodies, mice were immunized with formalin killed whole cells of *L. monocytogenes* serotype 4b strain LI0521. A total of 15 MAbs reactive to serotype 4b isolates were shown to recognize a ~77 kDa surface antigen subsequently identified by mass spectrometry as surface associated autolysin, IspC. Epitope mapping experiments further revealed that each of the 15 MAbs bound to the cell wall binding GW domain of IspC and can be essentially divided into 4 major groups based on epitope localization. ELISA analysis of the reactivity of each of the MAbs with various *L. monocytogenes* serotypes indicated that several MAbs were 100% specific for serotype 4b isolates. Surface plasmon resonance experiments showed that the affinity constants for each of these MAbs fell within the range of 1.0×10^{-7} to 6.4×10^{-9} M. To determine whether IspC, shown to be well conserved among various serotype 4b strains, is a useful diagnostic marker with antibody-based methods, the expression of IspC was assessed in *L. monocytogenes* cultured under normal and stress conditions. A functional promoter directing the transcription of *ispC* gene was identified immediately upstream of the *ispC* open reading frame by constructing the promoterless *lacZ* gene fusion with the putative *ispC* promoter region and by 5'RACE analysis. Data obtained

with the *lacZ* reporter gene system and immunofluorescent microscopy revealed that IspC is expressed on the cell surface under all growth conditions tested (temperature, osmotic stress, pH, ethanol, oxidative stress, anaerobic conditions, carbon source and enrichment media) that allow for cellular division, although the level of *ispC* gene expression varies. In addition, a significant effort were put into elucidating the hydrolytic bond specificity of IspC by HPLC and mass spectrometry analysis of muropeptides released from IspC-mediated hydrolysis of *L. monocytogenes* peptidoglycan (PG). The results demonstrated that IspC functions as an *N*-acetylglucosaminidase capable of cleaving the β -1,4-glycosidic bond of the PG glycan strand. Furthermore, IspC was more efficient at hydrolysing fully *N*-acetylated PG from a PG deacetylase gene (*pgdA*) deletion mutant of *L. monocytogenes* than partially de-*N*-acetylated wild-type PG, indicating that modification of PG by de-*N*-acetylation of GlcNAc residues renders PG resistant to IspC hydrolysis. In conclusion, the surface autolysin IspC with the *N*-acetylglucosaminidase activity is a novel diagnostic marker for the 4b serotype strains, which can be explored , in conjunction with specific MAbs developed here, for detection and isolation of *L. monocytogenes* serotype 4b strains directly from food, environmental and clinical samples with the need for minimal or no culture enrichment.

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List of Abbreviations

A

Ab – Antibody
ABTS – 2,2-Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid)
ALO – Anthrolysin O
ATP – Adenosine triphosphate

B

BHI – Brain Heart Infusion
BLEB – Buffered *Listeria* enrichment broth
BSA – Bovine serum albumin

C

cAMP – Cyclic adenosine monophosphate
CAZy – Carbohydrate-Active EnZyme database
CBD – Cell-wall binding domain
CDCs – Cholesterol-dependent cytolysins
CHP - Cumene hydroperoxide

D

DAP – Diaminopimelic acid
DEPC - Diethylpyrocarbonate
DNA – Deoxyribonucleic acid

E

EDTA – Ethylenediaminetetraacetic acid

F

Fab – Fragment antigen binding
Fc – Fragment (crystallisable)
FDA – Food and Drug Administration
FEA – Flagella export apparatus
FPE – Frimbrilin-protein exporter
FPLC – Fast protein liquid chromatography

G

GASP – Growth advantage in stationary phase
GFP – Green fluorescent protein
GH – Glycoside hydrolase
GlcN – Glucosamine
GlcNAc – *N*-acetylglucosamine
GSP – Gene specific primer
GW domain – Glycine Tryptophan domain

H

HSPG – Heparin sulphate proteoglycans
HGF/SF – Hepatocyte growth factor / scatter factor
HPLC- High performance liquid chromatography

I

Ig - Immunoglobulin
InlA – Internalin A
InlB – Internalin B
IR – Inter-repeat
IspC – Immunogenic surface protein C

L

LEB – *Listeria* enrichment broth
LRB – *Listeria* repair broth
LLO – Listeriolysin O
LTA – Lipoteichoic acid
LB – Luria-Bertani

M

MAB – Monoclonal Antibody
MS – Mass spectrometry
MALDI TOF – Matrix assisted laser desorption/ionization time of flight
MALDI PSD – Matrix assisted laser desorption/ionization post source decay
MurNAc – *N*-acetylmuramic acid

O

OD – Optical density

P

PAb – Polyclonal antibody
PBS – Phosphate-buffered saline
PCR – Polymerase chain reaction
PFO – Perfringolysin O
PG – Peptidoglycan
PgdA – Peptidoglycan deacetylase
 Δ PgdA – *pgdA* deletion mutant
PlcA – Phospholipase A
PlcB – Phospholipase B
PRR – Proline rich-repeat

Q

qPCR – Quantative polymerase chain reaction

R

RACE – Rapid amplification of cDNA ends

rIspC – Recombinant immunogenic surface protein C
RNA – Ribonucleic acid
RP-HPLC – Reverse phase-high performance liquid chromatography
RT- Room temperature
RTE – Ready to eat

S

SDS – Sodium lauryl sulfate
SDS-PAGE – Sodium lauryl sulphate – polyacrylamide gel electrophoresis
Sec – Secretion apparatus
 Δ SecA2 – *secA2* deletion mutant
SLO – Streptolysin O
SPR – Surface plasmon resonance
SRP – Signal recognition particle

T

TA – Teichoic acid
Tat – Twin-arginine translocation
TFA – Trifluoroacetic acid

U

UVM – University of Vermont Media

W

Wss – WXG100 secretion system
WT – Wild-type

Chapter I

Introduction

1. Introduction

1.1 Rationale

Listeria monocytogenes is the causative agent of listeriosis, a food borne infection implicated in many serious outbreaks (75). Listeriosis is a concern for the ready-to-eat (RTE) food-processing industry since *L. monocytogenes* can multiply at refrigeration temperatures and RTE foods are consumed without cooking. Although listeriosis is rare, it can be fatal in certain populations with suppressed immunity such as neonates, the elderly and pregnant women (229). *L. monocytogenes* is currently divided into 12 serotypes; however, 98% of human illness is caused by serotype 1/2a, 1/2b and 4b strains (79). *L. monocytogenes* serotype 4b is of particular importance for several reasons: it accounts for more cases of human listeriosis than serotypes 1/2a and 1/2b combined; it is most often implicated in patients with meningoencephalitis which is more serious than infection limited to the blood stream, and patients with a serotype 4b infection suffer a 26% mortality rate compared to the 16% mortality rate suffered by patients infected with other serotypes (79, 95, 250, 264). Despite the clear need, there are currently no rapid tools specific for the isolation and detection of *L. monocytogenes* serotype 4b from food matrices. Molecular techniques such as PCR are very valuable as a rapid detection step to reduce the turnaround time from sampling to test results; however, they face the challenge and obstacle of a lengthy sample preparation time, which sometimes includes culture enrichment to increase the number of target pathogens prior to detection. In addition, if PCR is performed without culture enrichment there is no way to determine if the contaminant pathogenic bacteria are viable. These inherent drawbacks prompted us to seek methods for rapid isolation and detection of this important *L. monocytogenes* serotype 4b.

Immunological methods developed to date have shown some great promise in the isolation or detection of foodborne pathogens from food matrices. An example is immunomagnetic separation where monoclonal antibodies (MAbs) are coated on magnetic beads and are used to capture bacterial cells usually from an enrichment culture sample. Additional immunological applications include using MAbs to detect target pathogens through ELISA, biosensors or flow cytometry. However, all these techniques require high affinity, highly specific MAbs for *L. monocytogenes* serotype 4b strains, which are not currently commercially available to fulfil this need.

1.2 Hypothesis

Each *L. monocytogenes* serotype has a different surface proteome which allows for the serotypes to be defined serologically (121). The overall hypothesis of this thesis is that there is a surface protein or proteins unique to *L. monocytogenes* serotype 4b which is present in all serotype 4b isolates, but absent from isolates of other serotypes. MAbs against the surface proteome of a *L. monocytogenes* serotype 4b strain may not only be useful as diagnostic reagents themselves, but may also allow identification of a surface protein antigen(s) unique to serotype 4b strains. Such surface antigens could be suitable diagnostic markers to target *L. monocytogenes* serotype 4b isolates if they were demonstrated to be expressed during growth in a variety of stress and normal conditions. Work will be performed to test the above hypotheses. In addition, characterization of MAbs to the identified antigen(s) with respect to their affinity and epitopes, as well as biochemical characterization of the identified antigens, should provide useful information for test development and thus form a significant part of my thesis.

1.3 Research Goals

This research aims to identify and characterize a cell surface protein as a diagnostic marker for *L. monocytogenes* serotype 4b isolates using a panel of surface-binding MAbs previously generated against a serotype 4b strain LI0521 (151). In parallel, these MAbs will be characterized and assessed for their diagnostic potential. Specific aims are:

- (i) Elucidate the molecular identity of the target antigen(s) being recognized by the MAbs using mass spectrometry and N-terminal sequencing
- (ii) Localize the epitopes for each of the MAbs by generating recombinant protein fragments
- (iii) Determine the affinity constant of the association between each of these MAbs and their respective epitopes by surface plasmon resonance analysis
- (iv) Assess cross-reactivity of the MAbs with various strains of *L. monocytogenes* serotypes
- (v) Identify the promoter directing the transcription of the gene coding for the identified protein antigen and investigate the gene/protein expression during growth in normal and stress environments
- (vi) Investigate the secretion system used to transport the identified protein antigen to the cell surface
- (vii) Conduct biochemical characterization of the identified protein antigen

Chapter II

Literature Review

2. Literature Review

2.1 *Listeria monocytogenes*

Listeria monocytogenes is a Gram-positive, non-spore forming, facultative anaerobe which is a significant foodborne pathogen. Since *L. monocytogenes* is widespread in nature, hardy and can actively divide at refrigeration temperature, it poses a serious safety concern for RTE foods, which are consumed without cooking. Although *L. monocytogenes* accounts for a low proportion of all foodborne outbreaks, it has the highest rate of mortality for a food borne infection (225). Certain high-risk groups, such as pregnant women, neonates, the elderly and those with suppressed T-cell mediated immunity, such as AIDS, transplant or cancer patients, are more prone to developing serious symptoms associated with *L. monocytogenes* infection (225).

2.1.1 *Listeria monocytogenes* taxonomy

The genome of *Listeria*, for which there are now many sequences available, places it in the phylum Firmicutes, reveals a low G+C content (39%) and shows a close relation to *Bacillus*, *Clostridium*, *Enterococcus*, *Streptococcus* and *Staphylococcus* (97). The genus *Listeria* contains 8 species: *L. grayi*, *L. innocua*, *L. ivanovii*, *L. marthii*, *L. monocytogenes*, *L. rocourtiae*, *L. seeligeri*, and *L. welshimeri*. Genomic data suggests that all *Listeria* species have evolved from a common pathogenic ancestor and some members have lost genes essential to infection through adaptation to life as a saprophyte (39). *L. monocytogenes* and *L. ivanovii* are the only species which are known to be pathogenic, although *L. ivanovii* generally infects ruminants and human infections with this species are extremely rare (264). Currently, *L. monocytogenes* is divided into 12 serotypes, which are defined by agglutination with sera against somatic and flagellar antigens: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e

and 7 (121). The serotypes 1/2a, 1/2b and 4b account for over 98% of diagnosed *Listeria* infections. *L. monocytogenes* serotype 4b is associated with most food borne outbreaks, although, 1/2a and 1/2b isolates are more frequently isolated from food (95, 122). The highest case mortality rate associated with *L. monocytogenes* infection occurs when patients are infected with a serotype 4b isolate (95). *L. monocytogenes* has been grouped into three phylogenetic lineages based on multilocus enzyme electrophoresis, pulsed-field gel electrophoresis (PFGE), ribotyping, and more recently analysis of DNA sequences. The serotypes fit nicely within 3 defined lineages: lineage I represents 1/2b, 3b and 3c; lineage II contains 1/2a, 1/2c and 3a; serotypes 4a and 4c comprise lineage III and serotype 4b is split between lineage I and lineage II (221).

2.1.2 *Listeria* lifestyles and infection

L. monocytogenes switches between lifestyles as an environmental organism, a persistent food-processing plant contaminant and an intracellular pathogen. It is widespread in the environment and is commonly found in fecal matter, soil, silage, and decaying vegetation (121). *L. monocytogenes* can also persist in food processing plants and shows resistance to conditions designed to control bacterial contaminants, such as metal ions, high salt and refrigeration temperature (252). In addition, *Listeria* can form biofilms, making eradication from contaminated food processing equipment difficult (142). The genome of *L. monocytogenes* codes for many surface and secreted proteins, transport systems and transcriptional regulators, which may explain why *L. monocytogenes* can rapidly switch between life as a planktonic saprophyte, as part of a biofilm or as an intracellular parasite (97).

2.1.2.1 Life as a saprophyte

In the environment *L. monocytogenes* is able to survive in a variety of harsh conditions including temperatures of 2-45°C, pH of 2.5-9.6 and salt concentrations up to 10%; although, active cell division may not occur in all extreme conditions (226). Genome sequencing has shown the presence of genes necessary for the use of a variety of carbon sources (97) and research using defined media has demonstrated growth and division on multiple carbon sources (208). Genes for the metabolism of plant sugars are also present, leading to the hypothesis that as a saprophyte *Listeria* feeds off decaying plant material (85, 97, 181).

L. monocytogenes is able to undergo growth advantage in stationary phase (GASP), without negatively affecting virulence potential (35). GASP was first discovered in *Escherichia coli* and describes a phenotype with the ability to acquire mutations during stationary phase that produces an organism with optimized fitness and ability to out compete members of the same species from a younger culture (285). GASP allows *Listeria* to have long-term survival in stationary phase, without losing its ability to cause infection (35). These features all contribute to the success of *L. monocytogenes* in the environment, where it has been demonstrated to persist for at least a year (197).

It is still unclear if, while circulating in the environment, *L. monocytogenes* acts as a parasite to lower eukaryotic organisms, such as fungus, protozoa, or nematodes (85). Although, amoebae have been shown to feed on *L. monocytogenes*, there are conflicting reports on the ability of *L. monocytogenes* to invade cells and multiply within this host (3, 157). It has been demonstrated that *L. monocytogenes* is able to sustain cell-to-cell spread in *Drosophila melanogaster*, an insect species, and cause a lethal infection; however, it is unclear how often this happens in the environment (159). It is tempting to speculate that

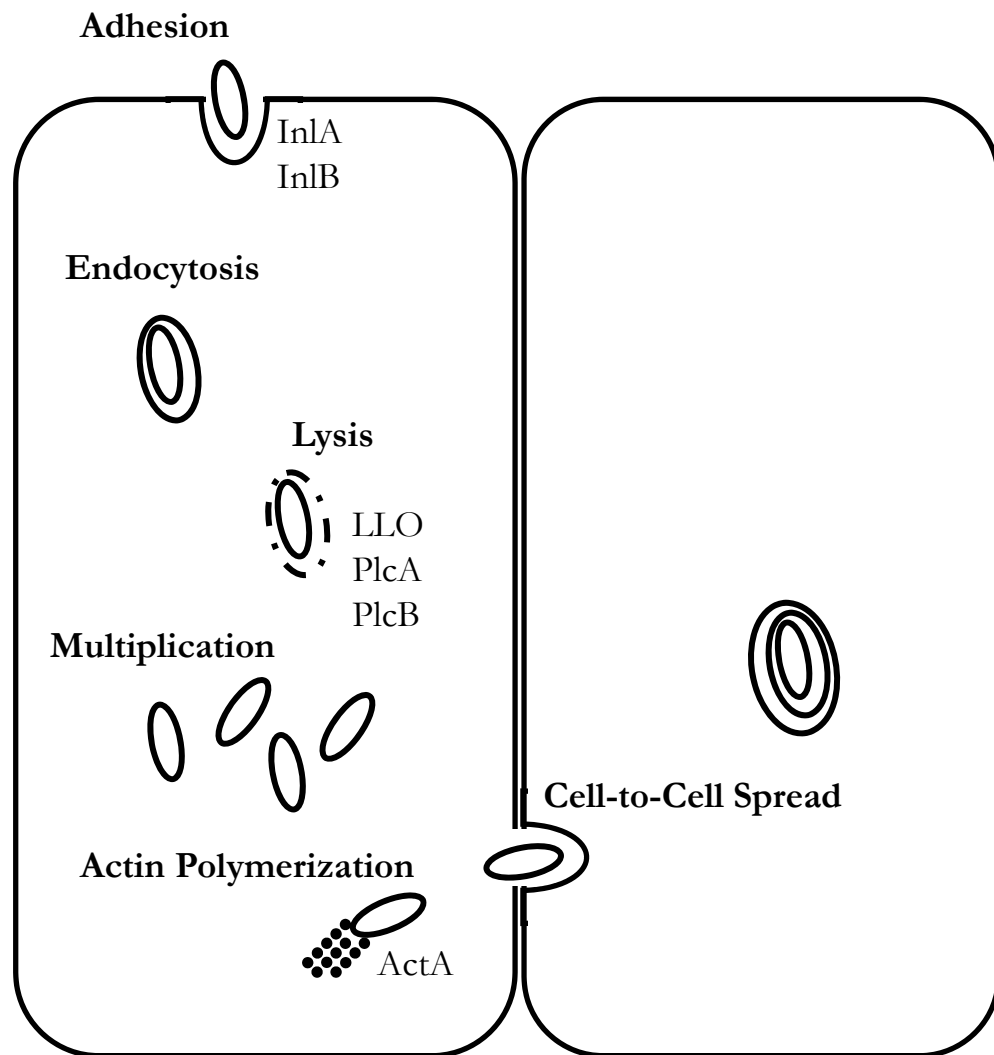
insects and lower eukaryotes may serve as transient hosts to *Listeria*, and help to maintain the presence of virulence genes during extended periods of time in the environment between periods of infection in higher organisms.

2.1.2.2 Life as an intracellular parasite

L. monocytogenes gains entry to the host through ingestion of contaminated food products and enters into a lifestyle as an intracellular pathogen. Listeriosis has a broad range of clinical outcomes, including asymptomatic infection, gastrointestinal symptoms, septicaemia, meningitis and spontaneous abortion. While this range of clinical outcomes is dependent on the dose, virulence and the immune status of the host; it is also a result of the ability of *L. monocytogenes* to cross three tight barriers: the intestinal barrier, the fetoplacental barrier and the blood brain barrier. The ability to cross tight barriers is likely a result of unique adhesions, internalization by non-phagocytic cells and the ability to spread directly from cell-to-cell.

Bacterial internalization and cell-to-cell spread take place through the coordinated regulation of a set of virulence factors, regulated by the PrfA regulon, including: InlA, InlB, LLO, PlcA, PlcB and ActA (Figure 2-1). Internalins (InlA and InlB) mediate adhesion to host cells through specific receptors and signal uptake into otherwise non-phagocytic cells (101). The phospholipases C (PlcA and PlcB) and the pore-forming toxin listeriolysin O (LLO) release *Listeria* from a membrane bound vesicle into the cytosol (204, 205). In the cytosol, *L. monocytogenes* is able to actively divide and express ActA. ActA is recruited asymmetrically to one pole of the bacteria and causes host actin molecules to polymerize and form a tail that propels the bacterial cell in one direction causing a protrusion into neighbouring cells until it is phagocytized in double layered vacuole (128). PlcB and LLO

Figure 2-1. *L. monocytogenes* infectious life cycle and cell-to-cell spread. *L. monocytogenes* adheres to permissive cells through interactions mediated by either InlA, InlB or both. Signalling cascades occur in permissive host cells after InlA or InlB bind to host cell receptors and induce endocytosis. After endocytosis *L. monocytogenes* is contained in a vacuole. The combined activity of LLO, PlcA and PlcB leads to lysis of the vacuole and release of *L. monocytogenes* cells into the cytoplasm where they multiply. Polarized expression of ActA causes actin to polymerize at one end of the bacterial cell and results in the propulsion of cells through the cytoplasm. Actin based propulsion continues until the bacterial cell is pushed up against the cell membrane causing it to protrude into neighbouring cells. Eventually the protrusion breaks off and the *L. monocytogenes* cell is contained in a double-layered vacuole in the neighbouring cell. The cycle then repeats itself.



are able to digest the double layered vacuole and the infectious cycle repeats (Figure 2-1) (245). Internalization by nonphagocytic cells and cell-to-cell spread allows for deep tissue infection as well as access to the bloodstream. Macrophages carry *L. monocytogenes* to mesenteric lymph nodes, the spleen and liver, where, in the absence of a strong immune response, the bacteria establish a serious infection (137). Upon systemic infection, *L. monocytogenes* shows tissue tropism for the placenta and the central nervous system (79).

InlA and InlB are members of the internalin family of proteins which contain an N-terminal signal peptide followed by a variable number of leucine rich repeats (LRRs) and a conserved inter-repeat (IR) region (101). Internalins are a special group of adhesins which bind eukaryotic host receptors associated with signalling cascades that are able to cause mechanical rearrangement of the actin cytoskeleton, at the site of attachment, leading to bacterial internalization by non-phagocytic cells (198). Internalins are divided into three subfamilies based on their association with the bacterial cell wall. InlA is part of the largest subfamily which is covalently anchored to the cell wall through an LPXTG motif at the C-terminal end. InlB is in a subfamily which interacts noncovalently with the cell-wall through interactions between lipoteichoic acid (LTA) and the cell wall binding domain (CBD) composed of GW-repeats. The third subfamily lacks an anchoring domain and is predicted to be secreted (101).

The involvement of InlA and InlB in host cell invasion has been demonstrated through multiple lines of research. Expression of recombinant InlA and InlB causes internalization of normally non-invasive bacteria such as *L. innocua* and *Streptococcus faecalis*. Latex beads coated in recombinant InlA and InlB are also internalized into permissive cell lines (32, 33, 139, 175). Deletion mutants of InlB (63, 153), InlA (153) or

both (89) are all severely impaired in host cell invasion. InlA is necessary and sufficient for invasion of intestinal epithelial cells (140) and is required for internalization into the Caco-2 epithelial cell line (63, 87). InlB is not required for intestinal cell internalization (125), but InlB is required for entry into hepatocytes (63), while InlA is not. InlB and InlA are both required to cross the fetal-placental barrier and cause neonatal listeriosis (57). The role of InlA and InlB in breaching the blood brain barrier is still poorly understood (24) and should be a focus in future research.

The host-cell receptor for InlA is E-cadherin (24) and Met is the receptor for InlB (125). InlA and InlB have stringent species-specific binding which is based on the sequence of the hosts E-cadherin and Met. InlA interacts with human, guinea pig and rabbit E-cadherin; however, mice and rats have a substitution of glutamate to proline at position 16 and are not functional receptors for InlA (101, 138). Mouse and human Met both interact with InlB, but guinea pig and rabbit do not (101). The long-standing lack of an appropriate animal model created a major hurdle for studying *Listeria* infection *in vivo* until two recent breakthroughs: the development of a mouse model expressing human E-cadherin (140) and the discovery that wild gerbils are permissive to both InlA and InlB (57). Better animal models have allowed advances in the understanding of *in vivo* internalin mediated phagocytosis and *Listeria* infection in general.

There is a common frame shift mutation that results in a truncated InlA lacking the LPXTG motif. This mutated InlA does not attach to the bacterial cell-wall and is found secreted into the extracellular milieu (115). Epidemiological evidence showing that InlA is an important virulence factor was provided by a study which showed that 100% of pregnant

women with listeriosis were infected with a strain expressing the full length InlA, while only 65% of food isolates expressed this protein (111).

The receptor for InlB, Met, is a protein tyrosine kinase that is almost ubiquitously expressed in human cells (24). It is suspected that InlB plays the primary role in infecting cells beyond the gut epithelia (24). Hepatocyte growth factor/scatter factor (HGF/SF) is the natural ligand for Met. Both InlB- and HGF-Met interactions induce cytoskeletal rearrangements; however, InlB induces a more potent activation of the Met cascade. The difference in activation strength likely results from InlB and HGF/SF having distinct binding sites on the Met protein (185, 239, 267) and InlB having two additional receptors. A complement component C1q receptor gC1qR (31) and heparin sulphate proteoglycans (HSPGs) have been shown to interact with InlB (116) and the interaction between InlB and HSPGs has been shown to enhance InlB-induced Met signalling (11, 116). The GW-repeats of InlB are also partially responsible for the interaction with coreceptors gC1qR and HSPGs (31, 116, 267) as evidenced by experiments showing that recombinant InlB molecules only containing the LRR and IR domains are not able to fully activate the Met pathway (11, 185).

LLO is the primary molecule responsible for the escape of *L. monocytogenes* from the phagosome (64). LLO knockout mutants are attenuated and do not escape the phagosome to multiply in the cytosol (12, 88, 133, 205, 236). Incubation with an LLO neutralizing MAb blocks passage of *L. monocytogenes* cells from the phagosome to the cytosol in macrophages (74). This provides additional evidence that LLO is required for phagosome escape. LLO also appears to be sufficient for phagosome escape. When *Bacillus subtilis*, a non-pathogenic soil organism, is transformed with recombinant LLO, it is able to escape from the phagosome and multiply in the host cytosol (18). LLO is also required for escape from the

double-membrane phagosome that is formed upon infection of adjacent cells during cell-to-cell spread (91).

LLO is a member of the cholesterol-dependent cytolysins (CDCs) family, which includes a number of pore-forming toxins from Gram-positive bacteria including LLO from *L. monocytogenes*, streptolysin O (SLO) from *Streptococcus pyogenes*, anthrolysin O (ALO) from *Bacillus anthracis* and perfringolysin O (PFO) from *Clostridium perfringens* (230).

There are two non-exclusive models of how LLO participates to dissolve the phagosome. The first model hypothesizes that LLO forms pores in the phagosome and PlcA and PlcB move through these pores to gain access to phospholipids which results in the dissolution of the phagosome (259). The second model suggests that LLO acts to slow phagosome maturation and gives PlcA and PlcB time to help to degrade the phagosome before lysosome fusion (105). Portnoy suggests that these models can be combined and speculates that LLO pores prevent maturation of the phagosome, which prevents lysosome fusion, thus providing a favourable environment for PLCs to participate in phagosome disruption (230).

Activity of LLO must be restricted to the phagosome to prevent lysis of the host plasma membrane which releases *L. monocytogenes* into the extracellular milieu, where it can be killed by the immune system. Since LLO activity on the host plasma membrane leads to bacterial attenuation, it is not surprising that there are multiple levels of regulation including transcriptional, translational and post-translational. Transcription of LLO is mostly regulated by PrfA and is up-regulated under conditions which increase expression of the PrfA regulon such as intracellular growth (132, 238). Although translational control is not fully understood, it is linked to the amino acid sequence of LLO, especially the unique N-terminus which

represses LLO translation. N-terminus deletion mutants have a 10 000-fold decrease in virulence due to lysis of the host cell membrane (55, 146). However, there are differences between the ability of the N-terminus to suppress LLO expression. The the N-terminus of LLO has variable ability to suppress green fluorescent protein (GFP) expression when the two are fused together. This suggests that the downstream sequence of LLO also plays a role in its own down-regulation (238). LLO activity is regulated at the protein level in several ways. LLO contains an intrinsic pH sensor which causes the protein to unfold at pH 7.4 and at temperatures above 37°C, however, LLO remains in the active conformation at pH 5.5 (233). This prevents pore formation in the host cell membrane. LLO present in the host cell cytosol is degraded by the proteasome, representing another mechanism of post-translational control (231). Ubiquitination and phosphorylation of LLO also seem to play a role in regulation, although, it is yet not fully understood how this operates (231).

The breakdown of the phagosome by LLO is facilitated by PlcA and PlcB (204). PlcA is a phatidylinositol-specific phospholipase (141, 165, 245) and PlcB is a broad range phospholipase (245, 263). It has been suggested that phospholipases are responsible for dissolving the inner membrane of the double-layered phagosome created after cell-to-cell spread, but they are not sufficient for disruption of the outer membrane if LLO is absent (4). A comprehensive study using a PlcA knockout mutant, a PlcB knockout mutant and a double knockout mutant showed that PlcA is involved in escape from the primary vacuole and $\Delta PlcB$ mutants are unable to escape from the double membrane phagosome formed in cell-to-cell spread (245). $\Delta PlcA$ mutants have a two-fold reduction in virulence, $\Delta PlcB$ mutants are 20 times less virulent and the double knockout mutants are reduced in pathogenicity by a factor of 500 (245).

ActA is required for cell-to-cell spread of *L. monocytogenes* through actin motility (50, 130, 253). $\Delta ActA$ deletion mutants are able to infect cells and escape the primary phagosome to enter the cytosol, however, they are unable to polymerize actin and move by actin-based motility and are therefore unable to spread from cell-to-cell (34, 58, 128). ActA is required and sufficient for host cell actin polymerization as evidenced by two lines of research. Directional movement of recombinant ActA coated polystyrene beads through cytoplasmic extract was observed (45). In addition, when ActA is expressed in *L. innocua*, which normally lacks actin based-motility, this protein is able to induce actin assembly and move unidirectionally (130). ActA controls actin polymerization at all steps in the process including: initiation of actin polymerization, initiation of actin-based propulsion and the rate of movement (246).

The ActA protein is composed of three domains: an N-terminal domain, a central proline rich-repeat (PRR) region and the C-terminal domain which anchors ActA into the bacterial cell membrane (50). The N-terminus is the active part of the protein involved in actin-polymerization. The first 156 amino acids of the N-terminus can polymerize actin and the PRR region controls the rate of actin-based movement. There is a linear relationship between the number of PPRs and the speed of bacterial propulsion (246).

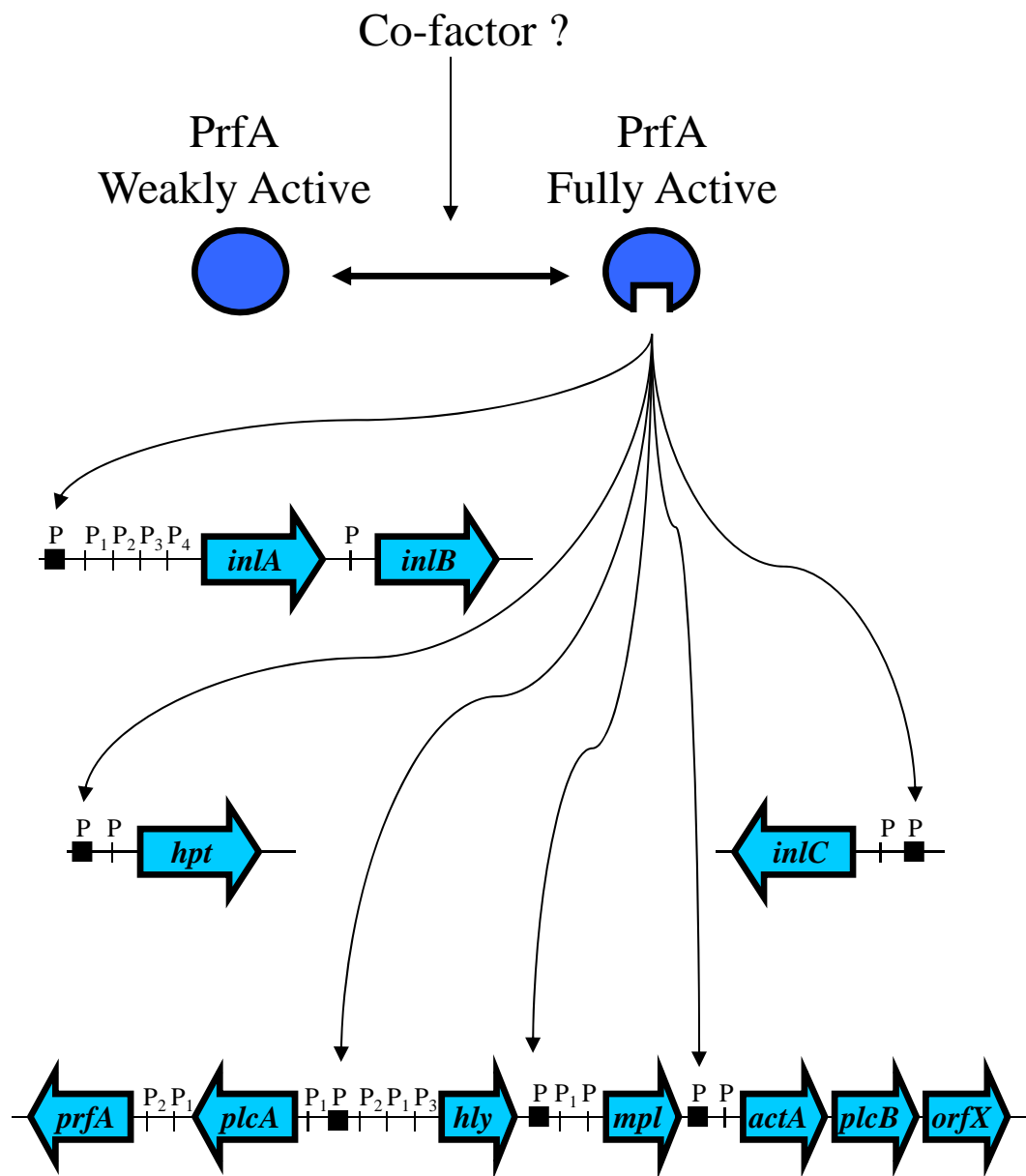
Polarized localization of ActA molecules is required for unidirectional movement of *L. monocytogenes*. There is a linear relationship between ActA polarity and propulsion speed (212). Polarization of ActA happens successively over generations. Initially ActA proteins accumulate at the sides of the bacteria. After one round of cell division ActA localizes its expression to the pole not involved in division (129, 213). This causes ActA distribution to become more polarized after each successive generation (214).

In addition to its involvement in actin-based movement, ActA also participates in epithelial cell invasion. Mutants which constitutively express the PrfA regulon (PrfA*) are hyperinvasive in epithelial cells. PrfA* mutants which also contain an ActA deletion are non-invasive but if ActA alone is over expressed the mutants are hyperinvasive (248). There are currently two theories explaining ActA involvement in epithelial cell invasion. The first is that Act A interacts with HSPGs and induces cell signalling leading to cytoskeletal rearrangement and phagocytosis (248). The second hypothesizes that ActA N-terminal fragments may be released and taken up by cells leading to the destabilization of the host cell actin cytoskeleton allowing for phagocytosis (5).

2.1.3 The *prfA* Regulon

The *prfA* regulon is essential for *L. monocytogenes* pathogenesis and controls the expression of genes whose products mediate intercellular parasitism. PrfA is the key transcriptional activator involved in regulation of the *prfA* regulon. The C-terminus is a DNA-binding helix-turn-helix domain, which promotes transcription of PrfA controlled genes when it binds to a 14 bp palindromic nucleotide sequence, referred to as the PrfA box, with the consensus sequence: tTAACanntGTaAa (234). PrfA is responsible for the expression of *plcA*, *plcB*, *mpl*, *hpt*, *actA*, *hly* (LLO), *inlA*, *inlB* and *inlC*, as well its own expression (132) (Figure 2-2). Although, these 10 genes are the only ones which have been experimentally shown to be directly regulated by PrfA, an analysis of the *L. monocytogenes* transcriptome revealed that as many as 145 other genes are indirectly regulated by PrfA (169). The strength of expression of genes in the PrfA regulon relies on each of three factors: the concentration of PrfA, the affinity for the promoter - based on the conformance to the PrfA box and the conformation of PrfA (234).

Figure 2-2. The PrfA Regulon. The virulence genes directly responsible for mediating *L. monocytogenes* infection *inlA*, *inlB*, *plcA*, *plcB*, *hly* and *actA* are each part of the PrfA regulon. Each of these virulence genes can be transcribed from any of several promoters (P₁-P₄) found upstream of their ORFs. However, upon infection, these genes are primarily transcribed from PrfA promoters also known as PrfA boxes (■). These PrfA boxes are the binding sites for the PrfA transcriptional activator protein. The PrfA protein is the primary regulator of virulence gene expression in *L. monocytogenes*. Prior to infection, PrfA is transcribed from a σ^A promoter. PrfA transcripts from the σ^A promoter contain a temperature sensitive loop that prevents translation. This loop melts at temperatures above 30°C. Upon infection (37°C) the temperature sensitive loops dissolve and the transcripts are quickly translated to protein resulting in an exponential increase in the availability of the PrfA activator protein. Since PrfA also controls its own expression via a PrfA box upstream of the PrfA ORF, this positive feedback loop contributes to prolonged PrfA production during infection. PrfA transcripts which are transcribed from the PrfA box do not contain a temperature sensitive loop. PrfA also appears to undergo an allosteric shift from a weakly active to a highly active state upon interaction with an unknown cofactor. This figure was based on information found in figures in Kreft (132) and Scotti (234).



Regulation of PrfA expression is multi-level and not completely understood.

Expression of PrfA takes place from at least 3 promoters: the P1prfA promoter is controlled by σ^A , the P2prfA promoter is controlled by σ^B and PplcA is controlled by PrfA. PrfA is able to activate its own transcription via a positive-feedback auto-regulatory loop. Expression from the *PplcA* promoter directs the expression of a bicistronic plcA-prfA transcript (177, 217, 234). The σ^B is involved in stress response and likely stimulates *prfA* expression as a response to stresses encountered in the gut (54). However, σ^A is associated with a constitutive promoter and transcripts from this promoter are always present. Transcripts from the σ^A contain a RNA hairpin thermoswitch up-stream of the translation start site, which blocks ribosome binding at temperatures below 30°C. This secondary structure melts at 37°C and allows translation of the PrfA protein (113). This latter mechanism of regulation is very advantageous, as it allows the *L. monocytogenes* cell to accumulate *prfA* transcripts which are not transcribed until the cell enters the host and warms to 37°C, where a large amount of the PrfA protein can be made quickly.

In addition, small-regulatory RNAs have recently been shown to down-regulate PrfA translation by binding with the 5' UTR of *prfA* mRNA. However, since this causes only a 2 or 3-fold down regulation, its biological significance is still unclear (155). PrfA is also allosterically regulated and has an off and on conformation. Deletion mutants have provided evidence of allosteric regulation, since deletion of various amino acids can lead to a perpetually active conformation (219, 220, 240). A change in PrfA conformation is likely the result of interaction with a co-factor, since the conformational changes which occur in deletion mutants leading to an active-state are similar to the changes seen in a related molecule Crp when it binds to its co-factor cAMP and changes into its active form (75, 266).

In addition, since all known factors of PrfA regulation do not account for the changes in PrfA activity due to medium variation, additional factors have been proposed to affect PrfA activity including carbon source, stress response and motility (54).

2.1.4 Protein secretion systems

In bacteria, secreted proteins are involved in a number of processes such as cell wall turn over, cell-to-cell communication, nutrient uptake and environmental sensing. Pathogenic bacteria also use secreted proteins to mediate host-pathogen interactions such as adhesion, internalisation and evasion of the immune system. There are currently 16 known systems involved in protein export in bacteria (192). Protein secretion in Gram-negative bacteria is complicated since proteins must be secreted through the inner membrane, the periplasm and the outer membrane. In addition, the Type 4 and the Type 3 Secretion Systems have been described in Gram-negative bacteria, which are able to inject proteins directly from the cytoplasm of the bacterial cell into host cells (192). Fewer secretions systems have been described in Gram-positive bacteria and they are generally less complex since secretion is only required to cross the cytoplasmic membrane. Secreted proteins are extremely important in *Listeria*. Differences in the secreted proteome have been suggested to explain the variation in virulence between *L. monocytogenes* and *L. innocua* (257).

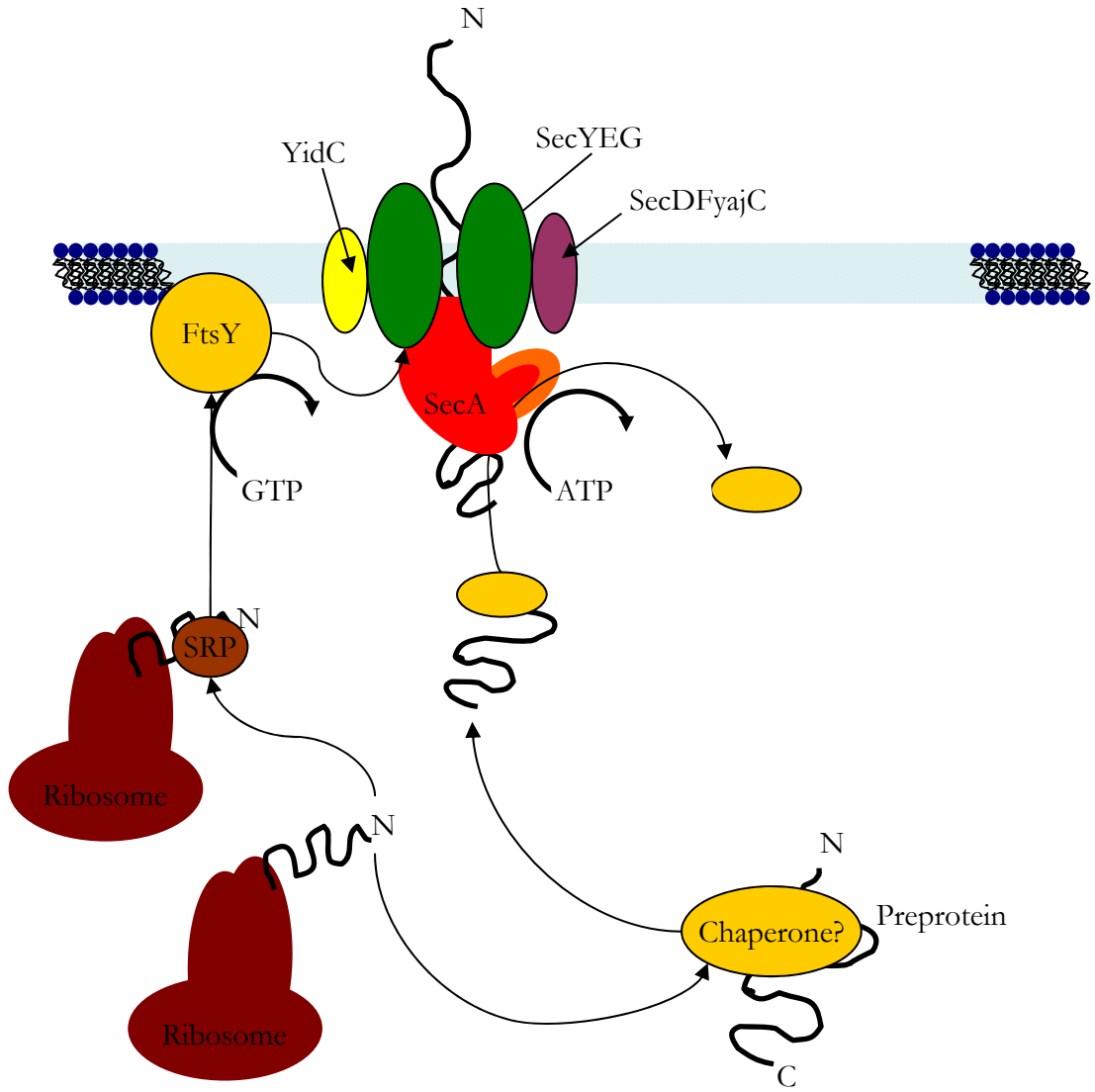
Six protein secretion systems are encoded by the sequenced genome of *Listeria* species: secretion apparatus (Sec), fimbriin-protein exporter (FPE), twin-arginine translocation (Tat), flagella export apparatus (FEA), WXG100 secretion system (Wss) and the Holins (56). Each of the above secretion systems are found in each of the sequenced genomes of *Listeria*, with the exception of the TAT pathway, which is not present in *L. monocytogenes* serotype 4b F2365 (56). Of the 531 proteins in *L. monocytogenes* which are

predicted to be secreted, only 508 are predicted to be exported via the Sec pathway (56). In a study which compared the exoproteome of 12 different *L. monocytogenes* isolates, only 60 proteins were experimentally demonstrated to be secreted. Of these 60 proteins 42 were predicted, by bioinformatics, to be secreted via the Sec pathway and the other 18 were predicted as being translocated by an unknown secretion system. Since such a high proportion of secreted *Listeria* proteins are secreted by the Sec pathway, it is ambiguous whether there are additional secretion systems that are functional in *Listeria* (68).

The genome of *L. monocytogenes* encodes all of the putative components of the Sec system. A large number of proteins present in the listerial genome contain a predicted Sec-dependent N-terminal signal peptide, several of which have been confirmed to be secreted (257). However, direct experimental evidence of a functional Sec system and complete characterization of its different components still does not exist for *L. monocytogenes* (56). The evidence accumulated to-date provides a compelling argument that SecA is functional and is the main secretion system used by *L. monocytogenes*. Given this, along with the high degree of observed conservation of the Sec system, the Sec secretion pathway will be discussed in detail in this section, using findings from closely related bacteria.

The Sec pathway is the major translocation system crossing the cytoplasmic membrane for Gram-positive bacteria (265) (Figure 2-3). There are three steps in secretion via the Sec pathway: protein targeting, translocation and protein release and maturation (192). Proteins transported by the Sec pathway require an N-terminal signal sequence to be targeted to the translocation machinery. Although the signal sequence has little to no conservation, all signal sequences display a tripartite structure, which can be detected by algorithms such as Signal P. A Sec signal sequence contains an N-terminus with positively charged amino acids,

Figure 2-3. SecA Secretion. Most surface proteins in *L. monocytogenes* are exported via SecA secretion. SecA secretion requires three sequential steps: protein targeting, translocation and protein release (192). Protein targeting can occur in a cotranslational manner using a signal recognition particle (SRP) or post-translation using either SecB or another unidentified chaperone (66). In the case of the SRP transport, a complex which contains the ribosome, a GTPase and an Ffh protein interacts with the FtsY protein at the cytoplasmic membrane. The FtsY protein interacts directly with the SecYEG channel and stimulates the hydrolysis of a GTP which in turn causes dissolution of the SRP complex and transfers the partially translated protein to the SecYEG complex. Other chaperones may be involved in an alternate protein targeting pathway, which may be functionally homologous to SecB transport, although, this pathway is unconfirmed in *L. monocytogenes*. SecYEG is the core component and serves as a protein conducting channel (66). SecA is the ATP dependent motor which provides the energy for translocation, however, the mechanism by which SecA generates energy is still under investigation (265). This Figure is modified from: (56), (66), and (265).



a central hydrophobic domain, and a C-terminus containing polar amino acids which serve as a signal peptidase site (66).

In *E. coli* preproteins can be targeted to the membrane associated Sec structures in one of two ways: in a cotranslational manner using signal recognition particle (SRP) or post-translationally by association with SecB or other chaperones (Figure 2-3) (66). In *L. monocytogenes* all components of the SRP pathway are encoded by the genome. SecB homologues have not been identified, but this does not rule out the possibility of other unidentified chaperones. SRP is a complex composed of the ribosome, a GTPase and Ffh (*Lmo1801*). The Ffh protein interacts with its receptor FtsY (*Lmo1803*) at the cytoplasmic membrane (66). FtsY has been shown to interact directly with the SecYEG channel (6). The interaction of FtsY and Ffh allows the complex to bind to and hydrolyze GTP. GTP hydrolysis initiates the dissociation of SPR and FtsY as well as the transfer of the partially translated protein to the SecYEG complex (209).

SecA is the ATP dependent motor which provides the energy for preprotein translocation through the SecYEG channel (265). The exact mechanism of how SecA generates energy and the nature of the SecA-preprotein interaction is unclear. It is, however, clear that SecA binds the N-terminal signal sequence of the preprotein and somehow provides the energy required for passage through the SecYEG pore. It is also clear that SecA transiently interacts with the SecYEG complex and this high affinity interaction activates the ATPase activity of SecA (102, 150). However, there are several models attempting to explain how SecA mediated ATP hydrolysis is coupled to a moving preprotein. The power-stroke model, predicts that ATP hydrolysis induces conformational changes in SecA that are translated into mechanical force which push the preprotein through the SecYEG channel.

This model proposes that ATP hydrolysis leads to insertion and de-insertion of SecA into the membrane at the SecYEG complex. The energy released by insertion and de-insertion leads to step-wise translocation linked to ATP-dependent conformational changes in SecA (72, 73, 229, 255, 262). An alternative model suggests that SecA conformational changes occur entirely on the cytoplasmic side of the inner membrane and directly feed the preprotein into the SecYEG channel. This model is based on the crystal structure of the SecA-SecYEG complex and suggests that the “fingertip” of SecA is inserted into the cytoplasmic opening of the SecY channel. The fingertip moves the preprotein through SecYEG when ATP binds and then releases the protein and resets upon ATP hydrolysis (77, 286). This model also explains step-wise ATP-dependent translocation. However, studies examining the transport kinetics of the SecA pathway, do not support step-wise translocation and propose an additional model. This latter model suggests that SecA is a gate keeper to prevent free access of preproteins to the SecYEG channel. ATP hydrolysis is required for opening the channel, and is not directly coupled to precursor movement. Under this model, the preprotein diffuses through the SecYEG channel upon the hydrolysis of a single ATP molecule required to open the channel (149). A similar model suggests that Brownian motion rather than diffusion is responsible for movement through the SecYEG channel (243).

The central component of the Sec pathway is the SecYEG complex which serves as a protein conducting channel and is very highly conserved among bacteria, eukaryotes and archaea (66, 265). SecY (*Lmo2612*) is the largest subunit of this complex and provides the structure for the protein conducting channel by spanning the membrane with 10 transmembrane domains (2). SecY is essential to the functionality of Sec secretion and subsequently SecY deletion mutations are fatal (265). When proteins are not being

translocated, a short helix plugs the periplasm side of the SecY pore; this is the closed state of the channel and the helix is displaced during translocation (261). SecY has been demonstrated to interact with SecE (*Lmo0245*), SecG (*Lmo2451*) and SecA (*Lmo2510*). SecE has been shown to provide stability to the SecY structure and in the absence of SecE, SecY is readily degraded (126). The fifth cytoplasmic loop of SecY likely provides docking for SecA and therefore mutations in this region are also fatal (174). SecE is also essential for Sec secretion and deletion mutants of this gene are fatal. The C-terminus is the only part of the SecE protein which is essential for SecY interaction, although, other parts of both proteins have shown evidence of additional interactions (131). SecG is not essential for a functional Sec secretion system and SecG deletion is not fatal; however, SecG makes translocation more efficient and deletion mutants have a cold-sensitive phenotype (27, 83). In *E. coli* SecG was shown to facilitate the activity of SecA translocation, especially at temperatures below 20°C (187). In addition, at low ATP concentrations (below 30 µM), ΔSecG mutants do not have functional Sec secretion (160). Experiments (based on the activity of PhoA) using a recombinant SecG-PhoA protein (PhoA is only active in the cytoplasm) have shown that SecG in complex with SecA has an inverted topology, which reverses upon SecA dissociation. This may play a role in providing the energy for SecA-dependent translocation (188). SecA can be mutated into a form that no longer requires SecG below 20°C, however, this mutation is disadvantageous to the cells because they lack regulation in channel gating and are unable to secrete proteins at low ATP concentrations (160).

In addition, there are auxiliary components of the Sec secretion system. A complex known as SecDFYajC has been found to co-immunoprecipitate with SecYEG (69). This complex is composed of SecDF (*Lmo1803*) and YajC (*Lmo1529*). The exact role of

SecDRYajC is unknown, but unlike other Sec pathway proteins SecDF and YajC constitute an operon. Deletion of both SecD and SecF have reduced growth and protein export, however, there is no detectable phenotype associated with YajC mutations (199). It has been suggested that SecDFYajC helps to stabilize SecA while it is inserted into the membrane (72). An anti-SecD antibody, which upon binding inhibits its activity, has provided direct evidence that SecD plays a role in releasing proteins which have been translocated across the inner membrane (161).

Recently it has been discovered that some, but not all, Gram-positive bacteria have two SecA proteins (SecA and SecA2). SecA is essential for survival, but SecA2 is not (144). SecA2 was discovered in *L. monocytogenes* by doing transposon mutagenesis screens for rough colonies (144). Since SecA2 is not essential, knockout mutants have been constructed for *L. monocytogenes* serotype 1/2a. Portnoy et al. (143) were able to isolate 25 extracellular proteins and 49 surface associated proteins from *L. monocytogenes*. Of these proteins 8 of the extracellular proteins and 19 of the surface associated proteins are SecA2-dependent for secretion (143). Some proteins exported via the SecA2 pathway did not appear to have signal sequences (143). Some proteins, such as P60 and NamA, were shown to be almost exclusively to be exported by SecA2, while only mild translocation deficits were shown in the SecA2 knockout for other proteins (143). There is likely a continuum that includes proteins which are entirely SecA secreted, some which are entirely SecA2 exported, and the rest falling somewhere in between (218). Some bacteria have an accessory SecY2, but *L. monocytogenes* does not. Therefore, it is predicted that in *L. monocytogenes*, at least, SecA2 shares SecYEG with SecA (218). Although SecA2 is found in both pathogenic and non-pathogenic Gram-positive bacteria (172), SecA2 clearly plays a role in virulence, at least for

L. monocytogenes (143). It is also required for the production of protective CD8⁺ T-cells during a listerial infection (176, 215). Differences in immune response were discovered to be due to SecA2 secreted proteins and not directly because of the presence or absence of SecA2 itself. Therefore, it is likely that SecA2 is important for the secretion of immunogenic proteins (176).

After translocation across the cytoplasmic membrane, proteins can be linked to the cellular envelope or secreted into the extracellular milieu. Each of these possibilities is discussed in detail in the surface protein anchoring mechanisms section below.

2.1.5 Surface protein anchoring mechanisms

Surface proteins must have a domain or region capable of interacting with the bacterial envelope. This domain is essential for anchoring the protein to the bacterial cell surface. There are three types of protein surface attachments identified in Gram-positive bacteria: covalent attachment to the peptidoglycan, attachment to the cytoplasmic membrane, and ionic interaction with cell wall polymers.

Several *L. monocytogenes* surface proteins are anchored to the cell wall peptidoglycan (PG) through a LPXTG motif. Proteins attached by this method have a characteristic C-terminal sorting signal that contains the LPXTG motif, followed by 20 hydrophobic residues and finally a stretch of positively charged amino acids (81). The LPXTG sorting signal is responsible for directing the covalent attachment to the PG (19). The LPXTG motif is the substrate for sortase A, which cleaves the LPXTG between threonine and the glycine residues and catalyzes the formation of an amide bond between the carboxyl group of the threonine and an amine group in the peptidoglycan peptide bridge (162, 256). *L. monocytogenes* encodes two sortases sortase A and sortase B. Sortase A is

involved with anchoring LPXTG proteins and a number of these are important surface proteins, including InlA (21). Sortase B is responsible for anchoring NXXTX proteins to the PG (210). The sortase B pathway is not used as frequently as sortase A. In *L. monocytogenes* strain EGD-e (serotype 1/2a), only two proteins were found to be sortase B-dependent: SvpA and Lmo2186 (20).

Another mechanism responsible for anchoring surface proteins to the bacterial surface is GW modules which anchor the protein to the cell wall through non-covalent interactions with lipoteichoic acids (LTA) (114). InlB is a common example of a surface protein which is anchored with GW modules. The c-terminus of InlB contains three conserved tandem repeats of ~80 amino acids each starting with the amino acid motif Gly-Trp, for which the GW modules are named (30). Several proteins, such as IspC (275), contain additional GW modules which create a stronger interaction with the cell wall (19). LTAs are anchored directly into the membrane through their diacylglycerolipid domain (180). GW modules also interact with eukaryotic molecules such as glycosaminoglycans, making any protein containing GW modules a suspect for mediating host cell interactions and attachment during infection (116). GW module containing proteins do not need to be processed by the cell to bind the LTA and if GW modules are added to a cell wall, or a whole cell mixture they will spontaneously attach (30). The interaction between a LTA and GW modules is specific. For example, GW modules from *Listeria* proteins will bind to the LTA of *Listeria*, however, they will not bind the LTA of *S. aureus* (114). GW modules are disproportionately responsible for anchoring autolysins to the cell wall. In *L. monocytogenes* EGD-e, seven of the 8 proteins which are predicted to be anchored via a GW domain also have a putative peptidoglycan hydrolase domain (19).

The LysM domain has been implicated in PG binding in other organisms, such as *Enterococcus faecalis* (71); however, this has never been experimentally demonstrated for *L. monocytogenes*. The LysM domain is common in autolysins and several proteins from *Listeria* have this domain. Two autolysins, involved in pathogenesis have LysM domains: p60 has two and MurA has four (43). Both of these proteins have been identified in the cell wall fractions of *L. monocytogenes* (43), suggesting that LysM anchors these proteins to the cell wall. However, p60 has also been identified as being secreted into the media, indicating it may have a weak attachment to the cell wall (143).

Hydrophobic tails are a mechanism of associating with the membrane through a stretch of hydrophobic proteins followed by a few charged residues (254). These charged residues act as a stop transfer signal for secretion. ActA is an example of a protein anchored to the cell through a hydrophobic tail (128). Lipoproteins also serve to anchor surface proteins through association with the cell membrane; however, this interaction is mediated by a covalent N-terminal lipidation (249). Lipoproteins have a lipobox with a conserved cysteine residue (249).

2.1.6 Expression of *L.monocytogenes* proteins in stress growth conditions

L. monocytogenes is very resistant to environmental stress compared to other bacteria and this resistance is partially due to several systems which mediate gene expression and allow the bacteria to adapt to changing environments. Protein expression in *L. monocytogenes* is also subject to regulation and is highly dependent on growth conditions (92, 93, 178, 179). In *L. monocytogenes*, the alternative sigma factor σ^B plays an important role in general stress response. Activity of σ^B increases in response to exposure to acid, ethanol, osmotic stress, heat and oxidation (80). σ^B upregulates the expression of several

proteins required for stress resistance and downregulates several genes such as those encoding flagellar or ribosomal proteins (184). Furthermore, σ^B has been shown to increase survival during stationary phase (13).

The proteome of *L. monocytogenes* has been analyzed in response to various stresses with the objective of better understanding mechanisms of resistance to stress growth. In response to osmotic shock the rate of synthesis of 42 proteins was altered, where 80% had increased expression in high-salt conditions (67). The expression of Cct, a general stress protein related to ribosomal proteins and YvyD, a protein that belongs to the 30S ribosomal family, are both up regulated by salinity and appear to be regulated by σ^B based on the presence of a σ^B promoter upstream (67). The expression of two proteins AckA and PdhD which are both involved in general metabolism are both downregulated by high salinity independently of σ^B (67). Changes in the proteome due to salt-stress may be indicative of an attempt by the cell to restore protein synthesis, which is depressed after osmotic shock (67). The synthesis of 45 proteins was upregulated by exposure to alkali conditions, while the synthesis of 45 additional proteins was downregulated under the same conditions (96). Growth in alkali conditions causes upregulation of proteins involved in phosphate uptake and utilization in *L. monocytogenes*. It was suggested that upregulating phosphatases may limit the transport of solutes through the cell membrane. Since most translocation reactions occur through phosphorylation/dephosphorylation, bacteria which limit these reactions may have an advantage in alkali conditions (96). In *L. monocytogenes*, 6 proteins were upregulated and 12 were down regulated due to heat shock at 60°C (1). Three proteins which were the most upregulated by heat shock, TcsA, Gap and AtpA, may have value as surface protein markers for identification of *L. monocytogenes* that have been heat stressed (1).

Growth of *L. monocytogenes* in stress conditions also affects the expression of surface antigens altering the ability of certain antibodies to detect bacterial antigens. The MAbs C11E9 and EM-7G1 were developed as diagnostic reagents for *L. monocytogenes* (17, 92, 93, 178, 179). The antigen recognized by C11E9 and EM-7G1 is an *N*-acetylmuramidase: MurA (*lmo2691*) (47, 92). This antigen is readily detected by C11E9 and EM-7G1 when cells are grown in *Listeria* repair broth (LRB), brain heart infusion (BHI) broth and *Listeria* enrichment broth (LEB), but is down regulated when cells are grown in University of Vermont media (UVM) or Fraser broth (92). MurA is also detected when cells are grown in BHI broth between 4 and 45°C and between 0.5 and 1.5% additional NaCl; however, the antigen becomes harder to detect above 3.5% additional NaCl (93). The effectiveness of polyclonal antibodies (PABs) to detect *L. monocytogenes* also varies dependent upon culture conditions despite being able to react to multiple antigens. A certain PAB which recognizes a 62 kDa protein (*lmo0610*), flavocytochrom C fumarate reductase (*lmo0355*), enolase (*lmo2455*), glyceraldehyde 3-phosphate dehydrogenase (*lmo2459*), and a hypothetical protein (*lmo0041*) was unable to detect cells which had been cultured in LRB or Fraser broth (92). However, this PAB readily detected cells grown in BHI, UVM and buffered *Listeria* enrichment broth (BLEB) (92); indicating that some or all of these proteins were differentially expressed in various enrichment media. Studies with PABs against virulence factors have demonstrated that these proteins can have altered expression in various growth media. A PAB against ActA was able to react with whole cells grown in BLEB, UVM and Fraser broth, but was unreactive to cells grown in BHI and LB broth (135). Alternatively, a PAB to InlB reacted strongly with cells grown in BHI and LB but the anti-InlB PAB did not react to cells grown in BLEB, UVM or Fraser broth (135). These findings demonstrate the

importance of validating antibodies used to detect *L. monocytogenes* with various growth conditions and media.

2.2 Listeria Diagnostics

2.2.1 Culture based methods

The conventional method for detection, isolation and identification of bacteria is by culturing the organism on a variety of selective and differential media. This may be exceptionally difficult with *L. monocytogenes* as most clinical, environmental and food specimens contain a small population of *L. monocytogenes* with a variety of other organisms (62). *L. monocytogenes* has non-specific nutritional requirements, and this has led to a focus on enrichment media that contain inhibitory substances to lower the growth of other bacteria. Initially, cold-growth (at 4°C) was used to select for *Listeria*, however, this procedure takes several weeks and is very labour intensive (99). This led to the development of several *Listeria* enrichment media, which can be used at an optimal growth temperature (30°C - 37°C) with different combinations of inhibitory substances such as: potassium tellurite, lithium chloride, nalidixic acid, acriflavin, polymyxin B, moxalactam and ceftazidime (62). The procedure recommended by Health Canada for the isolation and tentative identification of *Listeria* includes growth on UVM, Fraser and Oxford media (191). UVM contains nalidixic acid and acriflavine, which inhibits the growth of Gram-negative bacteria through interference of the DNA gyrase (62). Fraser is preferred by both Health Canada and the FDA as a secondary enrichment media, since *Listeria* will turn the broth black within 48 h of inoculation and provides a presumptive positive result (38). Fraser broth also contains lithium chloride, in addition to the inhibitory agents in UVM, which selectively amplifies *Listeria* in the presence of Gram-negative bacteria (62, 84). Finally Health Canada

recommends isolating colonies on Oxford Agar, which contains a variety of inhibitory substances including: colistin sulphate, fosfomycin, cefotetan, cycloheximide, lithium chloride, and acriflavine (51, 191). Oxford Agar also contains esculin and ferric ammonium citrate, on which *Listeria* produces black colonies. Although this procedure is recommended for the isolation and presumptive identification of *Listeria*, its drawback is that it is labour intensive and takes a minimum of 5 days to complete.

2.2.2 Nucleic Acid based methods

Several nucleic acid based methods are available to detect *L. monocytogenes*, however, polymerase chain reaction (PCR) is the most widely reported method. The availability of sequenced *Listeria* genomes has facilitated the development of specific primers for use in PCR-based detection. Commonly target genes include: *hly*, *iap* and *inlA* (reviewed (147)). Real-time PCR has also been proposed for use in the detection of *Listeria* and it has the advantage of having both detection and enumeration taking place in the same tube, eliminating steps and reducing the chances for contamination (109). Reverse transcriptase-PCR, which could also be used to detect *L. monocytogenes* has the advantage of only detecting live cells, since it targets mRNA for amplification and mRNA has the advantage of having a half-life of a few minutes (127). There are, however, some major downfalls to PCR detection. Food matrices contain a number of molecules which are inhibitory to the PCR reaction, such as proteins, fats, nucleases and chelators for magnesium. In addition, since these methods are extremely sensitive, there is a high risk of having false positives.

2.2.3 Immunological methods

Several monoclonal MAbs and PABs have been generated against *L. monocytogenes* and some have been extensively developed for diagnosis and detection. In addition, antibodies are used to serotype *L. monocytogenes*. Once antibodies are developed and characterized, they can be used in diagnostics on any of a number of platforms including: ELISA, immunomagnetic separation, column chromatography, immunofluorescence microscopy and flow cytometry. Currently, no antibodies with sufficient affinity and specificity to be used in diagnostics exist against *L. monocytogenes* serotype 4b strains.

2.2.3.1 Flow cytometry, biosensors, and real-time detection

Flow cytometry is a tool which may have applications in the diagnostics of food borne pathogens. It relies on fluorescent probes such as nucleic acid-binding dyes or fluorescently labelled antibodies to confer fluorescence to cells as they are carried by a liquid through an excitation light (62). Flow cytometry has the interesting advantage of being able to be automated and run at food processing plants in almost real time. Previous attempts have been made to use fluorescently labelled antibodies along with flow cytometry to detect *Listeria* in contaminated food samples (60, 61). However, the lack of specificity of the antibodies was a limiting factor and required that the method include a round of selective enrichment (61).

Biosensors are another method of real time detection, with the potential to be automated. In biosensors, a bioaffinity agent, such as an antibody, is attached to the surface of the biosensor. Upon injection of the sample, if the target comes in contact with this recognition element, it generates an electrical signal that is recorded by an output device (145). Biosensors have an advantage over flow cytometry in that they can provide label free detection in real time. Antibodies have already been investigated as a capture reagent to

detect *L. monocytogenes* in a biosensor, with encouraging results, although the focus of this study was to develop electrochemical detection and not to provide an exhaustive demonstration of a better diagnostic technique for *L. monocytogenes* (14). Biosensors and flow cytometry both have the potential to shorten the time it takes to detect *L. monocytogenes* in a food processing environment when compared to the traditional culture-based detection. However, both biosensors and flow cytometry are limited by the binding properties of the bioaffinity reagent used.

2.2.3.2 Monoclonal antibodies

There is a high demand for MAbs which exhibit different reactivity patterns against various groups of *Listeria* to be used in rapid detection methods (17, 106, 106, 178, 178, 179, 179). MAbs have been previously generated against a number of surface antigens of *L. monocytogenes* and depending on how conserved the target protein is among bacteria, the range of cross-reactivity of the antibodies can vary (Table 2-1). Some antibodies appear to have the ability to differentiate between *L. monocytogenes* serotypes (122) (Table 2-1).

Table 2-1. Summary of available monoclonal antibodies that react with *Listeria monocytogenes*^a

Antigen	Range of Reactivity	Antibody	Reference
Flagellum	<i>Listeria</i> spp. except <i>L. grayi</i> Did not react to <i>Listeria</i> grown above 37°C	MAb 16-5, E10, D5 and 400	(78)
InlA ^b	<i>L. monocytogenes</i> Minor reaction to <i>Listeria</i> grown at 20°C	M160, L244	(153)
InlB ^b	<i>L. monocytogenes</i> No reaction to <i>Listeria</i> grown at 20°C	IC100	(153)
ActA ^b	<i>L. monocytogenes</i> except serotype 3a and 4ab	Variety	(183)
p60	<i>L. monocytogenes</i>	p6007	(283)
p60	<i>Listeria</i> spp.	p6017	(283)
18 kDa Surface protein	<i>L. monocytogenes</i> , <i>L.</i> <i>innocua</i> , and <i>L. welshimeri</i>	P5C9	(244)
43 kDa and 94 kDa surface proteins	<i>Listeria</i> spp. when grown in BHI <i>L. monocytogenes</i> serotypes 1/2c, 3b, 4ab, and 7 when grown in UVM or Fraser media	EM-6E11	(17) (179)
Lmo2691 (autolysin with undefined activity)	<i>L. monocytogenes</i> Does not detect heat killed cells, cells grown in UVM or Fraser broths or cells grown at 5.5% NaCl, 45°C or 4°C	EM-7G1	(17) (178) (93)
Unknown	<i>L. monocytogenes</i> serotype 4b	c74.33 and c74.180	(122)
66 kDa surface protein	<i>L. monocytogenes</i> except serotype 4c and 4e	22D10 and 24F6	(106)
Lmo2691 (autolysin with undefined activity)	<i>L. monocytogenes</i> and some <i>L. innocua</i> Does not react with cells grown in 5.5% NaCl, 45°C or 4°C	C11E9	(93, 135)
Unknown	<i>L. monocytogenes</i> and <i>L.</i> <i>innocua</i>	B4	(247)

a - Table modified from Bhunia 1997 (16)

b - Generally virulence proteins are poor candidates for use in diagnostic tests as their expression is usually limited to *in vivo* conditions (65).

Many attempts have been made to generate MAbs specific for *L. monocytogenes*. An antibody able to detect all serotypes of *L. monocytogenes* would be very valuable. Attempts to generate such MAbs have all focused on virulence factors as an antigen, however, this approach has been largely unsuccessful because expression of virulence factors is extremely variable in *in vitro* growth (16, 23, 90, 167, 167, 220, 237). A particular MAb against p60, a protein required for cellular division in *L. monocytogenes*, was able to detect all isolates of *L. monocytogenes*, but also detected other *Listeria* spp. with the exception of *L. seeligeri* (283). In addition, very few of these studies provide extensive study of cross reactivity with all *Listeria* species and *L. monocytogenes* serotypes.

A problem generally encountered with antibody-based detection of *L. monocytogenes* is that the target antigens are often found to be unstable under different environmental conditions and their expression varies greatly between enrichment media (17, 178, 179). Obviously the instability in expression of the targeted antigens both under stress growth conditions and between various enrichment media creates an obstacle to using antibodies for real- or near real-time detection in either flow cytometry or biosensors. The ideal antibody for either of these methods of detection would target an antigen that is always displayed on the cell surface of *L. monocytogenes*.

The low avidity of antibodies to the cell surface of *L. monocytogenes* has been held responsible for the failure of immunomagnetic capture and flow cytometry in previous experiments (118). Any MAb that is proposed as a reagent for *L. monocytogenes* diagnostics should have high affinity to its target antigen.

2.3 Peptidoglycan hydrolases

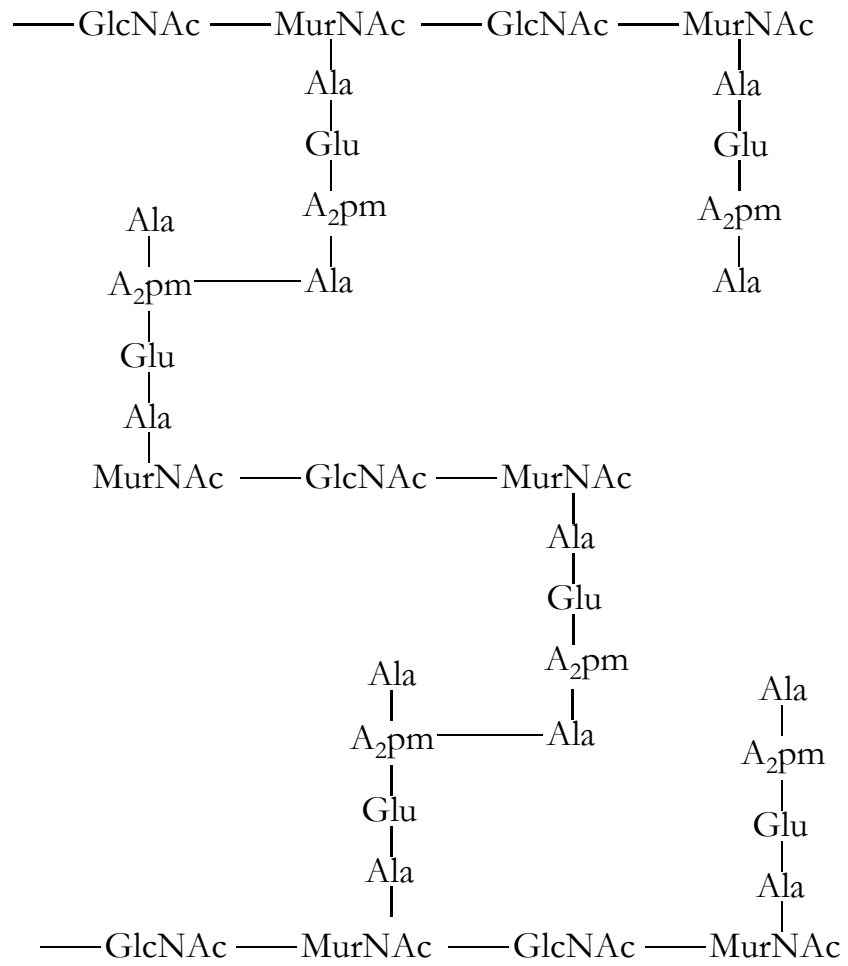
Peptidoglycan (PG) hydrolases are a class of enzymes capable of cleaving bonds within the bacterial cell wall PG. Mammalian cells often produce PG hydrolases, such as lysozyme, as an innate defence against bacterial pathogens. An autolysin, usually produced by bacteria, is a PG hydrolase which can break one of the bonds present in its own PG. The presence and activity of autolysins must be tightly regulated, because the uncontrolled activity of these enzymes can lead to lysis of the bacterial cell.

2.3.1 Peptidoglycan

Peptidoglycan is a major component of the Gram-positive bacterial cell wall. It is responsible for cellular strength and rigidity. If the integrity of the PG is compromised, the result is cell lysis due to internal turgor pressure (269). The canonical structure of PG is linear glycan strands, consisting of repeating *N*-acetylglucosamine (GlcNAc) linked by β -1, 4 glycosidic bonds to *N*-acetylmuramic acid (MurNAc), and cross-linked by tetrapeptide bridges. Peptide bridges are anchored to the glycan strands via substitution of the D-lactoyl group of the MurNAc with the tetrapeptide (280) (Figure 2-4). In most bacterial species, including *L. monocytogenes*, the peptide bridge composition is: L-Ala- γ -D-Glu-*meso*-A₂pm-D-Ala (120, 269). The position containing *meso*-A₂pm is highly variable and may contain: L-alanine, L-homoserine, L-diaminobutyric acid, L-glutamic acid or L-lysine, among others (280). Peptide bridges cross-link with each other between the terminal D-alanine and the *meso*-A₂pm of another tetrapeptide (280). However, variation in both the glycan strand and the peptide bridge exists between species and in the same species due to growth conditions, nutrients and the presence of antibiotics (269).

2.3.1.1 Post-assembly modification

Figure 2-4. Peptidoglycan Structure in *L. monocytogenes*. The canonical structure of PG in *L. monocytogenes* includes a glycan chain of *N*-acetylglucosamine (GlcNAc) β -1,4-linked to *N*-acetylmuramic acid (MurNAc). These glycan chains are cross-linked by peptide bridges which contain *L*-alanine (Ala), *D*-isoglutamic acid (Glu), and meso-diaminophmelic acid (A₂pm).



PG invariably gets modified or linked to other cell wall components after assembly; there is no example of simple unmodified peptidoglycan occurring in any bacterial species (268). Several types of structural modifications to the glycan strand have been reported and these include: de-*N*-acetylation, *O*-acetylation, glycosylation, the presence of both muramic acid δ -lactam residues, and 1,6-anhydride residues (268).

Bacterial pathogens have a high occurrence of de-*N*-acetylation of GlcNAc at the C-2 residue (53). De-*N*-acetylation of GlcNAc has been shown to occur at varying degrees in different bacteria, including: *B. anthracis* (88%) (287), *Bacillus cereus* (40-100%) (104, 287), *B. subtilis* (19%) (287), *Bacillus thuringensis* (88%) (287), *Streptococcus pneumoniae* (80%) (272), *Lactococcus lactis* (10%) (166), *Helicobacter pylori* (274) and *L. monocytogenes* (50%) (25). In addition, some species including *B. anthracis*, *B. cereus*, *B. subtilis*, *B. thuringensis*, and *S. pneumoniae* also de-*N*-acetylate the MurNAc residue (272, 287). De-*N*-acetylation likely occurs after PG assembly, as evidenced by the lack of identification of deacetylated PG precursors and the extracytoplasmic localization of enzymes known to be involved in de-*N*-acetylation (272).

De-*N*-acetylation seems to be an important post-translational regulatory factor for the activity of certain autolysins such as *N*-acetylglucosaminidases. For example, the autolysin AcmA from *L. lactis* is unable to hydrolyze de-*N*-acetylated peptidoglycan (166) and Auto, an *N*-acetylglucosaminidase from *L. monocytogenes*, prefers acetylated peptidoglycan (37). There are also multiple lines of evidence indicating that de-*N*-acetylation facilitates bacterial infection. De-*N*-acetylation has also been shown to provide resistance to lysozyme, a PG hydrolase produced by most mammals that is an important part of the innate immune system (7, 82). De-*N*-acetylation of GlcNAc confers a positive charge to the bacterial cell wall,

possibly affecting the binding of major virulence determinants and increasing resistance to anti-microbial peptides produced by the host (196). In addition, de-*N*-acetylation promotes intracellular survival in macrophages for *L. monocytogenes* and also limits the host cytokine response (25).

O-acetylation of MurNAc is very widespread in Gram-positive bacteria (268). *O*-acetylation also regulates the activity of bacterial autolysins especially lytic transglycosylases (107, 195). Like de-*N*-acetylation, *O*-acetylation provides resistance to lysozyme degradation (15). Recently, a *L. monocytogenes* double mutant lacking the ability to de-*N*-acetylate or *O*-acetylate its PG was constructed. This mutant was severely attenuated due to an increased occurrence of bacteriolysis in the macrophage cytosol. This phenotype was due to increased lysozyme sensitivity since infectivity returned to wild-type (WT) levels in lysozyme defective macrophages (211). *O*-acetylation also seems to provide a measure of resistance to the activity of penicillin, however, the mechanism behind this is poorly understood (242). Enzymes which de-*O*-acetylate *O*-acetylated MurNAc residues have also recently been discovered (278).

The presence of glycosylation in PG is not widespread and is generally only found in bacteria already containing mycolic acids such as *Mycobacterium* (10).

Muramic acid δ -lactam residues occur due to an amide bond forming between the carboxyl group of the lactyl at position 3 of MurNAc and the amino group at position 2 of the same MurNAc (268). This structure is so far found only in spores of *Bacillus* and *Clostridium* (200). Mutants lacking the enzymes required to form the Muramic acid δ -lactam residues are able to form spores with resistance to heat and dehydration equivalent to WT

bacteria. However, spores lacking muramic acid δ -lactam residues are unable to germinate because they are not hydrolysed by germination specific peptidoglycan hydrolases (200).

1,6-anhydro-*N*-acetylmuramic acid is commonly found at the terminal end of a glycan chain in Gram-negative bacteria (103, 107). In addition, this structure has also been found, in much less abundance, in the Gram-positive bacterium *B. subtilis* (8).

2.3.1.3 Peptidoglycan Deacetylases

Peptidoglycan deacetylases (PgdA) are enzymes responsible for de-*N*-acetylating the peptidoglycan (25, 201, 272). PgdA enzymes tend to have strict substrate specificity, either de-*N*-acetylating *N*-acetylglucosamine or *N*-acetylmuramic acid, but not both (86). In addition, PgdA enzymes are metalloenzymes and must be in complex with zinc for functionality (22). Since PgdA mediated de-*N*-acetylation of PG is an effective strategy for immune evasion, there are ongoing attempts to identify small molecule inhibitors of PgdA for possible use as antimicrobial drugs (40).

PgdA expression has been found to be regulated at the transcriptional level in response to conditions which damage the cell wall or in response to direct cell wall damage. In *L. monocytogenes*, DNA microarrays were used to examine genes that were up regulated during infections compared to expression levels during *in vitro* growth. PgdA was found to be up regulated 14-fold at 48 h post-infection (44). The *pgdA* gene was also found to be up regulated by oxidative stress *in vitro* in *H. pylori*, thought to mimic the oxidative stress encountered during uptake by phagocytes or interaction with antimicrobials at the mucosal surface *in vivo* (274). In *S. suis*, the *pgdA* gene was found to be up-regulated upon interaction with neutrophils (82). It would be interesting to know how the levels of de-*N*-acetylation of

GlcNAc vary between cells cultured in a stress environment and those grown in normal conditions.

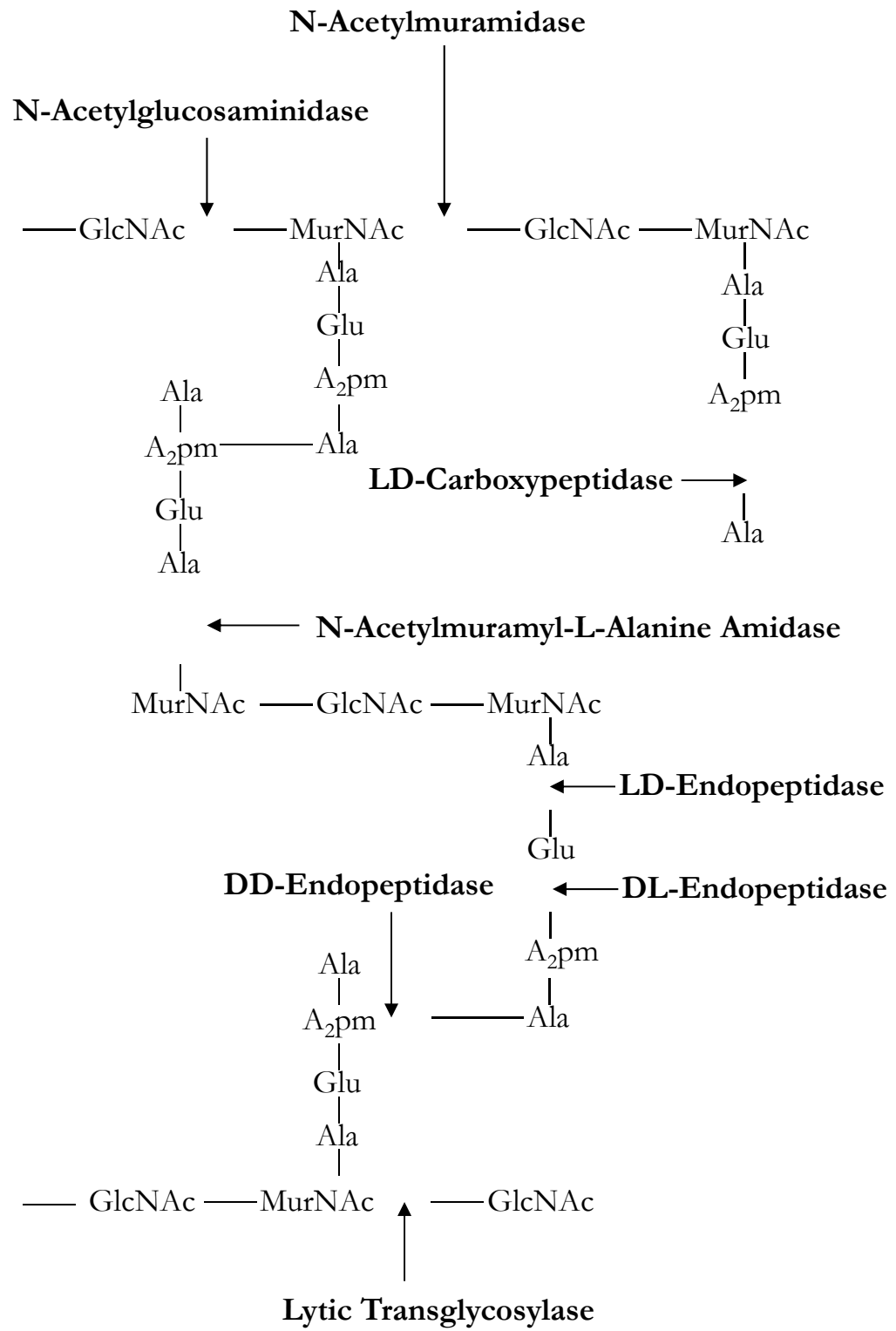
2.3.2 Biochemical activity of peptidoglycan hydrolases

PG hydrolases are categorized according to their cleavage site within the PG (Figure 2-5). There are three types of enzymes capable of cutting the glycan backbone, each resulting in a specific end product. *N*-acetylglucosaminidases cleave the bond between the C1 of the GlcNAc and the C4 of the MurNAc resulting in a terminal reducing GlcNAc residue. The bond between the C1 of the MurNAc and the C4 of the GlcNAc can be hydrolyzed by either *N*-acetylmuramidases or by lytic transglycosylases, resulting in a terminal reducing MurNAc or a 1,6-anhydro ring at the MurNAc residue respectively (271). *N*-acetylmuramyl-L-alanine amidases (or amidases) cleave the amide bond between the MurNAc residue and the L-alanine residue of the peptide bridge (271). Endopeptidases cleave the amide bonds linking amino acids in the peptide bridges. Carboxypeptidases remove the terminal L-alanine residue from pentapeptides resulting in tetrapeptides (271).

A current area of research is in experimentally verifying the specific catalytic activity of putative peptidoglycan hydrolases. It is important to experimentally demonstrate which bonds are hydrolyzed by PG hydrolases, as the predicted hydrolytic activity based on bioinformatics has been proven incorrect in a number of instances: LytG from *Bacillus subtilis* (279), Auto from *L. monocytogenes* (37) and AcmL from *L. lactis* (110). Discovering which type of activity an autolysin has may help to elucidate its role in bacterial cell physiology.

2.3.3 Peptidoglycan hydrolases in *Listeria monocytogenes*

Figure 2-5. Autolysins Categorized by Bond Specificity. *N*-acetylglucosaminidases hydrolyze the covalent bond between the C1 of GlcNAc and the C4 of MurNAc, while *N*-acetylmuraminidases cleave the bond between the C1 of MurNAc and the C4 of GlcNAc. This results in a reduced GlcNAc by hydrolysis with an *N*-acetylglucosaminidase and a reduced MurNAc by hydrolysis with *N*-acetylmuramidases. *N*-acetylmurmyl-L-alanine-amidases (also called amidases) hydrolyze the bond between the MurNAc residue and the Ala residue found in the peptide bridge. Various groups of endopeptidases and carboxypeptidases all hydrolyze bonds within the peptide bridges.



In *L. Monocytogenes*, seven autolysins have been experimentally demonstrated including: p60 (281), p45 (232), Ami (164, 170), MurA (47, 143, 143), Auto (42), Lmo0327 (203), and IspC (275). Of these autolysins, p60, Ami and IspC are the only ones which have been experimentally confirmed in *L. monocytogenes* serotype 4b (171, 224, 284).

p60 appears to occur in all *Listeria* species (36), and is the most well know autolysin in *Listeria*. It is responsible for septal PG splitting, as evidenced by formation of long chains in Δ p60 deletion mutants that can be disaggregated into individual cells by adding purified p60 to the culture (281). p45 from a *L. monocytogenes* serotype 4d isolate also shows PG hydrolase activity, with 38% sequence identity to p60 (232).

MurA, which has only been verified to occur in *L. monocytogenes* serotype 1/2a, appears to be involved in cell separation, as Δ MurA deletion mutants also display long-chain cell morphology and normal cell morphology can be restored by over expression of MurA from a plasmid vector (47). The autolytic activity of Lmo0327 has only been experimentally verified in *L. monocytogenes* serotype 1/2a, and it has been suggested that this autolysin is also involved in cell separation (203). FlaA, which is an important protein in flagellar movement, was also found to have PG hydrolytic activity in *L. monocytogenes* serotype 1/2a (202).

Interestingly, Ami, which has GW domains involved in cell wall binding, seems to mediate adhesion to eukaryotic cells (170). Initial observations also suggest that Ami has amidase activity (164). The *ami* genes is conserved between all *L. monocytogenes* serotypes, however, Ami from *L. monocytogenes* serotype 1/2a is composed of 917 amino acids and Ami from serotype 4b contains 770 amino acids (171). The catalytic domain is highly conserved between these two serotypes, but the GW domains are not. Ami contains eight

GW modules while Ami 4b only has six (171). The serotype 4b Ami was much more efficient at binding to eukaryotic cells (171).

Auto, which is found in *L. monocytogenes* serotype 1/2a, has been experimentally verified to act as an *N*-acetylglycosaminidase (37). Auto has four C-terminal GW modules which are predicted to facilitate entry into non-phagocytic cells (42). Auto is the first of the autolysins in *L. monocytogenes* that was shown to possess an autoinhibitory domain (amino acids 27-88); this domain must be cleaved to activate the enzyme (37). Auto also prefers fully acetylated PG as a substrate (37).

2.3.4 Immunogenic surface protein C (IspC)

IspC was first identified in a screen for immunogenic surface proteins that were induced or significantly up regulated during *Listeria* infection (284). This protein contains 774 amino acids and has a calculated molecular mass of 86 kDa. A strategy has been developed for efficient expression and purification of a recombinant form of IspC produced in *E. coli* (277). It has been experimentally demonstrated that the N-terminal region containing a PG hydrolase domain covering amino acids 58 to 197 has hydrolytic activity, and that the GW modules contain a cell wall binding domain (CBD) which spans from amino acids 197 to 774 and is responsible for anchoring IspC to the cell surface (275). IspC does not have a role in growth or division, as IspC knockout mutants do not display a noticeable change during in-vitro growth (276). However, IspC deletion mutants exhibit attenuated virulence in mice (276). In addition, the Δ IspC mutant cells were defective in adhesion, invasion and actin tail formation in a cell culture model (276). IspC is essential for full virulence in *L. monocytogenes* although the reasons are not fully understood.

Chapter III

Materials and Methods

3.1 Bacterial strains, plasmids and growth conditions

All *L. monocytogenes* isolates in this study (Table 3-1) were grown in BHI broth or on BHI agar plates at 37°C, unless otherwise stated. *Escherichia coli* strains used in this study, DH5 α and Rosetta DE3/(pLysS), were cultured in Luria-Bertani (LB) broth or LB agar plates. *L. monocytogenes* cell concentrations were estimated by measuring optical density (OD) at 620 nm with an OD₆₂₀ value of 0.61 being equivalent to 1 x 10⁹ cells/ mL which was previously validated (152). Antibiotics were added to media as required. For *E. coli* strains containing a pET 30a plasmid (Novagen, Madison, WI, USA) or its derivatives, 50 μ g/ mL kanamycin was added to broth and agar plates. For *E. coli* containing pAUL-A or its derivatives, 300 μ g/ mL erythromycin was added to both broth and agar plates. For *L. monocytogenes* containing pAUL-A derivatives, 5 μ g/ mL erythromycin was added to broth or agar plates. For *E. coli* containing pTCV-lac or its derivatives, either 150 μ g/ mL of erythromycin or 50 μ g/ mL kanamycin was added to the media and for *L. monocytogenes* containing pTCV-lac or its derivatives, either 10 μ g/mL erythromycin or 50 μ g/mL kanamycin was added to the media.

3.2 Extraction of *Listeria* surface proteins

Surface proteins attached non-covalently to the cell wall were released from 8.7 x 10¹⁰ *L. monocytogenes* serotype 4b strain LI0521 cells by boiling in 20 mL of PBS containing 4% (w/v) SDS for 10 min. Proteins in the supernatant were separated from insoluble cell materials by centrifugation at 10 000 x g for 10 min. SDS was removed from the supernatant by chromatography using a column Extracti-Gel Detergent Removing Resin (Thermoscientific, Rockford, IL, USA). The resin was washed with one bed volume of deionized H₂O. The sample protein was passed through the column, and the flow-through

fraction containing extracted proteins was collected. The column was regenerated through a series of wash steps, including: one bed volume of deionized H₂O, two bed volumes of butanol, followed by a bed volume of pure ethanol. The resin was then washed with one bed volume of each of 75%, 50% and 25% (v/v) ethanol followed by one bed volume of deionized H₂O. This procedure was repeated until SDS was undetectable in the protein sample. The concentration of extracted proteins was then determined using Bradford assay (29) using the Bio-Rad Assay Dye-Reagent concentrate (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Bovine serum albumin (BSA) was used as a standard.

3.3 SDS-PAGE and Western Blotting

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (134), using a 4% stacking gel and a 12% resolving gel with the BioRad minigel apparatus, unless otherwise stated. Proteins separated by electrophoresis were visualized in the gel by Coomassie blue staining or were electrotransferred to a nitrocellulose membrane by using a Trans-Blot SD semidry transfer cell (Bio-Rad) according to the supplier's instructions. Prior to Western blot, a successful transfer was verified by staining the nitrocellulose membrane with 0.1% (w/v) Ponceau S in 5% (v/v) acetic acid for 5 min. The membrane was blocked for 1 h at room temperature or overnight soaking in PBS-T containing 3% (w/v) BSA. The Western blot was carried out using specific primary antibodies followed by peroxidase-conjugated goat anti-mouse IgG (H+L) (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), and was performed essentially as described previously (152). The substrate solution was prepared from the HRP Conjugate Substrate kit (Bio-Rad) and used according to the supplied instructions.

3.4 Immunoprecipitation

The M2799 MAb was selected for immunoprecipitation on the basis of its strong reaction with the 77 kDa band of interest during initial MAb screening experiments (151). M2799 was purified from tissue culture fluid by affinity chromatography on a Protein G Sepharose 4 Fast Flow column (GE Healthcare, Baie d'Urfe, QC, Canada), prior to its use in immunoprecipitation. Purified M2799 (20 µg) was combined with 26 µg of extracted surface proteins from *L. monocytogenes* in PBS and one cOmplete EDTA-free Cocktail Tablet (Protease Inhibitor) (Roche Canada, Mississauga, ON, Canada) and then incubated at 4°C for 2 h with constant agitation. Protein A sapharose 4B beads (150 µL) (Invitrogen, Burlington, ON, Canada), after washing with PBS, were added to the MAb-surface protein mix and incubated at 4°C for an additional 4 h. Beads were collected by centrifugation and washed multiple times with PBS containing a protease inhibitor cocktail. Beads were then suspended in 2x SDS-PAGE sample buffer and boiled for 10 min prior to analysis of the supernatant by SDS-PAGE.

3.5 Mass spectrometry

Identification of Immunoprecipitated Protein. The proteins which had been released from the sepharose beads into the supernatant by boiling were separated by SDS-PAGE and visualized by Commassie blue staining. The ~ 77 kDa antigen was excised from the gel and sent to the Ottawa Hospital Research Institute (ORHI) Proteomics Core Facility (Ottawa, ON, Canada) for protein identification by mass spectrometry (MS).

Identification of secreted and surface proteins. Proteins recovered from the culture supernatant, as well as SDS-extractable surface proteins from *L. monocytogenes* grown in BHI broth were separated by 12% SDS-PAGE. SDS-extractable surface proteins from

bacteria cultured in LB broth were separated on a 4%-20% gradient gel. Gels were silver stained according to the procedure of Shevchenko et al. (241). Briefly, the gel was placed directly into fixer solution (50% ethanol (v/v), 5% acetic acid (v/v)) and incubated for 30 min. The gel was then washed in 50% ethanol (v/v) for 10 min and 2 x in water for 10 min. Sensitizer solution (0.02% sodium thiosulfate (w/v)) was then added to the gel for 10 min. The staining solution (0.1% silver nitrate (w/v)) was added to the gel for 30 min. After staining the gel was washed for 1 min in water, before adding the developer solution (0.04% formalin (v/v), 2% sodium carbonate (w/v)) and incubation occurred until protein bands appeared. A 5% acetic acid (v/v) solution was added to stop the reaction, and gels were stored in 1% acetic acid (v/v) (see also Appendix 2). Resolved protein bands derived from the WT corresponding to SecA2-dependent secretory proteins were excised from the gel and sent to the OHRI Proteomics Core Facility for protein identification by mass spectrometry. MASCOT 2.3.01 software (Matrix Science, UK) was used to match the observed MS/MS spectra to protein sequences in the NCBI database (taxonomy limited to *firmicutes*).

Muropeptide Identification by Matrix Assisted Laser Desorption/Ionization – Time of Flight MS. Mass spectrometric analysis of muropeptides derived from *L. monocytogenes* PG was performed in a reflectron mode on a Biflex IV Matrix Assisted Laser Desorption/Ionization Time of Flight (MALDI-TOF) MS instrument (Bruker Daltonics, Kanagawa, Japan). Muropeptides, which were previously purified using reverse phase – high pressure liquid chromatography (RP-HPLC), and desalted using ZipTipC18 (Millipore) and lyophilized with CH₃CN and 0.1% Trifluoroacetic acid (TFA) (50:50). The analyte and matrix solutions [α -cyano-4-hydroxycinnamic acid, saturated concentration in CH₃CN and 0.1% TFA (50:50)] were mixed at a 1:1 (v/v) ratio. The resulting solution (1 μ L) was applied

to the stainless steel MALDI plate and air-dried. Positive and/or negative ion mass spectra obtained were used to determine muuropeptide structures.

Muropeptide Identification by Matrix Assisted Laser Desorption/Ionization – Post Source Decay. In MALDI- Post Source Decay (PSD) experiments, the timed ion selection was used to select the $[M+Na]^+$ value of the precursor ion. MALDI-PSD mass spectra were used to select the most likely muuropeptide structure when MALDI-TOF experiments indicated that more than one structure was possible.

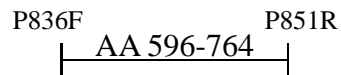
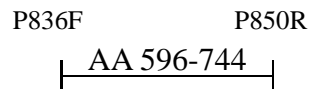
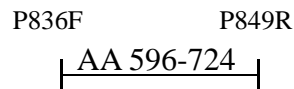
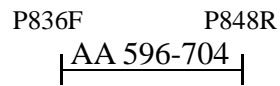
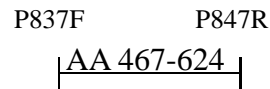
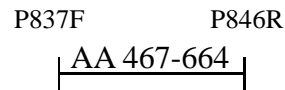
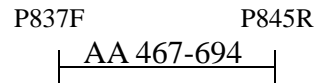
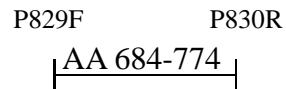
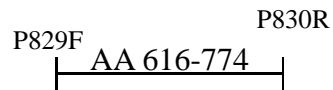
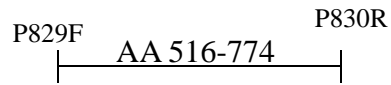
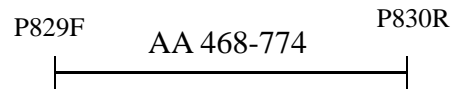
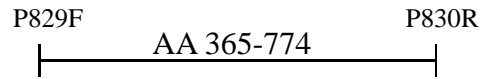
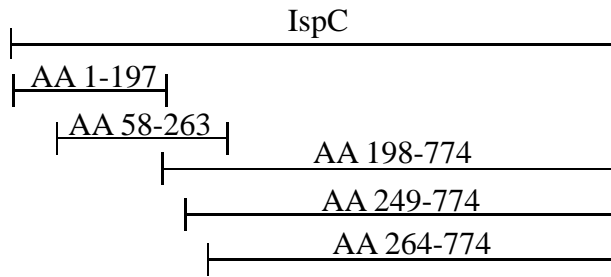
3.6 N-terminal Sequencing

The immunoprecipitated proteins which had been released from the sapharose beads into the supernatant by boiling were separated by SDS-PAGE and blotted onto a polyvinylidene fluoride membrane using a Trans-Blot SD semidry transfer cell (Bio-Rad), visualized by Commassie blue staining, and excised. The excised protein bands were sent to the protein core facility at Columbia University College of Physicians and Surgeons (New York, NY, USA) for N-terminal sequencing by Edman degradation.

3.7 Generation of Expression Constructs for IspC and truncated fragments

DNA manipulations were essentially performed according to the established procedures (227). A construct pIspC was previously produced by cloning the *ispC* open reading frame (ORF) into the NdeI and XhoI restriction sites of the pET 30a plasmid (275). Fragments of the *ispC* ORF, as shown in Figure 3-1, were amplified from pIspC by PCR with the primers listed in Table 3-2 and Pfu DNA polymerase. Each PCR product was purified using Wizard PCR Preps DNA purification system (Promega, Madison, WI, USA), digested by NotI and NdeI (New England Biolabs, Pickering, ON, Canada) and ligated into the corresponding sites of a double-digested pET 30a (Novagen, Madison, WI, USA).

Figure 3-1. Truncated IspC Fragments. Truncated IspC fragments were produced with the purpose of mapping monoclonal antibody epitopes. The fragments AA 1-197, AA 58-263, AA 198-774, AA 249-774, and AA 264-774 were previously produced by L. Wang (275). The additional fragments AA 365-774, AA 468-774, AA 516-774, AA 616-774, AA 684-774, AA 467-694, AA 467-664, AA 467-624, AA 596-704, AA 596-724, AA 596-744 and AA 596-764 were each amplified using the indicated primers (detailed in Table 3-2) and cloned into the corresponding sites of the pET-30a plasmid.



Recombinant plasmids were propagated in *E. coli* DH5 α cells and isolated from *E. coli* cells using the QIAprep Spin Miniprep kit according to the manufacturer's instructions (Qiagen, Toronto, ON, Canada). The recombinant plasmids were sequenced with the T7 promoter primer (5' –TAATACGACTCACTAT – 3') and the T7 terminator primer (5' – GCTAGTTATTGCTCAGCGG – 3') to ensure each of the inserts contained the correct sequence and was in-frame to the translation start site encoded on pET 30a. Each of the recombinant plasmids was then transformed into *E. coli* Rosetta (DE3)/pLysS (Novagen, Madison, WI, USA) cells for recombinant protein expression.

Table 3-2 – Oligonucleotide primers used in amplification of IspC truncated fragments

Primer	Nucleotide Sequence	Amplifies (Amino acids)	Position (Nucleotides)
P822F	5'- ACAC <u>CATATG</u> ATCTATCCGTATGATTCC -3' ^a	365-774	1093-1110
P824F	5'- ACAC <u>CATATGA</u> AGGGTAATGCTGTATGGACA -3' ^a	468-774	1402-1422
P825F	5'- ACAC <u>CATATGG</u> GCAAAGTAATCGGCTGGTTA -3' ^a	516-774	1546-1566
P827F	5'- ACAC <u>CATATGT</u> ACAACAAAGCGGTAAAC -3' ^a	616-774	1846-1863
P829F	5'- ACAC <u>CATATGT</u> TAGATAAAAAAGCTTTTGAT -3' ^a	684-774	2050-2070
P830R	5'- GTGGCGGCCGCTTTAACGTTTGTAAGCTC -3' ^b	Multiple	2303-2322
P837F	5'- ACAC <u>CATATGG</u> TGAAGGGTAATGCTGTATG -3' ^a	Multiple	1399-1418
P836F	5'- ACAC <u>CATATGG</u> ATGGTAAAGTCATTGGCTG -3' ^a	Multiple	1786-1805
P845R	5'- GTGGCGGCCGCAGAATCATATACATCAAAAGC -3' ^b	467-694	2062-2082
P846R	5'- GTGGCGGCCGCCGCTTCTCGAGTTATCTTAG -3' ^b	467-664	1973-1992
P847R	5'- GTGGCGGCCGCAGCATCTAAGTTAACCGCTT -3' ^b	467-624	1853-1872
P848R	5'- GTGGCGGCCGCAGTCATATTAATCGCTTTAT -3' ^b	596-704	2093-2112
P849R	5'- GTGGCGGCCGCGCCAATAACTCTATACGGCT -3' ^b	596-724	2153-2172
P850R	5'- GTGGCGGCCGCCTCGCGTATCAACTGTAAGT -3' ^b	596-744	2213-2232
P851R	5'- GTGGCGGCCGCCCAACCAACTATTTTACCAT -3' ^b	596-764	2273-2292

a- NedI restriction site underlined

b- NotI site underlined

3.8 Expression and preparation of protein fragments for epitope localization

Overnight cultures of *E. coli* Rosetta (DE3)/pLysS, grown at 37°C, harbouring one of each of the expression constructs were diluted 1:100 into 50 mL of LB broth containing 50 µg/mL kanamycin and subcultured at 37°C until the culture reached an OD₅₉₀ of 0.6 +/- 0.1. Isopropyl-β-D-thiogalactopyranoside (IPTG) (1 mM) was added to induce expression of the recombinant proteins. The culture was further incubated at 37°C for 3 h and then at 4°C for 16-18 h. After induction, the OD of the cultures was measured at 590 nm. The cells were pelleted by centrifugation at 11, 000 x g and re-suspended in PBS to a hypothetical OD₅₉₀ of 20. A volume of 2 x SDS-PAGE sample buffer equal to that of PBS was added to the cell suspension, which was boiled for 10 min and then stored at -20°C until use.

Expression of each IspC fragment was verified by SDS-PAGE followed by Western blotting with a Penta-His antibody (Qiagen) at a 1:2000 dilution. For epitope localization, the IspC fragments were analyzed by Western blotting probing with tissue culture fluid of anti-IspC MAbs at a 1:50 dilution.

3.9 Expression and purification of recombinant IspC

Recombinant IspC (rIspC), expressed from the construct pIspC in *E. coli* Rosetta (DE3)/pLysS cells, was purified essentially as described by Wang et al. (277), with some modifications. Briefly, an overnight culture was diluted 1:100 in LB containing kanamycin (50 µg/mL) and subcultured until an OD₅₉₀ of 0.6 +/- 0.1 was reached. After adding IPTG (1 mM), the culture was incubated at 37°C for 4 h and then at 4°C overnight. Soluble rIspC was purified by metal chelate affinity chromatography using Ni-NTA Superflow (Qiagen) followed by cation-exchange chromatography using SP Sepharose Fast Flow (GE Healthcare). rIspC used for surface plasmon resonance (SPR) analysis was purified using an additional step of size exclusion chromatography using a Superdex™ 200 column (GE

Healthcare) under the control of a AKTA™ fast protein liquid chromatography (FPLC), to reach the required purity.

3.10 Preparation of antibody Fab fragments

MAbs were purified from tissue culture fluid (TCF) by affinity chromatography using a column of CNBr-activated Sepharose 4B (GE Healthcare), conjugated with rIspC according to the manufacturer's instructions. Purified anti-IspC MAbs were digested with papain (Sigma, Oakville, ON, Canada) in a digestion buffer (100 mM glycine-HCl pH 7.0, 100 mM dithiothreitol and 50 mM EDTA), at an enzyme to antibody ratio of 1:100 (w/w) at room temperature for 2 h. The digestion reaction was quenched with 10 mM iodoacetamide; complete digestion of the MAbs was verified by SDS-PAGE. The digested antibodies were dialyzed overnight in 20 mM HEPES buffer. They were then passed through a column of Protein G resin to remove the Fc fragments from the Fab fragments. The column flow through fraction, containing the Fab, was analyzed by Western blot with HRP-conjugated goat anti-mouse IgG Fc γ fragment antibody (Jackson ImmunoResearch), to ensure the fraction was free of Fc fragments. The Fab flow through fraction was concentrated using an Amicon Ultra 10k (Millipore, Billerica, MA, USA). Fab fragments were stored in PBS at a neutral pH with 0.05% sodium azide at 4°C. Size exclusion chromatography was performed on all Fab samples immediately prior to SPR using a Superdex™ 75 column (GE Healthcare) controlled by a AKTA™ FPLC. Only peak fractions from the size exclusion column were used in affinity measurements.

3.11 Surface Plasmon Resonance Analysis

The binding of Fab fragments to immobilized IspC was determined by surface plasmon resonance (SPR) analysis using a BIACORE 3000 (GE Healthcare). Approximately

600 resonance units (RU) of IspC were immobilized on a research grade CM5 sensor chip. The immobilization was carried out at a concentration of 50 µg/mL IspC in 10 mM acetate at pH 4.0 using the amine coupling kit (GE Healthcare) according to the manufacturer's instructions. An ethanolamine blocked surface was used as a reference. For the binding studies, analyses were carried out at 25°C in 10 mM HEPES, pH 7.4 containing 150 mM sodium chloride, 3 mM EDTA and 0.005% surfactant P20. The flow rates were set at 20 µL/min and sample volumes were 200 µL. Data were analyzed with BIAevaluation 4.1 software.

3.12 Indirect ELISA

The MAbs were assessed for cross-reactivity with a variety of *L. monocytogenes* isolates (Table 3-1) by indirect ELISA essentially as described by Ronholm et al. (223). For preparation of the whole cell antigens, each isolate was grown overnight at 37°C in BHI broth, washed 3x in PBS, killed by overnight incubation in 30% formaldehyde (v/v) at room temperature, washed 3x in PBS and then stored in PBS with 50% glycerol (v/v) at -20°C. Formalin-killed cells were used to coat a 96 well NUNC plate (Thermoscientific, Rockford, IL, USA), at a concentration of 5×10^7 cells/ mL and 100 µL/ well, overnight in 50 mM carbonate buffer (pH 9.6). A predetermined dilution of each MAb TCF and an irrelevant MAb against *E. coli*, used as a negative control, were added to the plate wells and incubated at room temperature for 1 h. HRP-conjugated goat anti-mouse IgG Fcγ fragment antibody (Jackson ImmunoResearch) was used to detect bound MAbs. After incubation with the substrate solution [citrate buffer containing 0.3% (v/v) hydrogen peroxide and 0.1% ABTS (w/v)] for 10 min, the OD₄₁₄ values were measured. Each MAb was analysed in duplicate for binding to the positive control isolate (LI0521) and the test isolate on each plate. Three

Table 3-1. *L. monocytogenes* isolates used in this study

Isolate ID Number	Serotype
HPB 5327	1/2a
HPB 4705	1/2a
OLF 09016	1/2a
OLF 09033	1/2a
OLF 09011	1/2a
OLF 09049	1/2a
OLF 09015	1/2a
HPB 6036	1/2a
HPB 6095	1/2a
HPB 5330	1/2b
HPB 4857	1/2b
OLF 090271	1/2b
OLF 09060	1/2b
OLF 09040-1	1/2b
HPB 5328	1/2b
HPB 5913	1/2b
HPB 6027	1/2b
HPB 1869	1/2c
HPB 5121	1/2c
OLF 09013	1/2c
OLF 09022-1	1/2c
HPB 2972	1/2c
HPB 2768	3a
HPB 3058	3a
OLF 09005	3a
OLF 09039	3a
HPB 5665	3a
HPB 4909	3b
HPB 1031	3b
HPB 61	3c
HPB 3501	4a
HPB 5041	4a
HPB 5058	4a
LI0521	4b ^a
HPB 3449	4b
HPB 5251	4b
HPB 5364	4b
HPB 5816	4b
HPB 5906	4b
HPB 6024	4b
HPB 6092	4b
HPB 5248	4c
HPB 4497	4c
HPB 4706	4c
HPB 5248	4c
HPB 18	4d
HPB 4534	4d
HPB 1848	4e
HPB 1861	4e

a– Strain used to immunize mice for production of monoclonal antibodies.

independent experiments were performed for every MAb / isolate combination. A positive reaction was identified for a MAb if its reaction with the isolate being tested was at 25% or more of that observed for the same MAb on the *L. monocytogenes* serotype 4b LI0521 positive control.

3.13 RNA Extraction

All solutions, water, glassware and utensils used for RNA extraction were treated with 0.1% diethylpyrocarbonate (DEPC) overnight at 37°C and autoclaved. Total RNA was extracted from 1.5 mL of mid-log *L. monocytogenes* culture. Bacterial cells were lysed, after being treated with RNAprotect Bacteria reagent (Qiagen), by digesting with 13 000 units of lysozyme (Sigma) for 30 min at 37°C, followed by mechanical disruption using Lysing Matrix B in the FastPrep system (MP Biomedicals) according to the manufacturer's instructions. RNA was purified from the cell lysate with the RNeasy Mini Kit (Qiagen) as per the supplier's instructions. The integrity of the RNA sample was confirmed by agarose gel electrophoresis.

3.14 5' Rapid Amplification of cDNA Ends (5' RACE) Analysis

The 5' RACE protocol was carried out to identify the transcriptional start site for the *ispC* gene using a 5'/3' RACE kit (Roche) according to the manufacturer's instructions with the *ispC* gene specific primers (Table 3-3). Briefly, a reverse transcriptase reaction was performed on total *L. monocytogenes* RNA to create cDNA using the primer P998. The reaction was carried out at 55°C for 60 min and then stopped by incubation at 85°C for 5 min. The cDNA was purified with the High Pure PCR Product Purification kit (Roche) according to the supplier's instructions. Poly (A) tailing of the purified cDNA strand was accomplished by incubating the cDNA with terminal transferase at 37°C for 20 min and then inactivating

the enzyme though incubation at 70°C for 10 min. PCR amplification of the cDNA took place using a gene specific primer (GSP) 1 and the PCR poly-T primer included in the kit. The product of this primary PCR (10 µL) was used as a template for a nested secondary PCR using GSP2 and the PCR anchor primer. Similarly, the product from the nested secondary PCR was used as a template to conduct another nested PCR using GSP2 and the PCR anchor primer. The product of the third PCR was ran on an agarose gel to check for purity and was sent for sequencing using the primer GSP3.

Table 3-3. Oligonucleotide primers used in 5'RACE experiments and promoter activity experiments

Primer	Nucleotide Sequence
P998 (cDNA synthesis)	5' – CTTTGTGTAGCAACAGATACTA – 3'
P995 (GSP1)	5' – GAGGCTCCATTGCAGTTACTTTA – 3'
P996 (GSP2)	5' – GCGGAATTCAGCATCAATTTTAA – 3'
P997 (GSP3)	5' – CCCCAACCAGATTCTAGAATTCG – 3'
P920	5' – AGAGAATTC <u>AAAA</u> TATCAAAAAGAGCATAA – 3' ^a
P921	5' – CGCGGATCC <u>AA</u> TTTGTTTATTGTCCTAATT – 3' ^b
P918 (Vlac1)	5' – GTTGAATAACACTTATTCCTATC – 3'
P919 (Vlac2)	5' – CTTCCACAGTAGTTCACCACC – 3'

a- EcoRI site underlined

b- BamHI site underlined

3.15 Construction of pTCV-*PispC* transcriptional fusions

The pTCV-lac plasmid vector (207), a low copy number and broad-host range plasmid that contains a promoterless *lacZ* gene, was used to evaluate the *ispC* promoter activity in various growth conditions. A 454 bp DNA fragment containing the *ispC* promoter was amplified by PCR using the primer pair P920/ P921 (Table 3-3) from *L. monocytogenes* genomic DNA. After digestion with BamHI and EcoRI, the amplified promoter region was cloned into BamHI and EcoRI site of the pTCV-lac plasmid, resulting in a recombinant

plasmid pTCV-*PispC*. The recombinant plasmid was sequenced with the Vlac primers (P918 and P919) to verify the inserted sequence. pTCV-*PispC* was subsequently introduced into *L. monocytogenes* by electroporation as described (276).

3.16 Stress Growth Conditions

Various growth conditions were evaluated for their effects on *ispC* gene expression, including: low temperature (4°C), high temperature (42°C), salt (1-10%), acidity (pH 4), alkaline growth (pH 10), sub-lethal ethanol (2-5%), oxidative media, anaerobic growth, alternate carbon sources and various *Listeria* enrichment media. *L. monocytogenes* harbouring pTCV-*PispC* was grown from frozen stock bacteria on a BHI agar plate and subsequently stored at 4°C. A single colony was inoculated into BHI and incubated for 16-18 h at 37°C. A 1:100 dilution of the overnight culture was sub-cultured into the media associated with each test condition (below). As a control, a promoterless plasmid was also introduced into *L. monocytogenes* and tested under the same conditions. Each condition was examined in triplicates on each day tested. Each condition was also replicated on a minimum of 3 separate days. Samples were taken and assayed for β -galactosidase activity at 150 min, 350 min, and 24 h, except for the 4°C culture where samples were collected after 168 h, 336 h, and 504 h of growth.

For temperature evaluation, BHI media were each equilibrated to the pre-determined temperatures (4°C, 37° and 42°C) prior to inoculation. The effects of high salt were examined over a 24 hr period by supplementing BHI broth with 1-10% NaCl (w/v) with 1% intervals. The effects of pH were monitored by buffering BHI broth with HCl or NaOH to the desired pH value (pH 2, 4, 6, 8, 10, 12). Ethanol was tested by adding 2%, 5% or 10% CH₃CH₂OH directly to BHI broth. The effects of highly oxidative conditions on *IspC* expression were

tested by adding 7 mM cumene hydroperoxide (CHP) to BHI. Anaerobic conditions were created by autoclaving BHI in serum bottles. The bottles were sealed flushed by 5 cycles of vacuuming then bubbling with a gaseous mix of 80% nitrogen, 10% carbon dioxide and 10% hydrogen. Resazurin (0.005% v/v) was added to verify anaerobiosis of the medium. To determine if carbon source affected IspC expression, *L. monocytogenes* was grown in the minimal media developed by Johnson et al. (208) and supplemented with glucose, mannose or fructose.

Selective enrichment media have been shown to affect the expression of antigens (92). The effects of various growth media on IspC expression were examined using three common enrichment culture broths: University of Vermont Modified Enrichment Broth (UVM) (BD Diagnostics), Fraser broth (Oxoid) and Palcam broths (Oxoid).

For each condition, sub-cultured cells were grown for a total of 24 h, with the exception of the 4°C culture where the growth time was expanded to 504 h to compensate for the long generation time at this temperature.

3.17 β -Galactosidase assay

β -galactosidase assays were carried out essentially as described by Miller (168), with the exception that cells were collected by centrifugation for 2 min at 16 100 x g and resuspended in assay buffer (0.06M Na₂HPO₄, 0.04M NaH₂PO₄, 0.01M KCl, 0.001M MgSO₄ and 0.05M β -mercaptoethanol (BME) at pH 7) to remove background from the growth media prior to the β -galactosidase assay. Enzyme activity is expressed in Miller Units [1000x (OD₄₂₀-1.75 x OD₅₅₀)/(incubation time x volume of culture x OD₆₀₀)]. Experiments for β -galactosidase analysis were carried out in three independent experiments to ensure reproducibility.

3.18 Statistics

The variability in the activity of the IspC promoter was analyzed between the various growth conditions in comparison to the positive control (growth in BHI broth at 37°) using the Mann-Whitney U test (SYSTAT 10 software). *P*-values of <0.001 were considered significant. Significance of the differences in hydrolysis of WT and Δ PgdA mutant PG by IspC were also calculated in the same way, however, *P*-values of <0.05 were considered significant.

3.19 Immunofluorescent staining

Immunofluorescent staining was carried out essentially as described in (152). Briefly, cells were grown in each of the tested growth conditions for 24 hr (504 hr for the 4°C culture) and collected by centrifugation for 2 min at 16 100 x g to remove the culture supernatant. Cell pellets were blocked by resuspending in PBS containing 5% bovine serum albumin (BSA) for 1 hr. Cells were then suspended (for 1 h) in the primary antibody (M2773) tissue-culture fluid at a dilution of 1:50 in PBS containing 5% BSA. After washing twice with PBS, cells were incubated with a 1:2000 dilution of Dylight 488 conjugated goat anti-mouse IgG (H + L) (Jackson ImmunoResearch) in PBS containing 5% BSA for 1 hr. Cells were washed 3 times with PBS and resuspended in PBS. Cells were visualized with a microscope first under a phase contrast and then fluorescence modes. Images were captured using the QCapture Pro software (Q Imaging).

3.20 Construction of a *secA2* in-frame deletion mutant

An in-frame deletion of the putative *secA2* gene (*LMO*2365_0612) from the chromosome of a *L. monocytogenes* serotype 4b strain LI0521 was created through homologous recombination using the shuttle vector pAUL-A (48). Briefly, a 459 bp sequence upstream of the *secA2* ORF including the first three codons and a 541 bp sequence downstream of the *secA2* ORF were amplified by PCR using the primer pairs P898/P899, and P900/P901 (Table 3-4), respectively. The two amplicons were spliced together by PCR using P898 and P901, resulting in a deletion of 2343 bp internal to the *secA2* ORF. The fused DNA fragment was inserted into the EcoRI and HindIII sites of the pAUL-A plasmid to create pAUL-A Δ *secA2*, which was introduced into *L. monocytogenes* LI0521 by electroporation as described (193). Bacteria were screened for the targeted gene deletion using the methods described by Schaferkordt et al. (228). Suspected Δ *secA2* mutants were screened by PCR analysis of their genomic DNA with a primer pair (P903 and P904) internal to the deletion region and a primer pair (P902f and P902r) external to the deletion region and verified by sequencing the PCR product derived with P902f and P902r from their genomic DNA.

Table 3-4. Oligonucleotide primers used to create an in-frame Δ *secA2* knockout

Primer	Nucleotide Sequence	Purpose
P898	5'- ATAGA <u>ATT</u> CTAGCCGCGATAGTTCGTTTT -3' ^a	up-stream forward
P899	5'- TTAAGCCGTCTTGTCTCATTATATAACATCC -3'	up-stream reverse
P900	5'- AATGAGACAAGACGGCTTAATCCAAGGCTAA -3'	down-stream forward
P901	5'- GCGA <u>AAGCTT</u> AAGCTCGAGCGACTGAACTC -3' ^b	down-stream reverse
P902f	5'- TGCGTCAACAGTAGTCCCTTT -3'	internal forward
P902r	5'- AAGTAGAATAAAACAAGCGGCGTA -3'	internal reverse
P903	5'- CGATCCACATACTGGTCGTG -3'	external forward
P904	5'- GCTAGTCCGCCAAGCTTATG -3'	external reverse

a- EcoRI restriction site underlined

b- HindIII restriction site underlined

3.21 Construction of a *pgdA* in-frame deletion mutant

An in-frame deletion of the putative *pgdA* gene (LMOF2365_0434) (181) from the chromosome of a *L. monocytogenes* serotype 4b strain LI0521 was done through homologous recombination using the shuttle vector pAUL-A (250). The genomic DNA was prepared using DNAzol (Invitrogen) as per the manufacturers' instructions. A 512 bp DNA sequence located upstream of the *pgdA* ORF including the first three codons was amplified by PCR from the genomic DNA with the primer pair P831 and P832 (Table 3-4). A 465 bp DNA sequence located downstream of the *pgdA* ORF containing the last 8 codons of the C-terminus was also amplified using the primer pair P833 and P834 (Table 3-4). The two fragments were spliced together by PCR with the primer pair P831 and P834 using a 1:10 dilution of the amplified products as the template. This results in a PCR amplicon containing a deletion of 1368 bp internal to the *pgdA* ORF. The spliced DNA fragment was cut with BamHI and HindIII and ligated into the corresponding sites of pAUL-A, creating pAUL-A Δ *pgdA*. This recombinant plasmid was introduced into competent *L. monocytogenes* through electroporation as previously described (193). Bacteria were screened for *pgdA* gene deletion as described by Schaferkordt et al. (251). The Δ *pgdA* mutant was identified by PCR analysis of its genomic DNA using a primer pair internal to the deletion region: P835 and P836 and a primer pair external to the deletion region: P837 and P838 (Table 3-5). In addition, the PCR product derived from the genomic DNA of the mutant with the primers P837 and P838 was sequenced to confirm the *pgdA* gene deletion.

Table 3-5. Oligonucleotide primers used generate a *ApgdA* in-frame knockout

Primer	Nucleotide Sequence	Purpose
P831	5' – ATCGGATCCACCAATTTT CGCTGTAATTGG – 3' ^a	up-stream forward
P832	5' – TTGAATCTGTAATTTTCACATTATGCACCTC – 3'	up-stream reverse
P833	5' – TGTGAAAATTACAGATTCAAGAATGGTGAAA – 3'	down-stream forward
P834	5' – <u>AAAAAGCTT</u> GTACCGATATCTCCAAATATT – 3' ^b	down-stream reverse
P835	5' – CAAAGTCGCGCAACAAAGTAA – 3'	inside deletion forward
P836	5' – GTAGCGTCAAGGCTCGCAGCA – 3'	inside deletion reverse
P837	5' – TTCTGGTATGGCCAACTTTCA – 3'	outside forward
P838	5' – AATACAGAACTCAGTGTACCA – 3'	outside reverse

a – BamHI restriction site underlined.

b – HindIII restriction site underlined.

3.22 Preparation of *L. monocytogenes* peptidoglycan

PG was prepared from mid-log phase WT and Δ *apgda* strains of *L. monocytogenes* as previously described (119) with minor modifications to the procedure. Briefly, bacterial cells from 1 L of culture were collected by centrifugation at 10 000 x g for 20 min and resuspended in 4% (w/v) SDS and boiled for 30 min. Cells were then frozen for 12 h at -80°C and broken by 3 passages through a French press at 1500 lbs /in². The unbroken cells were removed by centrifugation at 3000 x g for 5 min and the remaining insoluble fraction was collected by centrifugation at 10 000 x g for 20 min. The pellet was resuspended in 13 mL of 100 mM Tris-HCl and treated with 0.7 mg α -amylase at 37°C for 2 h. After digestion, magnesium sulfate (20 mM), DNase I (100 μ g) and RNaseA (500 μ g) were added to the solution along with 1 mg trypsin. The mixture was incubated at 37°C overnight. To inactivate the enzymes, 1% (w/v) SDS was added to the mixture and boiled for 15 min. The insoluble fraction was collected by centrifugation at 10 000 x g for 20 min, washed 1 x with 8 M lithium chloride and then rinsed 3 x deionized H₂O. The insoluble fraction was resuspended in 48% (v/v) hydrofluoric acid and gently agitated at 4°C for 48 hr to remove teichoic acid. The PG was collected by centrifugation, washed multiple times with deionized H₂O, and then suspended in 10 mL NEB Buffer 3 (New England BioLabs). After incubation

with 50 units of alkaline phosphatase at 37°C overnight, the solution was boiled for 5 min. The insoluble PG was spun down by centrifugation, washed with deionized H₂O and lyophilized.

3.23 Digestion of *L. monocytogenes* peptidoglycan

L. monocytogenes PG was digested by mutanolysin (Sigma) in a 10 mM sodium citrate buffer (pH 6.7) containing 10 mg/mL sodium azide and 2500 units mutanolysin for 16 hr at 37°C. Digestion by IspC was carried out in 10 mM phosphate buffer (pH 8.0) containing 10 mg/mL sodium azide and 24 µg of recombinant IspC for 96 hr at 37°C. The digestion volume was 0.5 mL.

3.24 Separation of Muropeptides by High-pressure liquid chromatography

Soluble muropeptides obtained from PG digestion with mutanolysin or IspC were reduced by sodium borohydride and separated by RP-HPLC on a C18 Hypersil ODS column (250 x 4.6 mm i.d.; 5µm) (ThermoHypersil) at 45°C. The muropeptides were eluted with a 200 min linear gradient of Buffer A (50 mM sodium phosphate buffer containing 5% methanol, pH 2.5) to Buffer B (50 mM sodium phosphate buffer containing 30% methanol, pH 2.8) at a flow rate of 0.5 mL/min. Peaks of muropeptides were monitored at 206 nm. Peak fractions were collected manually.

3.25 Determination of the IspC cleavage site within peptidoglycan using HPLC

(Performed by Ikue Hayashi)

The soluble fraction of IspC hydrolyzed *L. monocytogenes* PG contains reducing sugars, when IspC cleaves a glycosidic bond within the glycan strands. To differentiate between *N*-acetylglucosaminidase and *N*-acetylmuramidase activity, the hydrolysis products were treated with alkali sodium borohydride (0.1 M) for 2 hr at room temperature and dried

under reduced pressure using a rotary evaporator. The samples were then hydrolyzed with 3 M hydrochloric acid at 100°C for 3 hr. Residual hydrochloric acid was evaporated. The samples were treated with triethylamine solution (methoanol:triethylamine:water, 2:1:2) to neutralize any residual hydrochloric acid. Derivatization was carried out for 20 min in phenylisothiocyanate reagent (methanol:triethylamine:water:phenylisothiocyanate; 7:1:1:1). After evaporation, the samples were redissolved in water and subjected to HPLC analysis. The phenylisothiocarbamyl derivatives of amino sugar alcohols were separated on a STR ODS II (Shimadzu, Kyoto, Japan) C18 reverse phase column (4.6 x 150 mm, 5µm) and monitored by a UV spectrometer at 254 nm. Solvent A contained 140 nM sodium acetate (pH 5.7), 200 mM boric acid and 0.7 ml/L triethylamine. Solvent B contained 60% acetonitrile in water. The gradient was 98% Solvent A at the time of sample injection, 93% Solvent A after 5 min, 50% Solvent A after 20 min and 0% Solvent A at 25 min at a flow rate of 1 mL/min and a column temperature of 40°C. Standards (a mixture of *N*-acetylglucosamine and *N*-acetylmuramic acid) were followed by the above procedures except for treating with (reduced standard) or without (non-reduced standard) alkali sodium borohydride (0.1 M).

3.26 Comparison of peptidoglycan hydrolysis

Renaturing SDS-PAGE. Renaturing SDS-PAGE was carried out essentially as described by Potvin et al. (206). Cell wall material was prepared by autoclaving overnight cultures of *L. monocytogenes* serotype 4b LI0521 or the *ApgdA* mutant and collected by centrifugation for 10 min at 10 000 x g. After 4 washes with deionized H₂O, the cellular material was lyophilized for 24 hr. Purified recombinant IspC was run on a 12% SDS-PAGE gel containing either 0.1% (w/v) WT or *ApgdA* cell wall material. The gels were washed with

deionized H₂O for 30 min after electrophoresis and incubated for 16 hr at room temperature in renaturation buffer (25 mM Tris-HCl pH 7.5, 1% v/v Triton-X). After renaturation, gels were washed in H₂O for 30 min and then stained with 0.1% (w/v) methylene blue (Fisher Scientific, Ottawa, ON, Canada) in 0.01% (w/v) KOH for 1 hr at room temperature with agitation. Gels were destained with H₂O until areas of hydrolysis showed up as clear bands on a dark blue background.

Cell wall turbidity assay. Autoclaved and lyophilised bacteria cells were dissolved in phosphate buffer (pH 7.9) to an approximate OD₆₆₀ of ~ 0.5. Mutanolysin (250 units) or purified rIspC (24 µg) were added to the suspension and the volume was made up to 1 mL with buffer. The sample was incubated for 24 hr or 96 hr for mutanolysin and IspC respectively, at 37°C with constant agitation. The absorbance was monitored at 660 nm at pre-determined intervals during digestion.

3.27 Precipitation of secreted proteins from the culture supernatant

Secreted proteins were precipitated from the culture supernatant of WT or ΔSecA2 strains of *L. monocytogenes* grown in BHI media using the protocol described by Lenz et al. (144). After removal of bacterial cells from 500 mL of growth media by centrifugation at 11000 x g for 20 min, trichloroacetic acid at 6% (v/v) was added and the mixture was incubated on ice for 30 min. Precipitated proteins were collected by centrifugation at 8000 x g at 4°C for 40 min. The precipitated proteins were then dissolved in 1 mL PBS and 1 mL 2 x SDS-PAGE sample buffer added. The protein samples were stored at -20°C until use.

Chapter IV

Characterization of Monoclonal Antibodies recognizing *L. monocytogenes* serotype 4b

Some of the data in this chapter has been published in:

Lin, M., Armstrong, S., Ronholm, J., Dan, H., Auclair, M.-E., Zhang, Z., and Cao, X. (2009) Screening and characterization of monoclonal antibodies to the surface antigens of *Listeria monocytogenes* serotype 4b. *Journal of Applied Microbiology*. 106(5): 1705-1714.

Ronholm, J., VanFassen, H., McKenzie, R., Zhang, Z., Cao, X., and Lin, M. (2013) Monoclonal antibodies recognizing the surface autolysin IspC of *Listeria monocytogenes* serotype 4b: epitope localization, kinetic characterization, and cross-reaction studies. Submitted to PLOS ONE.

4.1 Introduction

L. monocytogenes infection is very serious for certain demographics including: neonates, pregnant women, the elderly and those with impaired T-cell mediated immunity such as HIV or transplant patients, since outbreaks involving these individuals can lead to extremely high fatality rates. This makes *L. monocytogenes* a concern for consumers of RTE food products which are consumed directly without cooking. In addition, the presence of *L. monocytogenes* in food processing plants is difficult to control because it is frequently found in the environment, can multiply at refrigeration temperatures, and is able to survive in a wide range of salt concentrations, temperatures, and pH conditions (59).

Although *L. monocytogenes* is divided into 13 serotypes, 98% of human illness is caused by serotype 1/2a, 1/2b and 4b strains (79). Serotype 4b strains account for more cases of human listeriosis than serotype 1/2a and 1/2b isolates combined, although 1/2a and 1/2b strains are much more commonly found in foods and the environment (79, 250, 264). This suggests that serotype 4b strains may be specifically adapted to infecting human hosts (79, 250, 264). Serotype 4b strains are also more often isolated from patients with meningoencephalitis than from patients where the infection has been limited to the bloodstream (250). Listeriosis patients also suffer a 26% case fatality rate when infected with a serotype 4b strain compared to a 16% case fatality rate in patients infected with a serotype 1/2a or 1/2b (95). These latter observations suggest that serotype 4b may be more virulent in humans than other serotypes. Therefore, the development of a diagnostic test specific for *L. monocytogenes* serotype 4b strains is important.

Current culture based methods for detecting *L. monocytogenes* are labour intensive and take 5-7 days for detection and serotyping. PCR methods produce faster results, but have

several inherent disadvantages: large scale recalls, which are financially costly, are unlikely to occur based on a PCR result since false-positives are common. Determining if the organism from which the DNA came was alive or killed is also impossible with the PCR procedures. Antibody-based methods have been demonstrated to be very promising for the rapid isolation and detection of *L. monocytogenes* from food and environmental samples (189, 216). Antibodies with certain binding characteristics, such as a high affinity and specificity for a surface localized protein of *L. monocytogenes* make them especially useful in immunoassays which aim to capture live cells. With the advent of new technologies, culture enrichment may not be necessary for detection. Currently, there are commercially available antibodies which are able to detect *Listeria* spp. in food and environmental samples (90). For example, the monoclonal antibody (MAb) used in the VISAS LMO assay (bio-Merieux, Marcy-Etoile, France), can discriminate between *L. monocytogenes* and other species of *Listeria*, making it a useful screening tool in food pathogen testing laboratories (124). Attempts to develop MAbs against *L. monocytogenes* serotype 4b have been made (122); however, the surface antigens recognized by those MAbs remained unidentified and diagnostic tests performed with these MAbs had inconsistent results (122). Therefore, there is currently no antibody available to specifically detect *L. monocytogenes* serotype 4b.

The purpose of this work was to fully characterize the MAbs produced against *L. monocytogenes* serotype 4b. This involved elucidating the cell surface proteins which are recognized, and characterizing each of the MAbs as potential diagnostic reagents including epitope localization, kinetic characterization, and determining the range of cross-reactivity with other *L. monocytogenes* isolates.

4.2 Results

4.2.1 Western blot analysis of total cellular protein antigens

Mice were immunized with formalin killed *L. monocytogenes* serotype 4b LI0521 cells without an adjuvant. Thirty-nine days after immunization, the mice were sacrificed and spleen cells fused with Sp2/0-Ag14 myeloma cells. The culture supernatants from the resultant hybridoma cells were tested for *Listeria*-reactive MAbs using ELISA. Twenty-nine *L. monocytogenes* serotype 4b reactive clones were identified. The 29 MAbs from these positive clones were analyzed by Western blotting to reveal the antigenic protein components that reacted under denaturing conditions with total cellular antigens of *L. monocytogenes* (Figure 4-1). Twelve MAbs (M2773, M2774, M2775, M2777, M2778, M2779, M2787, M2790, M2792, M2794, M2799, and M2800) each reacted strongly with a protein having an apparent molecular weight of 77 kDa, while four (M2781, M2785, M2795 and M2797) each reacted weakly with this protein. A total of thirteen MAbs (M2772, M2776, M2780, M2782, M2783, M2784, M2786, M2788, M2789, M2791, M2793, M2796, and M2798) failed to react with any proteins on the Western blots.

4.2.2 Molecular identification of the antigen

The M2799 MAb, selected for immunoprecipitation due to its strong and specific reaction (Figure 4-1), was used to immunoprecipitate the 77 kDa antigen. After immunoprecipitation, the 77 kDa band was resolved by SDS-PAGE, excised and sent for protein identification by MS and N-terminal sequencing (Figure 4-2A). MS analysis and N-terminal sequencing indicated that the 77 kDa antigen recognized by M2799 was IspC (Figure 4-2B). The 10 amino acid N-terminal sequence of the immunoprecipitated protein aligned perfectly with amino acids 46 to 55 of IspC (Figure 4-2C). This demonstrates that

Figure 4-1. Western Blot Analysis of *L. monocytogenes* Total Cell Proteins with MAbs. Total cell proteins equivalent to 1.6×10^8 cells were loaded into each well of an SDS-PAGE gel, electrophoresed and probed with each of 29 MAbs. Reactions were visualized using HRP-conjugated goat anti-mouse IgG and the corresponding substrate solution.

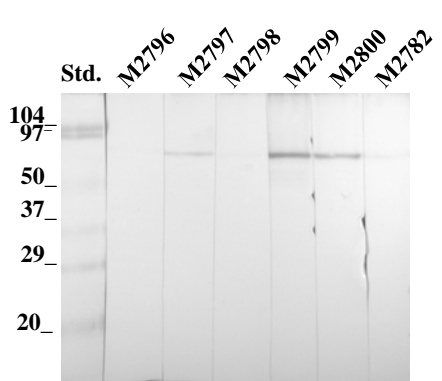
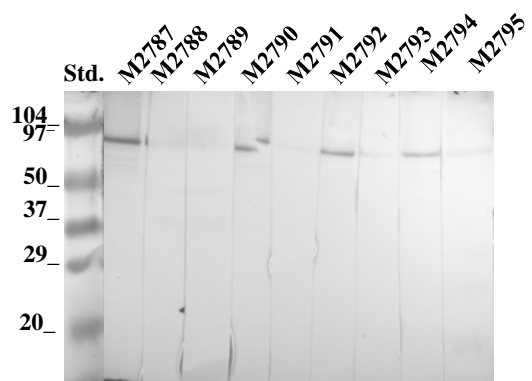
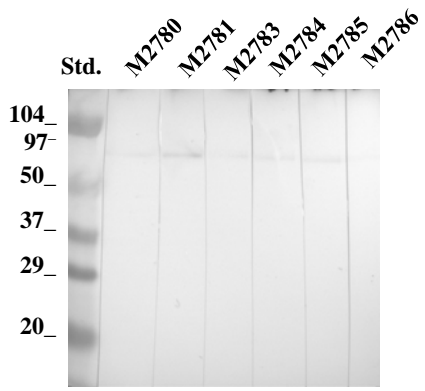
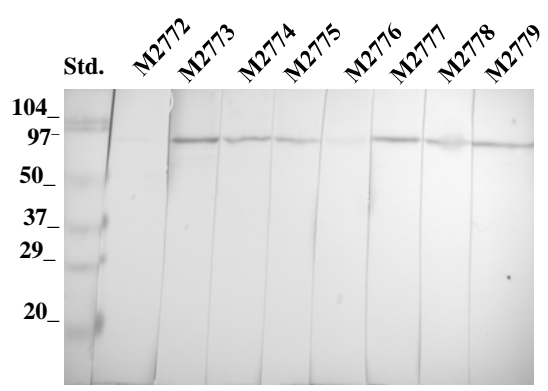
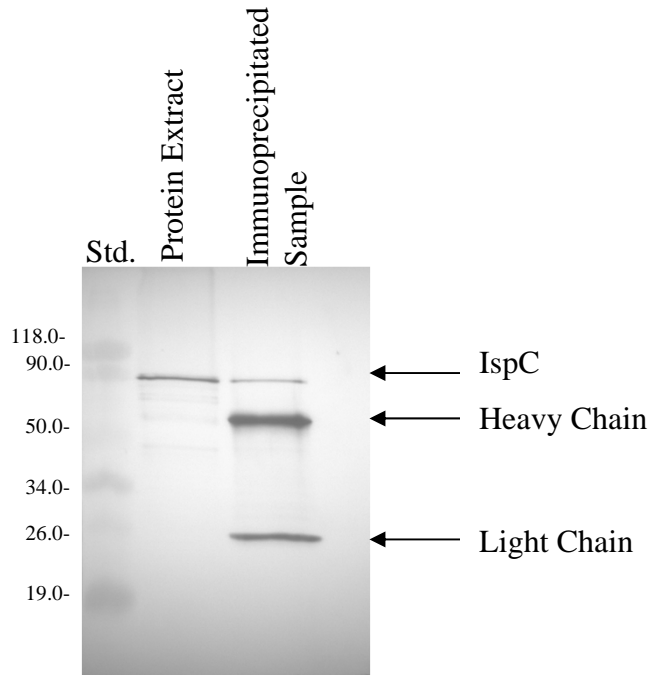


Figure 4-2. Molecular identification of the antigen. A) Surface proteins were extracted from *L. monocytogenes* serotype 4b cells by boiling 8.7×10^{10} cells in 4% SDS for 10 min. Insoluble cell material was removed by centrifugation. SDS was separated from the proteins in the sample by chromatography with Extracti-Gel D Detergent Removing Resin (Thermoscientific). Protein G purified M2799 was used in conjunction with protein A coated sepharose beads to immunoprecipitate the antigen. To release the antigen, the beads were boiled for 10 min in SDS-PAGE loading buffer. The proteins were separated by SDS-PAGE and protein bands were visualized by Western Blot. Lane 1 shows the sample pre-immunoprecipitation and lane 2 shows the sample after immunoprecipitation, where the heavy and light chains of the antibody are clearly visible. B) The immunoprecipitated protein was also excised directly from the SDS-PAGE gel after visualization with Coomassie blue staining and sent for MS protein identification at the Ottawa Hospital Research Institute (OHRI). A MASCOT search of the NCBI database identified IspC. Residues which were identified by MS/MS spectra and match the predicted sequence of IspC are shown underlined and in red. C) The immunoprecipitated protein was blotted onto a PVDF membrane, excised after Coomassie blue staining and sent to the protein core facility at the Columbia University for N-terminal Edman sequencing. The N-terminal sequence aligns perfectly with amino acids 46 to 55 of the predicted IspC amino acid sequence, based on its ORF. This provides evidence that IspC has a signal sequence which is cleaved prior to protein maturation.

A



B

1 MINKKWMKIV MIPMLVVPMY GLTTVGGQLQ DSLTGENSEFVKEVEAATTAS
 51 QQAFIDKIAPAAQASQEKYH LLSSITLAQA ILESGWGKSGLATOGYNLFG
 101 IKGKYNGQSV IMTTSEYVNG EWIK IDAEFRKYPSWNESVTDHTLLLVNGT
 151 SWNK DLYKKV VDATDYKVA MELQKAGYATSPTYGASLIQVIENYDLAKY
 201 DVL YDKILTQ KSTSGK ATVTSPTGNGVWTLPYK VKGVQSVSPASTYANKD
 251 IDLVSVAATTKR GTYYQFKYN GKVVGWVDGK ALTIYDSVNDKVNVR AKI
 301 TSPVSNGIWSKPYNVYGREFVTNATTYAQQEIK LLREAQAKGTYYQFSI
 351 NNKTIGWIDKRALTYPYDSIISKNVNLDGQITNPTGNGIWTKAYKL
 401 TTSVAQATK YANKDVKISQOIETOHGTYYNISIDGK AIGWLDNRNAILTYD
 451 QEEYNK TV AIDAVVK NVK GNAVWTEPYR TVGTK LIGPAETYLNKEVEVVR
 501 EAKTPKGTYYQFKSGGK VIGWLDKKAFDVYDNINYNKAVNLDAVVENVTG
 551 NAVWTAPYK SKGVKLV TSAATYK GKATKITREAQTSR GTYYEFSVDGKVI
 601 GWLDKKA FDVYDNINYNKAVNLDAVVENVTGNAVWTAPYKSKGVKLV TSA
 651 ATYKDKATKITREAQTSRGTYYEFSVNGKVIGWLDK KAFDVYDSIEYNKA
 701 INMTGLLSNAPGNGIWTPEYR VIGTKNVGQATAYANKTVQLIREAKTTR A
 751 TYYQMSVNGK IVGWVDKRAFTNVK

C

IspC Precursor:

45 ▼ 46

NH2-MINKKWMKIVMIPMLVVPMYGLTTVGGQLQDSL TGENSEFVKEVEAATTASQQAFI

Mature IspC

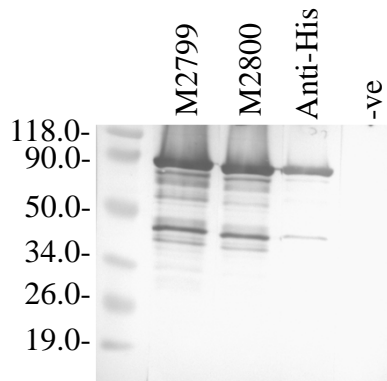
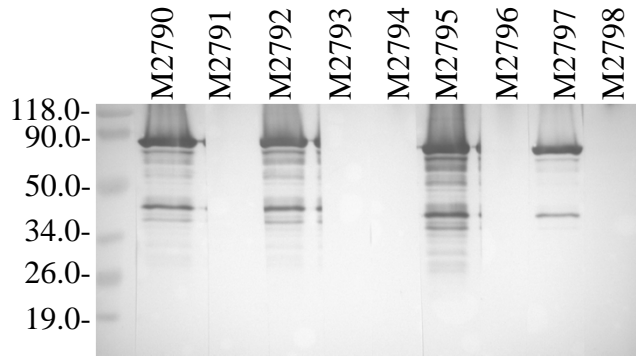
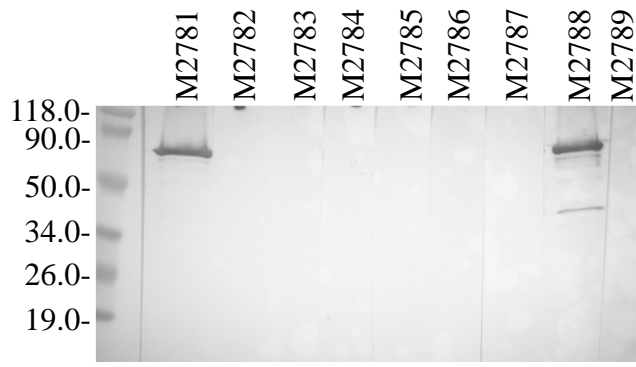
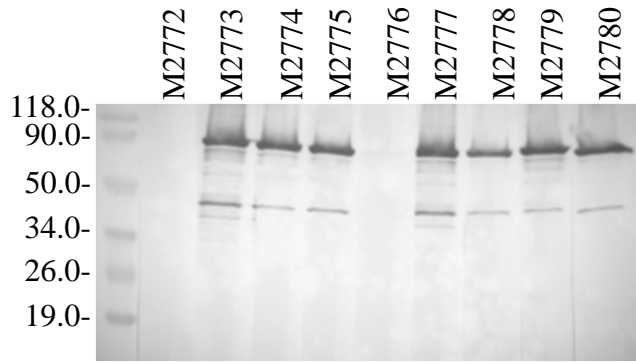
NH2- ATTASQQAFI

IspC has a 45 amino acid signal-sequence which is cleaved prior to protein maturation. The molecular weight of IspC, calculated based on its amino acid sequence, is 80.8 kDa after the signal sequence is cleaved. This is in agreement with the apparent weight based on the original Western blot (Figure 4-1). To determine which of the MAbs, in addition to M2799, react with IspC, recombinant IspC (rIspC) (275) was loaded into an SDS-PAGE gel, electrophoresed and probed with each of the 29 MAbs created in the original fusion (Figure 4-3). Fifteen MAbs (M2773, M2774, M2775, M2777, M2778, M2779, M2780, M2781, M2788, M2790, M2792, M2795, M2797, M2799, and M2800) reacted with rIspC (Figure 4-3) and were targeted for further characterization. These results do not correlate perfectly with the results shown in Figure 4-1. While M2787, M2785 and M2794 react well with IspC extracted from the cell surface of *L. monocytogenes*, these MAbs reacted weakly with rIspC. In contrast, M2780 and M2788 reacted weakly with IspC from *L. monocytogenes*, but reacted well with rIspC.

4.2.3 Epitope localization using recombinant IspC fragments

Epitopes were mapped for 15 MAbs capable of recognizing the denatured rIspC (Figure 4-3), by constructing a series of His-tagged recombinant proteins with deletions of defined amino acid (AA) sequences of IspC (left panel, Figure 4-4A). Reactivity of the recombinant protein fragments with each MAb was analyzed by Western blotting and the results are summarized in Figure 4-4A (right panel). The epitopes recognized by all 15 MAbs were found to be within the C-terminal CBD, spanning AA 198 to 774 of IspC (Figure 4-4B). This observation was further supported by a lack of reactivity of two N-terminal protein fragments (A A1-197 and AA 58 -263) with any of the MAbs. The epitopes for each MAb were further localized to smaller segments within the region of AA 197 to 774 by N- or C-

Figure 4-3. Recombinant IspC probed with 29 MAbs. Recombinant IspC was expressed in *E. coli*. Purified rIspC was loaded into each lane of a SDS-PAGE gel, electrophoresed and probed with each of 29 MAbs. This was followed by reaction with HRP-conjugated goat anti-mouse IgG and detection with HRP-substrate solution (BioRad). Fifteen MAbs (M2773, M2774, M2775, M2777, M2778, M2779, M2780, M2781, M2788, M2790, M2792, M2795, M2797, M2799, and M2800) each reacted strongly with rIspC.



terminal deletions or double deletions from both ends, with the two exceptions that (i) all truncated fragments (with the exception of weak reactions with AA 467-694, AA 467-664 and AA596-764) were non-reactive to M2781 and (ii) M2799 which reacted with all the fragments generated with the region of AA 197 to 774.

Group 1 MAbs (M2773, M2788, M2792, M2795 and M2800) showed the same pattern of reactivity to the protein fragments located within the C-terminal CBD (AA 197-774), recognizing every fragment with the exception that they did not react with the fragment AA 684-774 (Figure 4-4A and Figures S1-S8). This indicated that the epitope(s) recognized by these five MAbs was between AA 616 and AA 684. However, these MAbs also recognized an epitope within the region of AA 467 to 624, as demonstrated by antibody reactivity to the three fragments AA 467-694, AA 467-664 and AA 467-624. Recognition of the two fragments AA 616-774 and AA 467-624 by these five MAbs suggests that a 9 AA stretch between AA 616 and AA 624 which is common to both fragments, is critical for antibody binding. However, it is likely, since a great deal of sequence homology exists in this region, that other residues are also important for optimal binding.

The epitope recognized by group 2 MAbs (M2775 and M2797) was mapped to a smaller C-terminal region (AA 684 to 774) of IspC. M2780 has a similar reaction profile except that while it reacts to AA 616-774, it does not react to AA 684-774. A small deletion of the last 10 C-terminal residues, as shown by the fragment AA 596-764, completely abolished the reactivity to M2775, M2797 and M2780, indicating that the last 10 C-terminal residues are critical for binding of these MAbs.

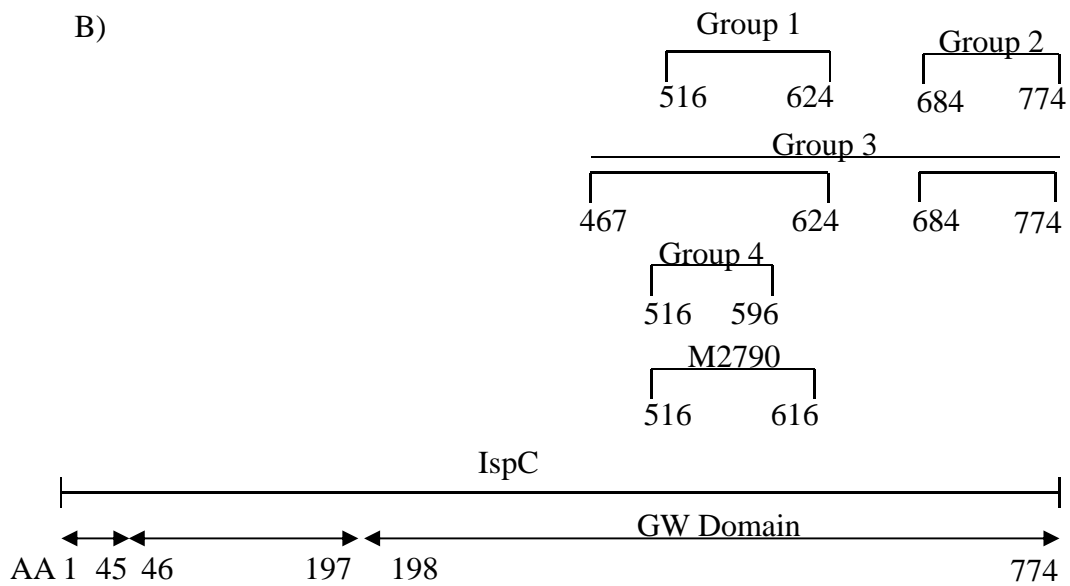
The epitopes for the group 3 MAbs, M2777 and M2778, were mapped to the region AA 684 to 764 commonly found in the reactive protein fragments AA 684-774 and AA 596-

Figure 4-4. Epitope Localization. A) The panel on the left provides a representation of each of the recombinant truncated IspC proteins that were produced in *E. coli*. The table on the right of the figure is a summary of the ability of the corresponding truncated protein to react with each of the MAbs as judged by Western blot. Positive reactions are indicated with a (+), no reaction is indicated by (-), and weak but detectable reactions are indicated by (w). Group 1 consists of M2773, M2788, M2792, M2795 and M2800. Group 2 is composed of M2775 and M2797. Group 3 consists of M2777 and M2778. Group 4 is composed of M2774 and M2779. The MAbs M2780, M2781 M2790 and M2799 are each in their own group since they are the only MAbs with their particular reaction profiles. B) An illustration of the approximate location of the epitopes for each of the major groups of MAbs. A representative sample of each of the Western blots summarized in this Figure be found in Appendix 3 Figures S1 – S8.

A)

	Group 1	Group 2	Group 3	Group 4	M2780	M2781	M2790	M2799
AA 1 IspC 774	+	+	+	+	+	+	+	+
AA 1-197	-	-	-	-	-	-	-	-
AA 58-263	-	-	-	-	-	-	-	-
AA 198-774	+	+	+	+	+	-	+	+
AA 264-774	+	+	+	+	+	-	+	+
AA 365-774	+	+	+	+	+	-	+	+
AA 468-774	+	+	+	+	+	-	+	+
AA 516-774	+	+	+	+	+	-	+	+
AA 616-774	+	+	+	-	+	-	-	+
AA 684-774	-	+	+	-	-	-	-	+
AA 467-694	+	-	+	+	-	w	+	+
AA 467-664	+	-	+	+	-	w	+	+
AA 467-624	+	-	+	+	-	-	+	+
AA 596-704	+	-	-	-	-	-	w	+
AA 596-724	+	-	-	-	-	-	w	+
AA 596-744	+	-	w	-	-	-	+	+
AA 596-764	+	-	w	-	-	w	+	+

B)



764. However, the serial deletions of 20 residues from the C-terminus resulted in two fragments AA 596-764, AA 596-744 which were only weakly reactive to M2777 and M2778 and additional C-terminal deletions created AA 596-724 and AA 596-704, which were not reactive. This indicates that the C-terminus is important to MAb binding. However, the strong reactivity of these two MAbs to the fragments AA 467-694, AA 467-664 and AA 467-624, suggests that the region of AA 467 to 624 contains a separate epitope with a similar sequence to the one contained in the region of AA 684 to 764.

Group 4 MAbs (M2774 and M2779) had an epitope within the AA 516-774 fragment. Further N-terminal deletions of 100 and 168 residues were made to form fragments AA 616-774 and AA 684-774, which did not react with group 4 MAbs. In addition, no reactivity to the four fragments AA 596-764, AA 596-744, AA 596-724 and AA 596-704 was observed for these two MAbs. The results indicated that the residues between AA 516 and 596 are critical to group 4 MAb binding. The reactions of these two MAbs with each of the fragments AA 467-694, AA 467-664 and AA 467-624, which each contain this hypothetical epitope, confirms this finding.

M2790 has a reaction profile similar to that of group 1 MAbs. However, M2790 does not react with AA 616-774 indicating that the epitope is between 516 and 616. This was confirmed by antibody reactivity to the three fragments AA 467-694, AA 467-664 and AA 467-624, all of which contain the AA 516-616 sequence. Reaction with the fragments AA 596-744 and AA 596-764 may indicate that the epitope is on a smaller section between AA 596 and 616, however, the weak reaction with AA 596-704 and AA 596-724 does not support this.

4.2.4 Kinetic analysis of IspC - MAb interactions

For fitting of the data to a 1:1 interaction model, the analyte must have only one binding site. Size exclusion chromatography showed that IspC elutes at a volume that corresponds to a molecular weight of 149.5 kDa, and since the estimated molecular weight of mature IspC based on its amino acid sequence is 80.8 kDa, this indicates that IspC likely forms a dimer in solution and is therefore not suitable to be used as the analyte (Figure 4-5 and Table 4-1). Therefore, to perform a kinetic analysis of these MAbs, which each have two antigen binding sites, they were digested with papain to produce Fab (fragment antigen binding) fragments. A preliminary trial, ran to determine the optimal ratio of papain to IgG and an optimal incubation time for complete IgG digestion, showed that at 1:100 ratio of papain to IgG (w/w) the heavy-chain was completely cleaved after 1 h (Figure 4-6A). Protein G column chromatography was performed to separate the Fab from the Fc fragment after papain digestion. Western blotting with a Fc specific antibody confirmed that Fc had been removed prior to final purification of Fab (Figure 4-6B and C). Each Fab was further purified by size exclusion chromatography immediately prior to kinetic analysis. Only peak fractions of Fab fragments were collected to ensure the highest possible degree of purity (Figure 4-7).

Figure 4-5. Purification of IspC by size exclusion chromatography. Size exclusion chromatography was performed on purified IspC. This was performed to achieve the desired purity of IspC prior to SPR. However, IspC eluted at approximately twice its expected size, strongly indicating that it forms a dimer in solution. IspC is therefore not suitable to act as the analyte in SPR experiments.

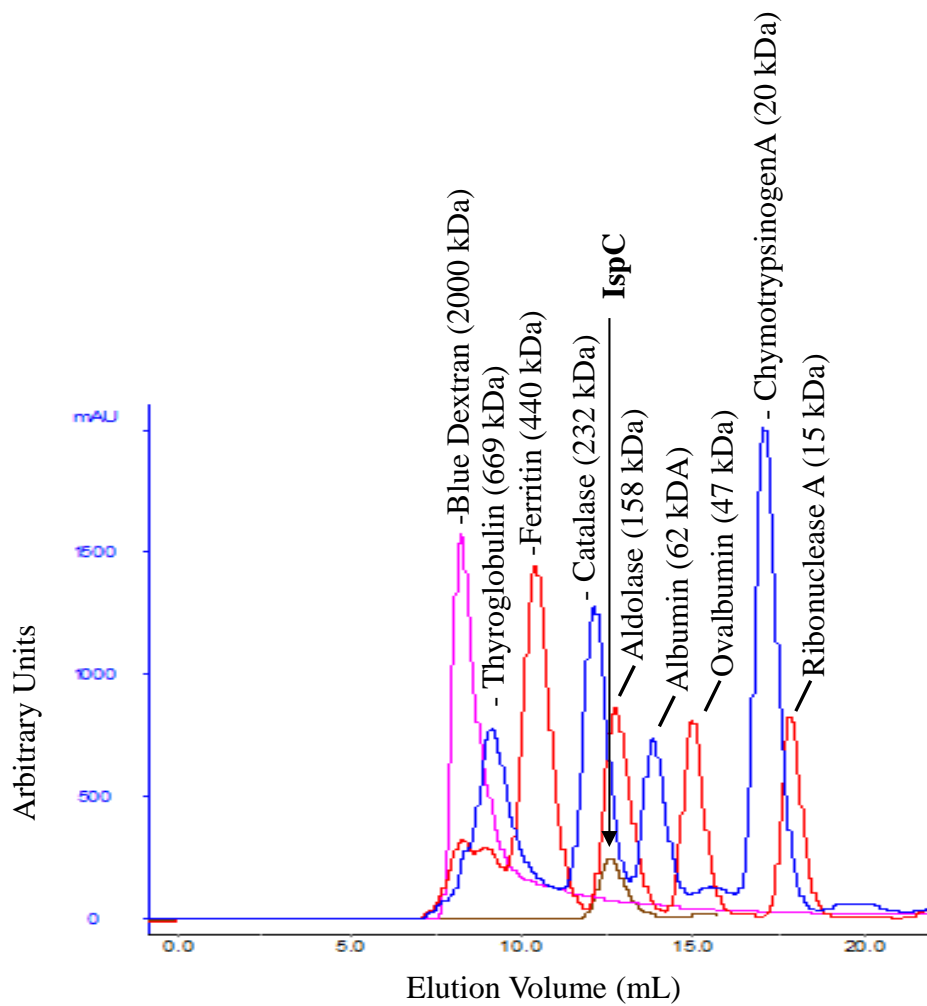


Figure 4-6. Preparation of antibody Fab fragments. Fab fragments were produced from each of the 15 mAbs prior to SPR kinetic analysis. A time course digestion was carried out at multiple ratios of papain to IgG. At a ratio of 1 mg of papain to 100 mg of IgG there was complete digestion after 60 min. Therefore, this time and ratio was used in all further experiments. A) A Comassie blue stain is shown to illustrate the presence of both the heavy and light chain from the MAb prior to the addition of papain. After 60 min, it is clear that there is no heavy chain remaining. The Fc and Fab which resulted from papain digestion were then separated using protein G chromatography. B) A Comassie blue stain shows the presence of the heavy and light chain in the MAb lane, the presence of the Fc portion of the heavy chain in the Fc lane, and the Fab portions of the heavy and light chains in the Fab lane. C) A Western blot using an Fc specific MAb shows the presence of the Fc in both the Fc and MAb lanes, while it is clearly absent from the Fab lane.

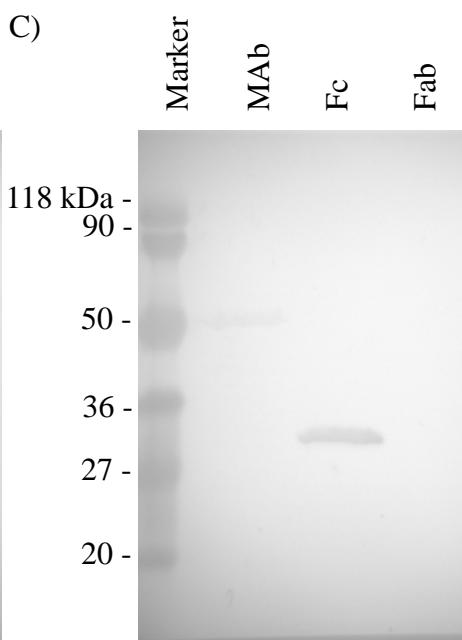
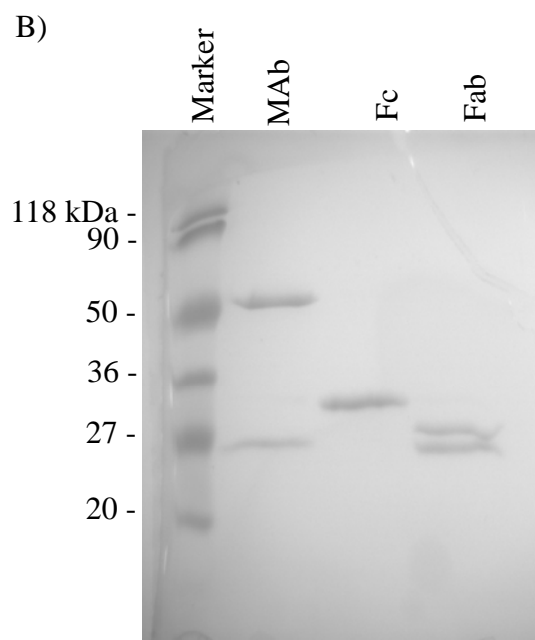
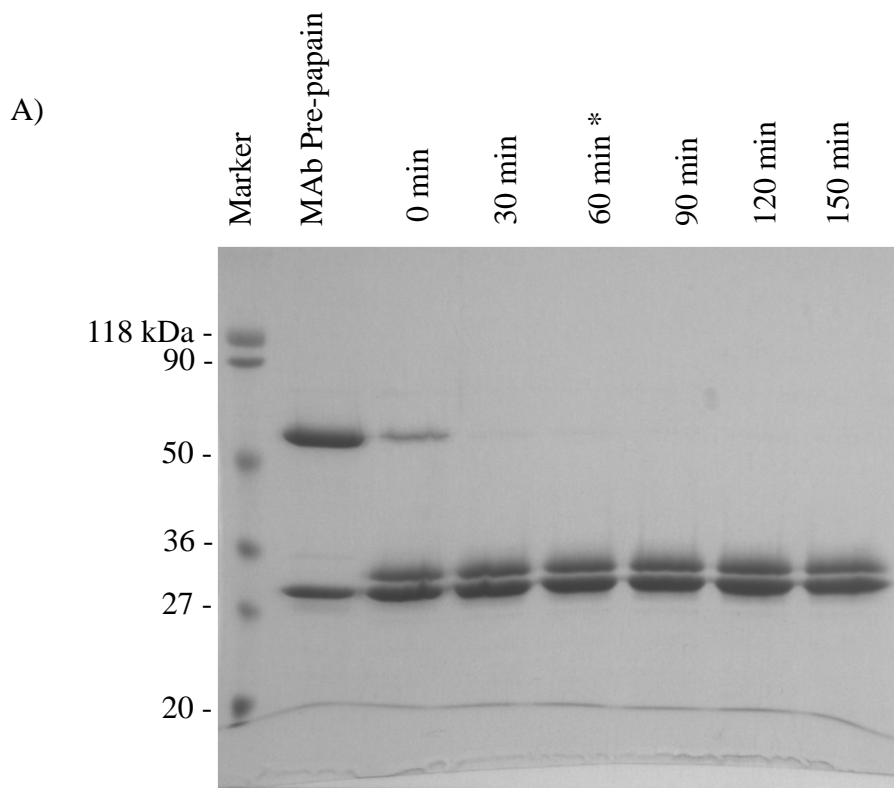


Figure 4-7. Size exclusion chromatography of Fabs. Size exclusion chromatography was performed on each Fab immediately prior to SPR to ensure maximum purity. Only peak fractions were collected and used for SPR.

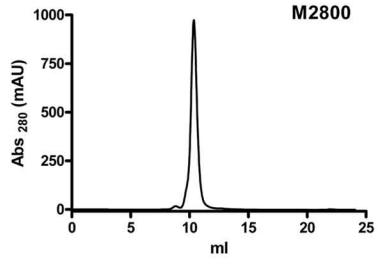
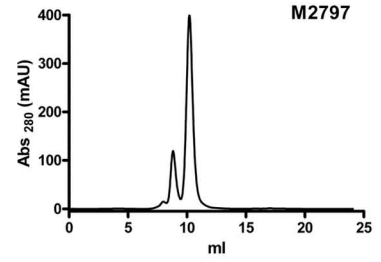
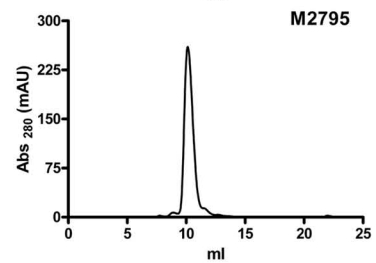
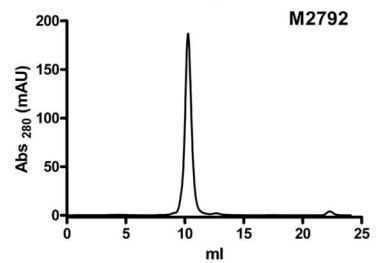
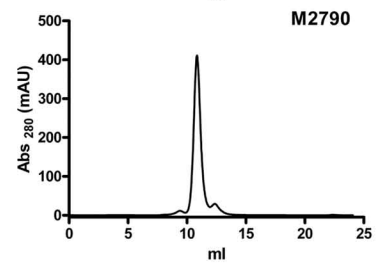
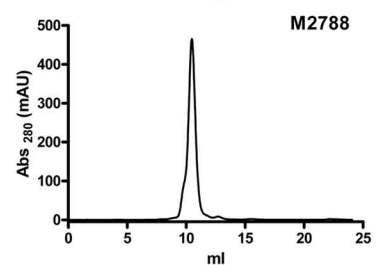
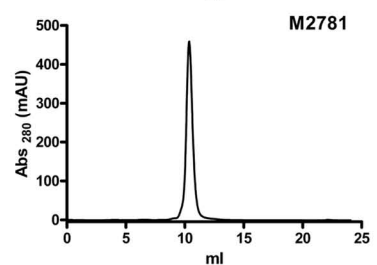
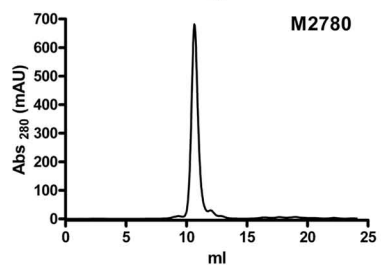
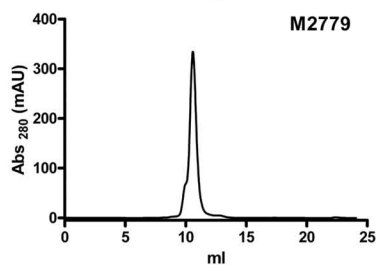
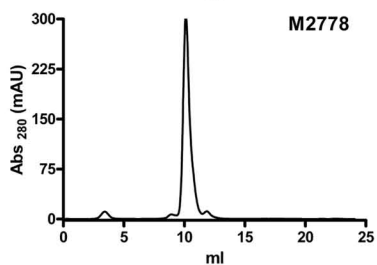
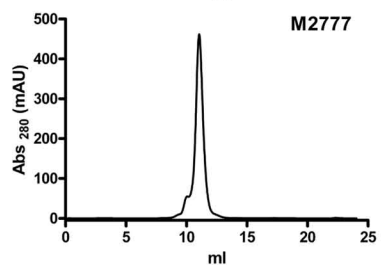
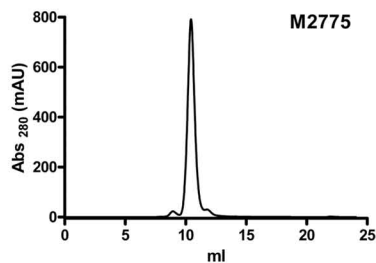
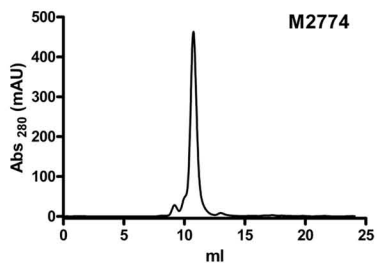
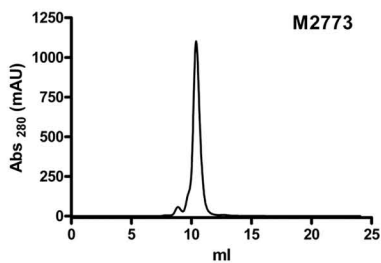


Table 4-1. Size Exclusion Chromatography of IspC.

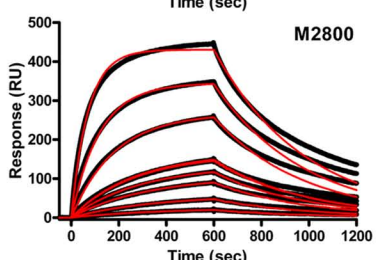
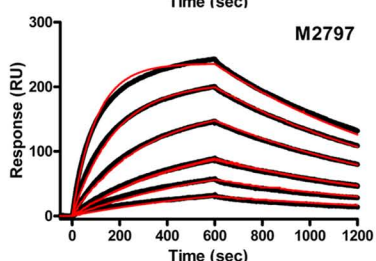
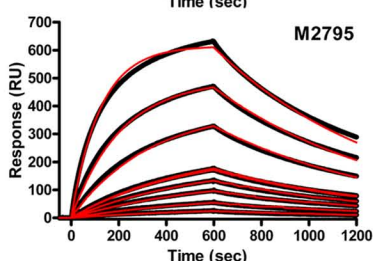
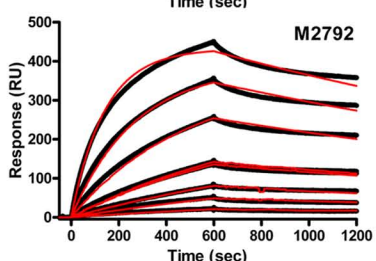
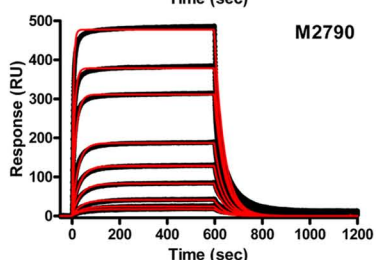
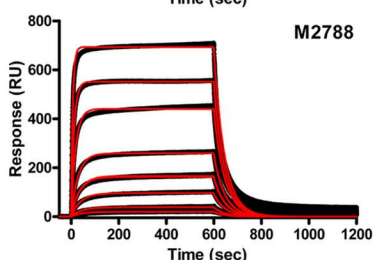
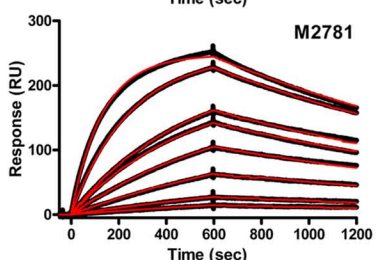
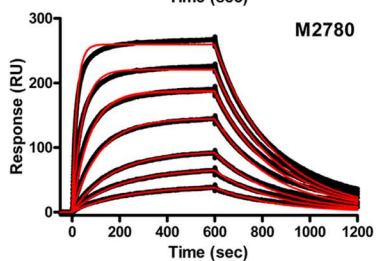
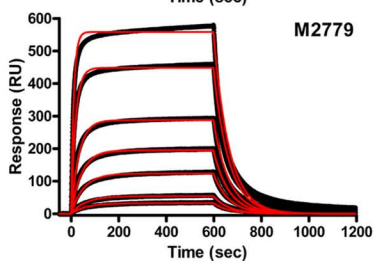
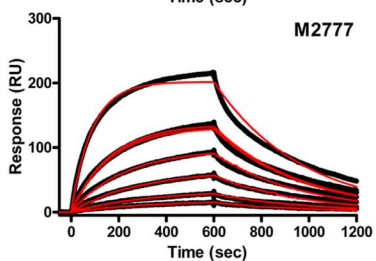
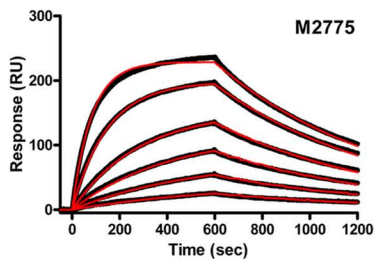
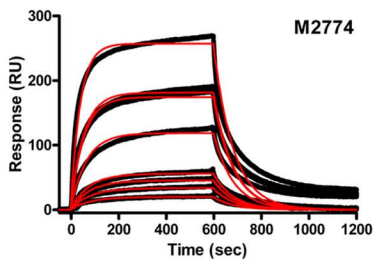
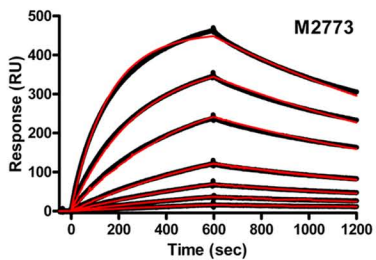
Protein	Formula Molecular Weight (kDa)	Elution Volume (mL)
Blue Dextran	2000 ^a	8.25
Thyroglobulin	669	9.13
Ferritin	440	10.41
Catalase	232	12.13
Aldolase	158	12.73
Albumin	67	13.84
Ovalbumin	43	14.99
Chymotrypsinogen A	25 ^a	17.08
Ribonuclease A	13.7 ^a	17.84
IspC		12.6

a - Indicates that values were not used in calculating the molecular weight of IspC, since they were out of range.

Surface plasmon resonance (SPR) analysis was performed on a Biacore 3000, with the immobilized rIspC as a ligand and the Fab as an analyte (a mobile molecule) to determine the equilibrium dissociation constant (K_D) of each MAb. Initial screening experiments, using only one Fab concentration, demonstrated that M2778 had a very low affinity. Therefore, it was not selected for further SPR analysis. Separation of the Fab and the Fc of M2799 by protein G chromatography proved to be difficult due to strong interactions of both the Fab and Fc with protein G. Thus, the purity of Fab required for SPR analysis could not be achieved for M2799. This MAb was not analyzed by SPR.

SPR was performed to determine the binding kinetics of purified Fab fragments to IspC. Different concentrations of purified Fab fragments passed over IspC, which was covalently immobilized to the dextran matrix on the sensorchip. Increase in the refractive index (caused by the interaction of the Fab fragments with the immobilized IspC) was measured in real time and displayed in a sensorgram in which resonance units (RUs) are plotted against time (Figure 4-8). The K_D , which is a measure of binding affinity, ranged

Figure 4-8. SPR Sensorgrams. Kinetic map summarising the kinetic analysis of IspC and Fabs interactions. SPR sensorgrams showing FAb binding to immobilized IspC at concentrations of: 1, 2.5, 5, 10, 10, 25, 50 and 100 nM for M2773, 2.5, 5, 7.5, 10, 25, 50, 50 and 100nM for M2774, 1, 2.5, 5, 10, 10, 25 and 50 nM for M2775, 0.5, 1, 2.5, 5, 10, 10 and 25nM for M2777, 5, 10, 25, 50, 50, 100, 250, and 500 nM for M2779, 2.5, 5, 10, 25, 50, 50, 100 and 250 nM for M2780, 0.5, 1, 2.5, 5, 10, 10, 25 and 50 nM for M2781, 5, 10, 25, 50, 50, 100, 250, 500, and 1000 nM for M2788, 2.5, 5, 10, 25, 50, 50, 100, 250, 500 and 1000 nM for M2790, 1, 2.5, 5, 10, 10, 25, 50 and 100 nM for M2792, 1, 2.5, 5, 7.5, 10, 10, 25, 50 and 100nM for M2795, 2.5, 5, 10, 10, 25, 50 and 100 nM for M2797, 1, 2.5, 5, 7.5, 10, 10, 25, 50 and 100 nM for M2800. Black lines represent raw data measurements and red lines represent fitted curve.



from 4.5 nM to 100 nM (Table 4-2). Five Fab fragments, derived from M2773, M2775, M2781, M2792 and M2797, all had particularly high affinities (Figure 4-9). The theoretical R_{max} for IspC is 592 RU based on the formula $R_{max} \cdot (MW_{analyte}/MW_{ligand}) \times \text{immobilize ligand amount (RU)} \times \text{stoichiometric ratio}$. The data showed good fitting to the 1:1 Langmuir model, except for M2788, M2795 and M2779 which, based on RU (Table 4-2), likely interact with more than one site on IspC.

Table 4-2. Association rate, dissociation rate and equilibrium affinity constants for each monoclonal antibody

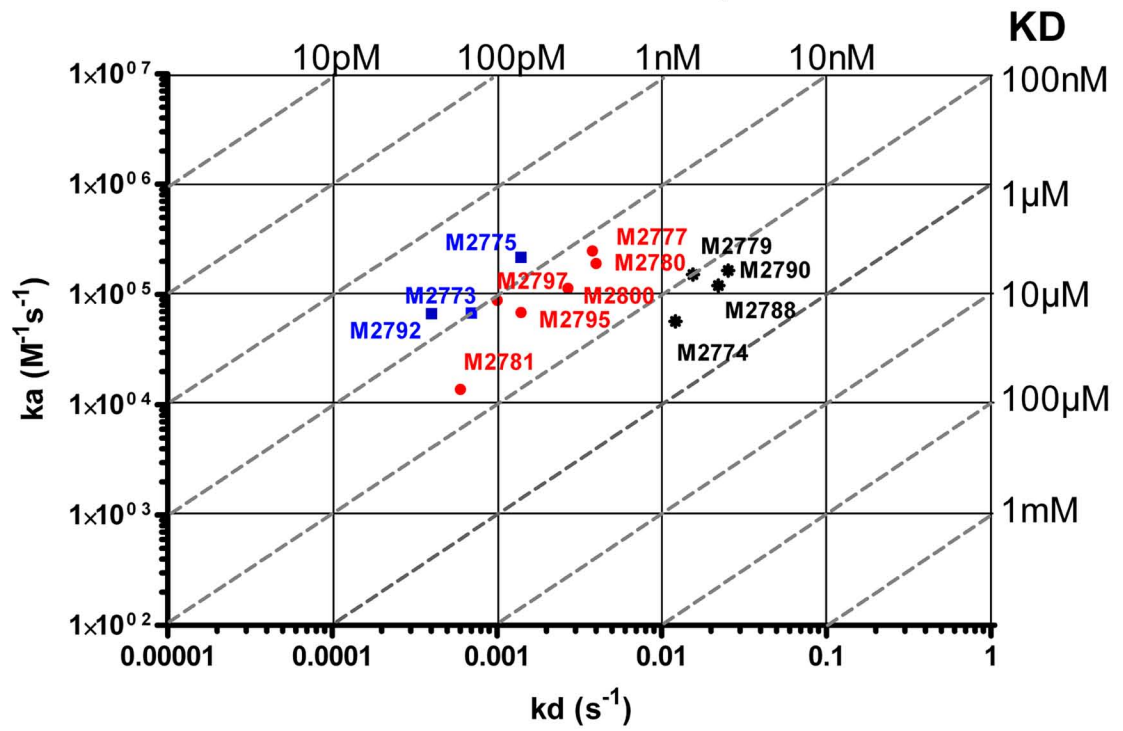
Monoclonal Antibody	k_a (1/Ms)	k_d (1/s)	K_D (M)	Resonance units (RU)
M2773	5.72×10^4	0.0007	1.2×10^{-8}	506
M2774	1.16×10^5	0.0121	1.1×10^{-7}	508
M2775	2.18×10^5	0.0014	6.4×10^{-9}	266
M2777	2.48×10^5	0.0038	1.6×10^{-8}	406
M2779	1.52×10^5	0.0155	1.0×10^{-7}	613
M2780	1.92×10^5	0.004	2.1×10^{-8}	285
M2781	1.38×10^5	0.0006	4.5×10^{-9}	340
M2788	1.21×10^5	0.0221	1.8×10^{-7}	753
M2790	1.65×10^5	0.0253	1.5×10^{-7}	521
M2792	6.70×10^4	0.0004	5.9×10^{-9}	460
M2795	6.87×10^4	0.0014	2.0×10^{-8}	731
M2797	8.91×10^4	0.0010	1.2×10^{-8}	281
M2800	1.14×10^5	0.0027	2.3×10^{-8}	552

4.2.5 Anti-IspC Mab cross-reactivity among the various serotypes of *L. monocytogenes*

Indirect ELISA was performed to assess MAbs for cross-reactivity with different *L. monocytogenes* serotypes (Table 4-3). The cross-reactivity of different isolates with each MAb was calculated as a percent of the OD₄₁₄ reading seen for the interaction between the MAb and *L. monocytogenes* serotype 4b LI0521 isolate. All 15 MAbs reacted very strongly to each of the 7 *L. monocytogenes* serotype 4b isolates tested, with many reactions exceeding 100%. This indicates that IspC is conserved in serotype 4b strains. A cross-reaction was

Figure 4-9. Rate plane plot with iso-affinity diagonals. This kinetic map summarizes the respective association rate, dissociation rate and affinity constants of each Fab to IspC as determined by SPR.

On / Off rate map



defined as any reaction with a non-serotype 4b isolate that exceeds 25% of the OD that would be seen between the particular antibody being tested and *L. monocytogenes* serotype 4b strain LI0521.

The MAbs M2795 and M2799 reacted with 2 and 4 of 9 *L. monocytogenes* serotype 1/2a isolates, respectively (Table 4-3). In addition, M2777, M2781, M2792, M2795 and M2799 were able to detect 6, 1, 1, 5 and 6 of 8 *L. monocytogenes* serotype 1/2b isolates, respectively. The MAbs M2774, M2775, M2780, M2790 and M2797 did not react with any isolates which were not serotype 4b (Table 4-3) these five antibodies were 100% specific for serotype 4b. Interestingly no cross-reactions were observed between any of the 15 Mabs and *L. monocytogenes* serotype 3a, serotype 3b, serotype 3c, or serotype 4d. Detailed cross-reaction profiles for each of the 15 Mabs can be found in Appendix 2 in Figure S9.

Table 4-3. Cross reaction of MAbs with *L. monocytogenes* serotypes

<i>L. monocytogenes</i> serotypes ^a	Number of Isolates Screened	Number of Positive Reactions ^b :														
		M2773	M2774	M2775	M2777 ^c	M2778	M2779	M2780	M2781 ^c	M2788	M2790	M2792 ^c	M2795 ^c	M2797 ^c	M2799 ^c	M2800
1/2a	9	0	0	0	0	0	0	0	0	0	0	0	2	0	4	0
1/2b	8	0	0	0	6	0	0	0	1	0	0	1	5	0	6	0
1/2c	5	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
3a	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3b	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3c	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4a	2	2	0	0	1	1	1	0	2	2	0	2	2	0	2	2
4b	9	9	8	9	9	9	9	9	9	9	8	9	9	9	9	9
4c	4	4	0	0	1	0	1	0	4	2	0	4	4	0	4	4
4d	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4e	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

a – Isolate details can be found in Table 3-1.

b – The interaction between each antibody-isolate pair was examined using ELISA in three independent experiments. Positive reactions were recorded if the average of the three OD measurements was >25% of the OD recorded for the same antibody when reacting *L. monocytogenes* serotype 4b LI0521.

c– Negative reactions are reported if the average OD of the three independent experiments was <25% of the OD recorded for the same antibody when reacting with *L. monocytogenes* serotype 4b LI0521. In most cases a negative reaction could also be defined by OD of all three experiments being below the 25% threshold. However, M2777, M2781, M2792, M2795, M2797 and M2799 each frequently had one or two of three of the measurements above the 25% threshold, even though the average remained below 25%. The variability of each of these MAbs makes them poor candidates for diagnostics. The detailed data from which this Table was constructed can be found in Appendix 3, Figure S10.

A BLASTp search was carried out to find proteins with sequence similar to the IspC protein. Only a few annotated *Listeria* hypothetical proteins in the database showed high homology with IspC. LMIV_0809, a putative protein from *L. monocytogenes* serotype 4a

FSL J1-208, showed 95% homology with IspC. The CBD of IspC also shares 89% identity with that of lin1064 and 90% identity with that of lwe1056, two hypothetical proteins from *L. innocua* and *L. welshimeri*, respectively. Collectively these data indicate that IspC is highly conserved in *L. monocytogenes* serotype 4b and can serve as a novel diagnostic marker for *L. monocytogenes* serotype 4b strains.

4.3 Discussion

This study provides a detailed characterization of fifteen MAbs that each recognizes linear epitopes located within the CBD of IspC. IspC was previously shown to be involved in virulence (276), and this work has shown that it is also well conserved within serotype 4b strains. The anti-IspC MAbs did not consistently react with any of the other *L. monocytogenes* serotypes tested, at the level at which they reacted with serotype 4b strains, providing evidence that IspC is unique to *L. monocytogenes* serotype 4b. This work is novel because it provides the most extensive characterization of MAbs produced against *L. monocytogenes* to date. In addition, the novel MAbs described in this work have the potential to be used as reagents in future research aimed at elucidating the role of IspC in virulence.

The 15 MAbs that recognize IspC were generated by immunizing mice with formalin killed *L. monocytogenes* serotype 4b cells (151). This immunization strategy produced a total of 23 MAbs that interacted with the *L. monocytogenes* cell surface; however, only 16 detected a protein band in a Western blot of total cellular proteins (151). Western blot analysis of rIspC has shown conclusively that this fusion produced 15 MAbs that recognize rIspC. Thus, immunization with formalin killed whole cells created a group of MAbs which targeted the same protein antigen, despite other potential antigens being readily available. An explanation may be that IspC is an immunodominant surface antigen of *L. monocytogenes*

serotype 4b. This interpretation is supported by a previous finding that IspC is a primary target of the humoral immune response to *L. monocytogenes* serotype 4b infection (284). Previous studies aiming to develop MAbs as diagnostic reagents have generally not identified the molecular identity of antigens being targeted by MAbs; and it is unclear if any were also against IspC. An earlier study that used *L. monocytogenes* whole cell lysate as an immunogen generated only 2 MAbs which recognized cell surface localized antigens, although, a total of 35 MAbs were produced (152). The 2 MAbs that recognized surface antigens did not detect protein bands in Western blots of total cellular proteins, indicating that the MAbs either recognised conformational epitopes or that the target antigen was non-protein in nature (152). Another study that used formalin killed whole cells for immunization generated MAbs that recognized cell surface antigens and were specific for *L. monocytogenes* serotype 4b (122). However, inconsistent Western blot results led to failure to provide an estimate of the MW of the antigens (122). Accurate predictions as to whether the MAbs from either of these studies also recognize IspC are impossible.

Epitope localization experiments demonstrated that all the MAbs investigated in this study recognize sequences within the C-terminal CBD of the IspC protein. IspC has a modular domain structure with an N-terminal signal sequence (AA 1-45), a peptidoglycan (PG) hydrolase domain (AA 46-197), and a C-terminal CBD (AA198-774) (Figure 4-4) (275). N-terminal sequencing showed that IspC contains a 45 AA N-terminal signal peptide which is cleaved prior to cell wall targeting. This is in contrast to earlier findings that IspC has an N-terminal signal sequence of 23 AA when produced in *E. coli* (277). The differences in signal peptide processing between *L. monocytogenes* and *E. coli* are not surprising, since the signal sequence of secreted proteins are generally longer in Gram-positive than in Gram-

negative bacteria (235). The CBD of IspC is composed of 7 tandem repeats called GW modules which are responsible for anchoring the protein to the cell wall through non-covalent interactions with lipoteichoic acids (LTA) (114). Previous work has demonstrated that the anti-IspC MAbs interact with the cell surface of live cells (151). These findings support a model where GW modules contained in the CBD are exposed at the cell-surface and not embedded in the PG. The lack of production of a MAb against the PG hydrolase domain provides the first evidence that this portion of the protein is hidden by the PG. Analysis of the IspC amino acid sequence provides additional support for this model, since the probability of cell-surface exposure is very low for the PG hydrolase domain but quite high for the CBD based on an Emini plot (76). Additional studies also suggest that the GW modules are surface-exposed. Antibodies produced specifically against GW modules are protective against *Erysipelothrix rhusiopathiae* (158). For antibody protection, GW modules would have to be cell-surface exposed and accessible to the antibodies.

The repetitive nature of the CBD complicated the interpretation of the epitope localization experiments in this study, since the homology of the GW modules made it possible for the MAbs to have multiple epitopes within the same protein (Figure 4-9). The RU values reported for M2779, M2788, and M2795 exceeded the theoretical R_{max} indicating multiple epitopes for these 3 MAbs. The high RU observed for other MAbs may also indicate multiple epitopes. Epitope localization experiments provide further evidence that some MAbs in this latter group react with more than one epitope.

Low affinity or avidity has been cited as a reason that antibody-based protocols, such as immunomagnetic separation or flow cytometry, fail to detect *L. monocytogenes* cells (118). A full kinetic analysis of the interaction between each MAb and IspC to select the MAbs

Figure 4-10. Alignment of GW-modules. The CBD of IspC contains 7 GW modules, named because each starts with a glycine (G) followed by a tryptophan (W). Each of the sequential GW modules has significant homology. Matching residues are shaded.

suitable for future diagnostic purposes was carried out. High affinity is a requirement of diagnostic tests, such as immunomagnetic separation or flow cytometry (186). The affinities of the MAbs from this study, in the nanomolar range, are within the biologically useful range for current diagnostic tests such as ELISA and M2773, M2775, M2781, M2792 and M2797 have a high affinity compared to other MAbs produced using similar methodology (148, 173, 190). In a clinical setting, high-affinity MAbs have a greater neutralizing potential than low-affinity MAbs during passive immunization (260). Future work with these selected MAbs against *L. monocytogenes* serotype 4b will concentrate on the ability of these MAbs to detect this important serotype using novel diagnostic platforms currently under development by our group.

Certain anti-IspC MAbs also have a high specificity for *L. monocytogenes* serotype 4b, particularly M2774, M2775, M2780, M2790 and M2797, which showed the most fidelity to *L. monocytogenes* serotype 4b and reacted with only 2 of the 41 tested non-serotype 4b isolates. Our detailed analysis of the cross-reactivity between anti-IspC MAbs produced against *L. monocytogenes* serotype 4b and other closely related isolates indicated that IspC is unique to the 4b serotype. BLAST analysis supports this finding, although, a highly homologous protein was found in *L. monocytogenes* serotype 4a, which may explain why there was a great deal of cross-reaction between some of the MAbs and this serotype. The CBD of IspC also shares 36 and 31% identity with the GW modules of Ami 4b (171) and InlB (114) respectively. Ami 4b is also unique to *L. monocytogenes* serotype 4b. Cross-reactions of the MAbs with Ami 4b would not be detected by ELISA. The observed homology with InlB conflicts with the low cross-reaction observed between anti-IspC MAbs and other *L. monocytogenes* serotypes given that InlB is conserved between serotypes. Low cross-

reactions between anti-IspC MAbs and InlB containing serotypes could indicate that homology is not considerable enough, the epitope is not surface exposed or that InlB is expressed poorly when cells are grown in BHI broth. GW modules have been suggested for inclusion in vaccine preparations since the homology among GW modules from different organisms would allow for cross-protection against related pathogens (194). In contrast, the MAbs against the CBD of IspC containing GW modules show little cross-reaction with related isolates, therefore MAb interaction is likely limited to the IspC protein.

Attempts have been made to generate *L. monocytogenes* specific antibodies by raising MAbs against the main virulence factors (InlA, InlB, LLO, ActA), however, most of these MAbs have failed in diagnostics since the targets are not expressed well during *in vitro* growth conditions (16). This study indicates that IspC is an excellent marker for *L. monocytogenes* serotype 4b and presents several high-affinity MAbs which could be used for bacterial detection and isolation. The dynamics of IspC expression during growth in various *in vitro* conditions is outlined in the next chapter. In addition, these MAbs have provided some interesting insights into the biochemistry of the IspC protein. We have proposed a model where most of the IspC CBD is cell-surface exposed, based on the observed interaction between anti-IspC CBD MAbs and the cell surface. These MAbs will continue to be useful in additional experiments aimed at probing the properties of the IspC protein.

Chapter V

Expression characteristics of IspC during growth under stress conditions

Some of the data in this chapter has been published in:

Ronholm, J., Cao, X., and Lin, M. (2012). Unveiling the expression characteristics of IspC, a cell wall-associated peptidoglycan hydrolase in *Listeria monocytogenes* during growth under stress conditions. *Applied and Environmental Microbiology*. 78(22): 7833-7840.

5.1 Introduction

In the environment, *L. monocytogenes* is extremely hardy and actively divides between 3 and 45°C (273), in up to 10% NaCl (163), at a pH of between 4.4 to 9.2 (94), and under anaerobic conditions (156). The ability of *L. monocytogenes* to grow in extreme environments makes it a particular concern for the food industry in food-processing plants where ready-to-eat foods are prepared. The MAbs which react specifically with *L. monocytogenes* serotype 4b were described in the previous chapter and shown to recognize a surface localized autolysin IspC (homologous to *LMOF2365_1093*). These MAbs, together with IspC as a surface maker, have the potential for use in diagnostic tests for *L. monocytogenes* serotype 4b strains. However, the surface expression of IspC at levels allowing these specific antibodies to bind *L. monocytogenes* cells originating from various growth condition is critical to the success in culture-independent antibody-based detection methods and remains to be assessed. Surface protein expression is unstable and generally dependent upon growth conditions (92, 93, 112, 178, 179). The variability of surface epitope expression has also been previously shown to limit the usefulness of antibodies in *L. monocytogenes* detection (178). Therefore, the objective of this work is to characterize the surface expression of IspC during growth under various physical and chemical stresses to determine if IspC is a useful marker for the development of antibody-based detection methods.

5.2 Results

5.2.1 Transcriptional start site (TSS) of IspC

The sequence upstream of the *ispC* open reading frame (ORF) was examined for the presence of regulatory elements. It should be noted that an ORF (*LMOF2365_1092*) for a putative teichoic acid ABC transporter, transcribed in the same direction as the *ispC* gene ends 224 bp upstream of the *ispC* translation start codon. Sequence analysis with a prokaryotic promoter prediction algorithm (www.fruitfly.org) revealed elements of two putative promoters and corresponding predicted TSSs with a perfect score of 1.0 within a region of 400 bp upstream of the *ispC* ORF. To determine if either of these putative promoters, or an alternative promoter within this region was active, a 473 bp DNA fragment immediately upstream of the *ispC* translation start codon was cloned into the pTCV-lac plasmid (207) containing a promoterless *lacZ* reporter gene, making pTCV-*PispC*. Approximately 40 Miller units of β -galactosidase activity were detected at mid-log phase (350 min) from *L. monocytogenes* serotype 4b strain LI0521 that had been transformed with pTCV-*PispC*. This indicated that a functional *ispC* promoter exists within the 473 bp region immediately upstream of the *ispC* ORF.

To define the *ispC* promoter more precisely, we mapped the TSS using 5' RACE (Figure 5-1) to be an adenine nucleotide 31 bp away from the *ispC* translation start codon (Figure 5-2). Surprisingly, this was not one of the two TSSs predicted for two putative promoters having a perfect score of 1.0. However, within this functional promoter region, another promoter was predicted with a score of 0.73, containing a predicted TSS one nucleotide away from the experimentally determined TSS (Figure 5-2). From this TSS, there are -10 (TGGTAAAAT) and -35 (TTGTTA) regions, as predicted by a bacterial promoter program BPROM (<http://linux1.softberry.com>). This promoter has a 19-bp space between the -10 element and the -35 element and is involved in transcribing *ispC* (Figure 5-2)

Figure 5-1. 5'RACE. The 5' RACE procedure was carried out to identify the transcription start site for the *ispC* gene using a 5'/3' RACE kit (Roche) A) according to the manufacturer's instructions with *ispC* gene specific primers. B) Total RNA was purified from mid-log *L. monocytogenes* cells and the purity and quality was checked using agarose gel electrophoresis. A reverse transcriptase reaction was performed on the purified RNA to create cDNA using GSPC. Poly A tailing of the purified cDNA strand was accomplished through incubating the cDNA with terminal transferase, supplied with the kit. PCR amplification of the cDNA took place using GSP1 and the dT-anchor primer provided in the 5' RACE kit. The product of this primary PCR reaction was used as a template for the secondary PCR GSP 2 and the PCR anchor primer provided in the 5' RACE kit. The band shown in C) is the product after the secondary PCR which was calculated to be approximately 685 bp. The product from the secondary PCR was used un-purified for a second nested PCR GSP3 which is shown in D) and is approximately 728 bp. This 728 bp product was excised from the gel and purified and sent for sequencing using GSP 3.

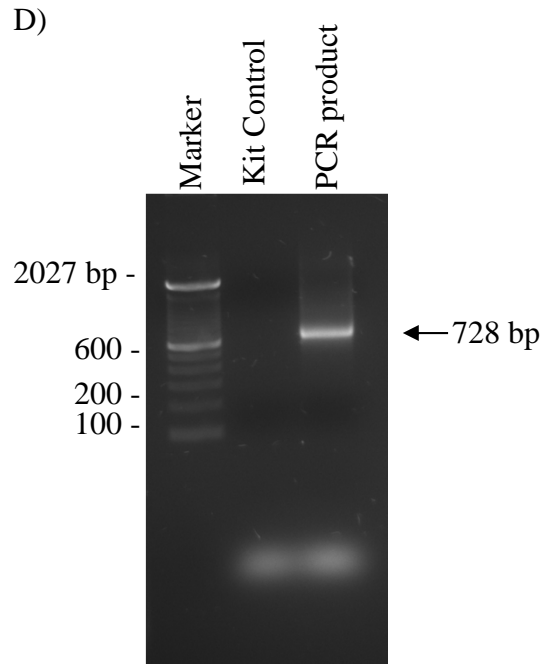
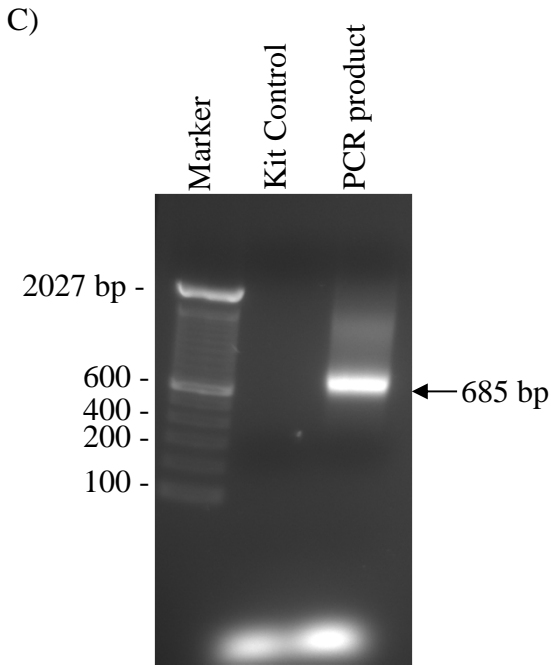
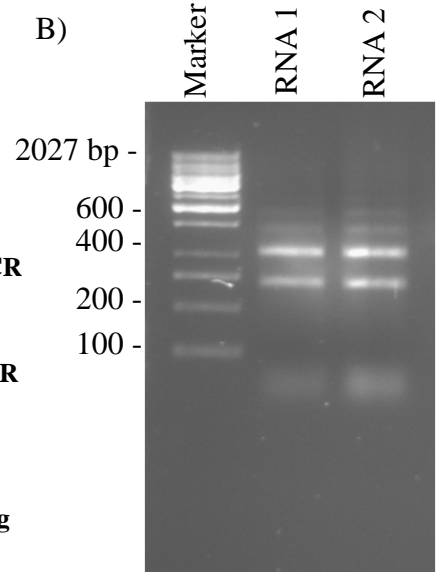
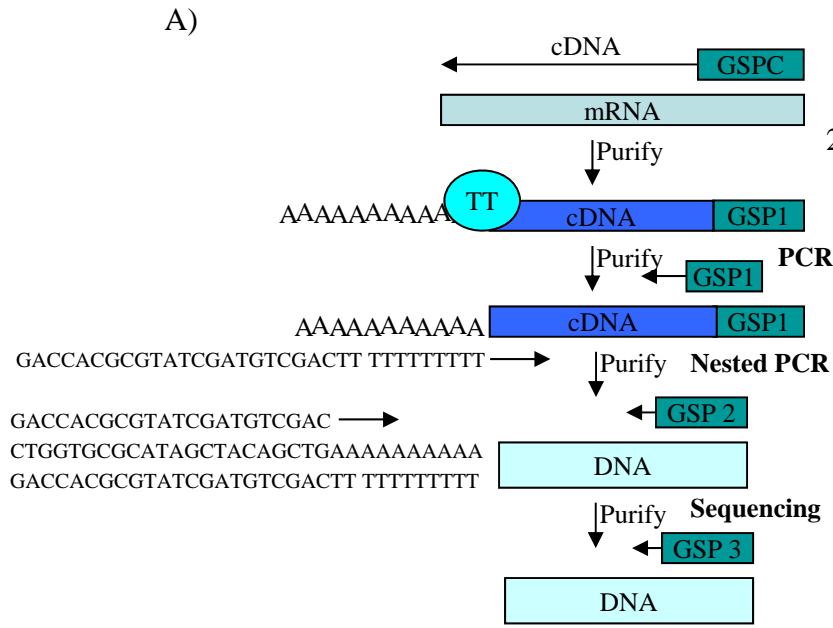
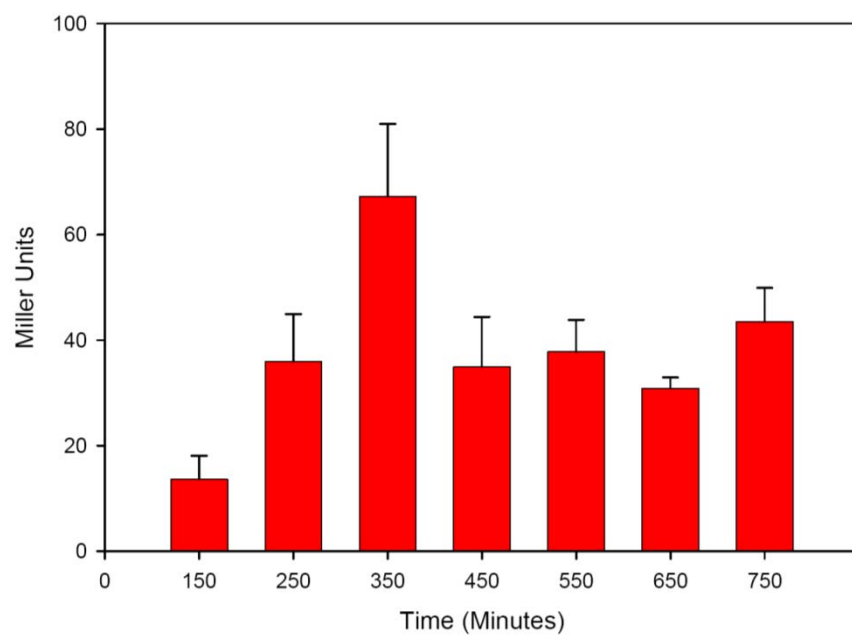


Figure 5-2. Transcription start site localization by 5' RACE. The amplicons from the 5' RACE experiments were sequenced using the GSP 3 primer. A) Shows sequencing reactions from the first trial. To insure that this was indeed the transcription start site and that there was no RNA degradation, RNA was re-purified and the experiment was repeated. B) Shows the sequencing reactions from the second trial, which agreed perfectly with the first trial. C) Shows the genetic sequence up-stream of the IspC ORF, including the transcription start site identified by 5' RACE. The -10 and -35 regions are shown in italics.

Figure 5-3. Transcription from the *ispC* promoter in BHI broth. Transcription was measured from the *IspC* promoter using a reporter gene assay. The predicted promoter region of *IspC* was cloned into the pTCV-lac plasmid up-stream of a promoterless β -galactosidase gene. Expression was measured in Miller units using a β -galactosidase assay. The ability to measure β -galactosidase in this assay strongly indicates that the promoter for *IspC* lies within the 473 bp region cloned into the plasmid. Also the peak in *IspC* expression at 350 minutes post-inoculation indicates that *IspC* is expressed at the highest level during exponential growth phase.



Examination of the sequence in this functional promoter region did not identify consensus sequence elements typical for a sigma B transcription factor (-35 GTTT and -10 GGGnAn) (123) in *L. monocytogenes* or for a PrfA box (TTAACAnnTGTTAA) (264), the binding site of the positive transcriptional activator PrfA. This indicates that IspC may be under the control of previously unidentified transcriptional regulators.

5.2.2 Effect of temperature on *ispC* expression

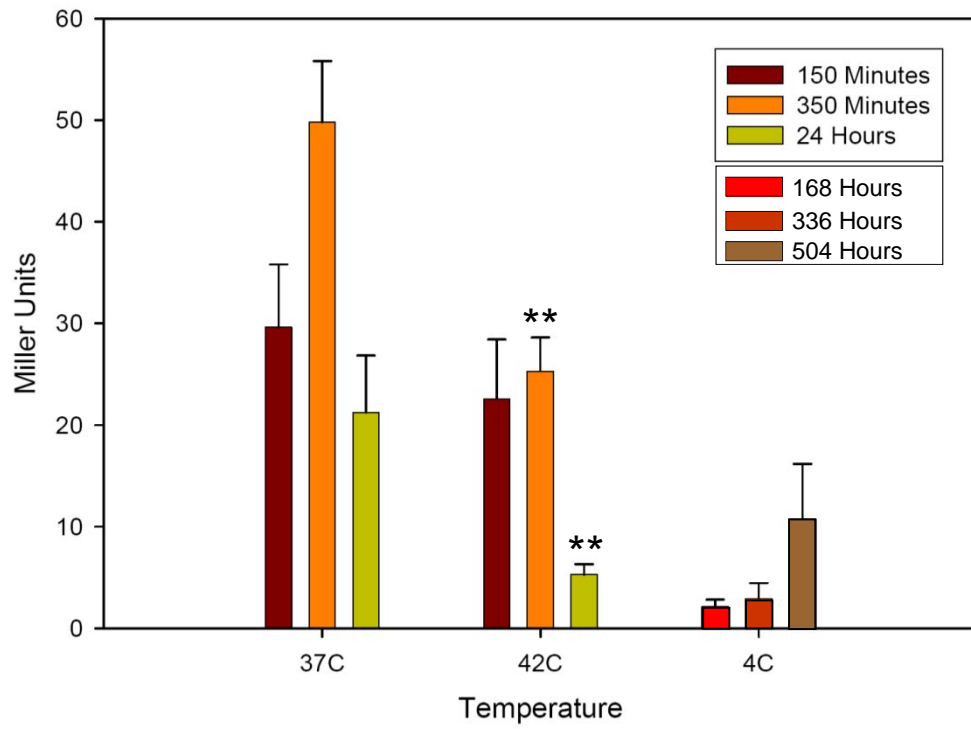
The *ispC* expression in response to growth temperature (37, 42, and 4°C) was investigated by assessing the β -galactosidase activity under the control of the *ispC* gene promoter with *L. monocytogenes* transformed with pCTV-*PispC* (Figure 5-4). The enzyme activity showed a significant decrease at 350 min and 24 h of growth at 42°C compared to the culture at 37°C ($p < 0.001$) (Figure 5-4A). Because of a long generation time at 4°C, the bacteria were allowed to grow for an extended period in order to obtain sufficient cells for accurate measurement of the β -galactosidase activity. The enzyme activity was substantially reduced when cultured at 4 °C in comparison to growth at 37 °C. These results indicate that the *ispC* gene expression is subjected to regulation by temperature. In spite of a low level of *ispC* gene expression after 24 h of growth at 42°C or at 504 h at 4°C (Figure 5-4A), the IspC protein was clearly detectable on the surface by immunofluorescence microscopy with the anti-IspC MAb, M2773 (Figure 5-4B and C).

5.2.3 Effect of Osmotic stress on *ispC* expression

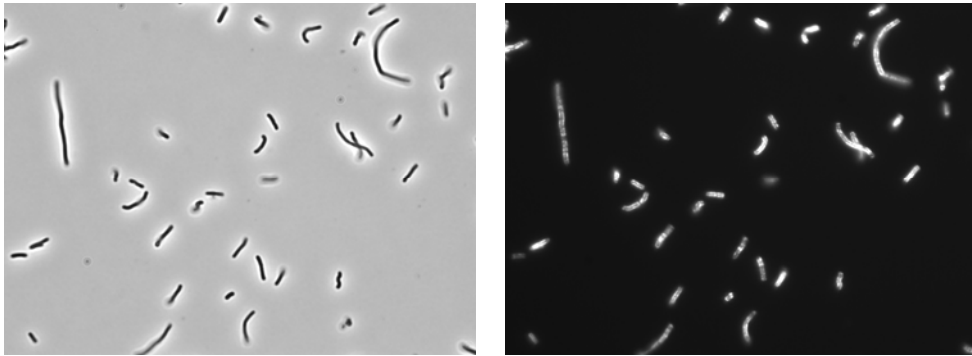
The expression of *ispC* in response to growth in high salt conditions (1-10% NaCl) was similarly investigated using the *L. monocytogenes* [pCTV-*PispC*] cells. After 150 min of growth, cells grown in 4 and 5% (w/v) NaCl had statistically significant reductions in enzyme activity ($p < 0.001$), compared to cells grown in BHI broth without additional salt.

Figure 5-4. Temperature dependent regulation of *ispC* expression. *ispC* promoter activity was measured using a *lacZ* reporter gene assay. A) After 150 min of growth at 42°C, there was no change in promoter activity when compared to growth at 37°C at the same time point. However, after 350 min and 24 hrs of growth at 42°C *ispC* gene expression was significantly less ($p < 0.001$) compared to cells grown for the same amount of time at 37°C. *ispC* expression in cells grown at 4°C was shown to be very low. Statistically significant changes ($p < 0.001$), in this and other figures in this chapter are indicated by (**). In all Figures in this chapter, bars indicate the median value of all observations and the error bars are used to indicate the interquartile deviation. Cells grown for 24 hrs at B) 42°C and cells grown for 504 hrs at C) 4°C were still detectable by an anti-IspC MAb by immunofluorescence. Phase contrast images are shown to illustrate the presence of *L. monocytogenes* cells.

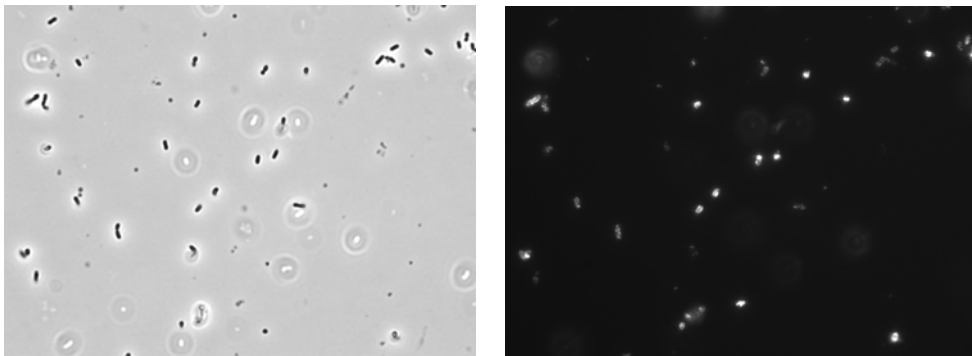
A



B



C



Cultures grown in the presence of 6-10% (w/v) NaCl did not show detectable levels of β -galactosidase activity (Figure 5-5A). Cells grown for 350 min in 5 -10% (w/v) NaCl had significantly reduced *ispC* expression ($p < 0.001$). After 24 hr of growth, cultures supplemented with 3 - 10% (w/v) NaCl had significantly reduced enzyme activity ($p < 0.001$) (Figure 5-5A). This indicated that the *ispC* gene expression is subjected to regulation by osmotic stress. Despite significantly decreased *ispC* promoter activity in osmotically stressed cells, the IspC protein was detectable on the surface of cells after 24 hr of growth in BHI containing various salt concentrations by immunofluorescence microscopy with M2773; the fluorescence signal decreased in a NaCl concentration dependent manner (Figure 5-5 B-K). Cell morphological changes, such as chaining, started to occur in bacteria grown at 5% (w/v) NaCl and became more prominent as the salt concentration increased. Chained cells were still able to express the surface IspC, detectable by immunofluorescence microscopy with M2773.

5.2.4 Culture pH has a minimal effect on *ispC* expression.

L. monocytogenes has been reported to grow in the pH range of 4.4-9.2 (94). However, our preliminary experiments indicated cellular division does not occur below pH 6 or above pH 10. Thus, the activity of β -galactosidase was investigated in *L. monocytogenes* [pTCV-*PispC*] only within the pH range 6-10. At the 150-min time point, the activity of the enzyme expressed under the control of the IspC promoter was equal in each culture. However, cultures at pH 6 and pH 10 had significantly lower enzyme activity than the positive control (pH 7.6) ($p < 0.001$) at the 350-min time point (Figure 5-6A). At 24 hr after inoculation, there was no difference in enzyme activity between cultures at different pH (Figure 5-6A). It follows that after 24 h of growth, immunofluorescence microscopy with

Figure 5-5. Sodium chloride dependent down-regulation of *ispC* expression. A) *ispC* promoter activity was measured in Miller units at 1-10% sodium chloride and concentration dependent activity suppression was observed at all time points, though significance (**) varied. Although cellular growth and division was also suppressed by osmotic stress, the IspC protein was detectable on the cell surface by immunofluorescence in BHI supplemented with B) 1%, C) 2%, D) 3%, E) 4%, F) 5%, G) 6%, H) 7%, I) 8%, J) 9% and K) 10% (w/v) additional sodium chloride. Phase contrast images are also shown to demonstrate the presence and abundance of *L. monocytogenes* cells. Significant cellular chaining was also apparent at 5% sodium chloride.

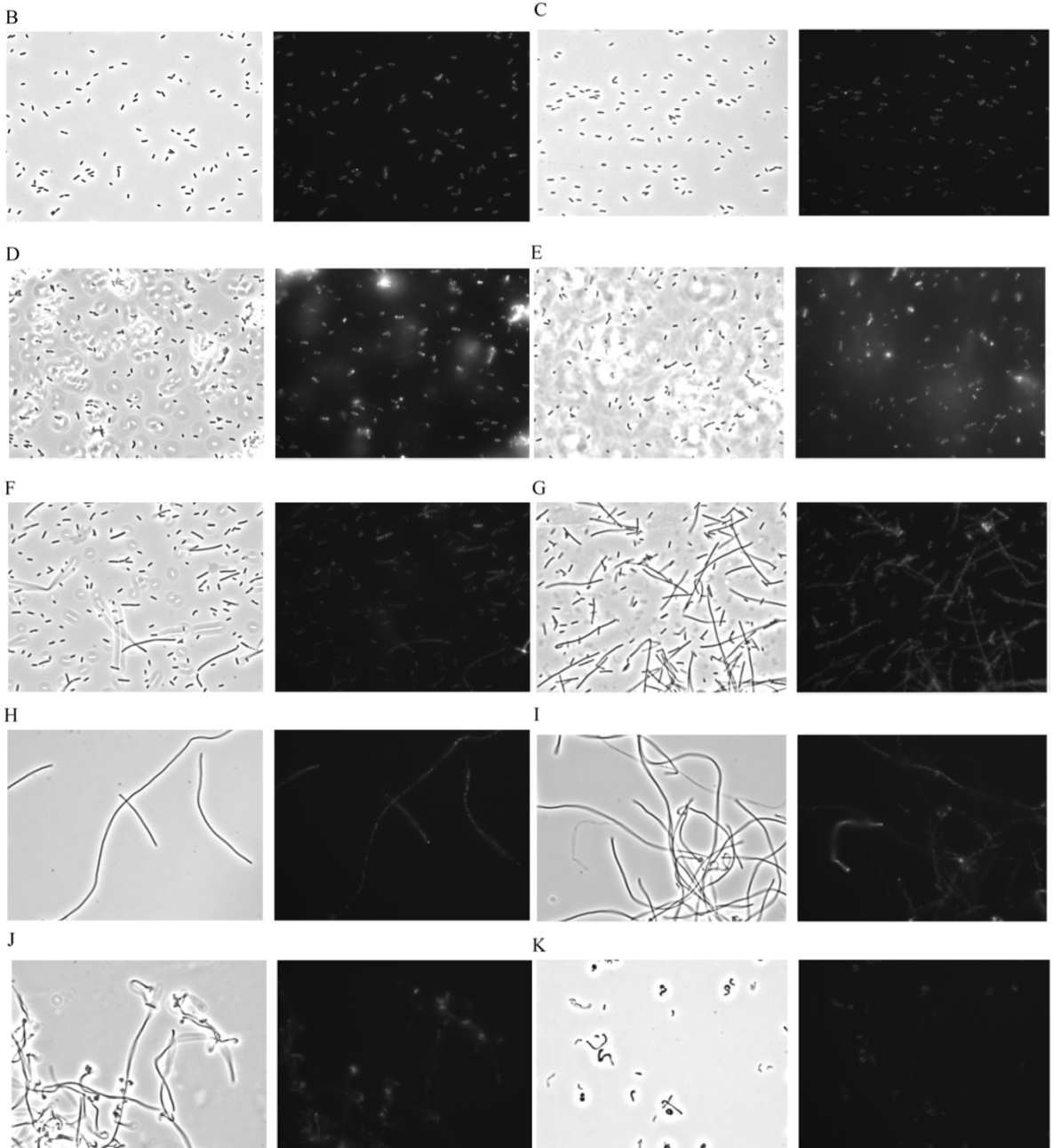
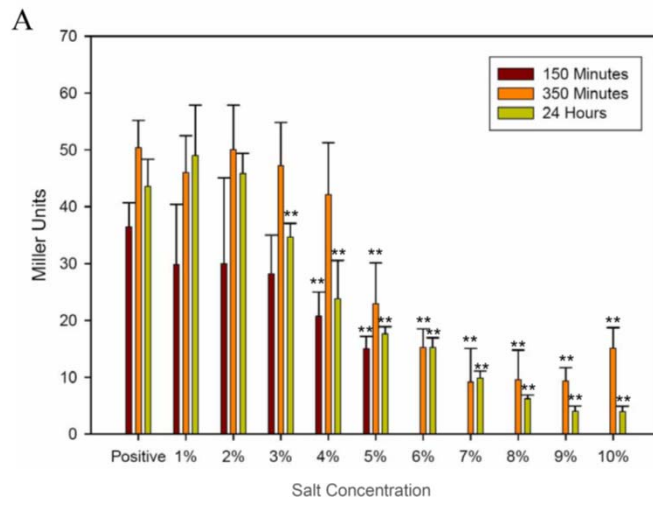
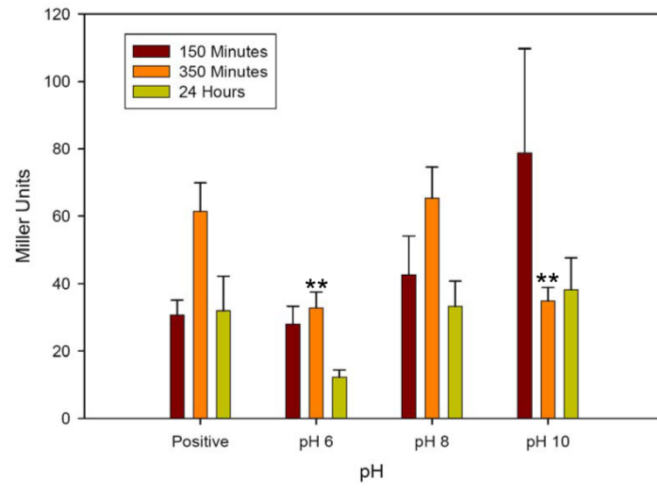
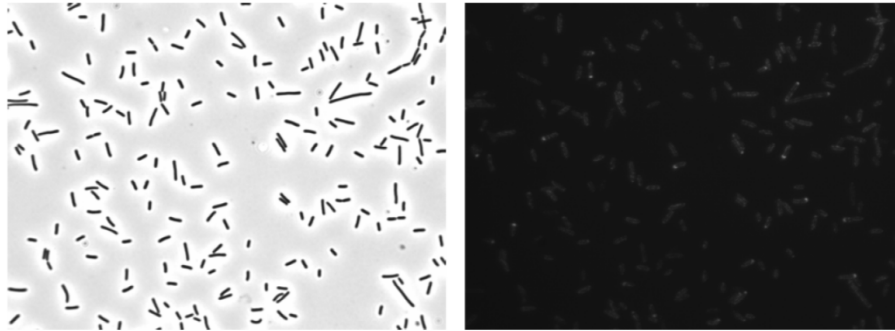


Figure 5-6. Effect of culture pH on *ispC* expression. Cultures with a pH outside of the range pH 6 - pH 10 could not actively divide within the time periods of this experiment, therefore only pH values of 6-10 were tested. A) Within this range, *ispC* expression was relatively constant, with statistically different ($p < 0.001$) expression only occurring at 350 min post-inoculation at pH 6 and p10. At 24 hrs of growth there was no significant difference in *ispC* expression between any of the cultures. IspC was also detectable on the cell surface using immunofluorescence microscopy at B) pH 6, C) pH 8, and D) pH 10.

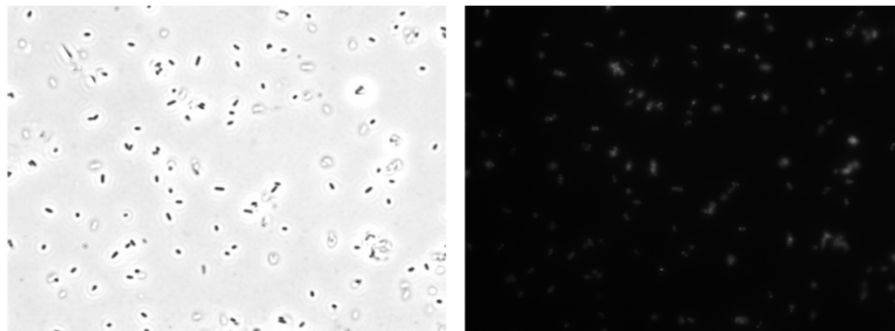
A



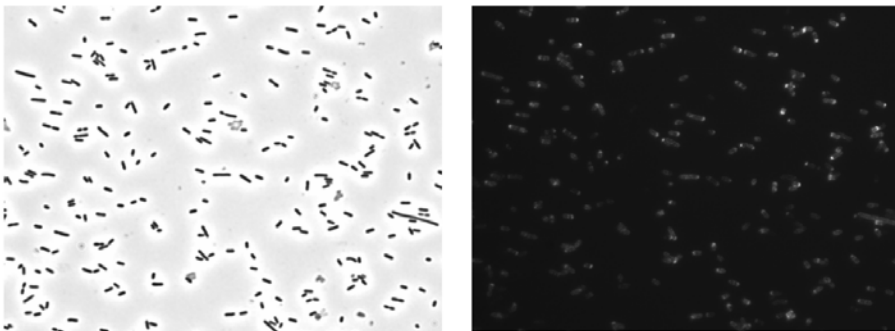
B



C



D



M2773 was able to detect the IspC protein on the cell surface under each condition, although qualitative analysis with immunofluorescence microscopy indicated that growth in alkaline conditions may make cells easier to detect (Figure 5-6B-D).

5.2.5 Effect of sub-lethal ethanol on *ispC* expression

β -galactosidase activity was assessed in *L.monocytogenes* [pTCV-*PispC*] cultivated in BHI containing sub-lethal ethanol concentrations (2%, 5%, and 10%, v/v). Ethanol at a concentration of 10% (v/v) was inhibitory to cell division and therefore this condition was eliminated from the study. Although, the generation time increased for bacterial cells in the presence of ethanol, the level of *ispC* expression was not significantly affected ($p>0.001$) by ethanol concentration used at any of the time points (Figure 5-7A) Therefore, expression of the *ispC* gene is not regulated by exposure to sub-lethal ethanol and cells grown in 2% and 5% ethanol were easily detected by immunofluorescence microscopy using the anti-IspC MAb M2773 (Figure 5-7B and C).

5.2.6 Effect of anaerobic growth on *ispC* expression.

L. monocytogenes [pTCV-*PispC*] grown in CHP to provide highly oxidative conditions and in anaerobic conditions was analyzed for β -galactosidase activity (Figure 5-8). Previous studies have shown that *L. monocytogenes* can survive in 13.8 mM CHP (80); however, our preliminary experiments indicated that *L. monocytogenes* cannot actively divide in CHP concentrations as low as 7 mM; therefore, this condition was eliminated from the study. Similar levels of enzyme activity were observed at 150- and 350 min time points for cells cultures under both aerobic and anaerobic conditions, revealing no effect of these conditions on the expression of *ispC*. However, after 24 h of growth, the *ispC* promoter showed more activity in aerobically than in anaerobic grown cells ($p<0.001$) (Figure 5-8A).

Figure 5-7. Effect of sub-lethal ethanol on *ispC* expression. A) Growth in the presence of 2 or 5% ethanol did not significantly ($p < 0.001$) effect *ispC* expression from that which is observed in the presence of 0% ethanol. IspC is readily detectable on the cell surface of *L. monocytogenes* cells grown in B) 2% and C) 5% ethanol.

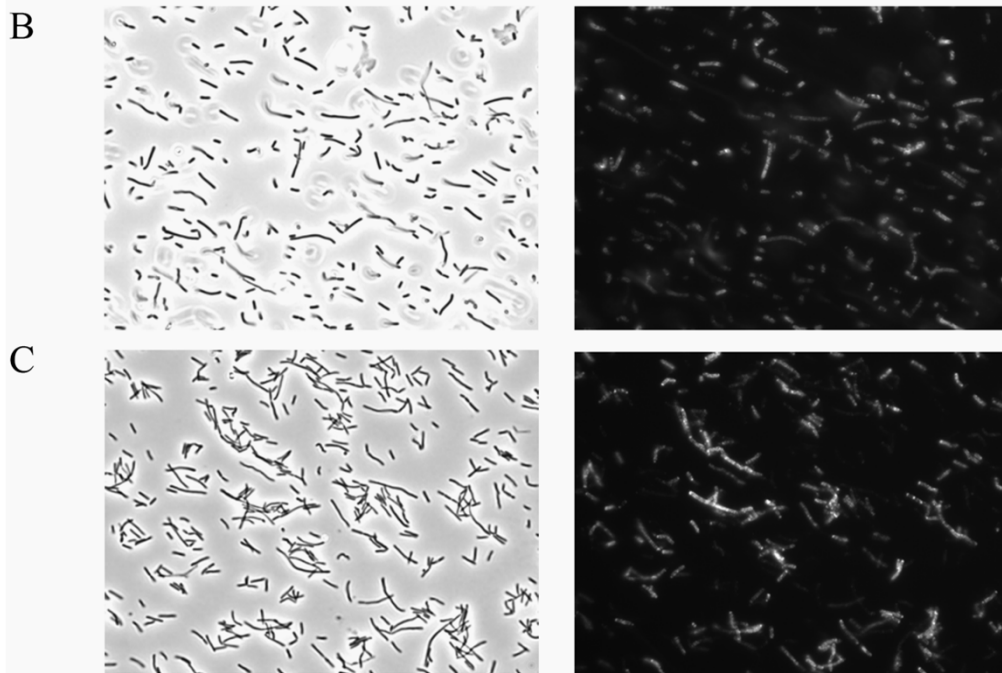
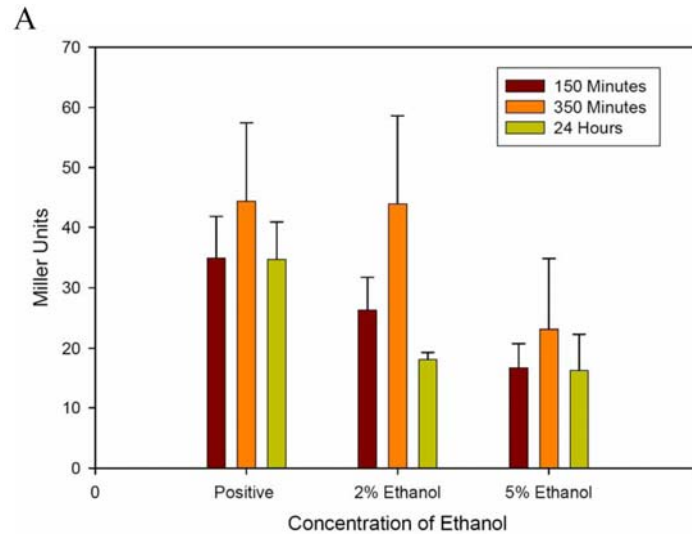
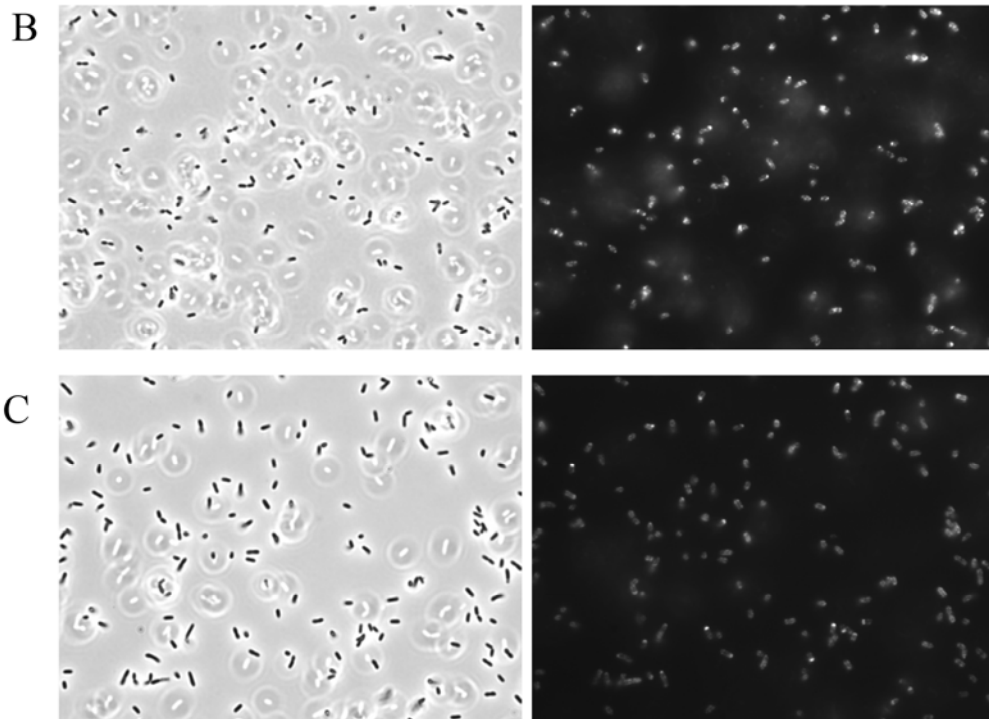
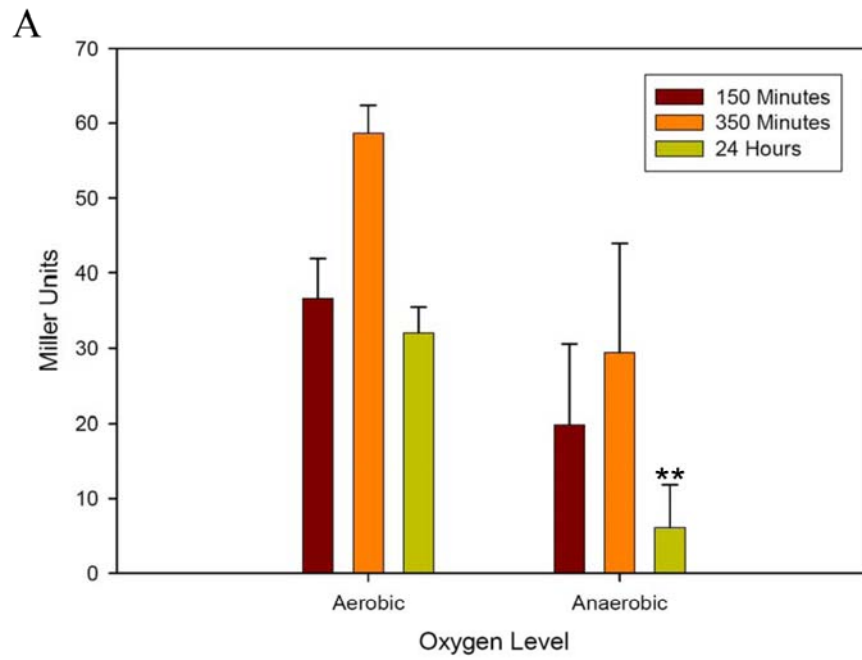


Figure 5-8. *ispC* is expressed in anaerobic conditions. A) *ispC* expression was measured from cells grown in an anaerobic culture and compared to cells grown in an aerobic culture. At 350 min after inoculation there was no difference in *ispC* expression. Although, *ispC* was also expressed at 24 hrs after inoculation, levels of expression in the anaerobic culture were significantly ($p < 0.001$) less in the anaerobic culture. However, IspC was detectable on the cell surface using immunofluorescence 24 hrs after inoculation on both B) aerobically and C) anaerobically grown cells.



Bacterial cells grown under both aerobic and anaerobic conditions expressed the IspC protein and were easily detectable by using immunofluorescence microscopy with the M2773 antibody (Figure 5-8B and C).

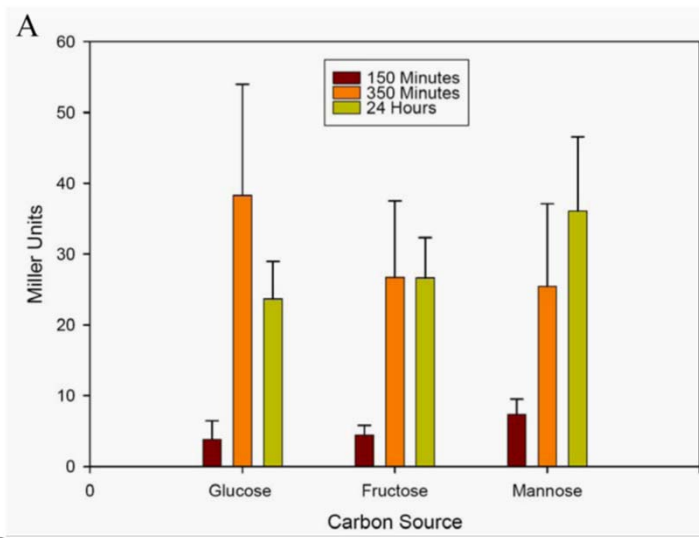
5.2.7 Carbon source does not regulate *ispC* expression.

The expression of the *ispC* gene was examined in response to three carbon sources (glucose, fructose and mannose). Carbon source has been cited as having a major effect on the expression of various proteins in *L. monocytogenes* (167, 208, 280). *L. monocytogenes* [pTCV-*PispC*] showed similar growth and β -galactosidase activity in the minimal media supplemented with each carbon source (Figure 5-9A). Therefore, the *ispC* gene expression is not regulated by carbon source. Cells grown with each carbon source were also easily detected by the presence of the IspC protein by immunofluorescence microscopy (Figure 5-9B-D).

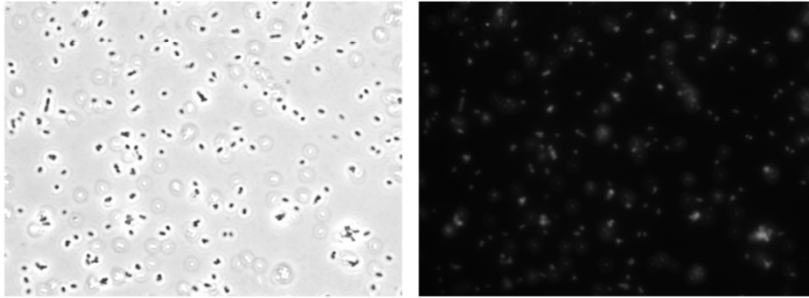
5.2.8 Effect of various *L. monocytogenes* enrichment media on *ispC* expression.

Several types of media are routinely used for the selective enrichment of *L. monocytogenes* in most *Listeria* methods prior to bacterial isolation and detection. To expand the potential of MAbs which recognize IspC beyond direct culture-independent detection into post-enrichment detection, the expression of the *ispC* gene, as assessed by the activity of β -galactosidase transcribed by the *ispC* promoter in *L. monocytogenes* [pTCV-*PispC*], was examined over the span of the growth curve in multiple media including BHI (Figure 5-10A), Fraser (Figure 5-10B), Palcam (Figure 5-10C) and UVM (Figure 5-10D). Although, the levels of β -galactosidase activity varied between media, they were detectable in all the media used. Expression of the *ispC* gene was significantly higher ($p < 0.001$) at all time points when cells were grown in BHI broth than when they are grown in UVM. The expression of the

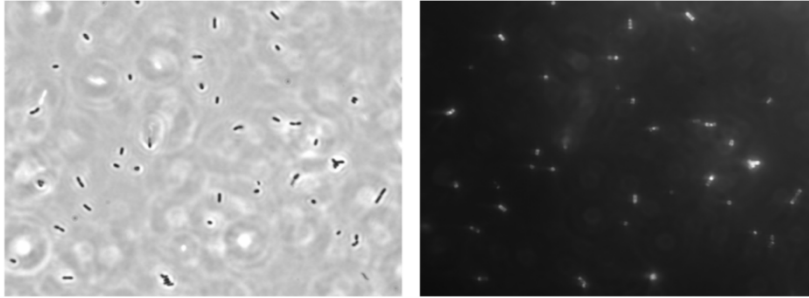
Figure 5-9. Carbon source regulation of *ispC* expression. A) *ispC* was expressed at the same level in minimal media when supplemented with a glucose, fructose or mannose carbon source. *L. monocytogenes* cells are also detectable by an anti-IspC antibody when grown in B) glucose, C) fructose or D) mannose.



B



C



D

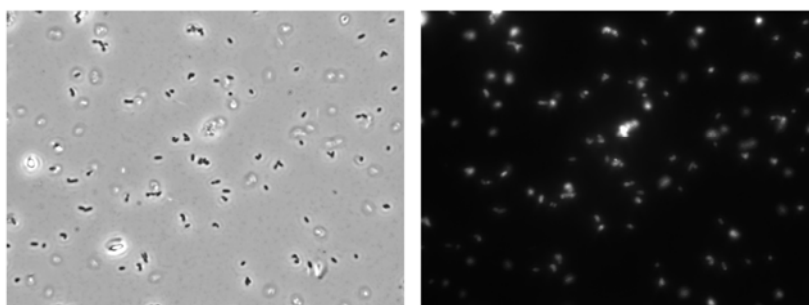
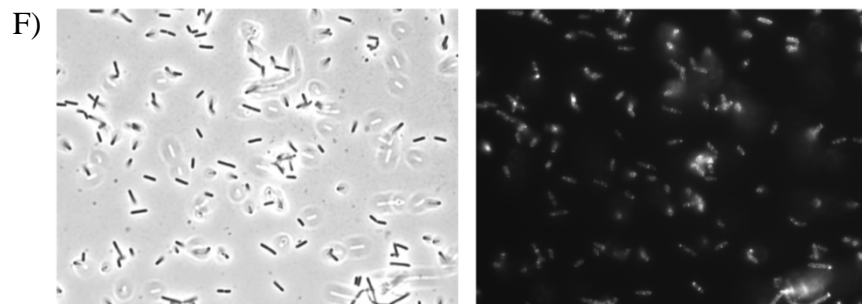
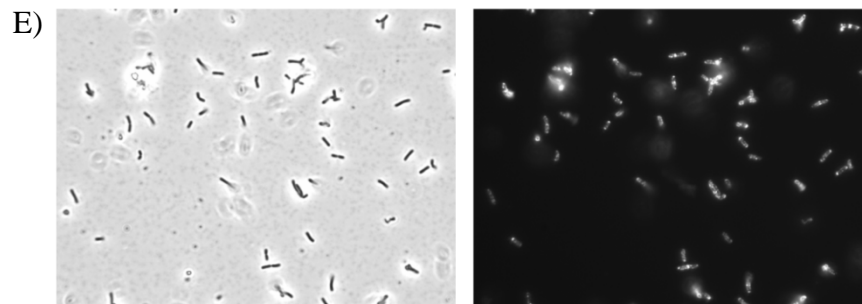
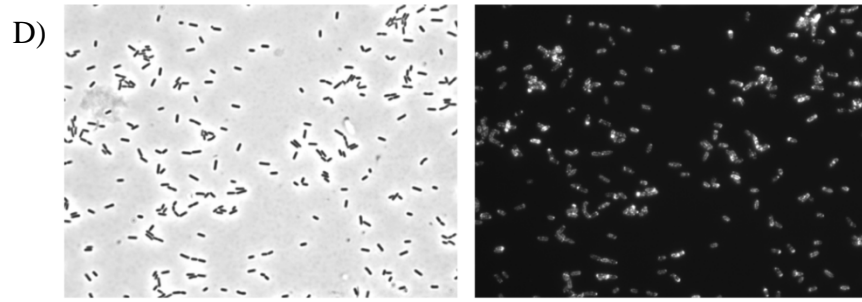
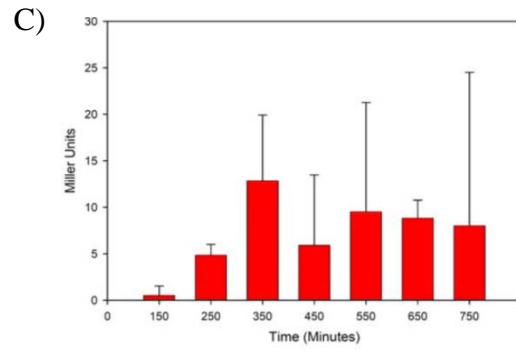
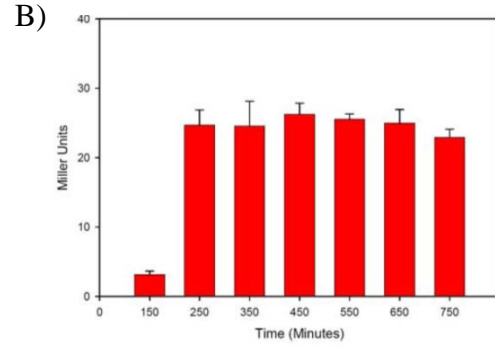
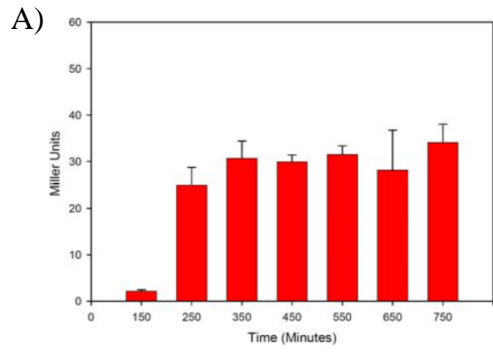


Figure 5-10. *ispC* expression in various enrichment media. Expression from the IspC promoter was measured in Miller units in *L. monocytogenes* cells grown from inoculation to lag phase in A) Fraser broth, B) Palcam broth and C) UVM. A clear peak in promoter activity during mid-log phase could be seen only in BHI broth. The presence of the protein IspC on the surface of cells grown in D) Fraser broth, E) Palcam broth and F) UVM broth were detectable by immunofluorescence microscopy. The phase contrast images of the same field of view are shown for comparison.



ispC gene was the same in cells grown in Fraser and Palcam broths ($p>0.001$), except for at the 750-min time point when the *ispC* gene was expressed more in Fraser broth ($p<0.001$). Expression from the *ispC* promoter was generally higher in cells grown in BHI broth than in cells grown in Fraser and Palcam broths, although the significance varied between time points. IspC was present on the cell surface at high enough levels to allow cells to be easily detected by immunofluorescence microscopy after 24 hr of growth regardless of growth media (Figure 5-10E-G).

5.3 Discussion

This work examined the expression characteristics of IspC, a surface-associated peptidoglycan hydrolase, through the use of the *lacZ* reporter gene system and immunofluorescence microscopy, for the purpose of assessing the value of IspC as a diagnostic marker for *L. monocytogenes* serotype 4b. A functional IspC promoter was active upstream of the *ispC* translation start site. This promoter remained active in *ispC* gene transcription in all environmental conditions tested, which are conducive to bacterial cell division, although the levels of its activity vary. We have further demonstrated that *L. monocytogenes* serotype 4b cells are capable of displaying IspC on the cell surface in each of the tested conditions, making cells detectable, by immunofluorescence microscopy using an anti-IspC MAb. Even in an extreme stress condition, where there was only weak expression of IspC, there was still a sufficient amount of IspC localized on the cell surface to allow detection using the anti-IspC MAb. This indicates that IspC is an excellent diagnostic marker for *L. monocytogenes* serotype 4b and can potentially be used, with the aid of anti-IspC antibodies, for detection or isolation of this important serotype of *L. monocytogenes*.

Transcription of IspC started from a TSS 31 bp upstream of the translation start site under the direction of its own promoter (Figure 5-2). Expression of *ispC* appears to peak, when bacterial cells are grown in BHI broth, during mid-log phase (350 min) (Figure 5-3). This finding correlated well with the results of a previous study which showed (by RT-PCR) that the IspC gene was transcribed at the highest levels during the early log phase (275). The IspC protein was detectable in all growth conditions examined in this study. However, very low levels of expression were observed during growth in BHI broth supplemented with 10% (v/w) NaCl or during growth at 4°C (Figure 5-5).

An anti-IspC MAb was able to detect the target protein on the cell surface by immunofluorescence microscopy, after 24 hr of growth (504 hr of culture at 4°C), regardless of growth conditions. These findings were an important addition to the *ispC* promoter activity analysis which suggested that IspC can serve as a good diagnostic marker because the gene expression is always observed in *L. monocytogenes*. In contrast, other studies conducted to determine which *L. monocytogenes* growth conditions allow for expression of the antigens targeted by various antibodies did not use cells grown entirely under a stress condition (92). In these studies, cells were either grown in a non-stressed condition followed by subculturing for a limited time in the stress condition (92), or in a stress environment and then rescued in an enrichment broth (92). The problem with growing cells for a limited time in the stress condition followed by transfer to enrichment broth is that residual protein from the original culture does not get degraded quickly enough to disappear after the short incubation time and is therefore still detected (J. Ronholm and M. Lin, unpublished observations). Our findings that IspC was detectable with this specific MAb, in all growth conditions tested, makes this protein antigen a novel diagnostic marker for diagnostics for *L.*

monocytogenes serotype 4b. Other researchers have used the methodology similar to the one presented here, and have found that their antigens were not expressed at detectable levels under all tested conditions (93, 135, 136, 178, 179). Since the primary objective of this study was to validate IspC as a marker for diagnostic use in pre-enrichment bacterial detection, possibly in a biosensor or a microfluidics-based flow cytometer, our methodology, where cells were grown entirely in the stress condition and detected by immunofluorescence signal, allowed us to examine IspC expression in cells from selected environments under detection conditions simulating those that would be present in a biosensor applied to the detection of *L. monocytogenes* directly from food or environment samples.

L. monocytogenes was shown to have chained morphology when subjected to extreme osmotic stress (Figure 5-5). This finding was in agreement with other studies (93, 117). Cellular chaining may indicate that the autolysins involved in cell division are either not expressed or not active in these conditions.

An anti-IspC MAb was able to detect *L. monocytogenes* cells grown in each of the selective enrichment broths, although, there was variation in the activity of the *ispC* promoter between broths (Figure 5-9). These experiments were carried out to assess if IspC can be detected by anti-IspC antibodies on the surface of *L. monocytogenes* after enrichment. Selective enrichment broths have been shown to affect the expression of several surface antigens (92). Similar studies, using different MAbs (EM-7G1, C11E9) which also recognize an *Listeria* autolysin (*lmo2691*) in *L. monocytogenes* serotype 1/2a, found that growth in UVM entirely inhibits the expression of the antigen, rendering these antibodies useless for detection when enrichment takes place in this media (92, 112, 178, 179). UVM was also found to reduce the expression of ActA making detection impossible using an anti-ActA

antibody after UVM enrichment (135). In agreement with these findings, we also showed that expression of IspC was most reduced in UVM when compared to the other media. However, despite weak activity of the *ispC* promoter, cells grown in UVM were still detectable by immunofluorescence microscopy with an anti-IspC MAb. This is likely because IspC is a very stable protein (J. Ronholm and M. Lin, unpublished observations) (275), and even minor expression during any growth phase leads to surface protein accumulation. The anti-IspC MAb was also able to detect cells grown in Fraser broth almost as well as cells grown in BHI. The EM-7G1 and C11E9 antibodies were unable to detect cells grown in Fraser broth (92, 178).

IspC is a peptidoglycan hydrolase enzyme with N-acetylglucosaminidase activity (222). It is involved in virulence, as evidenced by the attenuation seen in the *ΔispC* deletion mutant (276). Elucidating the mechanisms behind regulation of virulence associated autolysins is an important step in determining their role in pathogenesis, which appears to be separate from their roles in cellular division or growth (143). We are very interested in the biological significance of an autolysin which appears to be serotype 4b specific and is expressed over a broad range of environmental conditions. IspC is expressed at its highest levels during early-log phase. In addition, it is expressed in all cells which are able to divide; together these observations suggests that IspC has a role in cell division. However, previous work indicates that IspC is not directly involved in cellular division, since IspC knock-out mutants do not have the characteristic chaining phenotype that is seen in cells where essential division molecules have been knocked out (276). One possibility is that IspC has a role in cell division, however, this phenotype is not observed in the deletion mutant, because another autolysin with an overlapping function is able to functionally compensate for the

IspC knock-out. IspC expression is also highly up-regulated during rabbit infection (284), and based on the rate of cell division proportionate to its up-regulation, it is likely that there are requirements for IspC during infection besides those related to growth and division. The role of IspC in infection needs to be studied further.

Foodborne bacteria encounter a variety of environmental stresses which alter surface protein expression. This affects our ability to detect bacteria using antibody-based methods (178). Several studies have demonstrated that the ability of PAbs and MAbs to detect *Listeria* is highly dependent on culture conditions (92, 93, 100, 112, 121, 135, 178, 179, 221, 282, 284). Ideally, next generation rapid detection techniques will allow for specific pathogen detection without the need for enrichment. Developing technologies such as flow cytometry, immunomagnetic separation and biosensors have the potential to eliminate the need for cultural enrichment (70). However, a major hurdle to overcome for such technologies is the need for probes such as antibodies whose ligand is consistently expressed regardless of sample matrix or growth phase.

Our laboratory has produced MAbs which are specific to *L. monocytogenes* serotype 4b and bind to the cell wall-associate IspC with high affinity (151) (Ronholm et al., submitted). However, before these antibodies are used in diagnostics, it is crucial to evaluate the influence of stress on expression of the target protein and determine how this affects antibody reactions (92). We have shown that IspC is expressed in each of our tested conditions and that a MAb to IspC is able to detect bacterial cells in every tested growth environment including extreme stress condition and enrichment media. This supports the claim that IspC, together with its specific MAbs, has value in antibody-based diagnostics for *L. monocytogenes* serotype 4b.

Chapter VI

Biochemical Analysis of IspC Substrate Specificity

Some of the data in this chapter has been published in:

Ronholm, J., Wang, L., Hayashi, I., Sugai, M., Zhang, Z., Cao, X. and Lin, M. (2012) IspC is an *N*-acetylglucosaminidase in *Listeria monocytogenes* serotype 4b. *Glycobiology*. 22(10): 1311-1320.

6.1 Introduction

The N-terminus of IspC contains an active PG hydrolase domain between AA residues 24 and 197 (275). PG hydrolases are categorized according to the chemical bond within the cell wall PG they cleave. Glycan strands can be hydrolyzed by *N*-acetylglucosaminidases, *N*-acetylmuramidases, or lytic transglycosylases. *N*-acetylmuramyl-L-alanine-amidases (amidases) hydrolyze the bond between the MurNAc and the alanine residue of the peptide bridge; endopeptidases and carboxypeptidases degrade specific bonds within the peptide bridge (271) (Figure 2-6). Of the seven active PG hydrolases demonstrated in *L. monocytogenes*: p60, p45, Ami, Ami4b, MurA, Auto and Lmo0327, the catalytic specificity has only been investigated for Auto. Auto, cleaves the glycosidic bond between the GlcNAc and MurNAc at the glucosamine residue and is therefore categorized as an *N*-acetylglucosaminidase (37).

The bond specificity of IspC cannot be accurately predicted based on its sequence homology with other autolysins, since IspC has some degree of identity with various different PG hydrolases. The catalytic domain of IspC (residues 24–197) shares 44.2% identity with the catalytic domain of AmcB, an *N*-acetylglucosaminidase in *L. lactis* (residues 26-194) (110), 30.3% identity with the *N*-acetylmuramyl-L-alanine amidase domain of the bi-functional AtlL autolysin from *Staphylococcus lugdunensis*, (residues 195-798) and 24.6% identity with the *N*-acetylglucosaminidase domain of AtlL (residues 804-1275) (28). Several autolysins have also recently been demonstrated to have substrate specificity differing from that which was predicted based on amino acid sequence homology, including LytG from *B. subtilis* (108), Auto from *L. monocytogenes* (37), and AcMB from *L. lactis* (110). In addition, since IspC is clearly involved in pathogenesis (276) it would be

interesting to know if elucidating its catalytic activity would provide insight into the mechanisms behind its role in infection. Therefore, this work was undertaken to define the catalytic specificity of IspC.

6.2 Results

6.2.1 IspC hydrolyses covalent bonds in the glycosidic backbone

To determine the bond specificity of IspC, purified *L. monocytogenes* PG was digested with rIspC and the resultant soluble muropeptides were isolated by RP-HPLC and individually analyzed by MALDI-TOF MS. RP-HPLC separates muropeptides on the basis of polarity, a technique which provides high resolution for molecules with similar molecular weights (Figure 6-1). MALDI-TOF MS analysis of the muropeptides resolved by RP-HPLC allowed for identification, based on the mass to charge ratio (m/z), of the structure of the muropeptides released by rIspC hydrolysis (Figure 6-2). Where multiple possibilities existed, MALDI-PSD was used to determine a definite structure. The data in Table 6-1 demonstrate that muropeptides generated by IspC digestion all contain disaccharides. This suggests that IspC cleaves a bond within the glycan strands of PG.

To differentiate between the two main types of PG hydrolase activity that cleave the glycan strand, *N*-acetylglucosaminidases and *N*-acetylmuramidases, the sugar alcohol contained in the soluble fraction after digestion of the PG with IspC was analyzed by RP-HPLC. *N*-acetylglucosaminidases, which break the covalent bond between the C1 of the GlcNAc and the C4 of the MurNAc, result in a terminal reducing GlcNAc and the formation of glucosaminitol after reduction with NaBH₄. An *N*-acetylmuramidase breaks the bond between the C1 of the MurNAc and the C4 of the GlcNAc and results in a reducing MurNAc,

Table 6-1. Muropeptides produced from *L. monocytogenes* peptidoglycan digestion by IspC

Muropeptide Identification	Monoisotopic mass		
	Calculated [M+Na] ⁺	Wild-Type	
		Observed <i>m/z</i>	(Peak)
Monomers^a			
Di	721.3	721.0	6
Tri (deAc) (NH ₂)	850.4	850.0	1
Tri (NH ₂)	892.4	892.0	3
Tri	893.4	893.2	4
Tri (Ala, Dap), 2(NH ₂)	1134.5	1134.1, 1134.0	2
Tri (Ac)(NH ₂)	934.4	934.3	8
Tetra (NH ₂)	963.4	962.9	5
Tetra (Ac)	1005.4	1005.3	11
Tri (Ala, Dap, Glu, Ala) 2(NH ₂)	1334.6	1334.9	7
Penta (NH ₂)	1034.4	1034.3	9
Dimers			
Tri-Tri 2(NH ₂)	1741.7	1741.5, 1741.5	12
Tri-Tri 3(NH ₂), (deAc)	1699.7	1699.5	10
Tetra-Tri (deAc), 2(NH ₂)	1772.8	1772.5	13
Tetra-Tri 2(NH ₂)	1814.8	1813.9	14
Tetra-Tri (NH ₂)	1815.8	1815.4	15
Trimers			
Tri-Tri-Tetra (deAc), 2(NH ₂)	2622.2	2622.5, 2622.1	16

^a Abbreviations: Di, disaccharide dipeptide (L-Ala-D-iGln); Tri, disaccharide tripeptide (L-Ala-D-iGln-mesoDap); Tetra, disaccharide tetrapeptide (L-Ala-D-iGln-mesoDap-D-Ala); Penta, disaccharide pentapeptide (L-Ala-D-iGln-mesoDap-D-Ala-D-Ala) Disaccharide refers to one unit of GlcNAc-MurNAc. deAc indicates deacetylation and Ac indicates an extra acetylation in addition to the acetylation of Glc. NH₂ indicates amidation.

Figure 6-1. RP-HPLC chromatogram of muropeptides released by rIspC digestion of PG. Purified PG was digested by rIspC for 96 h, after which insoluble PG was removed by centrifugation. Soluble muropeptides were reduced by treating with sodium borohydride. The reduced muropeptides were loaded into a C18 Hypersil ODS column and eluted using a 200 minute linear gradient switching from buffer A (50 mM sodium phosphate containing 5% methanol, pH 2.5) to buffer B (50 mM sodium phosphate containing 30% methanol, pH 2.5) at a flow rate of 0.5 mL/minute. Muropeptide elution was monitored at 206 nm and peak fractions were collected manually. Labelled peaks correspond to muropeptide structures identified by MALDI-TOF MS and documented in Table 6-1, and representative examples of MS spectra are shown in Figure 6-2. Numbers labelled in red indicate de-*N*-acetylated glucosamine residues while black labelling indicates fully acetylated PG.

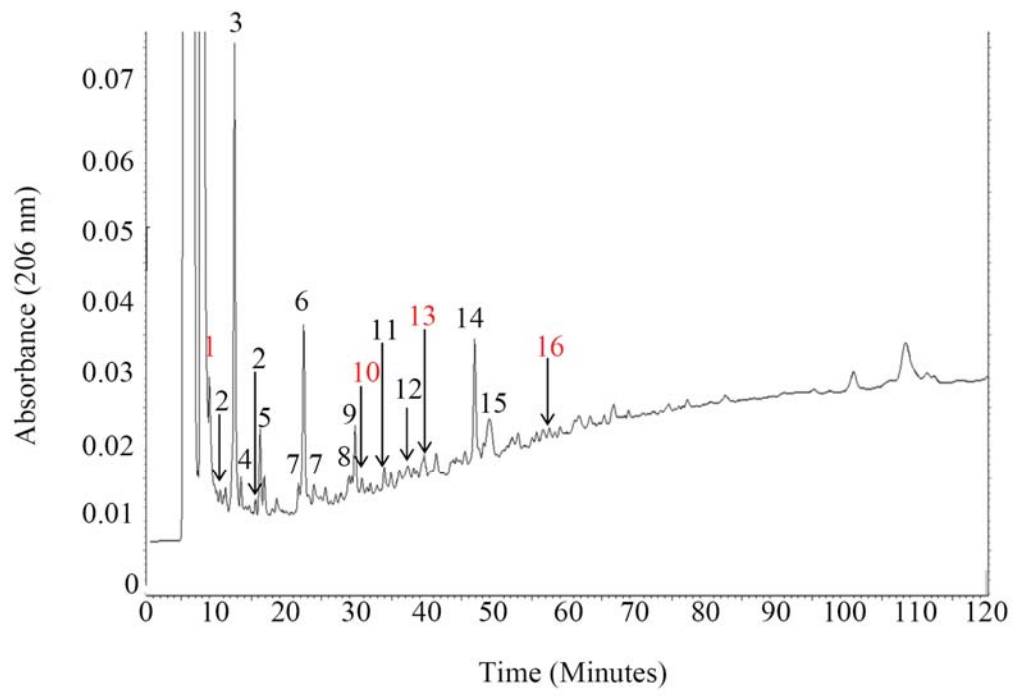
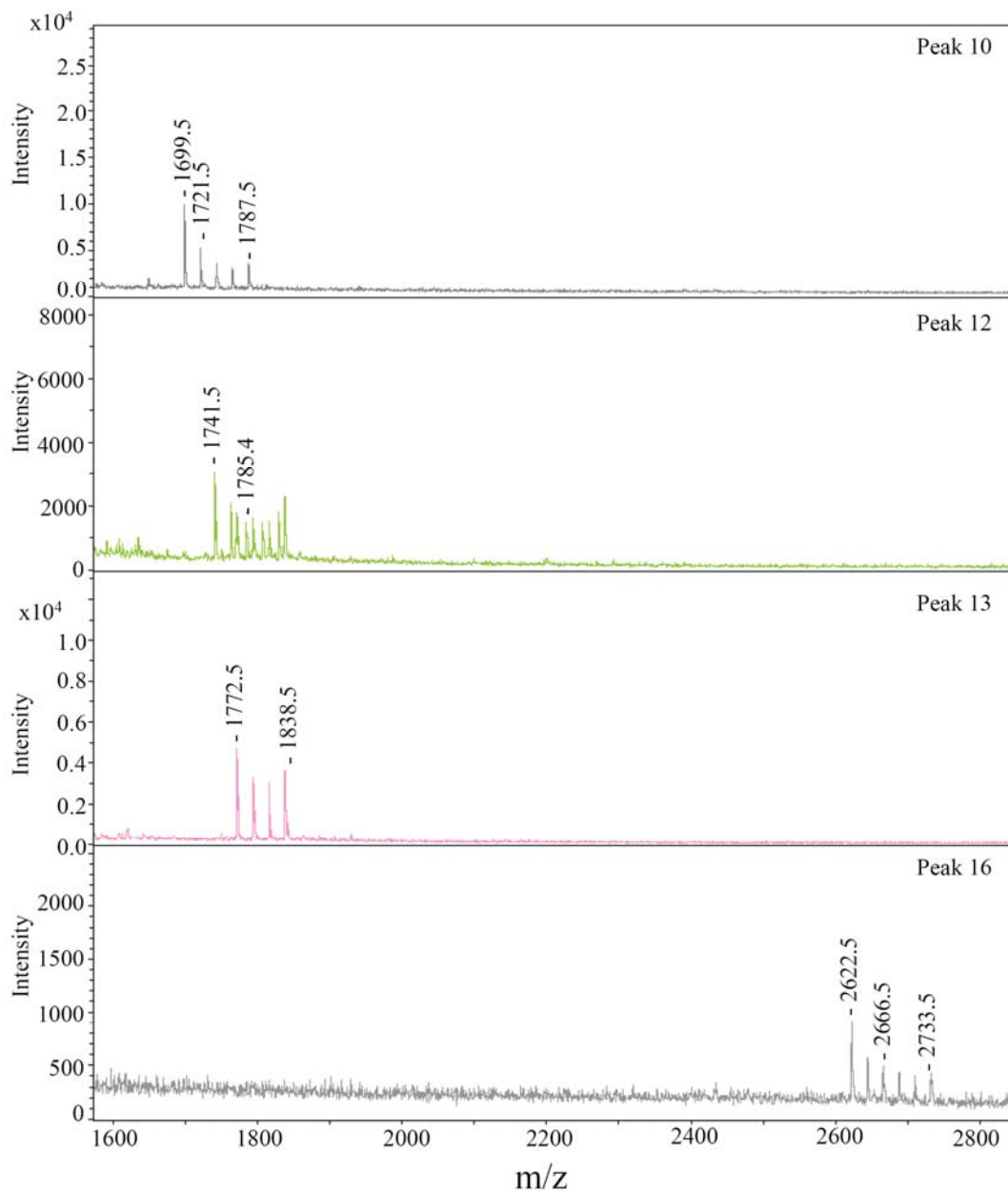


Figure 6-2. MALDI-TOF MS spectrums. Representative MALDI-TOF mass spectrums of selected mucopeptides created by rIspC hydrolysis of *L. monocytogenes* peptidoglycan.



and subsequent formation of muramicitol after reduction. Following IspC digestion, glucosaminitol but not muramicitol was identified (Figure 6-3) (Performed by Dr. Ikue Hayashi). This indicates that after IspC digestion there is a terminal reducing GlcNAc, and provides the first evidence that IspC has *N*-acetylglucosaminidase activity.

To provide additional evidence that IspC acts as an *N*-acetylglucosaminidase, MALDI-PSD was performed on the simplest structure produced by IspC hydrolysis of purified *L. monocytogenes* PG, disaccharide tri-peptide (m/z 892.2). This analysis showed several peaks corresponding with a loss of 223 Da, corresponding to the fragmentation of reduced GlcNAc (Figure 6-4A). The same analysis done with mutanolysin, a known *N*-acetylmuramidase, acted as a control. The findings, after MALDI-PSD MS was performed on the mutanolysin released muropeptides, were in direct contrast with those of IspC released muropeptides. Multiple peaks of fragmentation of which correspond to the m/z of a non-reduced *N*-acetylglucosamine (m/z 203) were observed with mutanolysin hydrolysis (Figure 6-4B). Collectively these data corroborate to indicate that IspC cleaves the bond between the C1 of GlcNAc and the C4 of MurNAc within the glycosidic chain, resulting in its classification as an *N*-acetylglucosaminidase.

Figure 6-1 shows that very few of the muropeptides released by IspC hydrolysis are de-*N*-acetylated at the glucosamine residue, however, WT *L. monocytogenes* PG was previously shown to contain 50% de-*N*-acetylated glucosamine residues (25). This may indicate that IspC shows substrate specificity for PG which is fully *N*-acetylated at the glucosamine residue.

6.2.2 Characteristics of the Δ PgdA knockout mutant

Figure 6-3. RP-HPLC chromatogram of reduced mucopeptides released by rIspC PG hydrolysis. The soluble fraction of purified *L. monocytogenes* peptidoglycan digested with rIspC was treated with alkali 0.1M NaBH₄ for 2 hrs and then dried using a rotary evaporator. The samples were hydrolyzed with HCl and then treated with triethylamine solution to neutralize the HCl. The phenylisothiocarbamyl derivatives of the amino sugar alcohols were then analyzed by HPLC using a linear gradient switching from Solvent A (0% acetonitrile) to 50% Solvent B (50% acetonitrile) over 20 min at a flow rate of 1 mL/min. The standards, which had a mixture of *N*-acetylglucosamine and *N*-acetylmuramic acid, were treated with the same procedures as outlined above, with the exception that the reduced standard was treated with alkali 0.1M NaBH₄ for 2 hrs while the non-reduced standard was not. Peaks showing glucosamine and muramic acid are clearly displayed in the non-reduced standard, while peaks showing glucosaminitol and muramicitol are present in the reduced standard. IspC digested PG shows peaks corresponding to glucosaminitol and muramic acid, indicating that glucosamine is the residue which is reduced after hydrolysis. This provides evidence that IspC acts as an *N*-acetylglucosaminidase.

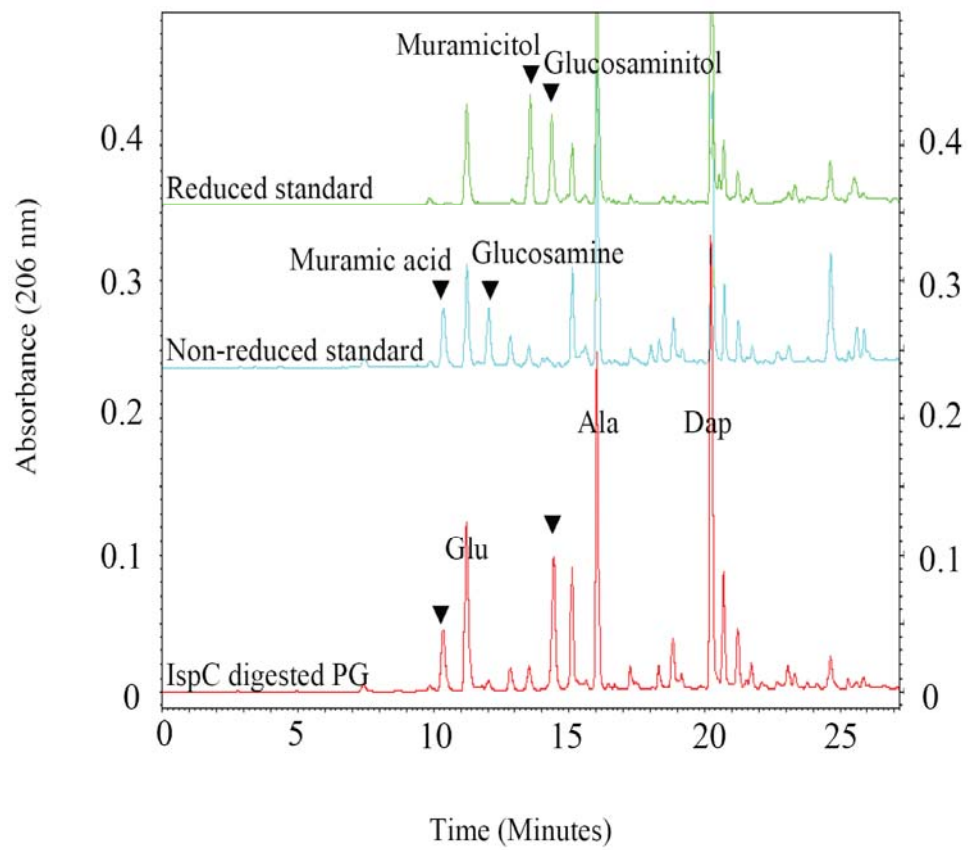
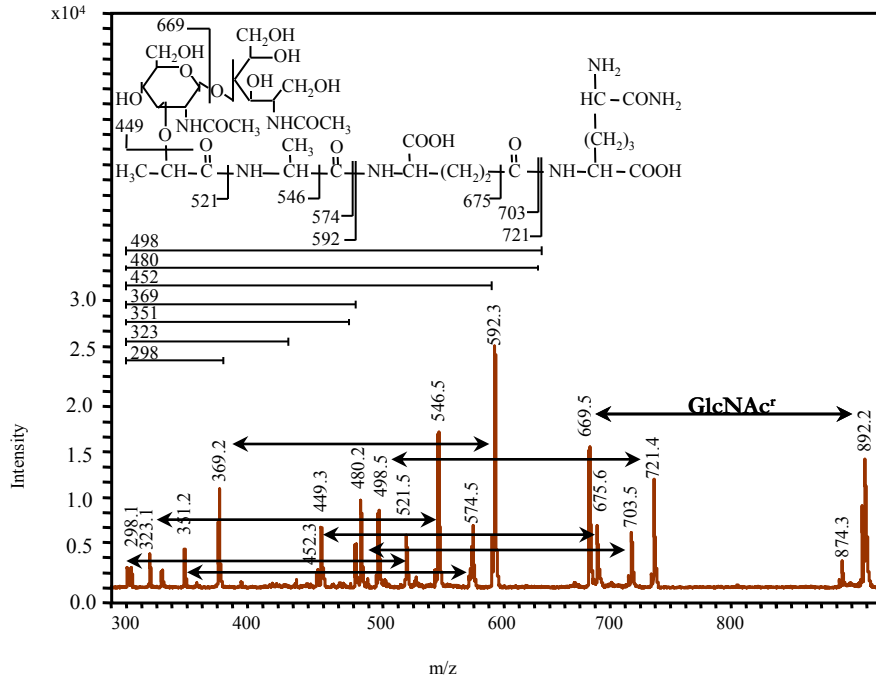
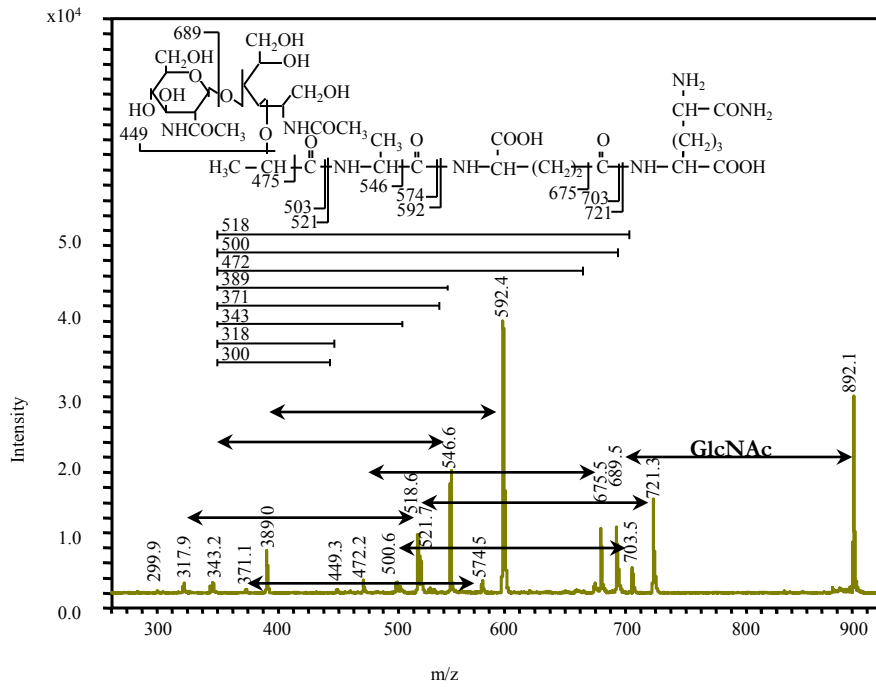


Figure 6-4. MALDI-PSD analysis of selected muropeptides. A) IspC digestion of *L. monocytogenes* PG produced a monomer tri-peptide (m/z 892.2), which was reduced with NaBH₄, and analyzed by MALDI-PSD. Upon PSD the monomer tri-peptide produced several peaks which correspond to the loss of a reduced GlcNAc (GlcNAc^r) residue (m/z 233.2). Each reduction which is indicative of a GlcNAc^r residue is shown by a double arrow. B) Mutanolysin is a known *N*-acetylmuramidase. As a control *L. monocytogenes* PG was digested with mutanolysin and the resultant muropeptides were reduced and subjected to the same procedures as muropeptides produced from IspC digestion. MALDI-PSD of muropeptides released by mutanolysin digestion revealed multiple peaks corresponding to the loss of a non-reduced glucosamine residue (m/z 202.6), which are indicated by a double arrow. This validates this procedure since glucosamine is not at the reducing end of the monomer tri-peptide when purified PG is digested by an *N*-acetylmuramidase.

A



B

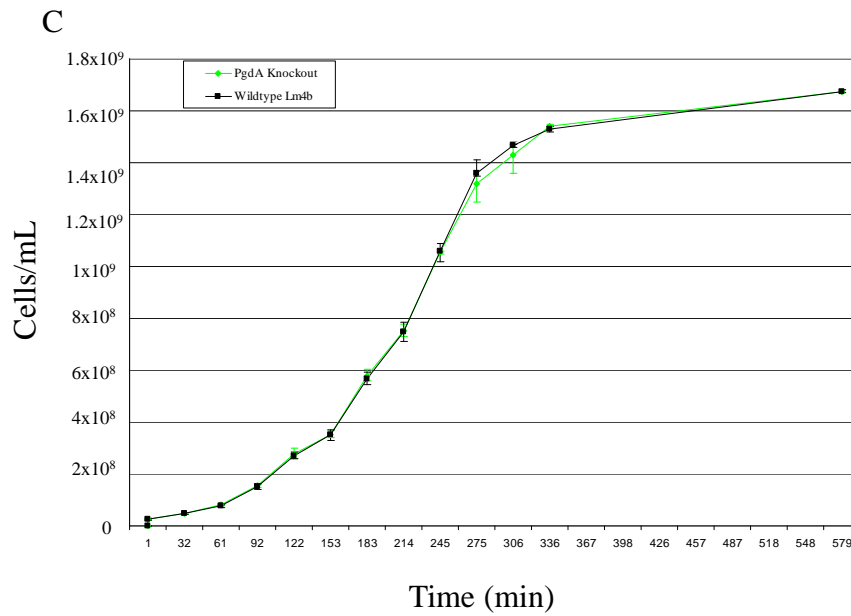
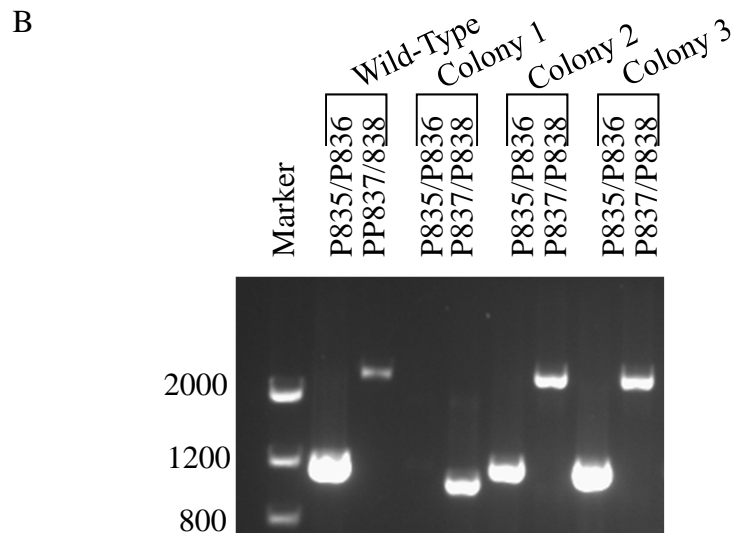
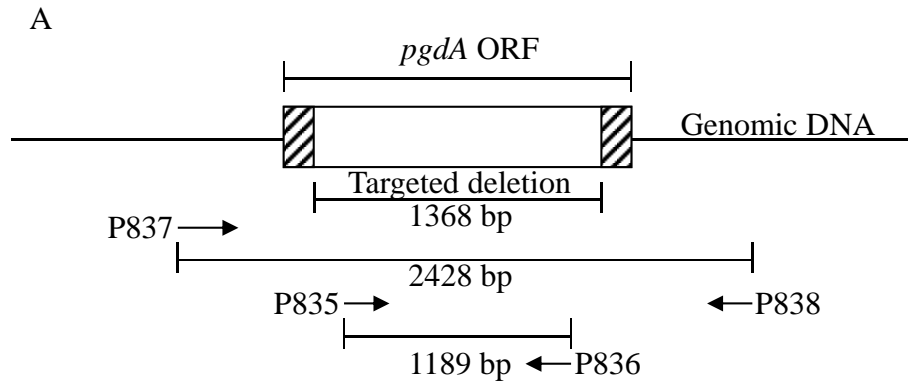


To examine if IspC had a preference for hydrolysing fully *N*-acetylated PG when compared to partially de-*N*-acetylated substrate, an in-frame PG deacetylase gene (*pgdA*) (*LMO*f2365_0434) deletion mutant of *L. monocytogenes* serotype 4b (LI0521), designated as Δ PgdA, was constructed using the pAUL-A-based integration-excision technique (48). The mutant was screened for successful targeted gene deletion by PCR using the genomic DNA of selected colonies and two sets of primer pairs (Figure 6-5A). The Δ *pgdA* mutant has an in-frame deletion of 1368 bp within the *pgdA* ORF (Figure 6-5A). PCR using primer pair P835/P836 will generate a 2428 bp amplicon with WT DNA while primer pair P837/P838 will amplify a 1189 bp fragment within the sequence targeted for deletion. PCR with primer pair P835/P836 and genomic DNA from Colony 1 amplified a 1060 bp fragment and primer pair P837/P838 will not produce an amplified product, indicating that Colony 1 has a clean knockout in the *pgdA* gene (Figure 6-5B). In-frame deletion was also confirmed by directly sequencing chromosomal DNA from Mutant 1 (Appendix 3, Figure S11). Mutants 2 and 3 produced amplicons similar to the WT with PP 835/836 and 837/838 and were not selected for further study (Figure 6-5B).

The growth of the Δ PgdA mutant was examined, in comparison to the WT, by constructing a 580 min growth curve for each isolate. No differences were observed, indicating that the Δ *pgdA* mutant did not have altered growth characteristics (Figure 6-5C).

To determine if IspC is able to interact equally with the cell-wall of live Δ PgdA mutant cells and WT cells, a recombinant fusion protein (GFPuv-CBD1) (276) containing the IspC cell wall binding domain (CBD) and GFP was assessed for its ability to bind to the WT and the Δ PgdA mutant. Fluorescence microscopy revealed the association of the fusion

Figure 6-5. Generation of a PgdA Deletion Mutant. A) Schematic representation of the *pgdA* (LMOF2365_0434) gene. A 1368 bp within *pgdA* gene was targeted for deletion. B) Agarose gel analysis of PCR products amplified by primer pair P835/P836 and P837/P838 from WT genomic DNA and genomic DNA from three selected colonies. Lane 2 shows the PCR product from WT DNA with P835/P836, while lane 3 shows the product from WT DNA with P837/P838. Lanes 4-9 show the products of the same primer pairs with the genomic DNA from the 3 selected colonies. Colonies 2 and 3 shows the same profile as the WT, while amplification of Colony 1 genomic DNA with primer pair P835/P836 does not show an amplification product and primer pair P837/P838 shows a smaller product indicating that Colony 1 has a successful *pgdA* knockout. C) The growth curve for wild-type *L. monocytogenes* is similar to the growth curve for the *L. monocytogenes* Δ *pgdA* knockout mutant.



protein GFPuv-CBD1 (276) with live WT and Δ PgdA mutant cells. Both cell types were essentially equal in their ability to bind to the GFP-CBD1 fusion protein (Figure 6-6).

6.2.3 Deletion of PgdA results in reduced PG de-*N*-acetylation

To determine the range of PG structural changes which occur due to knocking out the *pgdA* gene, a detailed analysis of both WT and Δ PgdA peptidoglycan was performed. Both types of PG were digested by mutanolysin and released muropeptides were separated by RP-HPLC (Figure 6-7). After manual collection of peak fractions, MALDI-TOF MS was carried out for each of the muropeptides, showing, in agreement with previous studies (120), that the basic repeating unit of *L. monocytogenes* peptidoglycan is GlcNAc cross-linked to MurNAc to form a repeating disaccharide unit, attached to a peptide chain composed of Ala, Glu and Dap (Figure 6-8, Figure 6-9, Table 6-2). Most of the peaks corresponding to de-*N*-acetylated muropeptides generated by mutanolysin digestion of WT PG were absent in the RP-HPLC chromatogram of the muropeptides released by the digestion of the Δ PgdA mutant PG (Figure 6-7, Table 6-2). Analysis indicated that about 50% of the WT GlcNAc residues were de-*N*-acetylated to Glc, whereas only 3% of the Δ PgdA mutant PG was de-*N*-acetylated. An additional acetyl group was present in some muropeptides (peaks 10, 13, 28 and 29 in Figure 6-8) obtained from mutanolysin digestion of the Δ PgdA mutant PG. This additional modification accounted for only 4.4% percent of the muropeptides observed. These data indicate that the only major structural change in PG due to the *pgdA* knockout is the change in de-*N*-acetylation.

6.2.4 Glycan strand termination with a 1,6-anhydromuropeptide

Anhydromuropeptides are common terminating residues found in the glycan strands of Gram-negative bacteria, but are much less common in Gram-positive bacteria (268).

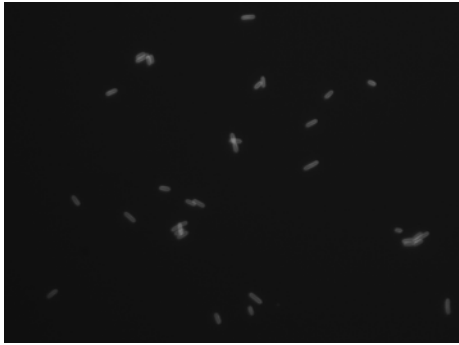
Table 6-2 - Muropeptides produced from *L. monocytogenes* peptidoglycan digestion by mutanolysin

Muropeptide Identification	Monoisotopic mass			
	Calculated [M+Na] ⁺	Observed m/z		Peak
		Wild-Type	ΔPgdA Mutant	
Monomers^b				
Di	721.3	721.2	721.2	9
Tri (deAc)(NH ₂)	850.4	850.3, 850.2	850.4	1
Tri (deAc)	851.4	851.2	--	2
Tri (NH ₂)	892.4	892.2	892.1, 892.1	3
Tri	893.4	893.2	893.3	4
Tri (Ala, Dap), 2(NH ₂)	1134.5	1134.2	1134.3, 1134.1	6
Tri (Ac)(NH ₂)	934.4	--	934.1	10
Tetra (deGlcNAc)	760.4	760.3	760.2	8
Tetra (deAc)(NH ₂)	921.4	921.2, 921.1	921.1	5
Tetra (NH ₂)	963.4	963.1, 963.1	963.2, 963.1, 963.1	7
Tetra (Ac)	1005.4	--	1005.1, 1005.1	13
Penta (NH ₂)	1034.4	1034.1, 1034.1	1034.1, 1034.1	11
Dimers				
Tetra-Tri 2(deAc), 2(NH ₂)	1730.8	1730.2, 1730.6, 1730.8	--	12
Tetra-Tri (deAc), 2(NH ₂)	1772.8	1772.3, 1772.2, 1772.2	--	14
Tetra-Tri (deAc)(NH ₂)	1773.8	1773.3, 1773.2, 1773.2	--	17
Tetra-Tri 2(NH ₂)	1814.8	1814.2, 1814.1, 1814.2	1814.2, 1814.2, 1814.2	16
Tetra-Tri (NH ₂)	1815.8	1815.2, 1815.2	1815.2, 1815.2, 1815.2	18
Tetra-Tri (deGlcNAc) 2(NH ₂)	1611.8	--	1611.4, 1611.5	15
Tetra-Tri 2(deAc), (NH ₂)	1731.8	1731.4	--	19
Tetra-Tri 2(deAc)	1732.8	1732.1	--	20
Tetra-Tri	1816.8	--	1816.2, 1816.2	21
Tetra-Tetra (NH ₂)	1886.8	--	1886.3	22
Tetra-Tri (Ac), 2(NH ₂)	1856.8	--	1856.2, 1856.2	28
Tetra-Tri (Ac), (NH ₂)	1857.8	--	1857.7, 1857.1	29
Anhydrides				
Tetra-Tri Anhydride(deAc), 2(NH ₂)	1752.8	1752.5	--	30
Tetra-Tri Anhydride 2(NH ₂)	1794.8	1794.2	1794.3, 1794.7	23
Tetra-Tri Anhydride(deAc), (NH ₂)	1753.8	1753.5	--	31
Tetra-Tri Anhydride (NH ₂)	1795.8	1795.2	1795.1	32
Trimers				
Tetra-Tetra-Tri 3(deAc), 4(NH ₂)	2610.2	2610.9	--	24
Tetra-Tetra-Tri 2(deAc), 4(NH ₂)	2652.2	2652.9	--	25
Tetra-Tetra-Tri (deAc), 4(NH ₂)	2694.2	2694.5	--	26
Tetra-Tetra-Tri 4(NH ₂)	2736.2	2737.5	2737.3, 2737.4	27

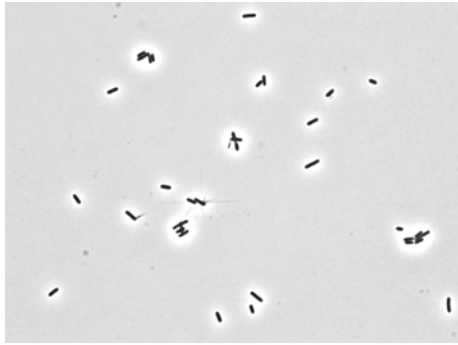
^b See Table 6-1 for a complete list of abbreviations.

Figure 6-6. Binding of the GFPuv-CBD1 fusion protein to live *L. monocytogenes*. Cells were incubated for 5 min with recombinant GFPuv-CBD1 or with GFPuv as a negative control. Molecules of GFPuv-CBD1 in association with the cellular surfaces were then visualized by fluorescence microscopy and images were captured using Q-capture pro. Both A) wild-type and C) Δ PgdA mutant cells appeared to have approximately equal interaction with the CBD. Phase contrast images of B) wild-type and D) Δ PgdA mutant cells are also shown for comparison. No fluorescence was observed in the negative control.

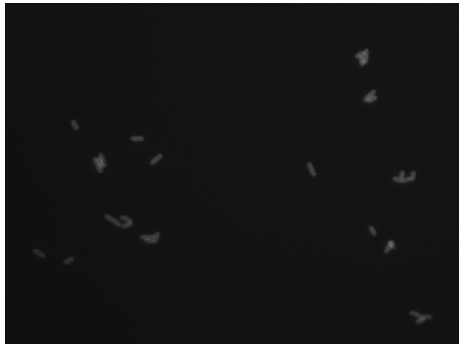
A



B



C



D

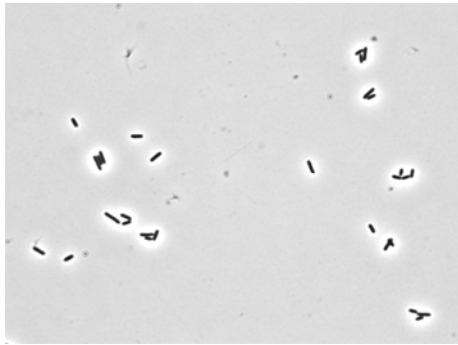


Figure 6-7. RP-HPLC chromatogram showing eluted muropeptides released by mutanolysin digestion of *L. monocytogenes* PG and *L. monocytogenes* mutant Δ PgdA PG. Purified PG was digested by mutanolysin for 24 hours, after which insoluble PG was removed by centrifugation. The soluble muropeptides were reduced by treating with sodium borohydride. The reduced muropeptides were then loaded into a C18 Hypersil ODS column and eluted using a 120 minute linear gradient switching from buffer A (50 mM sodium phosphate containing 5% methanol, pH 2.5) to buffer B (50 mM sodium phosphate containing 30% methanol, pH 2.5) at a flow rate of 0.5 mL/min. Muropeptide elution was monitored at 206 nm and peak fractions were collected manually. Labelled peaks correspond to muropeptide structures identified by MALDI-TOF MS and documented in Table 6-2. Numbers labelled in red indicate de-*N*-acetylated peptidoglycan, black indicates fully acetylated PG, yellow represents muropeptides with an extra acetyl group, and blue is used in denote a 1-6, anhydromuropeptide structure.

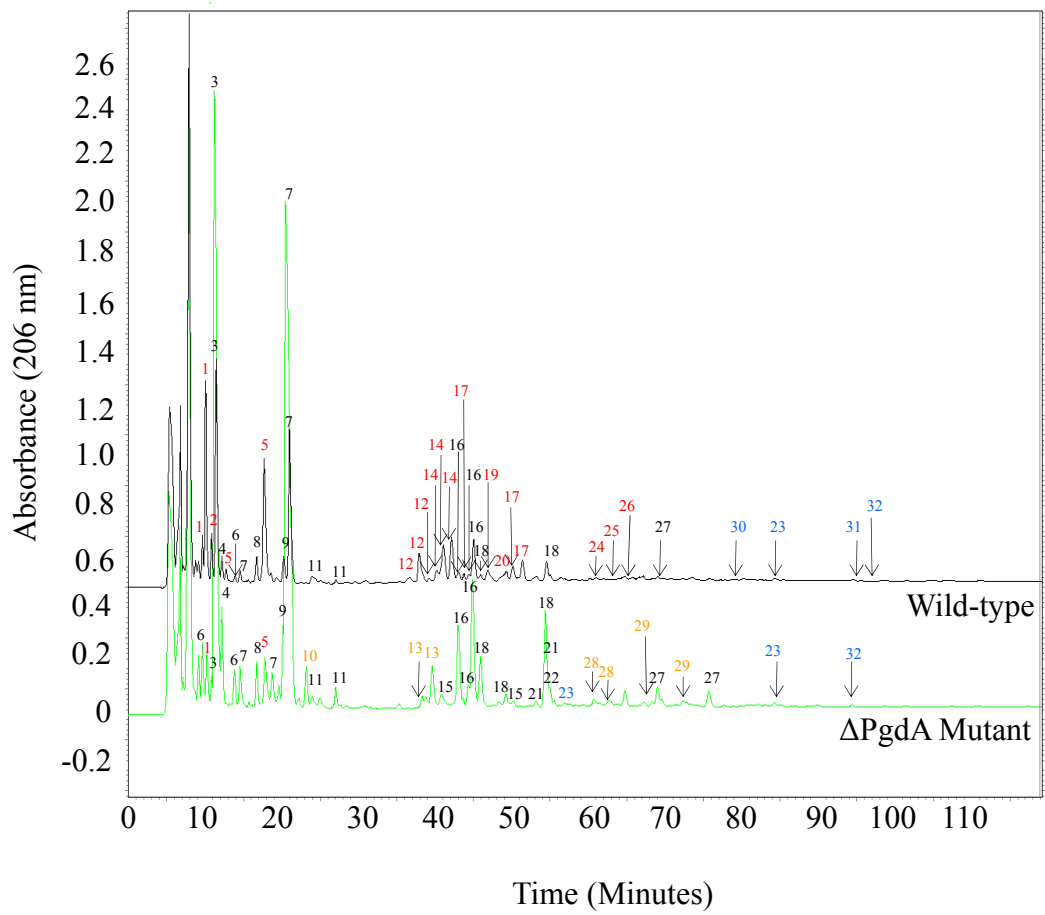


Figure 6-8. MALDI-TOF MS spectra of mucopeptide dimers. Representative MALDI-TOF mass spectra of selected mucopeptide dimers, shown to illustrate the observed high signal to noise ratio.

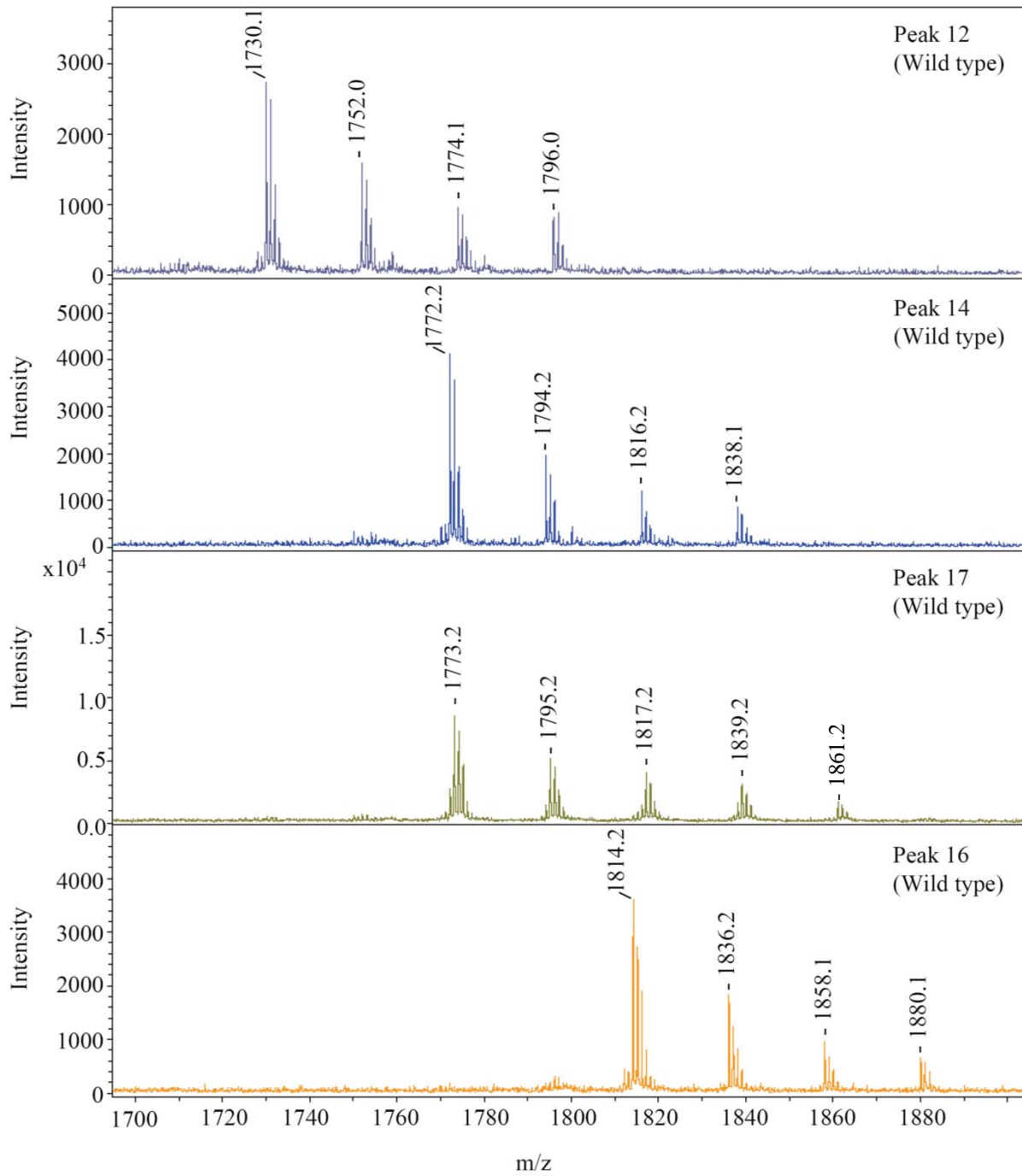
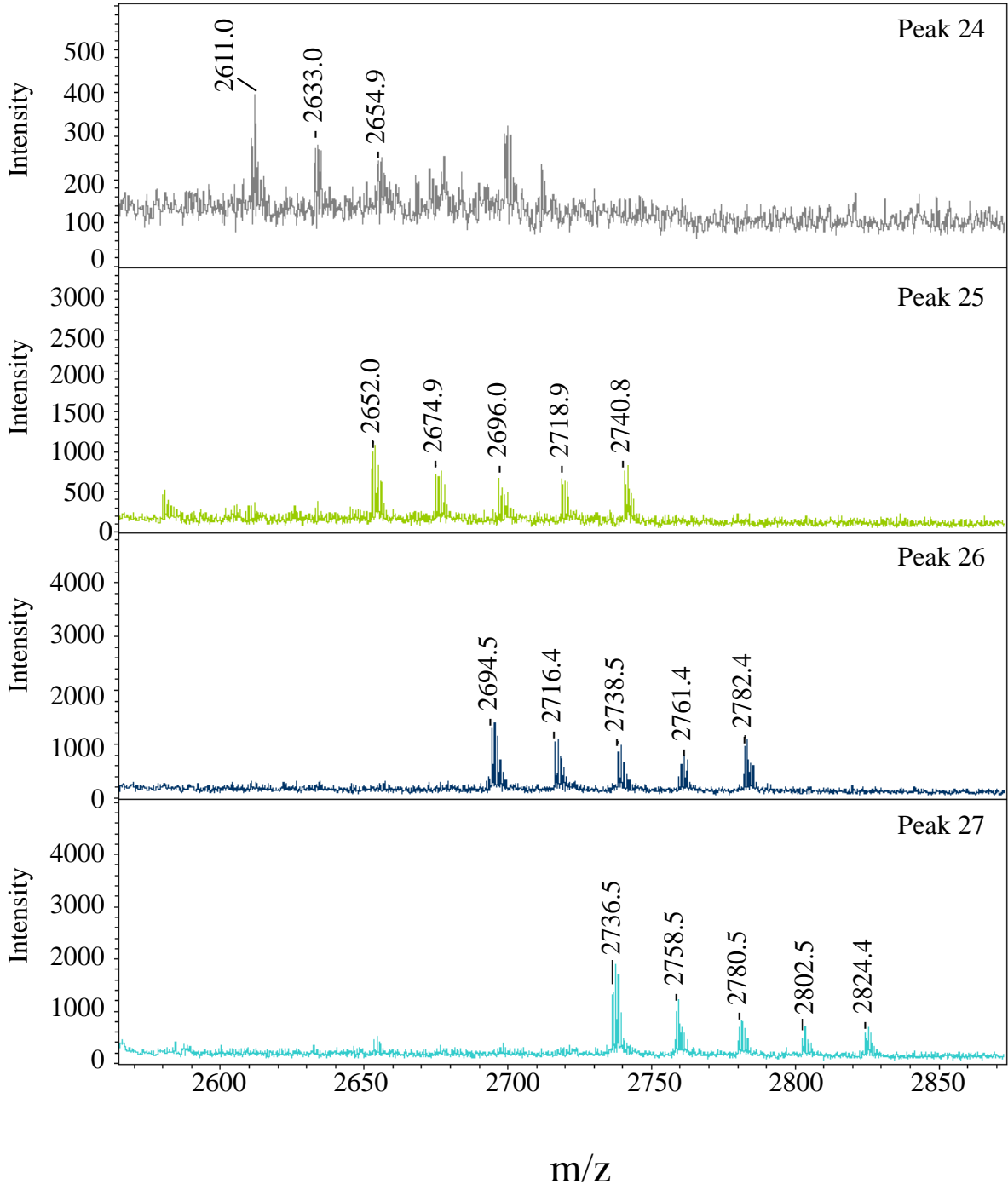


Figure 6-9. MALDI-TOF MS spectra of mucopeptide trimers. Representative MALDI-TOF mass spectra of selected mucopeptide trimers, shown to illustrate the observed high signal to noise ratio.



Approximately 0.34% of muropeptides (peaks 23 and 32 in Figure 6-6) released by digestion of the Δ PgdA mutant PG were identified by MALDI-TOF MS as 1,6- anhydromuropeptides (Figure 6-10). Similarly, 0.55% of WT muropeptides (peaks 23, 30, 31 and 32 in Figure 6-6 and Table 6-2) were found to contain 1,6-anhydride residues. This is the first report of the anhydride structure being observed in *L. monocytogenes* peptidoglycan.

6.2.5 IspC hydrolytic activity on de-*N*-acetylated PG substrate

For initial assessment of the hydrolytic activity of IspC on the WT and Δ PgdA mutant PG, re-naturing SDS-PAGE was performed. Although this technique is not quantitative, a lighter band indicates that the Δ PgdA mutant PG was more readily digested than the WT PG (Figure 6-11A). Monitoring of PG hydrolysis by IspC was performed by monitoring OD at 660 nm. This analysis indicates that IspC cleaves substrates which contain a higher amount of *N*-acetylated glucosamine more efficiently (Figure 6-11B). In contrast, mutanolysin showed a similar hydrolytic activity on both substrates (Figure 6-11C). This is likely explained by the difference in substrate bond specificity between IspC, which was found to be an *N*-acetylglucosaminidase, and mutanolysin, which is known to act as an *N*-acetylmuramidase.

6.3 DISCUSSION

Using RP-HPLC in combination with MALDI-TOF MS, we have conducted a detailed structural analysis of muropeptides released from *L. monocytogenes* PG by IspC-mediated hydrolysis. These results provide evidence that IspC is a *N*-acetylglucosaminidase, able to hydrolyse the β -1,4-glycosidic bond of the PG glycan strand. Muropeptides released from the WT PG by IspC hydrolysis were found to have disproportionately high levels of *N*-

Figure 6-10. MALDI-TOF MS mass spectra of 1,6- anhydromuropeptides.
Representative MALDI-TOF mass spectra of selected 1,6- anhydromuropeptides, shown to illustrate the observed high signal to noise ratio.

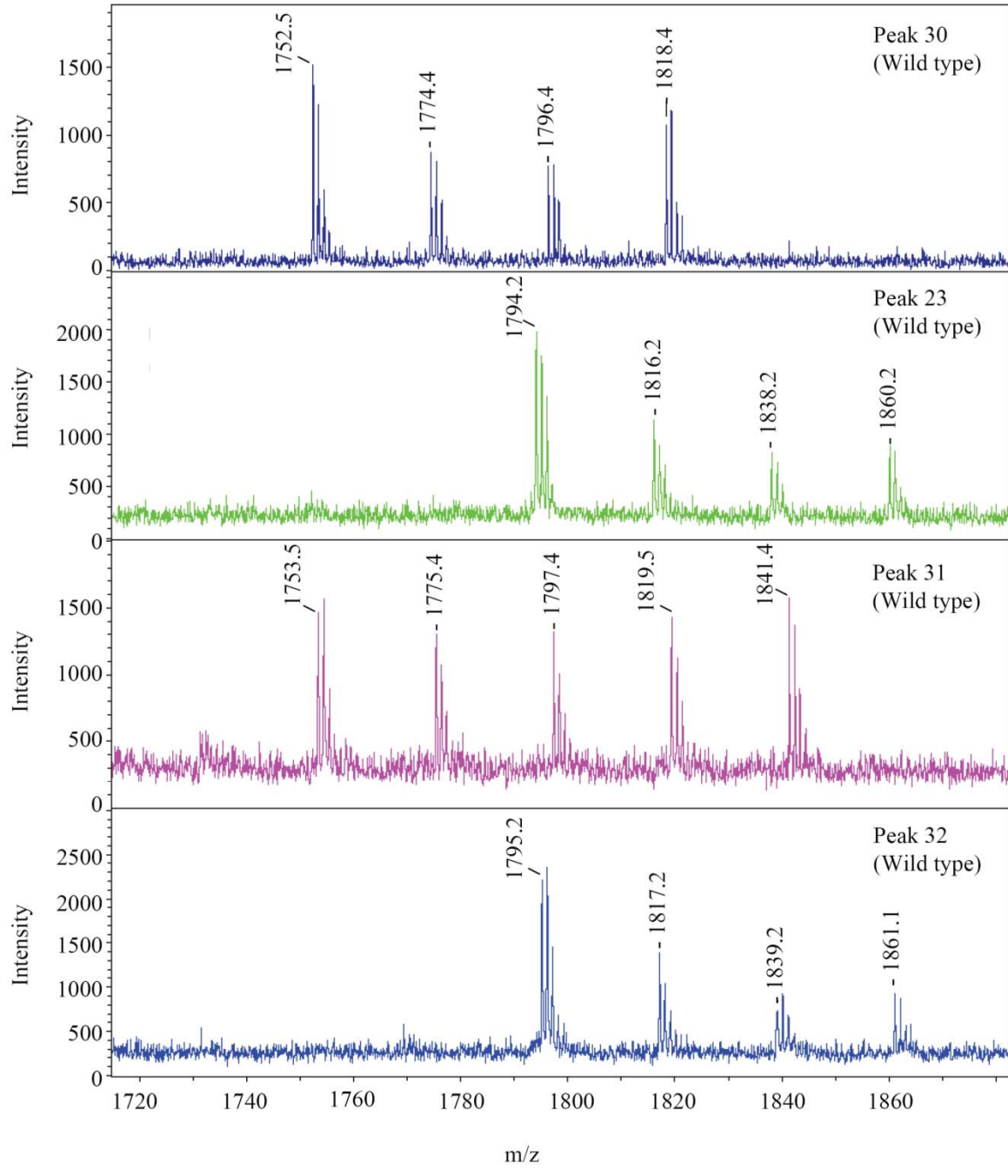
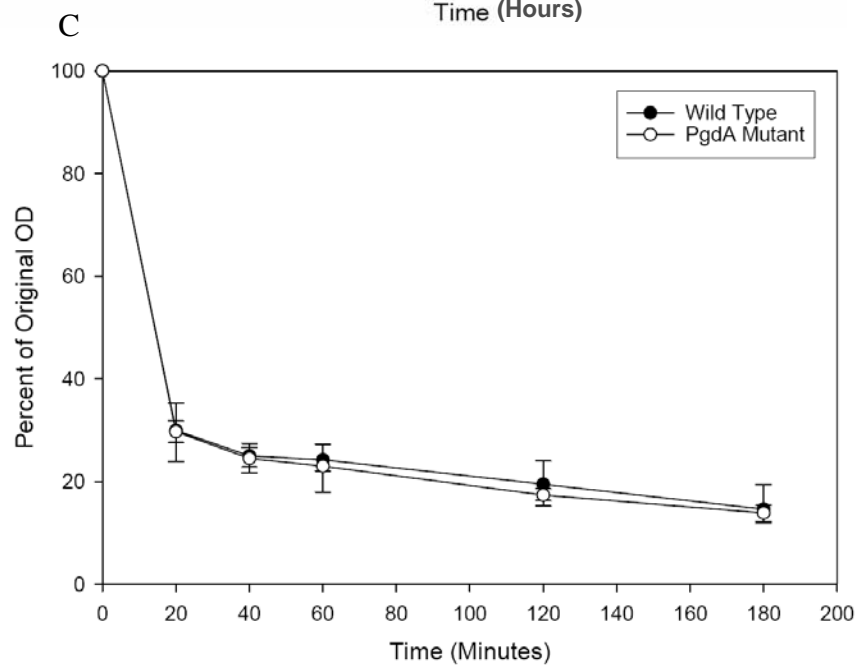
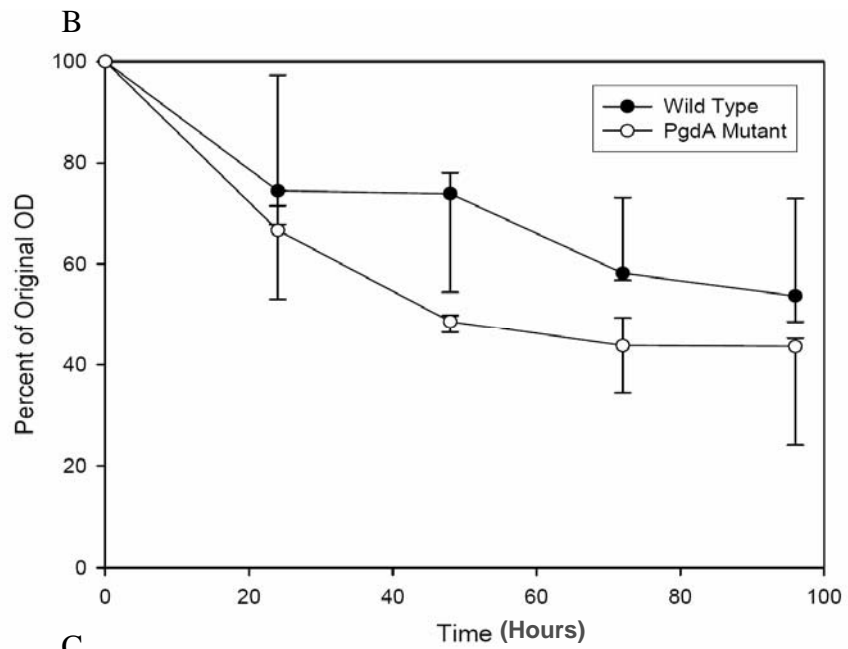
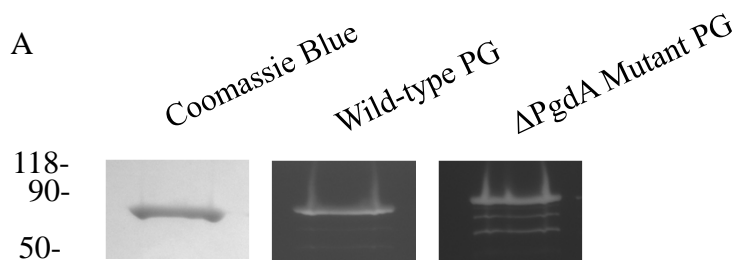


Figure 6-11. rIspC hydrolysis of wild-type and Δ PgdA mutant PG. A) rIspC was run on an SDS-PAGE gel and stained with comassie blue to insure purity. rIspC was analyzed for its ability to digest the cell wall material of WT and Δ PgdA mutant *L. monocytogenes* cells by re-naturing SDS-PAGE. Cell wall material was prepared by autoclaving bacterial cells of overnight cultures and harvested by centrifugation. After 4 washes with water the cellular material was lyophilized for 24 hours. Purified rIspC was run on a 12% SDS-PAGE gel containing either 0.1% (w/v) autoclaved and lyophilized WT or Δ PgdA mutant cell wall material. After renaturation, gels were washed in water for 30 minutes and then stained with 0.1% (w/v) methylene blue (Fisher Scientific) in 0.01% (w/v) KOH for 1 h at room temperature with agitation. Gels were de-stained with water until areas of hydrolysis became clear. B) Cell wall extract was also suspended in 10 mM phosphate buffer and rIspC was added. The suspension was measured spectrophotometrically at 600 nm at regular intervals. The OD₆₀₀ was calibrated so that the OD₆₀₀ prior to the addition of rIspC was 100% and the progression of hydrolysis was shown as a percentage of the original OD. The data is a combination of at least three separate experiments where the median is plotted and the error bars show the entire range of data. The Mann-Whitney U test was used to determine statistical significance. The difference between hydrolysis rates of wild-type and PgdA mutant PG where was statistically significant at 24, 48, 72 and 96 hrs ($p < 0.05$). C) *L. monocytogenes* cell wall extract suspended in 10 mM citrate buffer and mutanolysin was added. The same procedures outlined above for IspC were carried out for mutanolysin. No difference was found between the ability of mutanolysin to digest WT and Δ PgdA mutant peptidoglycan.



acetylation. As PgdA is the only enzyme predicted to de-*N*-acetylate PG in *L. monocytogenes* (201), an in-frame *pgdA* gene deletion mutant was created to investigate the rates of IspC hydrolysis on fully acetylated peptidoglycan compared with partially de-*N*-acetylated WT PG. MS analysis combined with time-course digestion of the PG substrates from both the WT and the Δ PgdA mutant revealed that structural modification of PG by de-*N*-acetylation of GlcNAc residues renders PG resistant to IspC hydrolysis.

IspC (homologous to *LMO*f2365_1093) is classified as a member of the glycoside hydrolase (GH) 73 family (Carbohydrate-Active EnZyme database (CAZy) at <http://www.cazy.org/>) (46). While there are 1934 bacterial enzymes assigned to this family based on both sequence alignments and secondary structure predictions, only 19 have been characterized. Most characterized GH73 enzymes have been shown to act as *N*-acetylglucosaminidases and this correlates nicely with our findings. However, there is also a report of a GH73 enzyme (Mur2) having *N*-acetylmuramidase activity (49), making experiments aimed at confirming the specificity of GH73 enzymes necessary. The crystal structure of Auto (*lmo*1076), another GH73 enzyme found in *L. monocytogenes* EGD-e which has *N*-acetylglucosaminidase activity, has recently been solved (37). Analysis of Auto has shown that the Glu122 and Glu156 residues were essential for PG hydrolysis (37). Amino acid sequence comparison reveals that these two important residues are conserved in IspC. Despite variation between the amino acid sequences of IspC and Auto the secondary structure is highly conserved, suggesting they may have a similar mechanism of action. An important difference between the two enzymes is that Auto has an N-terminal α -helix that blocks its catalytic site, causing autoinhibition and requiring proteolytic cleavage prior to

activation (37). IspC does not contain a regulatory helix, strongly suggesting it is not regulated in the same way as Auto.

Structural modification of PG by de-*N*-acetylation of GlcNAc results in less susceptibility to hydrolysis by IspC. De-*N*-acetylation also decreases the activity of other *N*-acetylglycosaminidases in the GH73 family, including: AcmA from *L. lactis* (166), NagZ from *B. subtilis* (154) and Auto (37). Two amino acids are generally required for glycosidic bond hydrolysis: an acidic proton donor and a nucleophile or base (52). While work on Auto indicates that the Glu122 acts as a catalytic residue (37), and this is also likely the case for IspC, it is possible that the *N*-acetyl group of the PG glucosamine residue acts as the nucleophile and is thus required for substrate-assisted hydrolysis. Our observations fit this model; in addition, this model would provide an excellent molecular explanation of why IspC favours fully-acetylated PG. However, additional experiments aimed at providing direct evidence that the *N*-acetyl group of glucosamine residue acts as the nucleophile in the hydrolysis reaction need to be performed. Autolysins can induce fatal cellular lysis; therefore, the activity of PG hydrolases is highly regulated and localized to specific areas of the cell wall, such as actively growing PG or the divisome, where PG hydrolysis is required. De-*N*-acetylation may be a mechanism of making the PG substrate unfit for hydrolysis, thereby controlling the location of PG hydrolysis.

To verify that the only major change to PG structure caused by the *pgdA* deletion of *L. monocytogenes* was the amount of *N*-acetylated glucosamine, we carried out a thorough study of the structure of both WT and mutant PG. In agreement with a published study (25), we found that WT PG was 50% de-*N*-acetylated, while de-*N*-acetylation was reduced to 3% in the Δ PgdA mutant. As expected, this was the only major change between the two types of

PG. Modifications of PG, including de-*N*-acetylation, *N*-glycolylation, *O*-acetylation and the presence of 1-6-anhydro rings are very common in Gram-positive bacteria. As more of the enzymes responsible for PG modification are identified, it is becoming increasingly clear that PG modification plays an important role in pathogenesis, sporulation and regulation of PG hydrolysis (269). Our study supports the basic structure of *L. monocytogenes* PG proposed by Kamisango et al., (120): a glycan polymer made up of disaccharide repeating units linked by a peptide bridge composed of L-alanine (Ala), D-isoglutamic acid (iGlu) and meso-diaminopimelic acid (Dap). In addition to de-*N*-acetylation, this study also found other PG modifications including *O*-acetylation and 1-6-anhydro rings. Several structures (Table 2, Figure 6-7) were identified as containing an extra acetyl group in addition to the *N*-acetyl group. Although, the exact location of this group was not determined, it is likely *O*-acetylation of the muramic acid residue. 1,6-anhydro-MurNAc was also found in both WT and Δ PgdA *L. monocytogenes* PG (Figure 6-10). This is the first report of 1,6-anhydro-MurNAc in *L. monocytogenes* (Figure 6-10). Anhydromuropeptides are proposed to be associated with the terminal end of a glycan strand in Gram-negative and some Gram-positive bacteria. They are the product of either a lytic transglycosylase or a synthetic transglycosylase and are commonly associated with PG turnover in Gram-negative bacteria (270), and with germination of spores in Gram-positive bacteria (8, 9). A putative transglycosylase (*LMO*f2365_0753) is encoded by the genome of *L. monocytogenes* serotype 4b strain F2365 (181). Anhydromuropeptides generally occur less often in Gram-positive bacteria than in Gram-negative bacteria. In *B. subtilis*, anhydromuropeptides account for only 0.4% of muropeptides (108) and they are not present in *S. aureus* (26). In *E. coli*, anhydromuropeptides account for 3.71% of muropeptides (98). Although data concerning the

proportion of anhydride residues in Gram-positive bacteria is limited, the finding in this investigation, that 0.55% of mucopeptides in *L. monocytogenes* are anhydromuropeptides, fits the current trend.

In conclusion, IspC functions as an *N*-acetylglucosaminidase in *L. monocytogenes*. Moreover, its catalytic activity is modulated by de-*N*-acetylation of the GlcNAc residues through the action of PgdA.

Chapter VII

Secretion of IspC is SecA2-independent

Some of the data in this chapter has been submitted for publication:

Ronholm, J., Cao, X., and Lin, M. (2013) Secretion of the surface autolysin IspC is SecA2-independent in *Listeria monocytogenes* serotype 4b. Submitted.

7.1 Introduction

L. monocytogenes has a complex lifecycle during which it alternates between life as an environmental saprophyte and as an opportunistic intracellular parasite. Extracellular proteins participate in both lifestyles, as well as in signalling when to switch from one to the other (19). In the environment, *L. monocytogenes* cells secrete proteins which participate in environmental sensing, degradation of substrates, and cell-to-cell communication (257). As a parasite, *L. monocytogenes* expresses surface proteins that mediate different stages of infection including: adhesion, internalization and immune evasion, and making some secreted proteins important virulence factors (218). Therefore, an understanding of bacterial protein secretion is critical to understanding pathogenesis in *L. monocytogenes*.

Most surface proteins in *L. monocytogenes* are secreted via the SecA pathway (56, 68). An accessory SecA paralog (SecA2) has been discovered in several Gram-positive bacteria, including *L. monocytogenes* (144). Unlike SecA which is essential for *L. monocytogenes* survival, the SecA2 pathway is not required for bacterial viability, however, several of the proteins secreted by SecA2 are involved in pathogenesis (143). SecA2-dependent proteins are necessary for persistent colonization of host cells by *L. monocytogenes* (143). Proteins secreted by SecA2 are required for the development of protective memory CD8⁺ T cells in response to an infection (215). Lenz *et al.* (143) suggest that autolysins are major products of SecA2-dependent secretion, as evidenced by their work with the *L. monocytogenes* 10403S, a serotype 1/2a isolate (143). However, the secretion pathway for IspC remains unknown. The aim of this work is to determine if IspC is SecA2-dependent, since SecA2-dependence has been demonstrated for other autolysins which are

involved in virulence, such as p60 and NamA, in *L. monocytogenes* 10403S (serotype 1/2a) (143).

7.2 Results

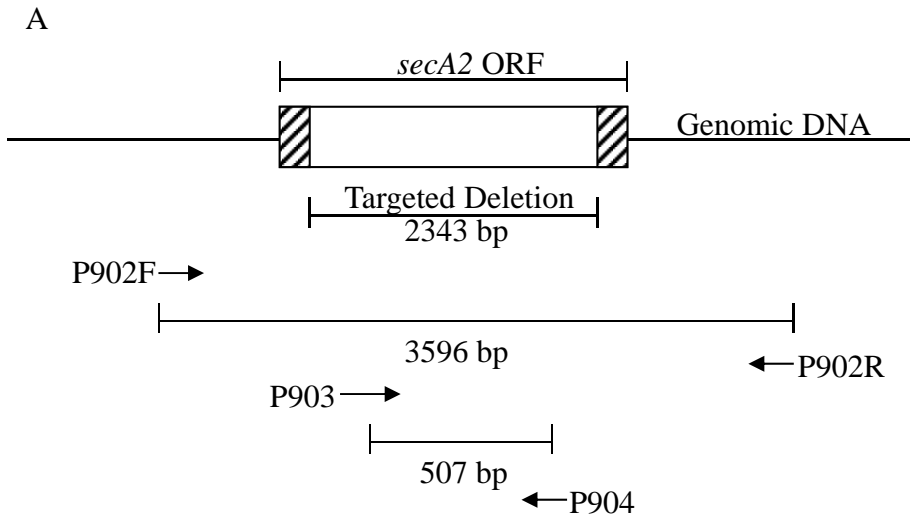
7.2.1 Generation of a Δ *secA2* in-frame deletion mutant

To determine if SecA2 has a role in IspC secretion an in-frame (Δ SecA2) (*LMO*f2365_0612) deletion mutant of *L. monocytogenes* serotype 4b (LI0521), designated as Δ SecA2, was constructed using the pAUL-A-based integration-excision technique (48). The mutant was assessed for successful targeted gene deletion by two sets of primer pairs (Figure 7-1A). The *secA2* mutant has an in-frame deletion of 2343 bp inside the *secA2* ORF (Figure 7-1A). The PCR with the primer pair P902f/P902r generated a 3596 bp amplicon from the genomic DNA of Colony 1, while PCR with the primer pair P903/P904 amplified a 570-bp fragment within sequence targeted for deletion. This indicates that a targeted deletion within the *secA2* ORF was not present in Colony 1. Colony 2 and 3 on the other hand were confirmed to have a clean knockout in the *secA2* gene, as the PCR product with primer pair P902f/P902r was reduced to about 1253 bp in each colony. Although contamination was observed in the PCR with primer pair P903/P904 using the genomic DNA from these two colonies, the PCR products expected for the WT were not present (Figure 7-1B). The in frame deletion of *secA2* was also confirmed by directly sequencing the PCR product from Colony 2 using P902f and P902r (Appendix 3, Figure S11).

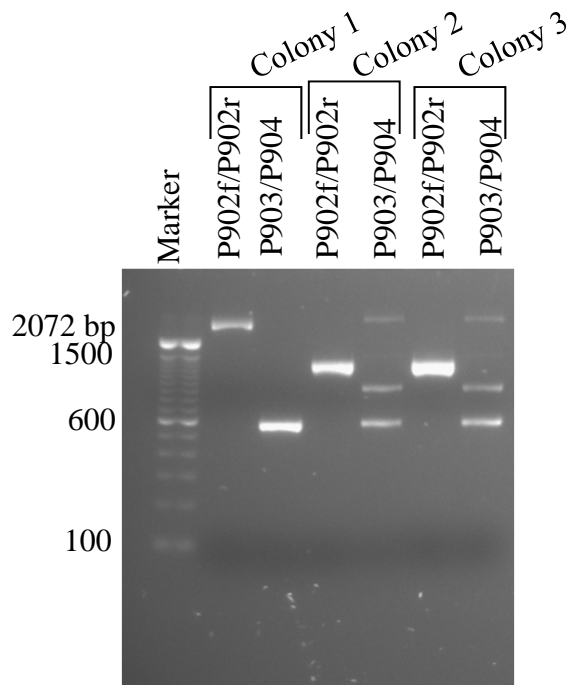
7.2.2 p60 and NamA are SecA2-dependent

Both WT and Δ SecA2 mutant cells were grown in LB and BHI broth to mid-exponential phase. Secreted proteins were recovered from the growth media by precipitation

Figure 7-1. Generation of an in frame *secA2* deletion mutant. An in frame deletion of the *secA2* ORF was created using a pAUL-A-based integration-excision technique. A) The approximate binding sites of each of the P902f, P902r, P903 and P904 primers and the size of the PCR amplicon derived from wild-type genomic DNA with these primers. B) PCR fragments amplified from the genomic DNA from each of the selected colonies are shown.



B



and surface proteins extracted from the cells by using SDS. Numerous protein bands were resolved from the BHI growth supernatant by SDS-PAGE analysis. However, only one protein showed a large reduction between the WT and the Δ SecA2 mutant (Figure 7-2A). Approximately 11 and 37 proteins were resolved from the WT bacteria grown in BHI and LB broth, respectively (Figure 7-2B and 7-2C). One of the 37 proteins was absent from the SecA2 mutant grown in LB broth and two proteins were absent from the mutant when grown in BHI. SecA2-dependent secretory proteins were identified by comparing the MS/MS spectra against a database using MASCOT. Two proteins were identified as exclusively SecA2-dependent secretory proteins: p60 (*LMOJ2365_0611*) and *N*-acetylmuramidase (NamA) (*LMOJ2365_2670*) (Figure 7-2).

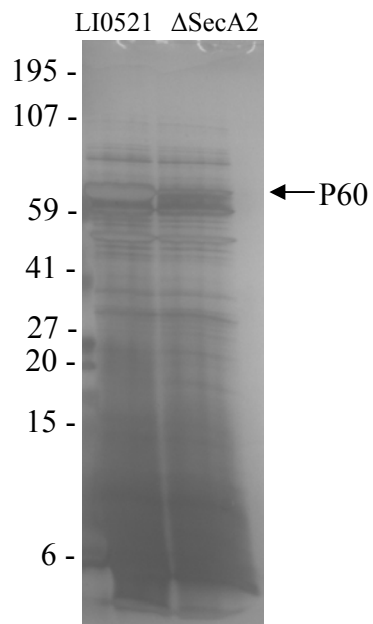
7.2.3 IspC is not SecA2-dependent

Secretion of IspC was not identified as being SecA2-dependent after SDS-PAGE analysis of SDS-extractable surface proteins from the WT when compared to that of the Δ SecA2 mutant. Therefore, to further provide evidence that IspC has SecA2-independent secretion, live WT and Δ SecA2 mutant cells were probed with an IspC specific MAb (M2773). IspC was found to be prominent on the surface of both types of cells (Figure 7-3A), indicating that IspC is not SecA2-dependent. In addition, SDS-extractable surface proteins were ran on an SDS-PAGE and Western blotted with M2773 (Figure 7-3B). IspC secretion was essentially equal between WT and Δ SecA2 mutant cultures, further providing evidence that IspC is SecA2-independent.

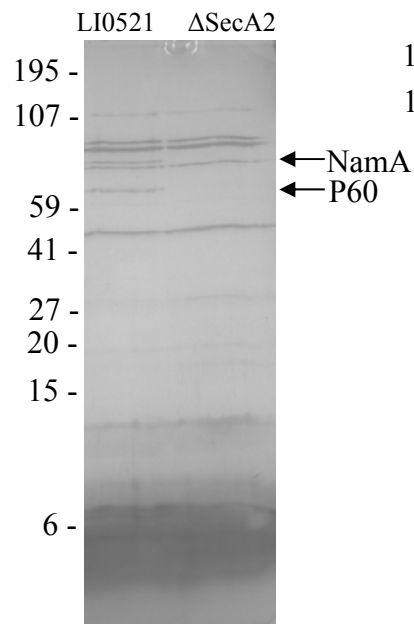
7.3 DISCUSSION

Figure 7-2. Silver stained SDS-PAGE gels showing SecA2-dependent secretion of proteins in *L. monocytogenes*. SecA2-dependent proteins that were precipitated from the culture supernatant (A) of *L. monocytogenes* cells grown in BHI broth or SDS-extracted from the cells surface of cells grown in BHI broth (B) or LB (C) broth are indicated. A total of 4 SecA2-dependent protein bands corresponding to two proteins: p60 and NamA were identified by mass spectrometry.

A



B



C

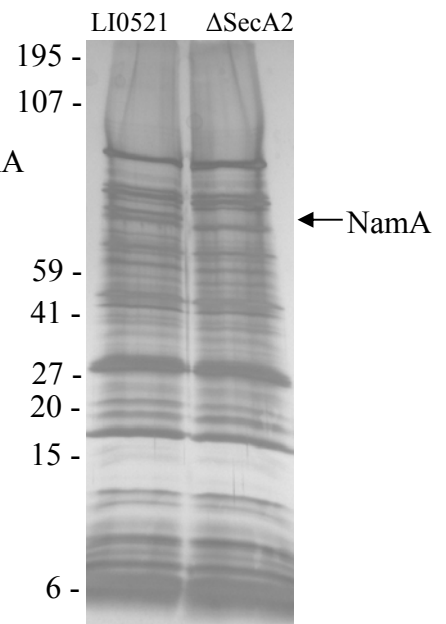
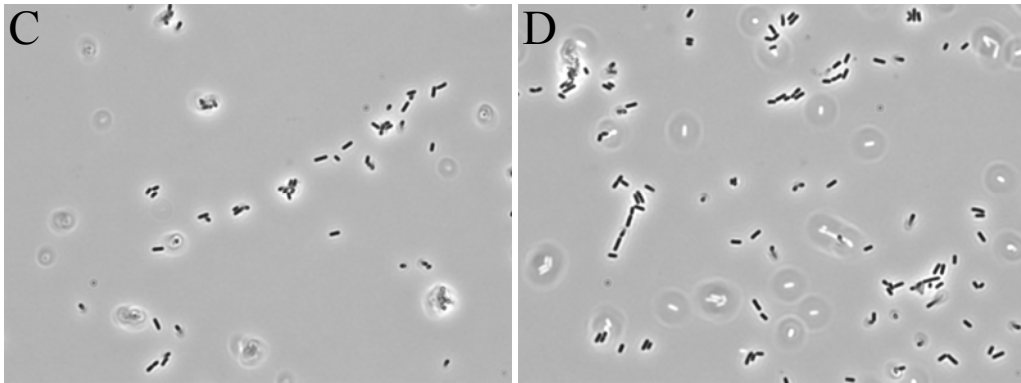
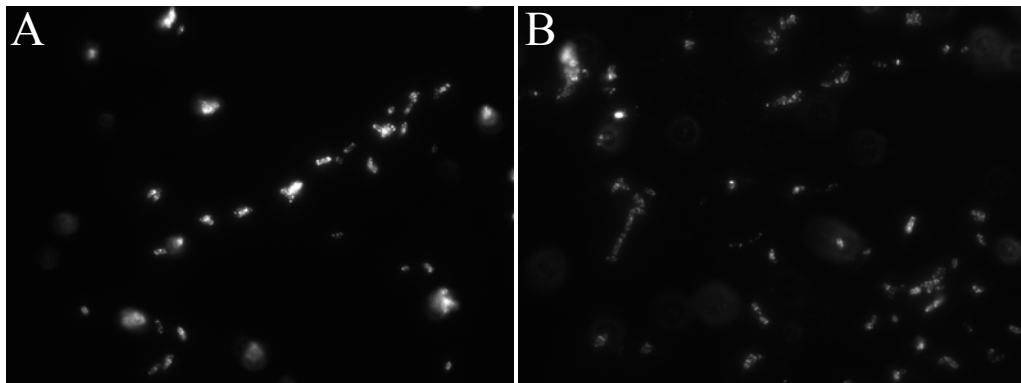


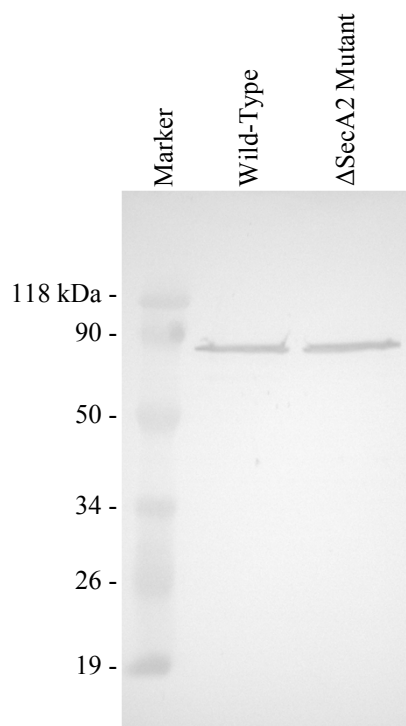
Figure 7-3. IspC secretion is not effected in the $\Delta secA2$ mutant. Immunofluorescence staining of (A) wild-type and (B) $\Delta SecA2$ mutant *L. monocytogenes* cells with an anti-IspC MAb indicates IspC is secreted in each cell type. Cells were probed with M2773, followed by reaction with FITC-conjugated goat anti-mouse IgG. Bacterial cells were visualized with a fluorescence microscope and fluorescent images were captured with an exposure of 3 sec to UV light. Phase contrast images of (C) wild-type and (D) $\Delta SecA2$ deletion mutant are shown to demonstrate the presence of bacteria. (E) Western blot analysis of SDS-extractable surface proteins from wild-type and $\Delta SecA2$ mutant cells using M2773 also indicates that IspC is surface extractable in the mutant.

Wild-type

Δ SecA2 Mutant



E



A previous study, aimed at identifying SecA2- dependent proteins showed that 17 secreted proteins including p60 and NamA that were at least somewhat SecA2-dependent in *L. monocytogenes* serotype 1/2a (143). The results from our current work correlate nicely with these previous findings, since these two proteins (p60 and NamA) were also identified in this study as SecA2-dependent secretory proteins in *L. monocytogenes* LI0521 (serotype 4b). Previous observations suggest that there is a continuum of secreted proteins, including some proteins that are entirely SecA-dependent, some proteins that are entirely SecA2-dependent, and most proteins that are secreted by each of the two systems at different ratios (218). Our study has indicated that p60 and NamA are mostly secreted via the SecA2 pathway, however IspC is mostly SecA2-independent.

IspC is a surface associated protein that has been experimentally demonstrated to have *N*-acetylglucosaminidase activity (222). It is up-regulated during infection and forms an important target of humoral immune response to listerial infection (284). In addition, IspC participates in adhesion to and invasion of non-phagocytic cells by *L. monocytogenes* and helps to facilitate bacterial cell-to-cell spread (276). Earlier studies have demonstrated that some proteins involved in similar processes are secreted in a SecA2-dependent fashion (41, 143, 143). IspC has several functional similarities to SecA2-dependent secretory proteins, however this work demonstrates that IspC shows little dependence on SecA2 for secretion.

Chapter VIII

General Discussion

8.1 General Discussion

L. monocytogenes serotype 4b is an important foodborne pathogen, as this serotype is associated with ~ 40% of listeriosis cases. The principle objective of this work was to identify and characterize novel diagnostic markers for this important serotype by using surface-binding MAbs which are able to react with all *L. monocytogenes* serotype 4b isolates, but not with isolates from other serotypes. Among 15 MAbs with this valuable characteristic, the MAbs M2774, M2775, M2780, M2790 and M2797 were most specific for *L. monocytogenes* serotype 4b and reacted with only 2 of 41 non-serotype 4b isolates tested. The target antigen, isolated by immunoprecipitation, was determined to be a surface autolysin, IspC (275) by MS analysis. Since the intended use of these antibodies is as diagnostic reagents, each MAb was intensively characterized with respect to its affinity and epitope. SPR was successfully used to determine that the affinity of these antibodies ranged from 1.0×10^{-7} to 6.4×10^{-9} M, which is within the biologically useful range for immuno-diagnostic tests. Recombinant fragments of the IspC protein were produced and tested for reaction against each of the MAbs to localize the epitopes, all of which were determined to be located within the C-terminal CBD composed of GW repeats. The CBD is responsible for anchoring IspC to the cell wall PG (276). ELISA analysis of the reactivity of MAbs with various isolates of *L. monocytogenes* suggests that IspC is unique to serotype 4b isolates.

IspC has previously been shown to be involved in virulence (276) and identified as a PG hydrolase (276). In this work, IspC was further shown to be an *N*-acetylglucosaminidase with higher hydrolytic activity on fully *N*-acetylated PG than on de-*N*-acetylated PG. In addition, this work aimed at gaining an understanding of factors which regulate the expression of IspC, has identified a functional promoter directing the transcription of the

ispC gene. Data obtained with a *lacZ* reporter gene under the control of the IspC promoter demonstrated that IspC was expressed, at varying levels, under each stress condition examined here. Immunofluorescence microscopy also revealed the surface expression of IspC in *L. monocytogenes* during growth under stress conditions. While data indicate that IspC expression is regulated by stress, there are sufficient levels of IspC, regardless of growth environment, to allow IspC to be targeted in culture-independent antibody-based detection methods.

The identification IspC as a surface-associated protein that is unique to *L. monocytogenes* serotype 4b has several important implications for diagnostics. The anti-IspC MAbs characterized in this study, are the first to specifically recognize serotype 4b strains. Now specific immunological diagnostic methods, using these MAbs as reagents, can be developed or explored for 4b strains (Figure S9 and Table 4-3). Several studies have shown that expression of some surface protein markers vary greatly in different culture conditions and sometimes are undetectable (93, 135, 136, 178, 178, 179, 179). IspC also appears to be unique, in that its expression was detectable in each of the conditions tested. This indicates that these MAbs may be useful in culture-independent capture and detection of *L. monocytogenes*. New technologies such as the microfluidics-based microchip flow cytometer, currently under development by our group, will be able to use these MAbs in low-cost, robust, reliable and high-throughput detection of *L. monocytogenes* allowing for more extensive bacteriological screening at the sources of food production.

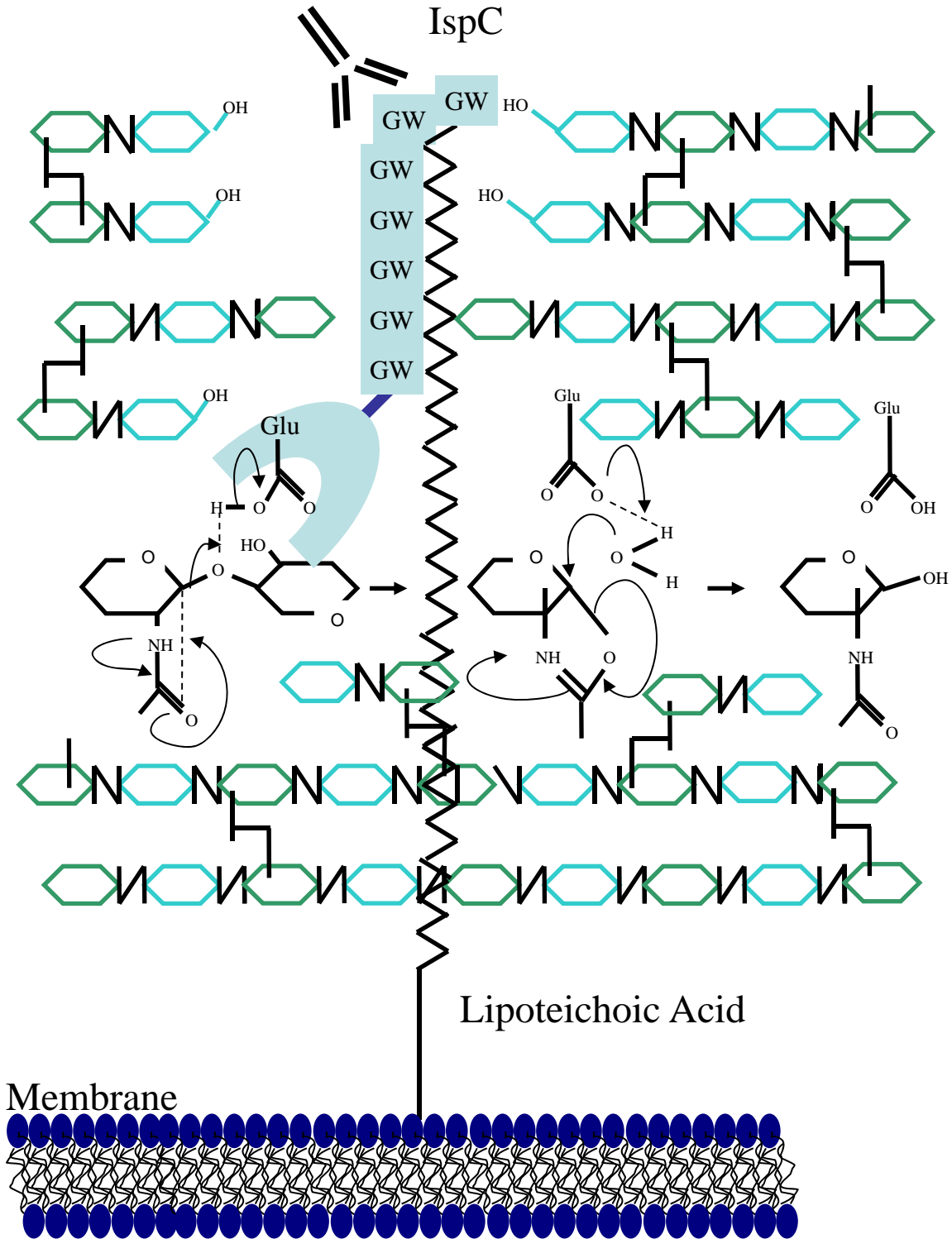
The characterization of MAbs performed in this work has led to the development of a model which explains observations about epitope localization and the ability of MAbs to consistently bind the surface of live cells (Figure 8-1). This model conflicts with the current

Figure 8-1. Proposed model of IspC cell wall association and hydrolytic activity.

This figure depicts the proposed model for the activity of IspC and the nature of its attachment to cell surface of *L. monocytogenes*. Each of the MAb characterized in this study bound to the cell surface of live *L. monocytogenes* cells. The MAbs also have epitopes spanning the length of the GW domain, indicating that each of the GW modules must be exposed at the cell surface. There are two ways in which this would be possible: (i)- that the entire GW domain is bound entirely to the extracellular portion of the LTA or (ii)- that the GW domain is able to diffuse down the length of the LTA molecule while the PG hydrolase domain digests the PG in the immediate vicinity. The latter model fits best with current knowledge of LTA and with the data described in this thesis. A schematic representation of the working hypothesis that IspC participates in substrate assisted catalysis of PG using the *N*-acetyl group as a nucleophile is also shown. This mechanism relies on the formation of an oxazolinium ion intermediate. The figure was modified from Twes et al., (258) and provides an excellent molecular explanation of why IspC favours acetylated PG for hydrolysis. Based on homology to other GH73 enzymes and conservation of the Glu residue in this family of enzymes (37), it is likely that the Glu residue acts as the catalytic residue in this PG hydrolysis reaction.

Anti-IspC MAb

IspC



dogma that the bulk of interaction of the GW module containing CBD with the LTA occurs buried within the PG (19). There are two ways in which the CBD of IspC could remain entirely surface exposed, and available for MAb interaction, while bound to the LTA: (i)- the interaction between the CBD and the LTA could take place entirely on the bacterial surface, providing the PG-hydrolase domain with access only to the outermost PG or (ii)- the CBD may diffuse along the length of the LTA molecule as the PG hydrolase digests PG in the vicinity of the LTA. In the second model, which was first proposed by Bublitz (37), PG hydrolysis occurs along the length of the LTA and creates an elongated pore around the LTA. This pore would likely allow for the interaction of the entire CBD with MAbs. The assumption that PG hydrolysis would allow the exposure of epitopes otherwise hidden by PG is reasonable, since observations from previous studies have shown that epitopes of proteins which are hidden within the PG become available after the PG is digested with mutanolysin (114). IspC has seven GW modules. Increasing the amount of GW modules has been found to increase the proportion of the protein exposed at the cell surface (114). The high number of GW modules found in IspC may provide evidence for the first model, but I favour the second model for the following reason: antibodies produced against LTA have a protective effect against infection, which demonstrates that part of this molecule is surface exposed (251). However, only a very small fraction of the non-lipid portion of the LTA molecule is predicted to extend beyond the cell wall (182). This makes it unlikely that the CBD containing 7 GW modules would be able to interact with a small portion of LTA molecule without creating steric hinderance, which would block some GW modules from MAb interaction.

The nature of the interaction between GW modules and LTA also provides evidence for the second model. Proteins attached to the cell wall through GW modules have been shown to dissociate from the cell wall and are found in the culture media (114). In addition, the covalent interaction between GW modules and LTA can occur spontaneously when proteins containing GW modules are added to a cell suspension (Figure 6-6) (30, 114). I propose that the association / dissociation interaction between the GW modules and LTA could be a mechanism by which IspC diffuses down the length of the LTA molecule. This would occur slowly, as the PG hydrolase domain opens up a pore around the LTA. This model fits nicely with previous observations about the role of IspC in virulence. IspC is not believed to be involved in cell division, since *ΔispC* deletion mutants do not have division defects. However, IspC does seem to play a role in the surface display of some proteins such as InlC2 and ActA (276), and ActA has an essential role in virulence (128). Perhaps, IspC is able to open a pore around the LTA large enough for ActA and other surface proteins to diffuse through, thus, facilitating their surface display.

A model for the substrate assisted catalysis of PG by IspC is also proposed, based on the presence of an oxazolinium ion intermediate (258) in Figure 8-1. The results outlined in Chapter 6 demonstrate that IspC acts as an *N*-acetylglucosaminidase and favours fully *N*-acetylated glucosamine for hydrolysis. A closely related PG hydrolase, also from *L. monocytogenes*, Auto, also displays *N*-acetylglucosaminidase activity and shows the same preference for *N*-acetylated peptidoglycan (37). In Auto, the catalytic acidic proton donor is Glu122 and a second Glu at position 156 is proposed to act as the nucleophile (37). The CAZy (www.cazy.org) database also indicates that members of the GH73 family, including IspC and Auto, use a Glu residue as the catalytic proton donor, while the nucleophile or base

is unknown (52). While both of these residues were demonstrated to be required for enzyme activity (37) and both are conserved in IspC, the model proposed by Bublitz does not adequately explain why these enzymes favour *N*-acetylated PG. Therefore, I favour a hypothetical model in which IspC participates in substrate mediated hydrolysis using the acetyl group as the nucleophile (Figure 8-1). While the data supports this model, experimental proof of substrate assisted hydrolysis by IspC has not yet been observed, and this should be a focus of future work in this area.

Despite the presence of other GW-domain proteins in *L. monocytogenes*, such as InlB, MAbs against the GW-domain containing CBD of IspC appear to be specific for *L. monocytogenes* serotype 4b. There are several possible explanations for this observation. The GW modules from different proteins may not share enough identity to preserve amino acid residues within epitopes critical for binding antibodies, since the GW domains of InlB and IspC share about 34% identity. It is possible that the epitopes for the MAbs are not part of the conserved region. Secondly, it has been demonstrated that portions of the InlB protein are buried in the peptidoglycan, and this may include the GW modules (114) which are therefore not available for interaction with MAbs. Third, like other virulence factors (65), InlB may not be expressed in BHI broth. The specificity of anti-IspC MAbs for *L. monocytogenes* serotype 4b strains, especially M2774, M2775, M2780, M2790 and M2797, are advantageous when designing a serotype 4b specific diagnostic test.

Several conclusions can be drawn from the findings presented in this thesis: (i)- IspC is a surface protein containing epitopes unique to *L. monocytogenes* serotype 4b; (ii)- the MAbs produced in this work, against the C-terminal CBD of IspC, have the potential to be used as reagents in culture-independent diagnostic tests for the 4b strains; (iii)- the *ispC* gene

is always expressed in growth conditions that allow cell division, albeit at varying rates; (iv)- IspC has *N*-acetylglucosaminidase activity, that hydrolyses the β -1,4-glycosidic bond of the PG glycan strand; (v)- IspC is SecA2-independent, and is likely secreted by the SecA system.

8.2 Future Work

The MAbs described in this work have been extensively characterized, however, questions surrounding their use in diagnostics still remain. IspC has been shown to be expressed, at varying levels, in each of the conditions tested that allow for cell division. Is IspC still detectable on the cell surface of viable, but not culturable cells? It is also unknown if IspC is detectable on the cell surface of cells taken directly from various food matrices. These questions will have to be addressed before these MAbs are used in diagnostic tests.

The model proposed in Figure 8-1 for IspC surface association and PG hydrolase activity fits nicely with data presented here, however, it lacks conclusive proof. Does IspC, through the action of its PG hydrolase domain, open up a pore around the LTA? If so, do surface proteins such as ActA also use this pore for secretion? These are both interesting questions for future study. LTA has also been found by several studies to regulate the activity of autolysins – repressing some and activating others (37). Does LTA play a role in the regulation of IspC activity? If it does, is it repressive or stimulatory?

Although the biochemical characterization of IspC led to the identification of the exact bond of PG that is cleaved by IspC, several questions remain. Does *N*-acetylated glucosamine act as a nucleophile in substrate assisted catalysis? Answering this question would have wide ranging implications, as it would likely answer the same question for each of the 1971 enzymes in the GH73 family. Currently, the nucleophile in the hydrolysis reaction mediated by the GH73 family is unknown (52). Bublitz (37) has proposed that Glu-

156 acts as the nucleophile GH73 mediating hydrolysis, since two Glu residues have been shown to be required for hydrolysis. Both Glu residues are also conserved in IspC. If the model proposed in Figure 8-1 for substrate mediated catalysis proves to be correct, what role does the second Glu have in hydrolysis? If substrate-mediated catalysis is not occurring, what role does the *N*-acetylated glucosamine play in regulating the activities of IspC and Auto?

Chapter IX

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Chapter X

Contributions of Collaborators

Chapter 4

Each of the immunizations and the fusions required to generate MAbs were carried out by the Monoclonal Antibody Unit at the Canadian Food Inspection Agency.

Biacore 3000 experiments were carried out in collaboration with Henk van Faassen and Roger MacKenzie at the National Research Council. I made and purified the fAbs as well as the rIspC. The final HPLC purification of the fAbs was carried out by both Henk and I. I measured the concentrations of the fAb after peak fraction collection. Then I calculated and made the serial dilutions of the fAb required for Biacore experiments. Henk and Roger decided on the appropriate approach and concentrations of fAb required for accurate measurement of these particular fAbs. Henk coated the Biacore chip with the IspC protein. Henk programmed the computer and loaded the samples into the Biacore machine. Henk interpreted the biacore data and generated Figure 4-9.

Teela O'Neill and I worked together to prepare each of the formalin killed whole cell samples used in ELISA to determine the range of antibody reactivity.

Chapter 6

Each of the experiments involving RP-HPLC and MALDI-TOF MS or MALDI-PSD MS were carried out by Dr. Ikue Hayashi a senior researcher at the University of Hiroshima and me. Dr. Moto Sugai, the principle investigator of the lab, gave a great deal of guidance in the design of these experiments as well as some assistance with data interpretation. Generally Dr. Hayashi and I would conduct each of the experiments together, taking turns doing different things, so unless directly specified everything we did was a joint effort. However, I made all of the samples (i.e. purified peptidoglycan and purified rIspC), and was responsible for making all of the buffers and doing all of the manual fraction collection. I also did most

of the actual MALDI-TOF MS and Dr. Hayashi did all of the MALDI-PSD. Dr. Hayashi did all of the zip-tip work. Dr. Hayashi figured out most of the mucopeptide structures based on the MALDI-TOF MS data; however, I did a few. Dr. Hayashi also did all of the RP-HPLC experiments and prep-work leading to the generation of Figure 6-3, and Linru Wang prepared the samples for this particular experiment.

Appendix 1

Common Buffers, Solutions, Media and Experimental Protocols

Appendix 1

Common Buffers, Solutions, Media and Experimental Protocols

Common Buffers, Solutions and Media:

SOC Media

Per 100 mL:

Tryptone	2 g
Yeast Extract	0.5 g
NaCl	0.05 g
MQH ₂ O	up to 100 mL

Sterilize this solution by autoclaving. Then add 2 mL of filter sterilized 1 M glucose.

Listeria monocytogenes Defined Media Reagents (modified from (208)):

Salt A 10x

Per 500 mL:

Sodium phosphate dibasic heptahydrate	81.9 g
Monopotassium phosphate	32.8 g

Dissolve in 500 mL of sMQH₂O and autoclave.

Salt B 10x

Per 100 mL:

Magnesium sulphate heptahydrate	0.2 g
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Dissolve in 100 mL of sMQH₂O and autoclave.

Glucose

Per 100 mL:

Glucose	20 g
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Glucose was dissolved in 100 mL of sMQH₂O, filter sterilized and stored at 4°C.

Fructose

Per 25 mL:

Fructose	5 g
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Fructose was dissolved in 100 mL of sMQH₂O, filter sterilized and stored at 4°C.

Mannose

Per 25 mL:

Mannose	5 g
---------	-----

Mannose was dissolved in 100 mL of sMQH₂O, filter sterilized and stored at 4°C.

Biosynthesis Amino Acids 100x

Per 20 mL:

L- leucine	0.2 g
L- isoleucine	0.2 g
L- valine	0.2 g

L- methionine 0.2 g
L- arginine 0.2 g
sMQH₂O 20 mL
Solution was filter sterilized and stored at 4°C.

Cystine 50x

Per 20 mL:
L- cystine 0.1 g
sMQH₂O 20 mL
Solution was filter sterilized and stored at 4°C.

Glutamine 50x

Per 20 mL:
L- glutamine 0.6 g
sMQH₂O 20 mL
Solution was filter sterilized and stored at 4°C.

Additional Amino Acids 100x

Per 20 mL:
L- Histidine 0.2 g
L- Tryptophan 0.2 g
sMQH₂O 20 mL
Solution was filter sterilized and stored at 4°C.

Ferric Citrate 100x

Per 100 mL:
sMQH₂O 100 mL
Ferric Citrate 0.439 g
The sMQH₂O was brought to a boil, and boiling was continued until ferric citrate was dissolved. This was cooled, filter sterilized and stored at 4°C.

Riboflavin 100x

Per 50 mL:
Formic acid 50 mL
Riboflavin 2.5 mg
This was filter sterilized and stored at 4°C.

Essential Vitamins 100x

Per 100 mL:
Thiamine 0.01 g
Biotin 0.005 g
100 x Thioctic Acid in Ethanol 0.1 mL
sMQH₂O 100 mL
This was filter sterilized and stored at 4°C.

***Listeria monocytogenes* Defined Media**

Per 100 mL:

Salt A	10 mL
Salt B	10 mL
Glucose, Fructose or Mannose	4 mL
Biosynthesis Amino Acids	1 mL
Cystine	2 mL
Glutamine	2 mL
Additional Amino Acids	1 mL
Ferric Citrate	1 mL
Riboflavin	1 mL
Essential vitamins	1 mL
sMQH ₂ O	67 mL

Ingredients were combined under sterile conditions and stored at 4°C until use.

25 x PBS

Per 1 L:

Sodium phosphate dibasic (Na ₂ HPO ₄)	27.5 g
Sodium phosphate monobasic monohydrate (NaH ₂ PO ₄ ·H ₂ O)	7.88 g
Sodium chloride (NaCl)	212.5 g
MQH ₂ O	up to 1 L

To make 1 x PBS, 40 mL of 25 x PBS was added to 960 mL MQH₂O.

50 x TAE

Per 1 L:

Tris	242.28 g
Acetic Acid (glacial)	57 mL
EDTA	14.61
MQH ₂ O	up to 1 L

To make 1 x TAE, 20 mL of 50 x TAE was added to 980 mL MQH₂O.

1.5% Agarose Gel

Per small gel:

Agarose	0.375 g
1 x TAE	25 mL

Bring solution to a boil over heat while providing constant agitation. Re-boil until agarose is dissolved. Cool. Add 0.25 µl SYBr Safe DNA stain (Invitrogen).

10 x TE Buffer

Per 1 L:

Tris	12.1 g
0.5 M EDTA	20 mL
MQH ₂ O	up to 1 L

To make 1 x TE buffer, 10 mL of 10 x TE buffer was added to 90 mL of MQH₂O.

4% Stacking Gel

Per 5 mL:

Acrylamide/bis (30%)	0.65 mL
0.5 M Tris-HCl pH 6.8	1.25 mL

sMQH ₂ O	3.05 mL
10% (w/v) SDS	50 µl
10% (w/v) APS	50 µl
TEMED	5 µl

12% Separating Gel

Per 10 mL:

Acrylamide/bis (30%)	4 mL
1.5 M Tris-HCl pH 8.8	2.5 mL
sMQH ₂ O	3.35 mL
10% (w/v) SDS	100 µl
10% (w/v) APS	50 µl
TEMED	5 µl

20% Separating Gel

Per 10 mL:

Acrylamide/bis (30%)	6.7 mL
1.5 M Tris-HCl pH 8.8	2.5 mL
sMQH ₂ O	0.65 mL
10% (w/v) SDS	100 µl
10% (w/v) APS	50 µl
TEMED	5 µl

2x SDS-PAGE Loading Buffer

Per 10 mL:

0.5 M Tris-HCl, pH6.8	2 mL
Glycerol	4 mL
β-Mercaptoethanol	2 mL
SDS	0.4 g
0.5% (w/v) Bromophenol Blue	400 µl
MQH ₂ O	1.6 mL

Store at -20°C.

10 x SDS-PAGE Running Buffer

Per 1 L:

Tris	30 g
Glycine	144 g
SDS	10 g
MQH ₂ O	up to 1 L

To make 1 x SDS-PAGE Running Buffer, 100 mL of 10 x SDS-PAGE Running Buffer was added to 900 mL sMQH₂O.

Protein Transfer Buffer

Per 1 L:

Tris	5.82 g
Glycine	2.93
MQH ₂ O	up to 1 L

Ponceau S

Per 100 mL:

Ponceau S	1 g
Acetic Acid (glacial)	100 mL

Coomassie Blue Stain

Per 100 mL:

Coomassie Brilliant Blue	0.5 g
Methanol	40 mL
Acetic acid (glacial)	10 mL

Make volume up to 100 mL with MQH₂O.**Coomassie Blue Destain soluton**

Per 100 mL:

Methanol	40 mL
Acetic acid (glacial)	10 mL

Make volume up to 100 mL with MQH₂O.**PBS-T**

Per 10 L:

25 x PBS	400 mL
Tween 20	5 mL
MQH ₂ O	9595 mL

PBS-TT

Per 10 L:

25 x PBS	400 mL
Tween 20	5 mL
Triton X-100	20 mL
MQH ₂ O	9575 mL

Wash Buffer A (Immobilized Metal Chelate Affinity Chromatography)

Per 1 L:

Sodium Phosphate monobasic	6.9 g
Sodium Chloride	17.4 g
Imidazole	1.36 g
MQH ₂ O	~500 mL

Adjust the pH to 8.0 using sodium hydroxide. Make volume up to 1 L by adding MQH₂O.**Wash Buffer B (Immobilized Metal Chelate Affinity Chromatography)**

Per 1 L:

Sodium Phosphate monobasic	6.9 g
Imidazole	17.0 g
MQH ₂ O	~500 mL

Adjust the pH to 8.0 using sodium hydroxide. Make volume up to 1 L by adding MQH₂O.

Wash Buffer C (Cation Exchange Chromatography)

Per 1 L:

pH 8.0 0.1M Phosphate buffer	50 mL
Glycerol	50 mL
MQH ₂ O	~900 mL

Wash Buffer C + 1 M NaCl (Cation Exchange Chromatography)

Per 500 mL:

Wash buffer C	500 mL
Sodium chloride	29.22 g

Buffer A (RP-HPLC)

Per 900 mL:

0.85 M Sodium Hydroxide	855 mL
Methanol	45 mL

The pH was buffered to 2.50 using hydro phosphoric acid.

Buffer B (RP-HPLC)

Per 300 mL:

0.85 M Sodium Hydroxide	210 mL
Methanol	90 mL

The pH was buffered to 2.80 using phosphoric acid.

Sucrose Electroporation Buffer

Per 500 mL:

HEPES	0.119 g
Sucrose	85.575 g
MQH ₂ O	~ 450 mL

Adjust pH to 7.0 and make volume up to 500 mL with MQH₂O. Immediately sterilize by autoclaving.

Carbonate buffer

Per 1 L:

Sodium Bicarbonate	3.8 g
Sodium Carbonate	1.93 g
MQH ₂ O	~ 950 mL

Adjust pH to 9.6 and make volume up to 1 L with MQH₂O.

Citrate Buffer

Per 1 L:

Citric Acid	4.6 g
Tri-Sodium Citrate	7.65 g
MQH ₂ O	~ 950 mL

Adjust pH to 4.5 and make volume up to 1 L with MQH₂O.

ABTS (2,2-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid))

For 10 mL measure the weight of ABTS as close to 0.2195 g as possible and then calculate the volume of water based on $V \text{ (mL)} = W \times (10/0.2195)$. Add required MQH₂O.

ELISA Substrate Solution

Per ~10 mL:

ABTS solution	0.5 mL
3% (v/v) Hydrogen Peroxide	0.1 mL
Citrate Buffer	10 mL

PG Digestion Citrate Buffer

Per 50 mL:

Sodium Citrate	0.129 g
Magnesium Chloride	0.129 g
Distilled water	~20 mL

The pH was buffered to 7.0 and then the volume was made up to 50 mL by adding distilled water.

PG Digestion Phosphate Buffer

Per 50 mL

Sodium phosphate monobasic	add 27.8 g to 1 L distilled water
Sodium phosphate dibasic septa hydrate	add 53.7 g to 1 L distilled water

Add 7.0 mL of sodium phosphate monobasic to 93 mL of sodium phosphate dibasic. If needed buffer to 7.96 with hydro phosphoric acid. Make volume up to 200 mL distilled water. Before use dilute original solution 1:10.

Re-naturation buffer

Per 1 L:

Tris	3.02 g
Triton X -100	1 mL

The pH was buffered to 7.5 and the volume was made up to 1 L with MQH₂O.

Silver Staining Reagents:**Fixing solution**

Per 100 mL:

Ethanol	50 mL
Acetic Acid (glacial)	5 mL
MQH ₂ O	45 mL

Wash solution

Per 100 mL:

Ethanol	50 mL
MQH ₂ O	50 mL

Sensitizer

Per 100 mL		
	Sodium thiosulfate	0.02 g
	MQH ₂ O	100 mL

Stain

Per 100 mL		
	Silver nitrate	0.1 g
	MQH ₂ O	100 mL

Developer

Per 100 mL		
	Formalin (37% formaldehyde)	0.04 mL
	Sodium Carbonate	2 g
	MQH ₂ O	up to 100 mL

Stop solution

Per 100 mL		
	Acetic Acid (glacial)	5 mL
	MQH ₂ O	95 mL

Storage solution

Per 100 mL		
	Acetic Acid (glacial)	1 mL
	MQH ₂ O	99 mL

DEPC- Treated water

Per 1 L:		
	Sterile MQH ₂ O	1 L
	DEPC	1 mL

Add DEPC to MQH₂O and incubate at 37°C overnight. Autoclave to remove DEPC.

Experimental Protocols:

Protein G Affinity Chromatography:

1. Set the range of the Econo Recorder (Bio-Rad) to 0.2 or 0.5 and the chart speed to 12 cm/hr.
2. Clean all tubing attached to the Econo Gradient Pump (Bio-Rad) with fresh MQH₂O and then 1 x PBS.
3. Attach the Protein G Sepharose (GE Healthcare) column to the correct tubing.
4. Equilibrate the column with 20 mL 1 x PBS.
5. Near the end of the equilibration press and hold the Auto Zero the Econo Recorder until it is zeroed.
6. Change the valve to the loading position.
7. Load the TCF containing the antibody at a rate of 0.5 mL/min.
8. Change the valve to the wash position. Wash the column with 20 mL 1 x PBS at a flow rate of 1 mL/min.

9. Place 20 glass tubes in the fraction collector (Bio-Rad). Set the fraction size to 1 mL.
10. Elute the antibodies with 20 mL 100 mM Glycine-HCl pH 2.5 at a rate of 1 mL/min.
11. Add 140 μ l 1 M Tris-HCl (pH 9.1) to neutralize each fraction and confirm neutralization using pH paper.
12. Pool fractions containing antibodies and place on ice.
13. Estimate the concentration using OD at 278 nm with the absorption coefficient $1.35 \text{ A.mL.mg}^{-1}$.
14. Store at 4°C.

Immobilized Metal Chelate Affinity Chromatography:

1. Lyse *E. coli* cells with the French Press at 1500 psi.
2. Centrifuge the homogenate at 27 000 x g for 20 min at 4°C.
3. Store supernatant on ice. Discard pellet.
4. Set the range of the Econo Recorder (Bio-Rad) to 0.5 or 1 and the chart speed to 12 cm/hr.
5. Clean all tubing attached to the Econo Gradient Pump (Bio-Rad) with fresh MQH₂O and then Wash Buffer A.
6. Attach the column (Qiagen) to the correct tubing.
7. Equilibrate the column with 25 mL of Wash Buffer A.
8. Near the end of the equilibration press and hold the Auto Zero the Econo Recorder until it is zeroed.
9. Change the valve to the loading position.
10. Load the protein sample at a rate of 0.5 mL/min.
11. Wash the column with 30 mL of Wash Buffer A (or until Econo Reader shows the flow-through is back to base-line absorbance).
12. Place 20 glass tubes in the fraction collector (Bio-Rad). Set the fraction size to 1 mL.
13. Elute the recombinant protein with 25 mL Wash Buffer B at 1 mL/min.
14. Store all the collected fractions at 4°C.
15. Analyze each fraction SDS-PAGE followed by Western Blotting with an Anti-His antibody.

RP-HPLC:

1. Attach column (Hypersil ODS)
2. Set column temperature at 45°C.
3. Program column to run 10 mL of Buffer A prior to injection of sample.
4. Inject sample.
5. Run a linear gradient at a flow rate of 0.5 mL per minute of 100% Buffer A to a 100% Buffer B over 200 minutes.
6. Manually collect peak fractions as they elute.

Size Exclusion Chromatography:

1. Attach column (SuperDex-200 or SuperDex-75 (GE Healthcare))

2. Program computer to run at 1 mL/min and equilibrate with 5 mL buffer before sample injection, have a fraction size of 1 mL, empty the loop with 10 mL buffer, wash the column with 2 column volume total, clean column with 10 mL buffer.
3. Inject sample
4. Run program.
5. Fractions are collected by fraction collector.

Cation Exchange Chromatography:

1. Pack a column with SP sepharose Fast Flow.
2. Set the range of the Econo Recorder (Bio-Rad) to 0.5 or 1 and the chart speed to 12 cm/hr.
3. Clean the tubing of the Econo gradient pump with water then Buffer C.
4. Attach the column to the gradient pump and equilibrate with 60 mL Buffer C.
5. Near the end of the equilibration press and hold the Auto Zero the Econo Recorder until it is zeroed.
6. Change the valve to the loading position.
7. Load the protein sample at a rate of 0.5 mL/min.
8. Wash the column with 30 mL of Wash Buffer C (or until Econo Reader shows the flow-through is back to base-line absorbance).
9. Place 40 glass tubes in the fraction collector (Bio-Rad). Set the fraction size to 1 mL.
10. Elute the protein using a 40 mL linear gradient starting with 100% Buffer C and ending with 50% Buffer C + 1 M NaCl.
11. Collect fractions.
12. Store all the collected fractions at 4°C.
13. Clean the column with 30 mL of 100 % Buffer C + 1M NaCl.
14. Analyze each fraction SDS-PAGE followed by Western Blotting with an Anti-His antibody

Bio-Rad Protein Assay Protocol:

1. Warm up the Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad) to room temperature.
2. Prepare BSA standards (0 µg/mL, 1 µg/mL, 2.5 µg/mL, 5 µg/mL, 7.5 µg/mL, 10 µg/mL, 12 µg/mL), to a total volume of 800 µl.
3. Dilute protein samples to be measured (dilution factor will depend on protein concentration), to a total volume of 800 µl.
4. Add 200 µl of dye to each standard and sample.
5. Incubate for 10 min at room temperature.
6. Blank the spectrophotometer with 0 µg/mL BSA and measure the absorbance of each sample at 595 nm.
7. Generate a graph of absorbance 595 nm vs concentration µg/mL of the standards.
8. Perform a linear regression to determine $x = (y-b)/m$ and solve for x.
9. Based on the dilution in step 3, use the formula $C_1V_1=C_2V_2$ to calculate the initial sample protein concentration.

Extraction of *L. monocytogenes* genomic DNA Protocol:

1. Inoculate *L. monocytogenes* in 2 mL of BHI broth and grow overnight at 37°C.

2. Place 1 mL of culture into a 1.5 mL tube and centrifuge at 16 100 x g for 2 min.
3. Re-suspend the cells in 100 µl 1x TE buffer.
4. Add 6 µl of 50 mg/mL lysozyme and digest at 37°C for 30 min.
5. Add 500 µl DNAzol (Invitrogen) and mix.
6. Add 250 µl of ethanol and invert 50 times.
7. Centrifuge at 16 100 x g for 1 min and discard the supernatant.
8. Wash pellet twice with 750 µl of 75% (v/v) ethanol.
9. Briefly air-dry the pellet.
10. Dissolve the pellet in MQH₂O.
11. Store at -20°C.

QIA MINIPREP Protocol:

1. Grow cells containing plasmid overnight on media with appropriate antibiotics.
2. Spin down 5 mL of cells.
3. Re-suspend the pellet in 250 µl P1 buffer.
4. Add 250 µl of P2 buffer and gently invert the tube 4-6 times.
5. Add 350 µl of P3 buffer and invert the tube 4-6 times.
6. Centrifuge at 16 100 x g for 10 min.
7. Apply the supernatant to the supplied spin column by pipetting.
8. Centrifuge at 16 100 x g for 60 s, discard the flow-through, add remaining supernatant and centrifuge at 16 100 x g. Discard the flow-through.
9. Add 500 µl PB buffer to the spin column, centrifuge at 16 100 x g for 60 s and discard the flow-through.
10. Add 750 µl of PE buffer to the spin column, centrifuge at 16 100 x g for 60 s and discard the flow-through.
11. Centrifuge at 16 100 x g for 60 s.
12. Place the spin column in a clean 1.5 mL tube.
13. Add 50 µl of EB buffer to the centre of the spin column membrane and let stand for 60 s.
14. Centrifuge at 16 100 x g for 60 s.
15. Store plasmid at -20°C until use.

Preparation of CaCl₂ Competent *E. coli* cells Protocol:

1. Grow *E. coli* in 5 mL of LB broth overnight.
2. Subculture at a 1/100 dilution in 40 mL LB broth and incubate at 37°C until the OD at 600 nm reaches 0.4 ± 0.1.
3. Chill the culture on ice for 10 min.
4. Centrifuge cells at 2700 x g and 4°C for 10 min.
5. Discard the supernatant.
6. Re-suspend the pellet in 24 mL of cold 80 mM MgCl₂ – 20 mM CaCl₂ and agitate gently on ice for 20 min.
7. Centrifuge at 2700 x at 4°C for 10 min.
8. Discard the supernatant.
9. Resuspend the pellet in 1.6 mL ice cold 0.1 M CaCl₂ in 10% (v/v) glycerol and agitate until re-suspended.
10. Dispense into 50 µl aliquots.
11. Chill aliquots at -20°C overnight and then store at -80°C.

Transformation of CaCl₂ Competent *E. coli* cells by Heat Shock Protocol:

1. Add 35-50 μ l of competent cells to a 1.5 mL tube.
2. Add 5-10 μ l of ligation reaction or 2 μ l of un-cut plasmid DNA to the tube.
3. Mix.
4. Incubate on ice for 30 min.
5. Place tube in 42°C water bath for 45 s.
6. Incubate on ice for 2 min.
7. Add 250 μ l SOC media to the tube.
8. Incubate with agitation at 37°C for 1 hr.
9. Make a 1:100 dilution of 2.5 μ l culture in 223.5 μ l LB broth and plate on an LB agar plate.
10. Plate the rest of the culture on an LB agar plate containing the correct antibiotics.
11. Incubate the plates overnight at 37°C.

Preparation of Electroporation Competent *L. monocytogenes* cells Protocol:

1. Inoculate LB broth with *L. monocytogenes* and culture at 37°C overnight.
2. Subculture at a 1:10 dilution in LB broth containing 0.5 M sucrose, until the OD at 600 nm is \sim 0.2.
3. Add penicillin G to a final concentration of 10 μ g/mL.
4. Incubate at 37°C for 2.5 hrs.
5. Calculate the cell concentration using a ratio of an OD at 620 of 0.61 is equal to 1×10^9 cells/mL.
6. Centrifuge the culture at 8000 x g at 4°C for 10 min.
7. Wash with sucrose electroporation buffer three times.
8. Re-suspend bacteria in sucrose electroporation buffer to a concentration of 1×10^{11} cells/mL.
9. Add sterile glycerol to a final concentration of 15% (v/v) and store cells at -20°C for future use.

Transformation of Electroporation Competent *L. monocytogenes* cells by Electroporation Protocol:

1. Remove electroporation cuvette (VWR) from the package and place on ice.
2. Add 50 μ l of electroporation competent *L. monocytogenes* cells to the electroporation cuvette.
3. Add 1-2 μ l of ligation reaction, or plasmid DNA to the electroporation cuvette.
4. Mix well.
5. Let the mixture sit on ice for 5 min.
6. Insert cuvette into the white slide and slide into the chamber until the cuvette completes the circuit between the two contacts at the base of the chamber.
7. Turn on the pulser (Bio-Rad).
8. Set the pulser to 2.5 kV.
9. Hold both the rectangular red pulse buttons until a continuous tone sounds. Release the buttons immediately.
10. Remove the cuvette and rapidly add 500 μ l SOC media to the cells, then place on ice.
11. Transfer the cells to a 1.5 mL tube.

12. Incubate the cells at 37°C with constant agitation for 1 hr.
13. Plate the cells on LB agar containing the appropriate antibiotics to select for the plasmid.
14. Incubate the plates at 37°C overnight.

Generation of *L. monocytogenes* mutants with a plasmid derivative of pAUL-A

1. After the pAUL-A plasmid has been prepared in a DH5 α cloning host the Bio-Rad *E. coli* pulser is was set up in the level 2 room.
2. A 0.2 cm electroporation cuvette was cooled on ice. The cuvette holder was also pre-cooled in the freezer.
3. 1 μ l of pAUL was added to 50 μ l of electroporation competent *L. monocytogenes* cells. This was held on ice for 1 minute.
4. The pulser was set to 2.5 kV. To charge the capacitor and release the pulse both of the rectangular red pulse buttons were depressed and held until a continuous tone was sounded. The buttons were released.
5. 1 mL of SOC media was immediately added to the cells.
6. The actual pulsing parameters were recorded.
7. The culture was added to a 5 mL culture tube and incubated at 30°C for 3 hours with gentle shaking.
8. The culture was divided into 4 250 μ l aliquots. Each was plated on a LB agar plate containing 5 μ g/ mL erythromycin.
9. The plates were incubated at 30°C for 3 to 6 days, or until colonies appeared (in both experiments around day 3 to 4).
10. 5 colonies were selected and streaked onto new LB agar plates containing 5 μ g/ mL ethromycin and to inoculate 5 mL LB broth cultures containing 5 μ g/ mL erythromycin. Both were incubated at 30°C overnight.
11. Cells were isolated from the liquid culture and plasmid DNA was extracted from them using standard procedures.
12. PCR was performed using the isolated plasmid as the template and the M13 F and M13 R primers.
13. Colonies which contained plasmids with the expected insert were streaked onto a fresh LB agar plate containing 5 μ g/ mL erythromycin and this was incubated at 42°C for 48 hours to encourage plasmid integration.
14. A singly colony was streaked onto a fresh LB agar plate containing 5 μ g/ mL erythromycin. This was further incubated at 42°C overnight.
15. A single colony was streaked onto a fresh LB plate containing 5 μ g/ mL erythromycin. This was further incubated at 42°C overnight.
16. A colony from this plate was used to inoculate 20 mL LB broth (without erythromycin) and this was incubated overnight at 30°C to encourage excision.
17. This culture was diluted (1:100) into 40 mL LB broth (without erythromycin) and this was incubated at 30°C overnight.
18. This culture was diluted (1:100) into 40 mL LB broth (without erythromycin) and this was incubated at 42°C overnight.
19. Cells were divided into 100 μ l aliquots and plated onto LB Agar plates (10 plates) and incubated at 37°C.

20. Each colony (a total of 25) was spotted onto two fresh LB agar plates, one containing 5 µg/ mL erythromycin and one without erythromycin. These plates were incubated at 30°C overnight.
21. Erythromycin sensitive colonies were used to inoculate one of each 5 mL LB broth and streaked onto an LB agar plate. These were each incubated at 37°C overnight.
22. Genomic DNA was extracted from cells in LB broth culture.
23. The LB agar plates were stored at 4°C.
24. The genomic DNA was analyzed by PCR using primers internal and external to the deletion region to determine which colonies contained a successful deletion.
25. Colonies which were identified by PCR to have a successful deletion were sent for sequencing to confirm
26. Successful mutants were stored in 15% glycerol at -80°C.

General PCR Master Mix:

Per Reaction :

sMQH ₂ O	39 µl
10 x Reaction Buffer	5 µl
dNTP (25 mM)	2 µl
Forward Primer	1 µl
Reverse Primer	1 µl
Template	1 µl
pFU (homemade)	1 µl

General PCR Program (times may have been adjusted for individual PCRs):

Purpose	Temperature	Time	Cycles
Denaturation	94°C	2 min	1
Denaturation	94°C	30 s	30
Annealing	58°C	45 s	
Elongation	68°C	1.5 min	
Final Elongation	68°C	7 min	1
Cooling	4°C	Loop	

RE-Digestion Reactions:

Per Reaction:

Plasmid/Amplicon DNA	40 µl
Restriction Enzyme 1	1 µl
Restriction Enzyme 2	1 µl
Buffer (NEB 1, 2, 3, or 4)	6 µl
sMQH ₂ O	12 µl

This solution was mixed by pipetting and incubated at the appropriate temperature based on the Restriction Enzymes for the appropriate amount of time based on DNA concentration.

Ligation reactions:

Per Reaction:

RE-Digested plasmid	8 μ l
RE-Digested amplicon	8 μ l
Ligation buffer (10x)	2 μ l
DNA ligase (NEB)	2 μ l

This solution was mixed by pipetting and incubated at 16°C overnight.

5' RACE Reverse Transcription Mixture:

Per Reaction:

cDNA synthesis buffer (kit)	4 μ l
deoxynucleotide mixture (kit)	2 μ l
cDNA synthesis primer	1 μ l
RNA	10 μ l (1.01 μ g)
Neo1 / control primer (kit)	1 μ l
control RNA (kit)	1 μ l
reverse transcriptase (kit)	1 μ l

5' RACE Poly A Tailing Reaction Mixture:

Per Reaction:

purified cDNA	19 μ l
reaction buffer (kit)	2.5 μ l
2 mM dATP	2.5 μ l
terminal transferase (kit)	1 μ l

5' RACE GSP1 PCR Mixture:

Per Reaction:

dA-tailed cDNA	5 μ l
oligo dT-anchor primer (kit)	1 μ l
GSP1	1 μ l
dNTP mixture	1 μ l
Taq DNAp	0.75 μ l
10 x reaction buffer	5 μ l
MQH ₂ O	36.25 μ l

5' RACE GSP2 PCR Mixture:

Per Reaction:

GSP1 PCR product	1 μ l
oligo dT-anchor anchor primer (kit)	1 μ l
GSP 2	1 μ l
dNTP mixture	1 μ l
Taq DNAp	0.75 μ l
10 x reaction buffer	5 μ l

sMQH₂O

40.25 µl

5' RACE Nested PCR Cycle:

Purpose	Temperature	Time	Cycles
Denaturation	94°C	2 min	1
Denaturation Annealing Elongation	94°C 58°C 68°C	10 s 30 s 1 min	10
Denaturation Annealing Elongation	94°C 58°C 68°C	15 s 30 s 2 min	20
Final Elongation	68°C	7 min	1
Cooling	4°C	Loop	

High Pure PCR Product Purification Kit Protocol:

1. 100 µl of binding buffer was added to the first-strand cDNA reaction and mixed by inversion.
2. The solution was added to the column provided in the kit.
3. This was centrifuged for 30 sec at 6000 x g. The flow through was discarded.
4. 500 µl of wash buffer was added to the column.
5. This was centrifuged for 30 sec at 6000 x g. The flow through was discarded.
6. 200 µl of wash buffer was added to the column.
7. This was centrifuged at 13 000 x g for 2 min. The flow through was discarded.
8. The column was placed in a sterile tube and 50 µl elution buffer was added directly on the filter.
9. This was centrifuged at 6000 x g for 30 sec.
10. Assess the purity of the product using both spectrophotometric measurements and by an agarose gel.

Silver Staining Procedure:

1. The gel was placed in fixer solution for 30 min.
2. It was then washed in the wash buffer for 10 min.
3. The gel was washed twice for 10 min water.
4. Then the gel was placed in the staining solution for 30 min, before being washed for 1 min in water.
5. A small amount of develop was added to the gel and swirled briefly then discarded.
6. More development buffer was added until protein bands appeared.
7. Then the gel was incubated for 5 min in stop solution.
8. The gel was stored in storage solution at 4°C.

Peptidoglycan purification:

1. Overnight cultures were diluted 1:100 in BHI broth and incubated at 37°C to mid-log phase.
2. Bacteria were collected at 3500 x g at 4°C for 10 min.
3. The pellet was re-suspended in 50 mL 4% (w/v) SDS and boiled for 30 min.
4. The suspension was centrifuged at 10 000 x g at room temperature for 20 min.
5. The pellet was washed 6 x with water.
6. The pellet was frozen at -80°C overnight.
7. These cells were broken by 3 passages through the french press at 1500 psi.
8. Unbroken cells were removed by centrifuging at 3000 x g for 5 min.
9. The supernatant was further centrifuged at 10 000 x g for 20 min.
10. The pellet was re-suspended in 13 mL of 100 mM Tris HCl (pH 7.5) and 0.7 mg α - amylase at 37°C for 2 hr.
11. 20 mM magnesium sulphate, 100 μ g DNase I and 500 μ g RNaseA were added and incubated at 37°C for 1 hr.
12. 10 mM calcium chloride and 1 mg trypsin were added and this was further incubated at 37°C for overnight.
13. SDS 1% (w/v) was added and this was boiled for 15 min.
14. The solution was centrifuged at 10 000 x g for 20 min.
15. The pellet was washed 2 x in water, 1x in 8 M LiCl and then 3 x in water.
16. The pellet was re-suspended in 10 mL 49% (v/v) hydrofluoric acid.
17. This was incubated at room temperature for 48 hrs.
18. The solution was centrifuged at 10 000 x g for 20 minutes and washed 3 x times in water.
19. The pellet was further neutralized with 100 mM Tris-HCl (pH 7.5) and washed 2 times in water.
20. The pellet was then suspended in 10 mL NEB Buffer 3 and 50 units of alkaline phosphatase was added. This was incubated at 37°C overnight.
21. The solution was boiled for 5 min to inactivate enzymes.
22. It was centrifuged at 10 000 x g for 20 min and washed 3 times with water.
23. The pellet was frozen overnight at -80°C.
24. The pellet was then lyophilized for 2 days.

Appendix 2

Supplementary Figures

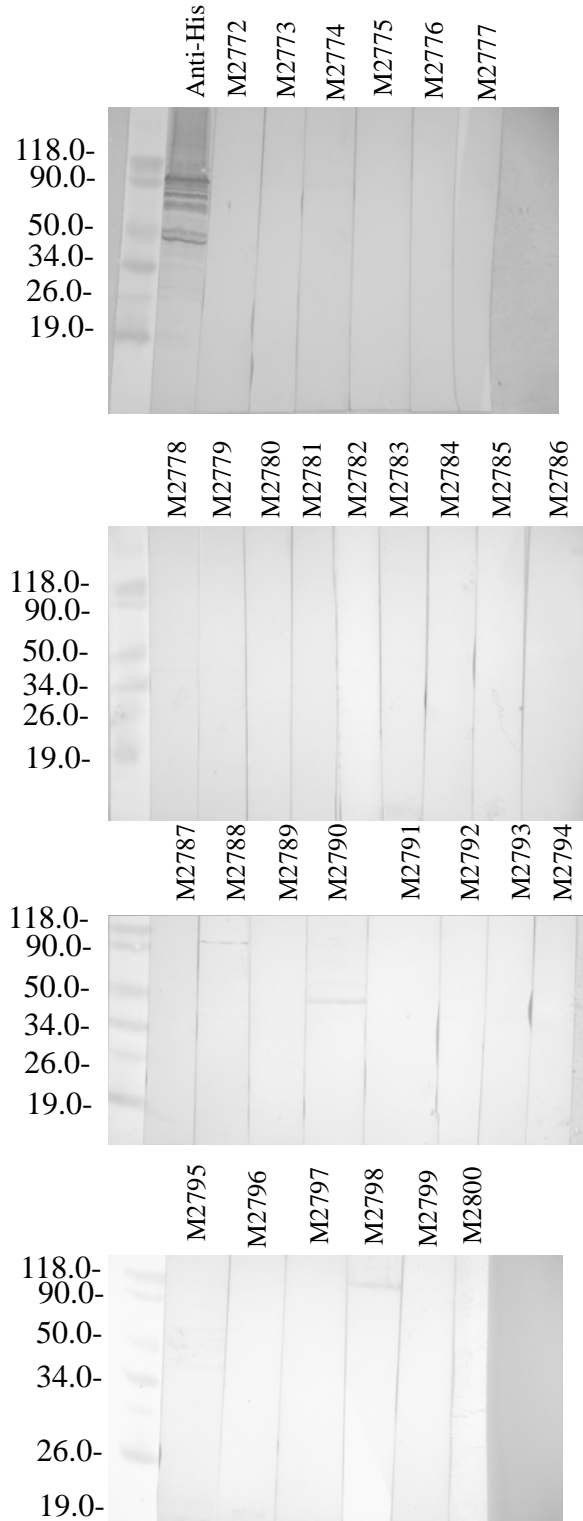


Figure S1. Western Blot of Monoclonal Antibodies with Recombinant IspC Fragment AA 1-197. The reaction of each of 29 MABs was analyzed by Western blot with a His-tagged IspC recombinant fragment AA 1-197. No reaction was observed for any of the 29 MABs. A very strong reaction was observed with the anti-his antibody, indicating ample protein expression.

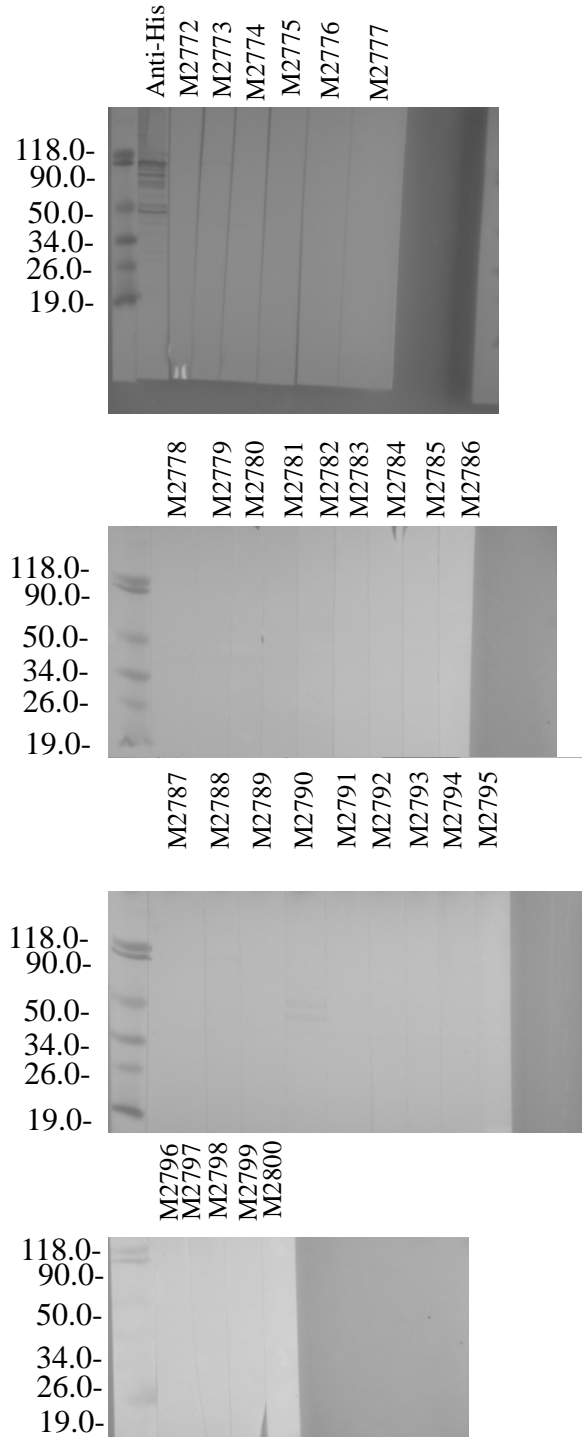


Figure S2. Western Blot of Monoclonal Antibodies with Recombinant IspC Fragment AA 58-197. The reaction of each of 29 MAbs was analyzed by Western blot with a His-tagged IspC recombinant fragment AA 58-197. No reaction was observed for any of the 29 MAbs. A very strong reaction was observed with the anti-his antibody, indicating ample protein expression.

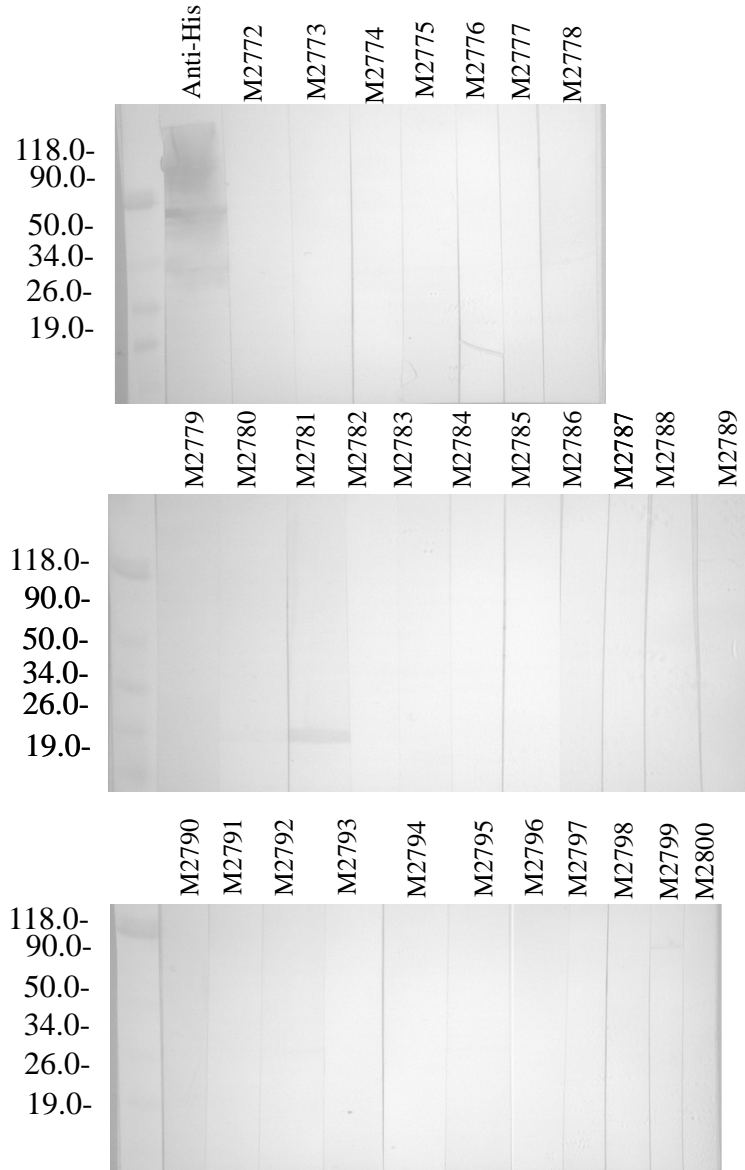


Figure S3. Western Blot of Monoclonal Antibodies with Recombinant IspC Fragment AA 58-263. The reaction of each of 29 MABs was analyzed by Western blot with a His-tagged IspC recombinant fragment AA 58-263. No reaction was observed for any of the 29 MABs. A very strong reaction was observed with the anti-his antibody, indicating ample protein expression.

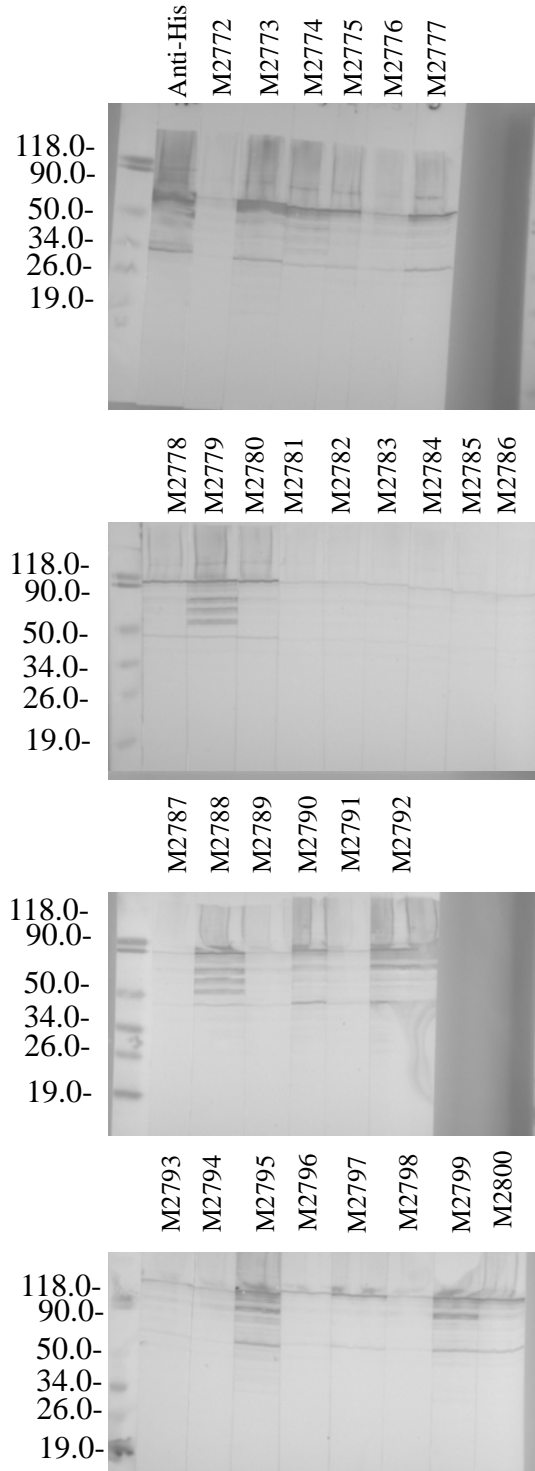


Figure S4. Western Blot of Monoclonal Antibodies with Recombinant IspC Fragment AA 198-774. The reaction of each of 29 MAbs was analyzed by Western blot with a His-tagged IspC recombinant fragment AA 198-774. A very strong reaction was observed with the anti-his antibody, indicating ample protein expression. A strong reaction was also observed for each of the antibodies that react to full length IspC.

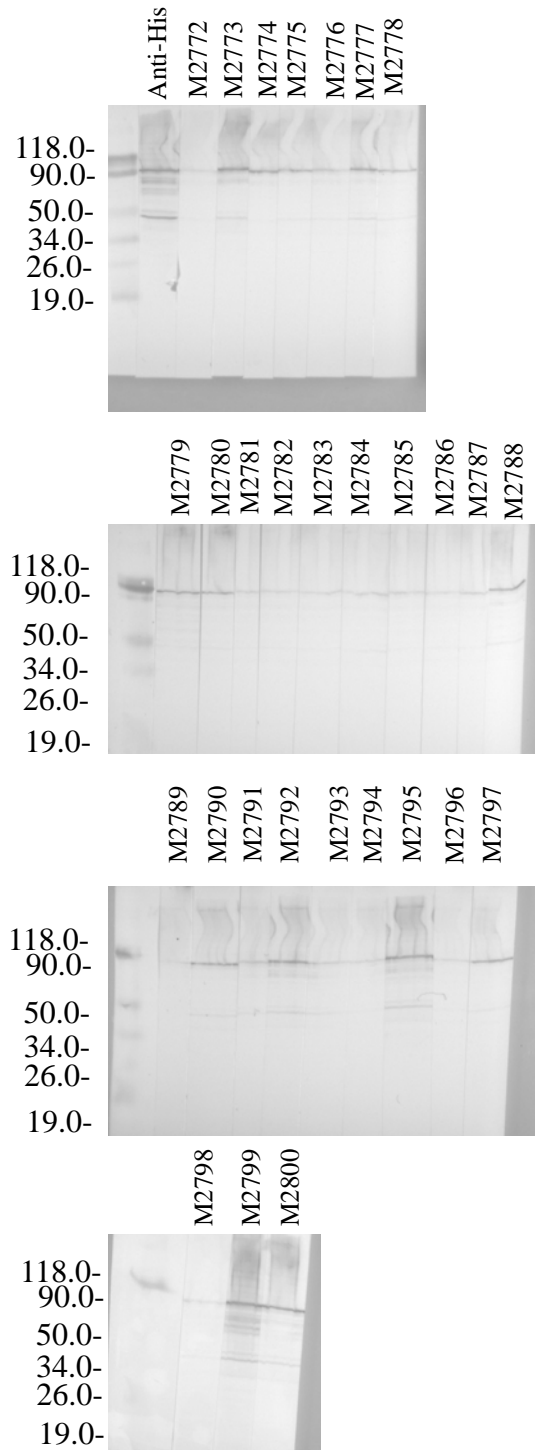


Figure S5. Western Blot of Monoclonal Antibodies with Recombinant IspC Fragment AA 264-774. The reaction of each of 29 MAbs was analyzed by Western blot with a His-tagged IspC recombinant fragment AA 264-197. A very strong reaction was observed with the anti-his antibody, indicating ample protein expression. A strong reaction was also observed with each of the antibodies that react with recombinant IspC.

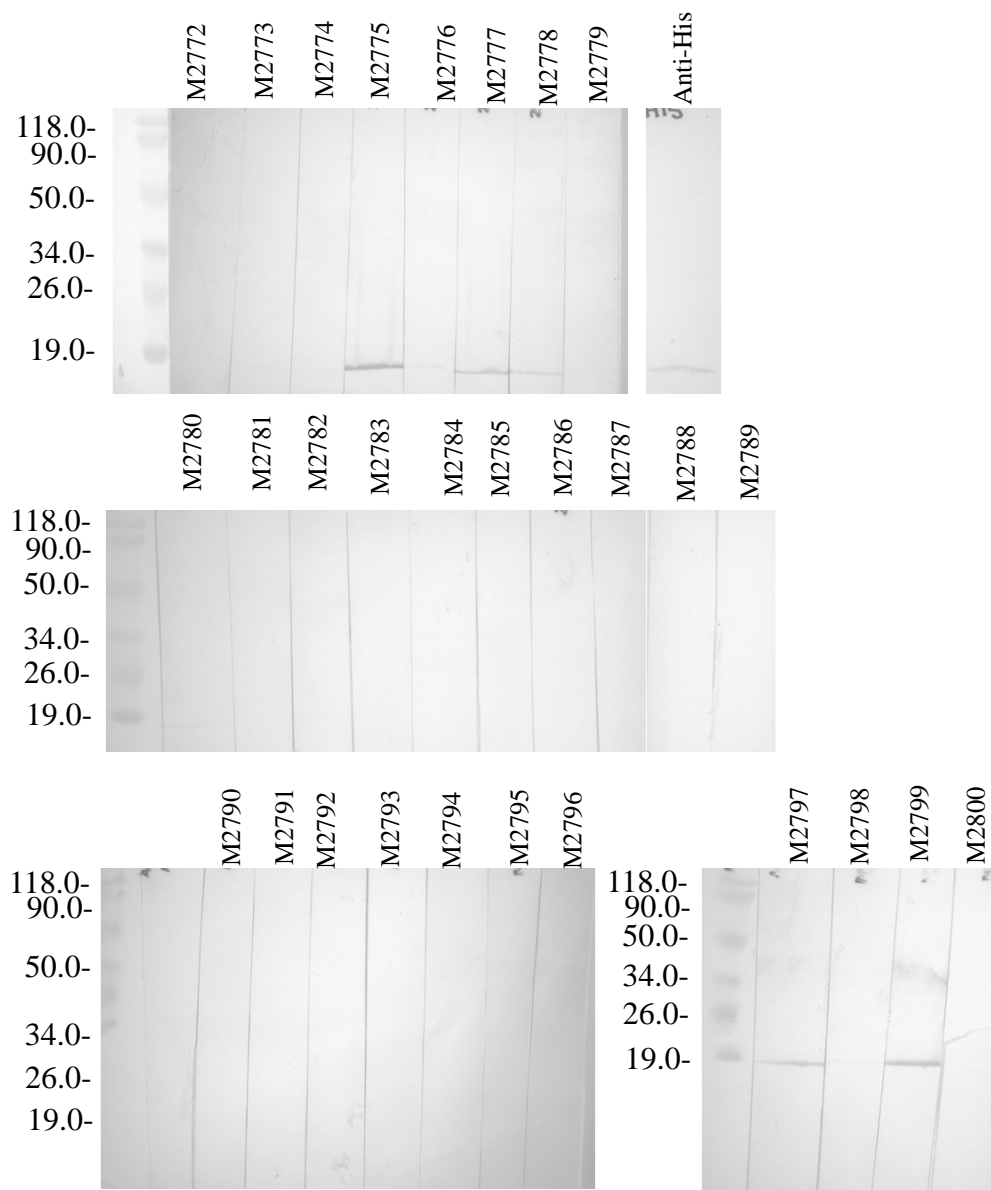


Figure S6. Western Blot of Monoclonal Antibodies with Recombinant IspC AA 684-774. The reaction of each of 29 MAbs were analyzed by Western blot with a recombinant protein containing a His-tag linked to amino acids 684-774 of the IspC protein.

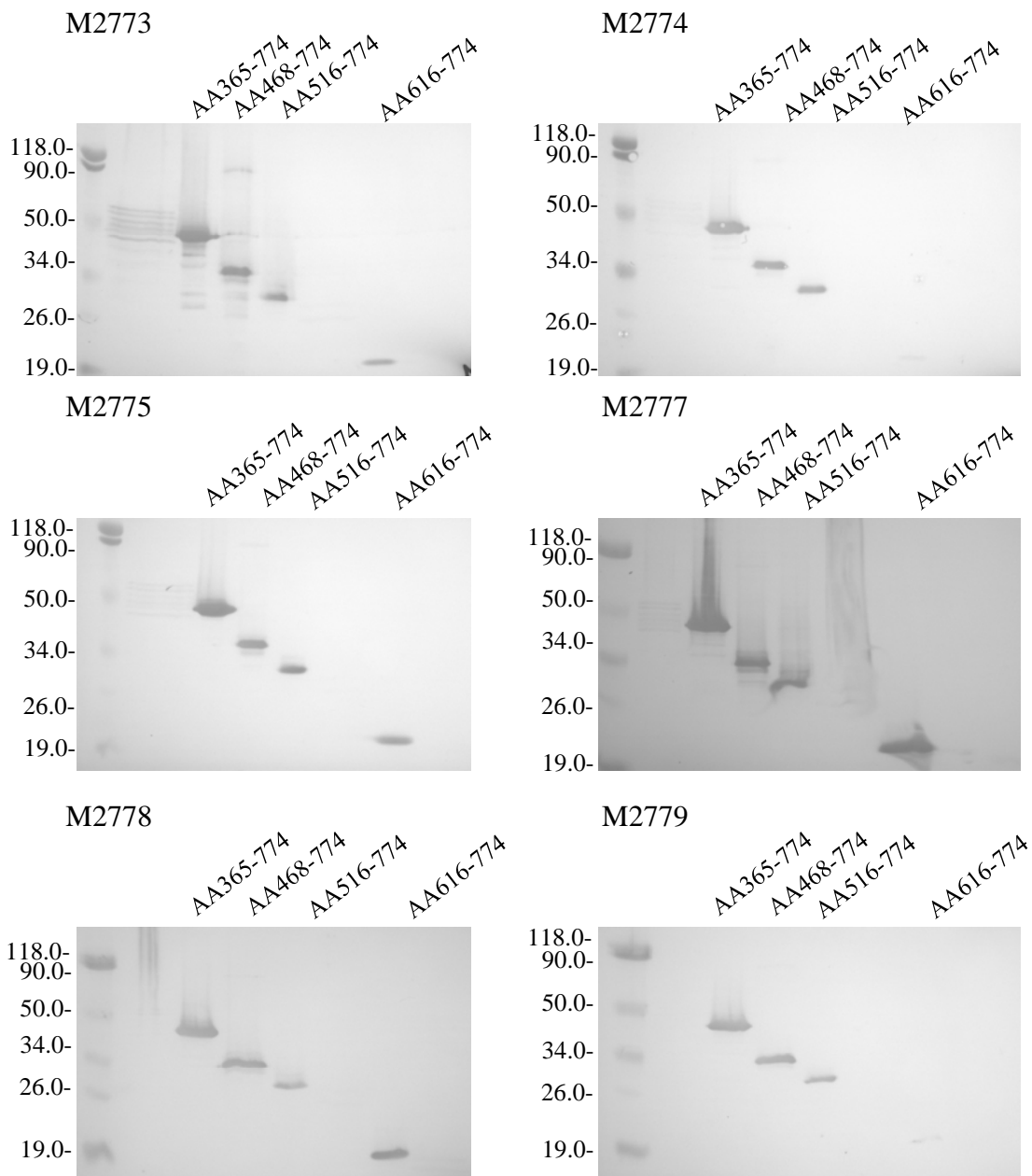


Figure S7. Western Blot of Monoclonal Antibodies with Recombinant IspC Fragments. The reaction of each of 15 MAbs known to react to recombinant IspC were analyzed by Western blot with several recombinant IspC fragments. Reaction of the protein fragments with an anti-His MAb indicates that ample protein was included in each of the lanes. Figure continued on next 2 pages.

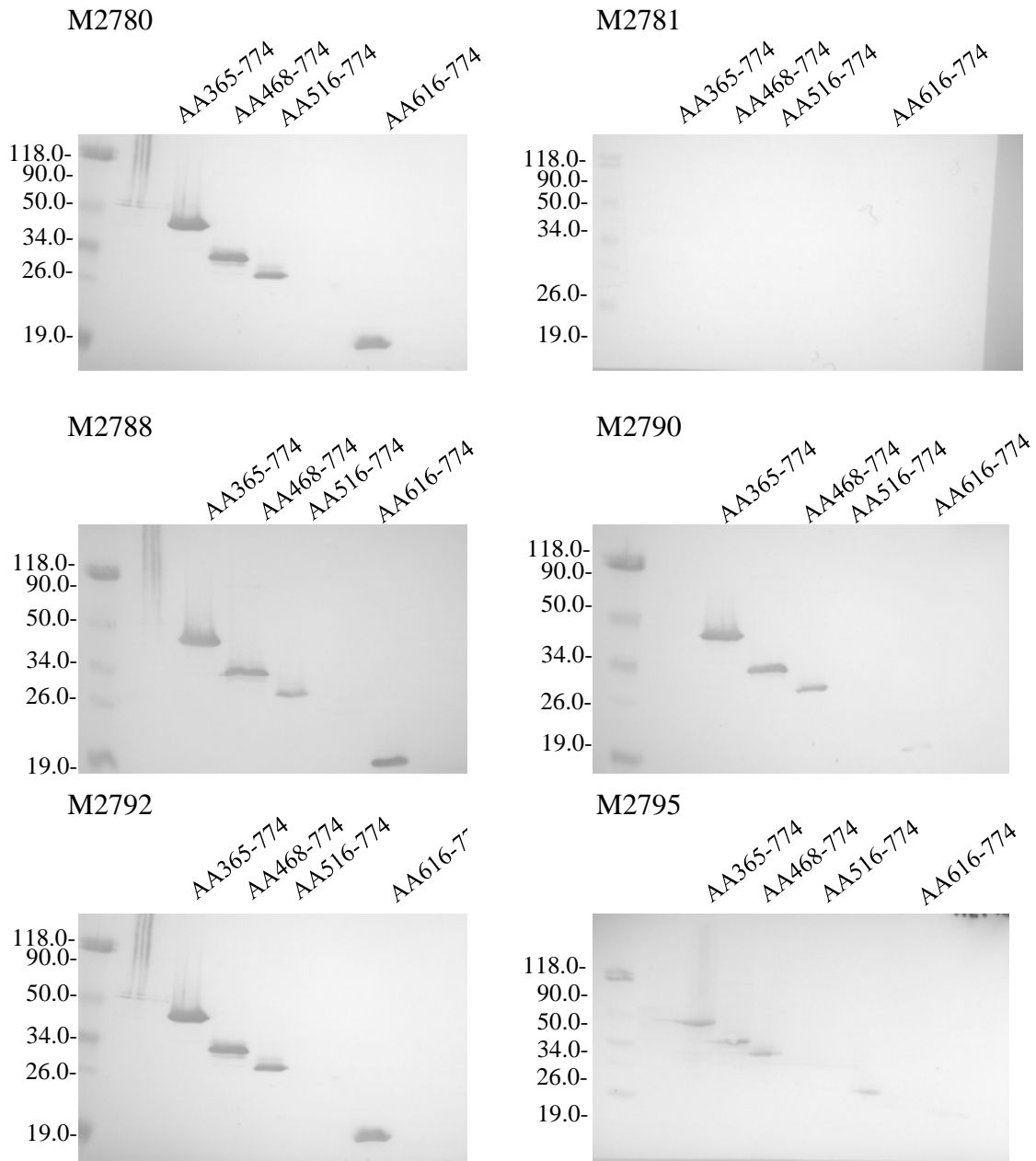


Figure S8. Western Blot of Monoclonal Antibodies with Recombinant IspC Fragments. (Continued)

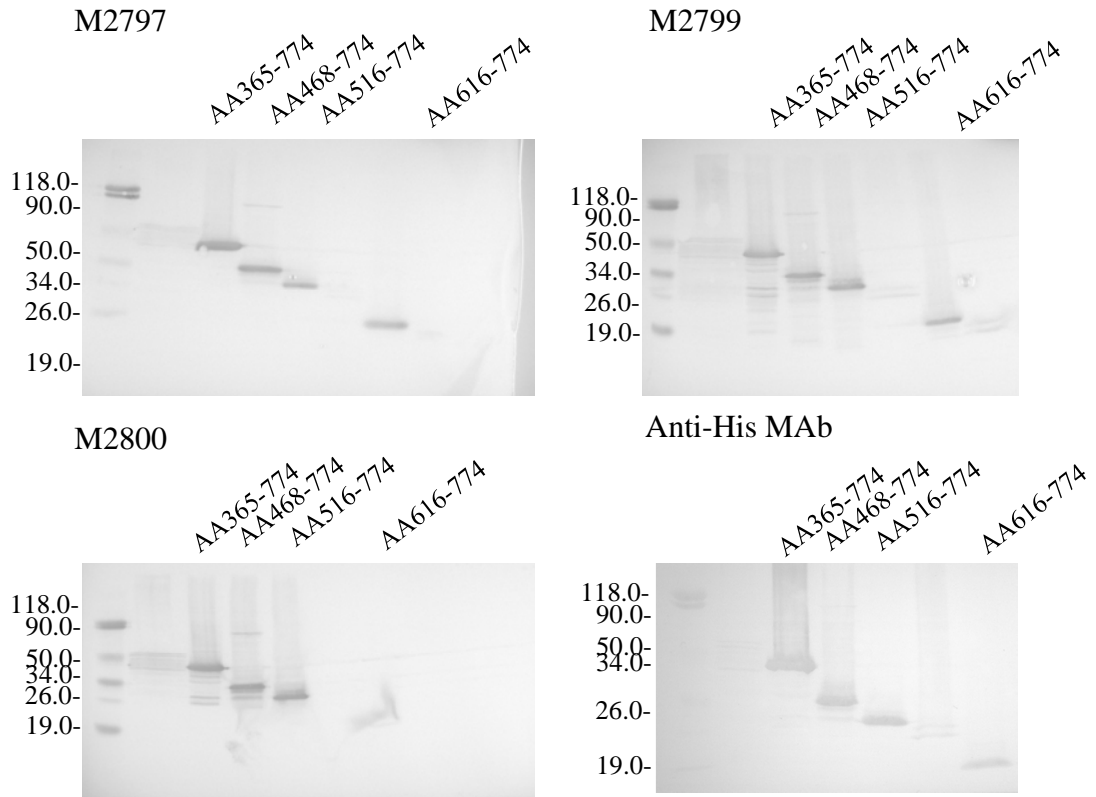


Figure S8. Western Blot of Monoclonal Antibodies with Recombinant IspC Fragments. (Continued)

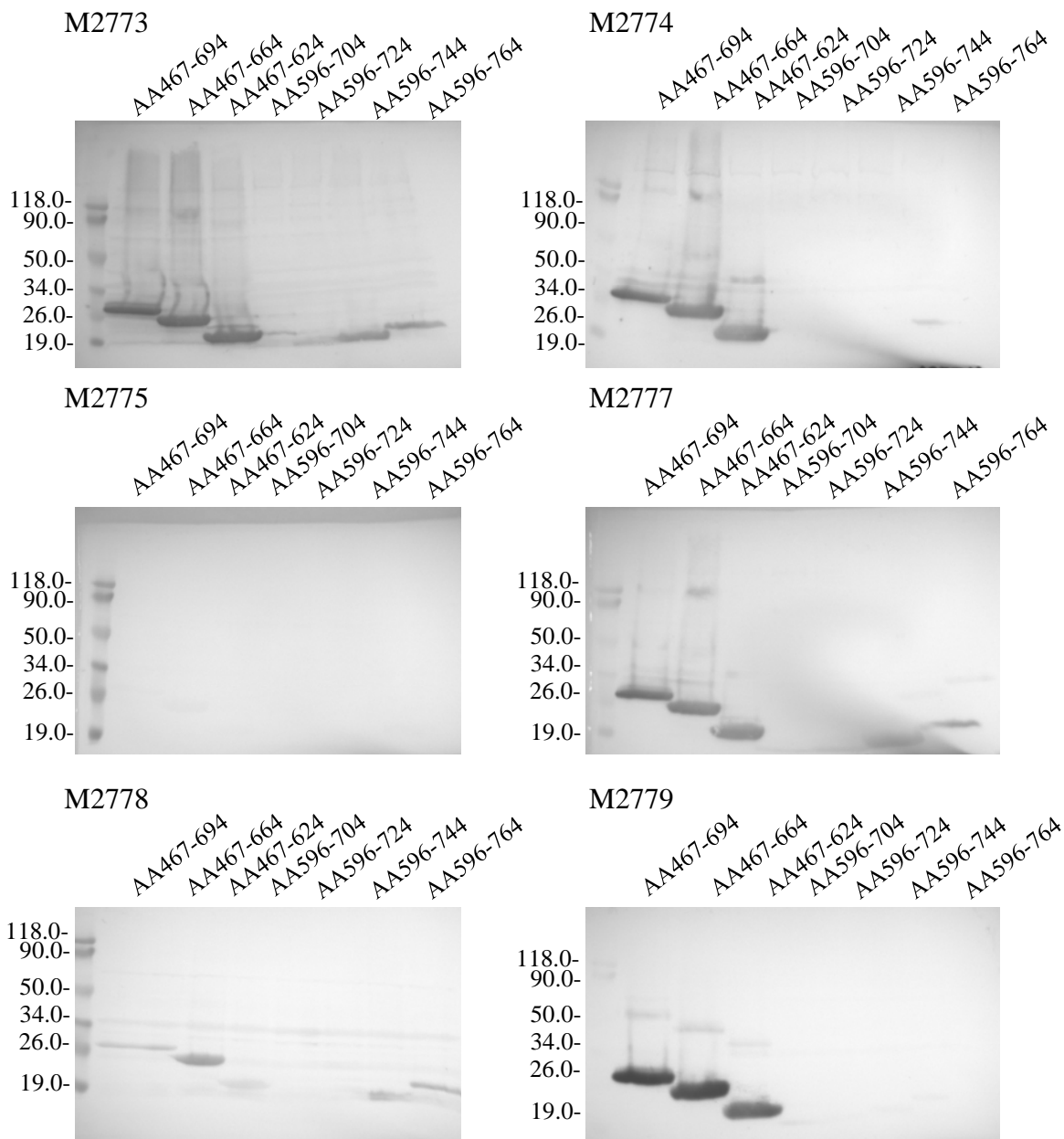


Figure S8. Western Blot of Monoclonal Antibodies with Recombinant IspC Fragments. The reaction of each of 15 MAbs known to react to recombinant IspC were analyzed by Western blot with several recombinant IspC fragments. Reaction of the protein fragments with an anti-His MAb indicates that ample protein was included in each of the lanes. Figure continued on next 2 pages.

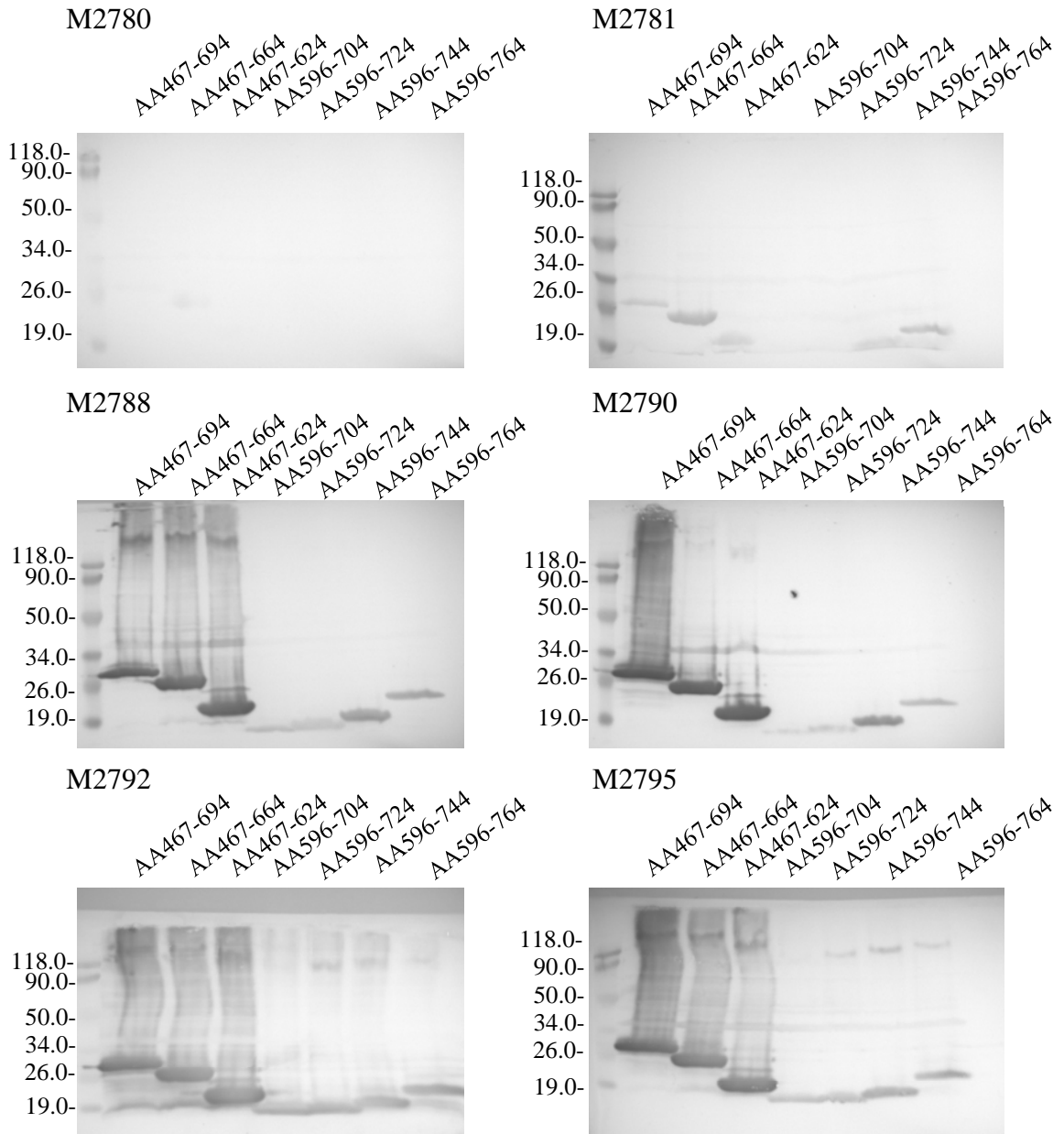


Figure S9. Western Blot of Monoclonal Antibodies with Recombinant IspC Fragments.

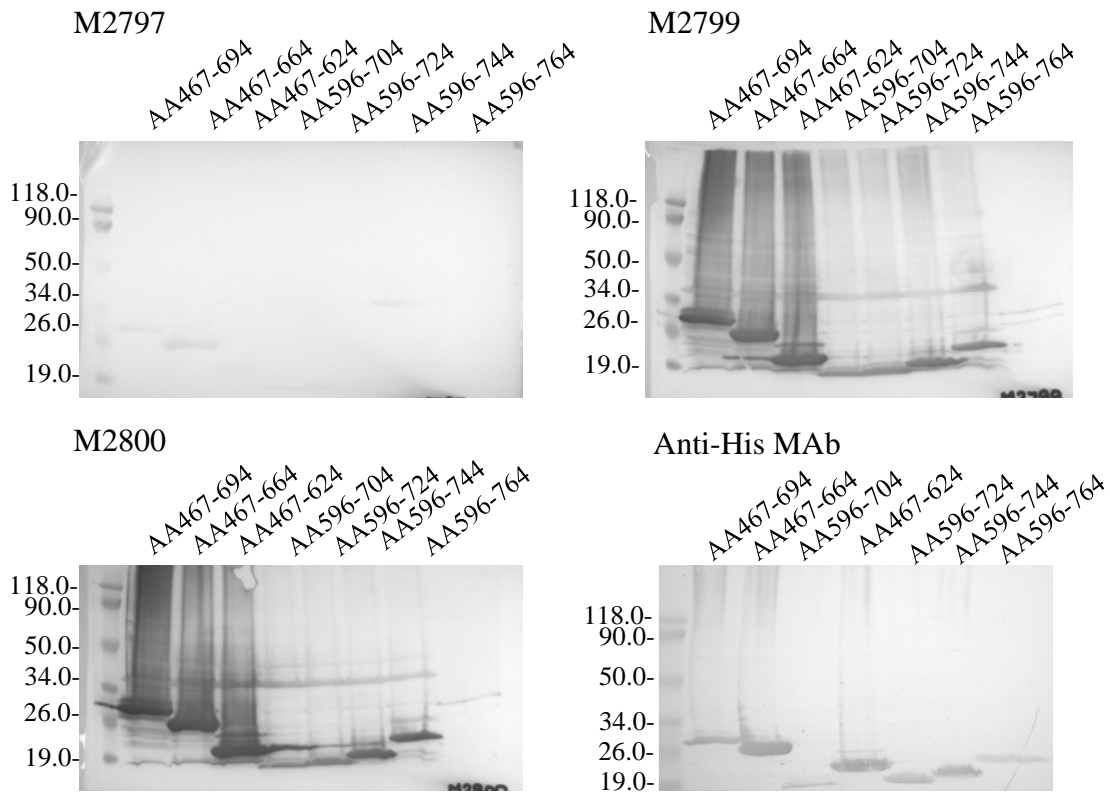


Figure S9. Western Blot of Monoclonal Antibodies with Recombinant IspC Fragments.

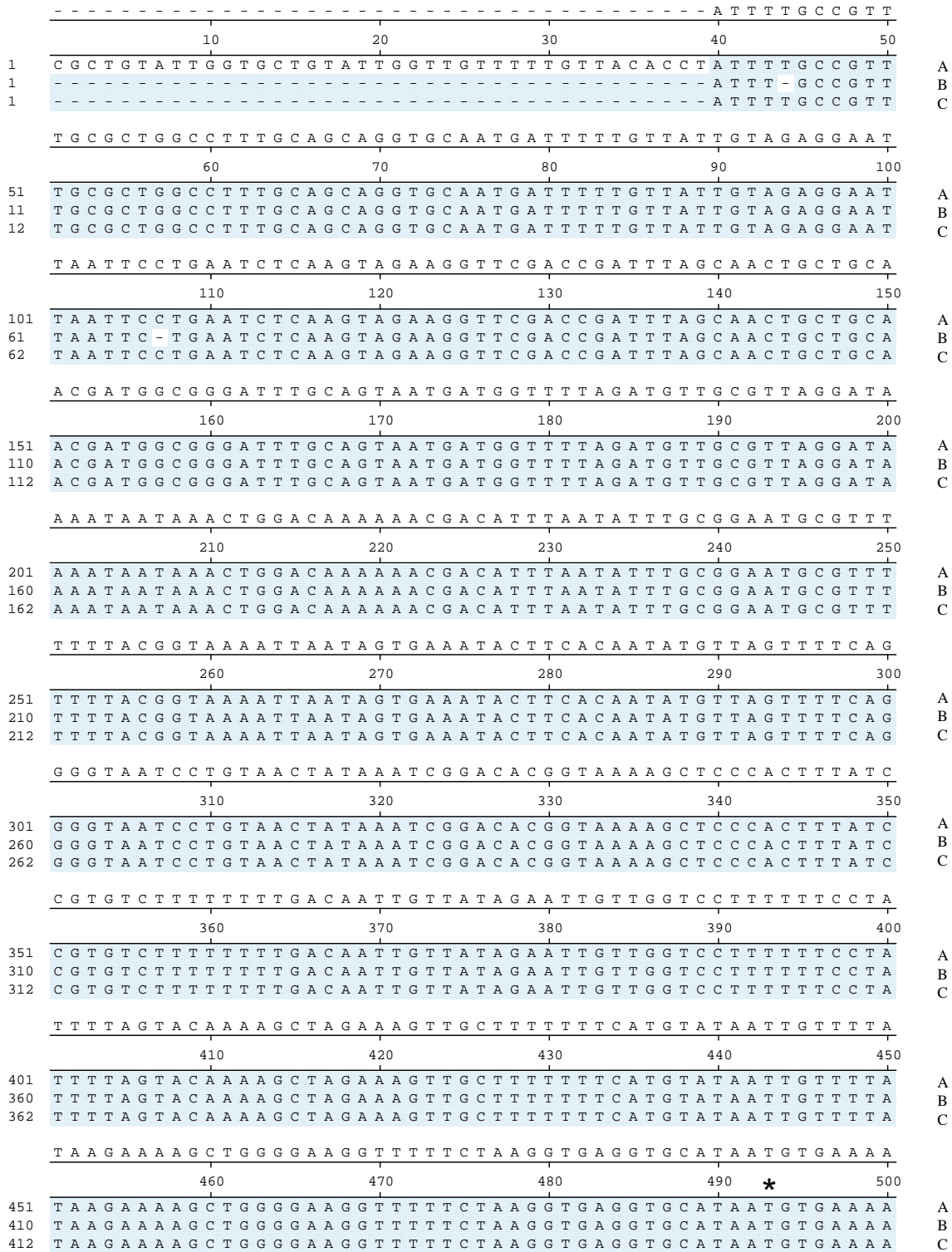


Figure S10– Alignment of *pgdA* in-frame deletion mutant sequencing reactions.

A) Forward sequencing reaction using the P837 primer. B) Reverse complement of the reverse sequencing reaction using the P838 primer. C) *pgdA* gene and *pgdA* promoter sequence from NCBI database. Results indicates clean knockout of the IspC ORF has been created. (*) Indicates the position of the *pgdA* start codon.

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		1010	1020	1030	1040	1050		
504	-----	-----						
463	-----	-----					A	
962	A A T C A A A C T A A A T T A A A T T T A A A A C A G C T G T T G A A G A A G A A C T G A T T A A						B	
	-----						C	
-----							-----	
		1060	1070	1080	1090	1100		
504	-----	-----						
463	-----	-----					A	
1012	A A C A G G G G T T T T T C T T T A A A G A T G T T G G G A A T C T T G G G A A A T T A A G A						B	
	-----						C	
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		1110	1120	1130	1140	1150		
504	-----	-----						
463	-----	-----					A	
1062	G T T T A G T G A A A T G G A A T C A A A C A G A T T T C G A A A T T A C G A A C T C T G A A C T A						B	
	-----						C	
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		1160	1170	1180	1190	1200		
504	-----	-----						
463	-----	-----					A	
1112	A T T T T G C C A G T A A A A A T A C C A G G T G C A C C G G A A C C G A A T A A A G T A A A A G T						B	
	-----						C	
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		1210	1220	1230	1240	1250		
504	-----	-----						
463	-----	-----					A	
1162	A A A A C T T G C T G A T A T T G C T A G T T C T G T G A A T A A A C G C T A C T T A C C A A G C A						B	
	-----						C	
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		1260	1270	1280	1290	1300		
504	-----	-----						
463	-----	-----					A	
1212	G T G T G A A A G T T C C A G A A G T G C C A A A A G C A A A A C C A A C A A G C G A A T T G C G						B	
	-----						C	
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		1310	1320	1330	1340	1350		
504	-----	-----						
463	-----	-----					A	
1262	C T T A C T T T T G A T G A T G G T C C A A G T G C C T C G G T A A C A C C A G G C G T A C T T G A						B	
	-----						C	
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		1360	1370	1380	1390	1400		
504	-----	-----						
463	-----	-----					A	
1312	T A C A T T G A A A C G C C A C A A T G T A A A A G C G A C A T T C T T T G T A C T T G G C T C T A						B	
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		1410	1420	1430	1440	1450		
504	-----	-----						
463	-----	-----					A	
1362	G T G T C A T A C A A A A T C C G G G T T T A G T A A A A C G T G A A T T A G A G G A A G G G C A C						B	
	-----						C	
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		1460	1470	1480	1490	1500		
504	-----	-----						
463	-----	-----					A	
1412	C A A G T T G G C A G C C A C T C A T G G G A T C A C C C G C A A T T A A C T A A A C A A T C G A C						B	
	-----						C	

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                1510                1520                1530                1540                1550
504 -----
463 -----
1462 A C A A G A A G T G T A C A A C C A - - A A T T T T A A A A C G C A A A A G C A G T A T T T G A
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                1560                1570                1580                1590                1600
504 -----
463 -----
1510 T C A A A C T G G A T A T T T T C C A A C T A C A A T G C G C C C T C C A T A T G G T G C A G T A A
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                1610                1620                1630                1640                1650
504 -----
463 -----
1560 A C A A A C A G G T G G C T G A A G A G A T T G G T C T T C C A A T A A T T C A G T G G T C T G T T
-----

                1660                1670                1680                1690                1700
504 -----
463 -----
1610 G A T A C A G A A G A T T G G A A A A C A A A A A T G C T G G T A T A G T A A C G A A A A A A G T
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                1710                1720                1730                1740                1750
504 -----
463 -----
1660 C C T T G C T G G T G C A A C G G A C G G A G C G A T T G T A C T A A T G C A T G A T A T T C A T A
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                1760                1770                1780                1790                1800
504 -----
463 -----
1710 A A A C C A C T G C T G C G A G C C T T G A C G C T A C A T T G A C T A A A T T A A A G A A T C A A
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                1810                1820                1830                1840                1850
504 -----
463 -----
1760 G G A T A T G A G T T T G T T A C T A T T G A T G A A C T T T A T G G C G A G A A A T T G C A A A T
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                C A G A T T C A A G A A T G G T G A A A T A A A A A T
                1860                1870                1880                1890                1900
504 -----
463 -----
1810 T G G G A A G C A A T A T T T C G A T A A A A C A G A T T C A A G A A T G G T G A A A T A A A A A T
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                C A G T A G C T A A G A T G A A T G A A T C A T T T T A G C T A C T T T T T T A T A C A A A A T G
                1910                1920                1930                1940                1950
531 C A G T A G C T A A G A T G A A T G A A T C A T T T T A G C T A C T T T T T T A T A C A A A A T G
490 C A G T A G C T A A G A T G A A T G A A T C A T T T T A G C T A C T T T T T T A T A C A A A A T G
1860 C A G T A G C T A A G A T G A A T G A A T C A T T T T A G C T A C T T T T T T A T A C G A A A T G
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                T A A A G T T T T C T T T A C G T G A A G C T G T A T T T A T T T T A T A A T A A C T G T A A A T A
                1960                1970                1980                1990                2000
581 T A A A G T T T T C T T T A C G T G A A G C T G T A T T T A T T T T A T A A T A A C T G T A A A T A
540 T A A A G T T T T C T T T A C G T G A A G C T G T A T T T A T T T T A T A A T A A C T G T A A A T A
1910 T A A A G T T T T C T T T A C G T G A A G C T G T A T T T A T T T T A T A A T A A C T G T A A A T A
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A
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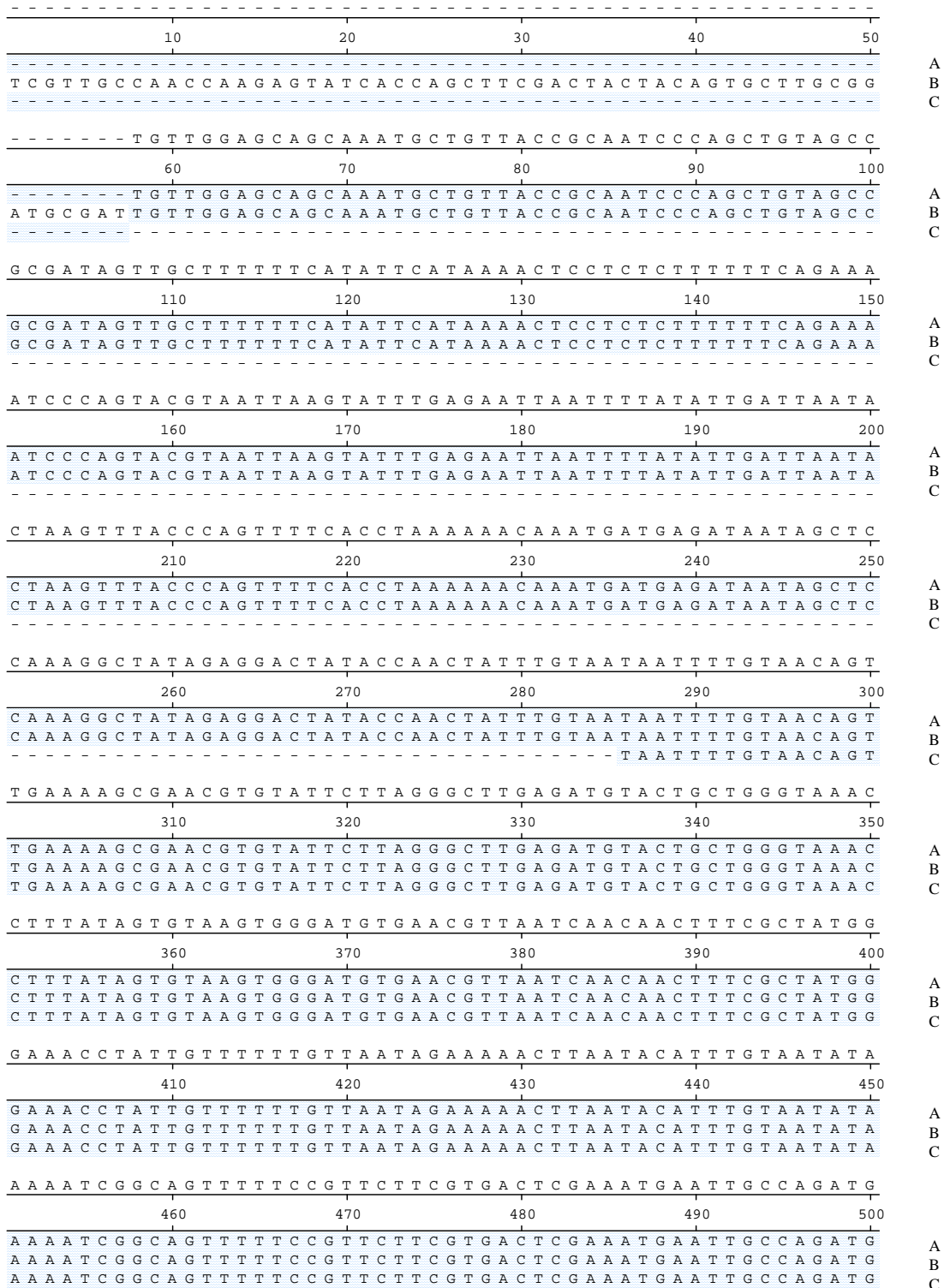


Figure S11 – *secA2* in-frame deletion mutant sequencing reactions. A) SecA2 ORF and promoter sequences from NCBI database. B) Forward sequencing reaction using the P902f primer. C) Reverse complement of the reverse sequencing reaction using the P902r primer. Results indicate a clean knockout of the IspC ORF has been created. (*) Denotes the start codon for SecA2

----- 1010 1020 1030 1040 1050 -----	A B C
G G T T T A G A C A T A G C C C A A A A A A A G G C C A T T T A T A C A G C A G A T G T T A T T T A ----- -----	
----- 1060 1070 1080 1090 1100 -----	A B C
T G G A A C G G C T T C T G A A T T T G G T T T T G A T T A C T T A C G C G A C A A T A T G G T A C ----- -----	
----- 1110 1120 1130 1140 1150 -----	A B C
G C C A A A A A G A A G A T A A A G T A C A A A G T G G A C T T G A T T T T G T T T T A A T A G A T ----- -----	
----- 1160 1170 1180 1190 1200 -----	A B C
G A A G C A G A T T C A A T T T T A A T A G A T G A A G C G A G A A C G C C C C T A C T G A T T T C ----- -----	
----- 1210 1220 1230 1240 1250 -----	A B C
T G A C C G T A A A G A A G A A G A T T T G T C C C T T T A T C A T A C A G C G A A T A A G C T T G ----- -----	
----- 1260 1270 1280 1290 1300 -----	A B C
T G A A G A A G A T G A T G A A A G A T G A T T A T G A G A T G G A A G A A C A T A A A C G C T T T ----- -----	
----- 1310 1320 1330 1340 1350 -----	A B C
G T T T G G T T G A A T G A T G C T G G A A T T G A G A A A G C C C A G A A G T T T T G G G G T G T ----- -----	
----- 1360 1370 1380 1390 1400 -----	A B C
C G A A T C A C T T T A T T C C G C A G A G G C G C A G T C A G A A C T T A G A A T T A C G A T G C ----- -----	
----- 1410 1420 1430 1440 1450 -----	A B C
T T T T A A T G C G A G C T C A T T T C T T G A T G C A T A A A G A T A A A G A T T A T G T G G T G ----- -----	
----- 1460 1470 1480 1490 1500 -----	A B C
C T T G A T G A T G A A G T A T T A A T T A T C G A T C C A C A T A C T G G T C G T G C G C T T C C ----- -----	

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A G G C C G G C G C T T T A A T G A C G G A C T C C A T C A G G C A A T C G A A G C A A A A G A G G				

1560	1570	1580	1590	1600
G C G T G G A A G T T A A A G A A G A A T C A C G T A C G C T A G C A A C A A T T A C A A T T C A A				

1610	1620	1630	1640	1650
A A C T A C T T C C G G A T G T A T A A G A A A A T T T C T G G A A T G A C A G G A A C G G C T A A				

1660	1670	1680	1690	1700
A A C C G A A G A A G A A G A A T T C C G T C A A A T T T A T A A C A T G G A T G T C G T A G T A A				

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T C C C C A C C A A T T T A C G T G T A A A A T C G G G A A G A T A T G C A A G A T G A T A T T T T C				

1760	1770	1780	1790	1800
T A T A C G A A A A A G G A A A A G G T C G T G C G A T T G T G T A T G A A G T A T C A T G G C G				

1810	1820	1830	1840	1850
C T A T G A A A A A G G A C A G C C A A C T T T A A T A G G A A C T T C T T C T A T T A A A A G T A				

1860	1870	1880	1890	1900
A T G A A T G G A T C A G T G G C T T A T T A G A C G C T G C T G G A A T T C C C A C C A A G T A				

1910	1920	1930	1940	1950
T T A A A T G C G A A A A A C C A T G C C C A A G A A G C G G A A A T A A T T G C C A A A G C T G G				

1960	1970	1980	1990	2000
G A A A C G C G G A A T G G T A A C T T T A G C A A C G A A C A T G G C T G G T C G G G G G A C A G				

A
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2110	2120	2130	2140	2150
T T C T G G A C G A C G G G G T G A C C C T G G T T T C A G T A A G T T T A T G A T T T C T T T A G				

2160	2170	2180	2190	2200
A A G A T G A T T T A T T A G A A C A A T T T G A A A G T A A G A G C T G G G A G A A A C T T T C C				

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G T T A C T A A T T T T T C T T T T A G T A G C A C T T G A T A T T T A T G T A T T A A C T A A G A	C
T T G C C T T T A T T T T T G A T C C G T T A A T G G T A A A T T C T T A A A A C C G T T G C T G C A	
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Appendix 3

**Manuscript - Monoclonal Antibodies to Lipopolysaccharide
Antigens of Salmonella enterica serotype Typhimurium DT104**

Monoclonal Antibodies to Lipopolysaccharide Antigens of *Salmonella enterica* serotype Typhimurium DT104

Jennifer Ronholm,^{1,2} Zhiyi Zhang,⁴ Xundong Cao,³ and Min Lin^{1,2}

Salmonella enterica subsp. *enterica* serotype Typhimurium is one of the major causative agents of human gastroenteritis. Here we raised a panel of 45 monoclonal antibodies (MAbs) against ser. Typhimurium DT104 by immunizing mice with formalin-killed bacteria and demonstrated that all the MAbs recognized the bacterial lipopolysaccharide (LPS) antigen. These MAbs were specific for group O:4 *Salmonella* with very little or no cross-reactivity with other closely related bacteria and were able to bind to the cell surface of live bacterial cells, making them potential candidates for capture and concentration of the pathogen in food and water samples. Epitope characterization revealed that the O:5 antigen present in the LPS of some serogroup 4 *Salmonella* is the critical factor for the binding of these MAbs to LPS. This study has provided some insights into the structure of the *Salmonella* LPS and its influence on the antigenicity of LPS.

Introduction

ACUTE FOODBORNE GASTROINTESTINAL INFECTIONS are commonly caused by the gram-negative, rod-shaped bacterium *Salmonella enterica* subsp. *enterica* serotype Typhimurium.⁽¹⁾ Although most individuals experience acute gastroenteritis, *Salmonella* infection in infants, the elderly, or immunocompromised patients can become systemic and result in death.⁽²⁾ In otherwise healthy patients, *Salmonella* infection can lead to additional complications such as chronic acute reactive arthritis.⁽³⁾

Salmonella enterica subsp. *enterica* is classified into over 1500 serotypes based on antigenic differences in lipopolysaccharide (LPS) (O) and flagellar (H) antigens. The H-antigenic determinants are carried in the flagellin protein, the main structural component of flagella. Through phase variation, *Salmonella* can express antigenically different flagella.⁽⁴⁾ Lipopolysaccharide is a major component of the outer surface of gram-negative bacteria, composed of a hydrophobic lipid A, which anchors LPS to the membrane, a core oligosaccharide region, and an O-polysaccharide polymer (O-chain) composed of oligosaccharide-repeating units. While the lipid A and the LPS-core region are relatively conserved among gram-negative organisms, there is a substantial difference in the composition of the O-chain-repeating units, which leads to a large antigenic diversity in O-antigens.

Monoclonal antibodies (MAbs) have been raised against the surface antigens of *Salmonella*, several of which were generated in an attempt to seek a MAb that would be specific for a certain serotype. The MAb 23D4 directed against the *Salmonella* H antigen appeared to be highly specific for ser. Typhimurium but did cross-react with monophasic [4,5,12:i:-] *Salmonella*.⁽⁵⁾ Other attempts to produce MAbs specific for the H antigen of ser. Typhimurium have been less successful, showing the ability of these MAbs to differentiate between the two phases (H:i, H:1,2), but cross-react with other serotypes.⁽⁴⁾ An interesting immunization approach using the outer membrane protein (OMP) extract from ser. Typhimurium led to the creation of 10 MAbs against a 38 kDa OMP, which were specific to serogroup 4 *Salmonella*.⁽⁶⁾

Monoclonal antibodies have also been raised against the structural components of *Salmonella* LPS. Antibodies against the core region of LPS tend to react with a broad range of *Salmonella* and other enterobacteriaceae. The T6 MAb directed against a highly conserved N-acetylglucosamine and glucose portion of the Ra-type core reacts with a broad range of *Salmonella*, although it does not react with several *Salmonella* isolates of subsp. *arizonae*, lacking the Ra-type core.^(7,8) The MAb M105 had slightly more success in recognizing a larger portion of *Salmonella* isolates by binding to both the Ra-type and less effectively to the Rb1 and Rb2-type core,⁽⁹⁾ although it does not react with all *Salmonella* isolates.⁽¹⁰⁾

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Attempts have been made to raise MAbs against the O-chain of LPS that may be able to recognize specific serogroups or even serotypes. *Salmonella* ser. Typhimurium belongs to the O:4 (formerly B) serogroup. The LPS of group 4 organisms contains the O:12 antigen, which is comprised of a repeating unit of mannose, rhamnose, and galactose in the O-chain, although this sugar arrangement is common to a number of serogroups. The defining factor of serogroup 4 LPS is the O:4 antigen, which is a bequose α -1, 3 linked to the mannose. The stereochemistry of this bequose residue is the sole determinant of the immunodominant epitope within the serogroup 4 LPS.⁽¹¹⁾ Acetylation of the 2-hydroxyl group of the bequose residue defines the O:5 antigen, which is not expressed by all Typhimurium strains. As the attachment of this acetyl group changes the stereochemistry of the bequose residue, it is predictable that the presence or absence of the O:5 antigen is critical in defining immunogenic serogroup 4 epitopes.⁽¹²⁾ Jaradat and Zawistowski reported that MAbs 4A8, 5B2, and 5A5 all react with O-acetylated variants of serogroup 4 *Salmonella* while failing to react with the non-acetylated counterparts.⁽¹³⁾ Schlauch and colleagues developed seven MAbs against ser. Typhimurium LPS, five of which recognized acetylated LPS exclusively while two recognized non-acetylated LPS exclusively.⁽¹²⁾ Luk and Lindberg reported a number of MAbs specific for various O-groups, including MABO-8 and MABO-10 directed against the serogroup 4 LPS.⁽¹⁴⁾ In contrast to MABO-10 that detects LPS in both the presence and absence of the O:5 antigen, MABO-8, which has a higher affinity for the LPS, only reacted with LPS containing O:5 antigen.⁽¹⁴⁾ The same specificity has also been demonstrated with polyclonal antibodies.⁽¹⁵⁾ The mechanism behind this was suggested in a detailed analysis of the SE115-4 binding site. Through crystallographic studies, antibody-antigen interaction as defined by hydrogen bonding was shown to be dominated by the bequose residue, despite the antibody binding site being complementary to a trisaccharide-sized epitope.⁽¹¹⁾ A number of MAbs recognizing surface antigens of *Salmonella* have been produced, although none is specific enough to be used in diagnostics or serotyping of certain *Salmonella* serotypes.

A study comparing the ability of participating laboratories to correctly serotype *Salmonella* found significant differences,⁽¹⁶⁾ which suggests that better antisera are required to get clear results in serological assays. The current serotyping antisera are polyclonal, created through immunization with *Salmonella* whole cells. Taking into account the similarities in cell surfaces between gram-negative organisms such antisera may contain many cross-reacting antibodies. Since LPS is known to carry distinct diagnostic markers, which aid in the ability to define serotypes, creation of new MAbs against LPS may increase the accuracy of serological assays. In addition, since this study indicates that LPS plays a critical role in the immune response to *Salmonella* infection, the creation of MAbs against LPS may be useful for mapping potential virulence epitopes.

Given the fact that *Salmonella* ser. Typhimurium is an important agent of foodborne gastrointestinal infections, a MAb specific for this serotype would be very useful in diagnostics. This study was undertaken to create and characterize antibodies directed against surface antigens of *Salmonella* ser. Typhimurium, which may be useful in diagnostics as well as the future study of *Salmonella*.

Materials and Methods

Chemicals and reagents

Horse radish peroxidase (HRP)-conjugated goat anti-mouse IgG (H+L) was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (H+L) was bought from Zymed Laboratories (San Francisco, CA). Protein standards (pre-stained), nitrocellulose membranes, and HRP conjugate substrate kit were obtained from Bio-Rad (Mississauga, Canada). Phenol extracted LPS from *Salmonella* ser. Typhimurium ATCC 7823 was obtained from Sigma (Oakville, Canada). All other chemicals were of analytical grade and commercially available.

Bacterial culture

The bacteria species used in this study (Table 1) were cultured at 37°C on Luria-Bertani (LB) agar or in LB broth, with the exception of *Listeria* spp., which was cultured on either brain heart infusion (BHI) agar or in BHI broth, for 16–18 h. Cell concentrations were estimated using OD₆₀₀ 1 = 1.6 × 10⁹ cells/mL for *Salmonella* species,⁽¹⁷⁾ OD₆₀₀ 1 = 1 × 10⁹ cells/mL *E. coli*, and OD₆₀₀ 0.61 = 1 × 10⁹ cells/mL for *Listeria*.⁽¹⁸⁾

Mouse immunization

A *Salmonella* ser. Typhimurium DT104 strain SA03-1907 was used as an immunogen. Bacterial cells were harvested from a 200 mL overnight culture by centrifugation at 10000 g for 10 min at 4°C, washed three times with PBS, and killed by resuspending in 30 mL of 0.3% (v/v) formalin solution in PBS for 24 h at room temperature. Killed cells were collected by centrifugation at 10000 g for 10 min at 4°C, washed twice with PBS, and then resuspended in PBS to obtain a concentration of 1 × 10⁹ cells/mL. Formalin-killed cells were stored at -20°C until use. Three ND4 and three BALB/c mice, after collecting pre-immune sera, were immunized intraperitoneally with 1 × 10⁸ cells in 0.5 mL of PBS at days 0 and 8. Two intravenous injections with 1 × 10⁸ cells in 0.1 mL of PBS were given at days 29 and 38. On day 42, three ND4 and two BALB/c mice were sacrificed for fusion with SP2/0-Ag14 myeloma cells as previously described.⁽¹⁸⁾ Animal immunization experiments were approved by the Animal Care Committee at the Ottawa Laboratory Fallowfield (Canadian Food Inspection Agency) and carried out under the guidelines of the Canadian Council on Animal Care.

Screening and isotyping of murine monoclonal antibodies

Culture supernatants from hybridoma cells were tested for *Salmonella*-reactive monoclonal antibodies using the indirect ELISA procedure as previously described.⁽¹⁸⁾ Formalin-killed *Salmonella* ser. Typhimurium DT104 cells were used as the coating antigen at a concentration of 1 × 10⁹ cells/mL in a volume of 100 µL/well. Hybridoma cell lines secreting reactive MAbs were identified by using an OD₄₁₄ cut-off of 0.3 under the conditions used. The subclasses of immunoglobulins (Ig) secreted by hybridoma cell lines were determined as previously described.⁽¹⁸⁾

TABLE 1. BACTERIA USED IN STUDY

Bacteria	Strain or isolate	O-Antigenic formula
<i>Salmonella</i> ser. Typhimurium DT104	SA03-1907	1,4,[5],12
<i>Salmonella</i> ser. Typhimurium P10	Unflagellated mutant.	1,4,[5],12
<i>Salmonella</i> ser. Typhimurium Passage 1	ATCC 14028	1,4,[5],12
<i>Salmonella</i> ser. Typhimurium DT108	02D1634 2-4	1,4,[5],12
<i>Salmonella</i> ser. Typhimurium Str. 324	ADRI 5040	1,4,[5],12
<i>Salmonella</i> ser. Typhimurium variant Copenhagen DT104 1	00X344-C5	1,4,12
<i>Salmonella</i> ser. Typhimurium variant Copenhagen DT104 2	01D5997 25-3	1,4,12
<i>Salmonella</i> ser. Typhimurium 14028 <i>ofaA127::</i> TN 10d- Km	MTI20	1,4,12
<i>S. enterica</i> subsp. <i>arizonae</i>	ADRI 15	48
<i>Salmonella</i> ser. Abortus equi	ADRI 37	4,12
<i>Salmonella</i> ser. Heidelberg	01D3969-14	1,4,[5],12
<i>Salmonella</i> ser. Heidelberg Type 8	01D5997 31-7	1,4,[5],12
<i>Salmonella</i> ser. Heidelberg	OLC#502	1,4,[5],12
<i>Salmonella</i> ser. Agona	ADRI 36	1,4,[5],12
<i>Salmonella</i> ser. Chester	OLC#320	1,4,[5],12
<i>Salmonella</i> ser. California		4,12
<i>Salmonella</i> ser. Thompson		6,7,[14]
<i>Salmonella</i> ser. Enteritidis		1,9,12
<i>Salmonella</i> ser. Paratyphi A	OLF#1836	1,2,12
<i>L. monocytogenes</i> ser. 4b	LI0521	
<i>L. grayi</i>	HPB29	
<i>E. coli</i> O157:H7	ATCC 43889	157

Cross-reactivity of anti-*S. Typhimurium* DT104 MAbs with other microorganisms

The MAbs, selected based on reactivity with *Salmonella* ser. Typhimurium DT104, were assessed for cross-reactivity with bacterial species (Table 1) by indirect ELISA using respective formalin-killed bacteria ($\sim 1.0 \times 10^8$ cells/well) as described.⁽¹⁸⁾

Immunofluorescence microscopy

The ability of the MAbs to bind to the surface antigens of live *Salmonella* ser. Typhimurium was assessed using immunofluorescence microscopy.⁽¹⁸⁾

Preparation of *Salmonella* LPS

LPS was prepared from *Salmonella* ser. Typhimurium DT104 through a procedure modified from Johnson and colleagues.⁽¹⁹⁾ Briefly, lyophilized bacteria were suspended in water to a concentration of 5% (w/v). The aqueous suspension was heated to 70°C in a water bath for 10 min. An equal volume of preheated 90% (v/v) phenol was added to the bacterial suspension. This was maintained at 70°C while being stirred vigorously for 15 min. The solution was quickly cooled to 10°C. The emulsion was centrifuged at 10,000 g for 30 min. The upper aqueous layer was removed and retained. The remaining phenol phase was suspended in one volume of water and extracted again. The aqueous layers from each extraction were combined and dialysed overnight against water. The dialysed sample was lyophilized, dissolved in PBS, and then centrifuged at 27,000 g for 30 min to remove insoluble material. The supernatant was treated with RNase (1 µg/mL) and DNase (1 µg/mL) in the presence of 10 mM MgCl₂ at 37°C for 1 h and ultra-centrifuged at 100,000 g for 10 h. The final pellet was dissolved in water and lyophilized.

Immunoblot analysis of *Salmonella* LPS using anti-*S. Typhimurium* DT104 MAbs

Binding of anti-*S. Typhimurium* DT104 MAbs to *Salmonella* LPS was analyzed by immunoblotting, as described previously.⁽²⁰⁾ Transfer of the LPS to nitrocellulose essentially took place as previously described⁽²¹⁾ using a Bio-Rad wet-electrotransfer apparatus (Hercules, CA). The purified LPS (Sigma) antigens were dissolved in SDS-PAGE sample buffer at 2.3 mg/mL, heated at 100°C for 10 min and loaded into wells of 12% SDS-PAGE gels (20 µL per well). The separated LPS bands were probed with monoclonal antibody-containing hybridoma cell culture fluids at a dilution of 1:20 in PBST containing 5% BSA.

SDS-PAGE analysis of purified *Salmonella* LPS

Analysis of purified *Salmonella* LPS by SDS-PAGE followed by silver staining were performed using the procedure modified from Tsai and Frasch.⁽²²⁾

Deacetylation of purified LPS

Hot phenol purified LPS was subjected to treatment with either 4 M acetate buffer (pH 4.6), 4 M sodium hydroxide (pH 9.6), or PBS (pH 7.0). Each LPS solution was either heated at 100°C for 30 min or held at room temperature for 30 min. Carbonate buffer (60 mM NaHCO₃-Na₂CO₃, pH 9.6) was used to dilute LPS to 10 µg/mL. This LPS (100 µL) was added to each well of an ELISA plate⁽¹⁸⁾ and used to assess the ability of the MAbs to react with chemically modified LPS.

Results

Identification of hybridoma clones secreting MAbs to *Salmonella* ser. Typhimurium

ELISA screening of culture supernatants of hybridomas generated by fusion of myeloma cell line SP2/0-Ag14 with

TABLE 2. SUMMARY OF ELISA DATA ON CROSS-REACTIONS OF MABS WITH SALMONELLA SEROTYPES

	ser. Typhimurium DT104	ser. Typhimurium Strain 324	ser. Typhimurium Passage 1	ser. Typhimurium P10	ser. Typhimurium DT108	ser. Typhimurium variant Copenhagen 1	ser. Typhimurium variant Copenhagen 2	ser. Chester	ser. Heidelberg 1	ser. Heidelberg Type 8	ser. Heidelberg 2	ser. Abortus equi	ser. Paratyphi A	ser. California	ser. Enteritidis	ser. Thompson	ser. Agona	subsp. Arizonae	<i>E. coli</i> O157:H7	<i>L. monocytogenes</i>	<i>L. grayi</i>
M3037	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
M3038	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
M3039	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
M3040	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
M3041	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
M3042	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
M3043	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
M3044	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
M3045	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
M3046	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
M3047	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
M3048	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
M3049	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
M3050	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
M3051	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
M3052	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
M3053	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
M3054	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
M3055	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
M3056	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
M3057	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
M3058	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
M3059	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
M3060	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
M3061	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
M3062	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
M3063	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
M3064	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
M3065	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
M3066	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
M3067	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
M3068	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
M3069	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
M3070	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
M3071	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
M3072	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
M3073	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
M3074	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
M3075	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
M3076	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
M3077	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
M3078	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
M3079	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
M3080	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
M3081	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■

ELISA optical density (OD) readings were determined at 414 nm: ■ represents an OD₄₁₄ of >1, ▣ represents an OD₄₁₄ of <1 but >0.5, ▤ represents an OD₄₁₄ of <0.5 but >0.05, □ represents an OD₄₁₄ of <0.05, which was comparable to the negative control. Negative controls were performed with an irrelevant MAb M2787 raised against *Listeria monocytogenes*.⁽¹⁷⁾ All ELISA OD values are the average of two determinations.

spleen cells from three Balb/c and two ND4 mice receiving formalin-killed *Salmonella* ser. Typhimurium DT104 identified 45 positive clones. ELISA analysis of MAb-containing culture supernatants revealed that all MAbs showed a reaction with the immunizing bacteria with an OD at 414 nm of greater than 1.0 (Table 2). All the MAbs were able to react with other *Salmonella* ser. Typhimurium isolates tested (Table 2). Antibody isotype analysis demonstrated that of these 45 MAbs, four were IgG1 (M3037, M3044, M3061, and M3068); 22 were IgG2a (M3038, M3040, M3041, M3045, M3046, M3049, M3050, M3051, M3054, M3059, M3060, M3064, M3065, M3069, M3070, M3071, M3072, M3073, M3074, M3077, M3078, and M3080), 13 were IgG2b (M3043, M3047, M3048, M3053, M3055, M3057, M3058, M3061, M3063, M3075, M3076, M3079, and M3081); and six were IgG3 (M3039, M3042, M3052, M3056, M3066, and M3067).

Binding of monoclonal antibodies to the surface of live Salmonella ser. Typhimurium

Immunofluorescent staining of live *Salmonella* ser. Typhimurium DT104 showed that all 45 MAbs were able to bind to the cell surface. The result of immunofluorescence staining was exemplified by binding of M3037 to the surface of *Salmonella*

ser. Typhimurium (Fig. 1). No fluorescence staining was observed with an irrelevant MAb M2787 raised against *L. monocytogenes*.⁽¹⁷⁾

Cross-reactivity of monoclonal antibodies within Salmonella serogroup 4

All 45 MAbs were tested for cross-reactivity by indirect ELISA with a number of non-Typhimurium isolates and three other bacterial species (Table 1) as well as 14 ser. Typhimurium isolates. All 45 MAbs strongly reacted with all 14 ser. Typhimurium isolates (data not shown). Two *Salmonella* ser. Typhimurium variant Copenhagen strains tested here showed a similar reaction pattern with all the monoclonal antibodies (Table 2): no cross-reaction with 19 MAbs (M3038, M3046, M3050, M3055, M3059, M3060, M3061, M3064, M3065, M3069, M3070, M3071, M3072, M3073, M3075, M3077, M3078, M3079, and M3080) with OD₄₁₄ readings comparable to the negative control; strong reaction with three MAbs (M3047, M3056, and M3066) with OD₄₁₄ readings above 0.5; and mild cross reactions, having OD₄₁₄ readings less than 0.5 but greater than the negative control with the remaining MAbs. Of seven other isolates from serogroup 4 (three *Salmonella* ser. Heidelberg isolates, *Salmonella* ser.

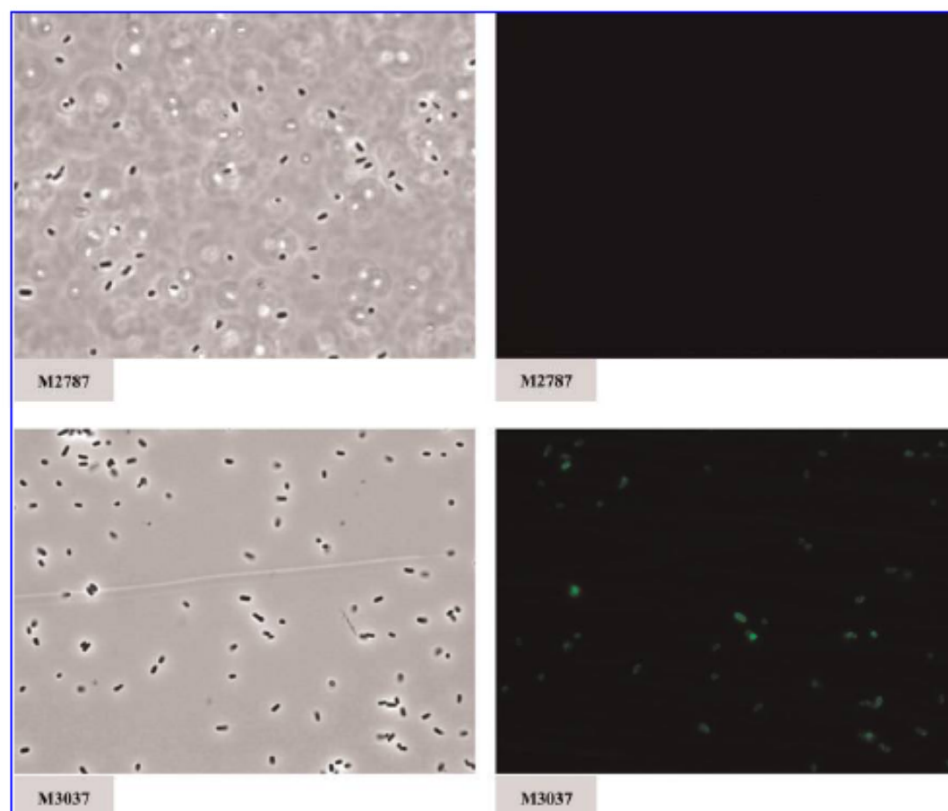


FIG. 1. Immunofluorescence staining of live *Salmonella* ser. Typhimurium DT104 cells with monoclonal antibodies. Bacteria cells were probed with all 45 MAbs followed by reaction with FITC-conjugated goat anti-mouse IgG as described in Materials and Methods. Examples of fluorescence images (right) and phase-contrast images (left) of the bacterial cells probed with two MAbs (M2787 and M3037) are shown.

Agona, *Salmonella* ser. California, *Salmonella* ser. Abortus equi and *Salmonella* ser. Chester), most showed negligible cross-reactions with all 45 MAbs. However, one of the three *Salmonella* ser. Heidelberg isolates tested and the *Salmonella* ser. Chester isolate cross-reacted significantly with these MAbs. Significant cross-reactions, having an OD₄₁₄ of greater than 0.5, were observed with *Salmonella* ser. Paratyphi A (a serogroup 1 isolate) and 13 MAbs (M3039, M3040, M3041, M3042, M3044, M3046, M3049, M3052, M3056, M3062, M3065, M3066, and M3067). Several other strains of *Salmonella* serotypes O:6 and O:9 exhibited negligible cross-reactions. All 45 MAbs showed no cross-reaction with the gram-negative bacterium *E. coli* O157:H7 and two gram-positive bacterial species of *Listeria*, *L. monocytogenes* and *L. grayi*.

Molecular nature of antigens recognized by monoclonal antibodies to Salmonella ser. Typhimurium

The ability of all 45 MAbs to bind purified *Salmonella* ser. Typhimurium LPS was demonstrated through immunoblotting. Although some MAbs gave a stronger binding signal than others, all 45 MAbs showed a similar band pattern characteristic of a LPS ladder on the immunoblot, each band differing from the next by one O-chain repeating unit (Fig. 2). The ability of the MAbs to bind equally to both the low molecular weight fragments and the high molecular weight fragments indicates that the antigen is likely the O-chain repeating unit.

Role of acetyl group in LPS in antibody-antigen binding

A highly purified preparation of LPS from *Salmonella* ser. Typhimurium as revealed by SDS-PAGE with silver staining (data not shown) showed strong reactions with all 45 MAbs by indirect ELISA (Fig. 2). This further confirmed that each MAb recognized LPS and also revealed that during the LPS purification process the integrity of the epitopes was not altered. The effect of various temperature and pH conditions on binding of all 45 MAbs to LPS was investigated using an indirect ELISA. No substantial change in the ability of each MAb to bind LPS was observed when LPS was pre-treated with PBS (pH 7.0) or acetate buffer (pH 4.6) at room temperature (Fig. 3a) or at high temperature of 100°C (Fig. 3b). However, LPS pre-treated with alkali conditions at room temperature retained only a moderate ability to interact with M3039, M3047, M3052, M3056, M3058, and M3066 and lost the ability to bind to all other MAbs. Pre-treatment of LPS in alkali conditions at 100°C completely abolished the ability of each MAb to bind LPS (Fig. 3b). Pre-treatment with alkali conditions removes the acetyl group from group B LPS while leaving the remaining structure intact.⁽²³⁾ A similar experiment was also carried out for other group 4 isolates, which appeared to cross-react with the MAbs. The same trend was observed with all other purified LPS from *Salmonella* ser. Heidelberg and *Salmonella* ser. Chester (data not shown). The results indicate that the presence of the acetyl group (O5 antigen) is required for binding of each MAb to LPS.

Role of lipopolysaccharide acetylase in binding of MAbs to Salmonella ser. Typhimurium

Binding of all 45 MAbs to an *acfA* gene inactivation mutant of *Salmonella* ser. Typhimurium was markedly reduced (Fig. 4)



FIG. 2. Immunoblot analysis of purified LPS from *Salmonella* ser. Typhimurium DT104. LPS (10 µg) from Sigma was loaded onto each lane of an SDS-PAGE gel, electrophoresed, electrotransferred onto nitrocellulose membranes, and probed with all 45 MAbs, followed by reaction with HRP-conjugated goat anti-mouse IgG. All 45 MAbs, exemplified here by M3037, showed reaction with LPS in a similar band pattern. Each additional band represents the addition or deletion of an O-antigen repeating unit from the band following or preceding it, respectively.

compared to *Salmonella* ser. Typhimurium DT104 (Fig. 5). The mutant lost the ability to interact with the majority of the MAbs and retained some binding to M3039, M3047, M3056, and M3058 with OD₄₁₄ readings around 0.5.

Discussion

In this study, 45 MAbs were generated against formalin-killed *Salmonella* ser. Typhimurium. These MAbs showed a high serological specificity for group 4 *Salmonella*, although they do not detect all group 4 isolates. Immunoblotting with purified LPS showed a distinctive ladder-like pattern under conditions used for carbohydrate antigens, confirming that the epitopes for these MAbs were located on the O-antigen of LPS (Fig. 2). Although the cells used for immunization were formalin killed, immunofluorescence microscopy demonstrated that the MAbs are capable of binding to live bacteria (Fig. 1). This indicates that the epitopes for these MAbs are surface exposed in live cells and were formalin resistant,

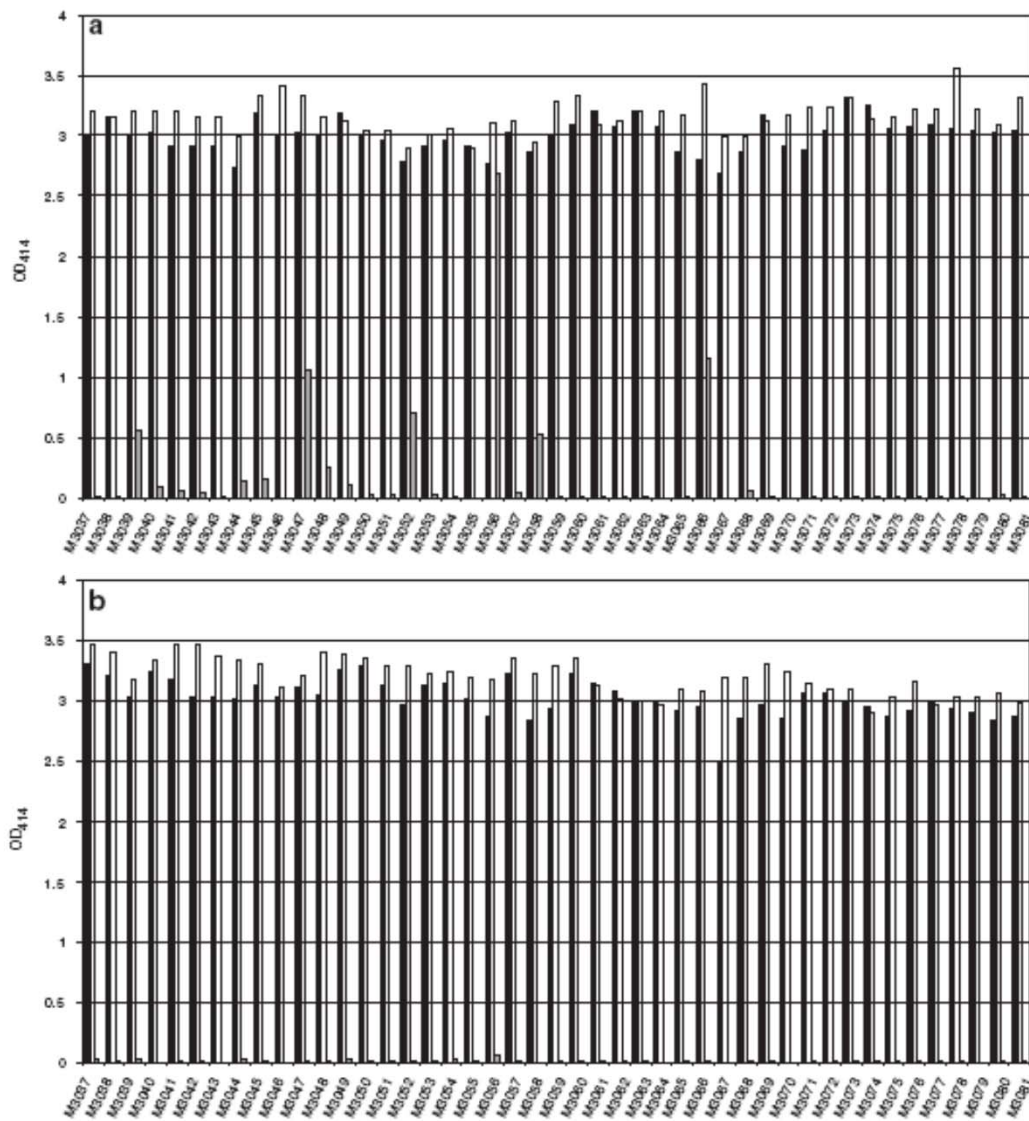


FIG. 3. ELISA analysis of monoclonal antibody interaction with chemically modified purified LPS. The first bar (■) represents LPS treated in acetate buffer at pH 4.6, second bar (□) represents LPS treated in PBS at pH 7.0, and the third bar (▨) represents LPS treated in NaOH at pH 9.6 for 30 min at room temperature (a) or 100°C (b). Each well of the ELISA plate were coated with ~1 µg of pre-treated LPS diluted in 100 µl of carbonate buffer. All ELISA OD values are the average of two determinations.

consistent with the nature of the epitope being the LPS O-antigen. This also suggests that these antibodies may be useful in capturing live *Salmonella* pathogen from food, water, or environmental sources.

Evidence that the presence of the acetyl group, which confers the O:5 serotype, is essential for the binding of all 45 MAbs to the *Salmonella* LPS was shown by assessing the ability of the MAbs to react with *Salmonella* ser. Typhimurium both before and after acetyl-group removal. The acetyl group

was removed using two independent methods: first through chemical removal of the acetyl group with heat and alkaline conditions and through creation of a mutant without a functional acetylase, which is required for the synthesis of the O:5 antigen.⁽¹²⁾ Although, this study delivers a novel set of 45 MAbs reactive to *Salmonella* ser. Typhimurium LPS, others have made similar findings concerning the significance of the O:5 antigen in serological assays with both MAbs and with polyclonal antisera.⁽¹¹⁻¹³⁾

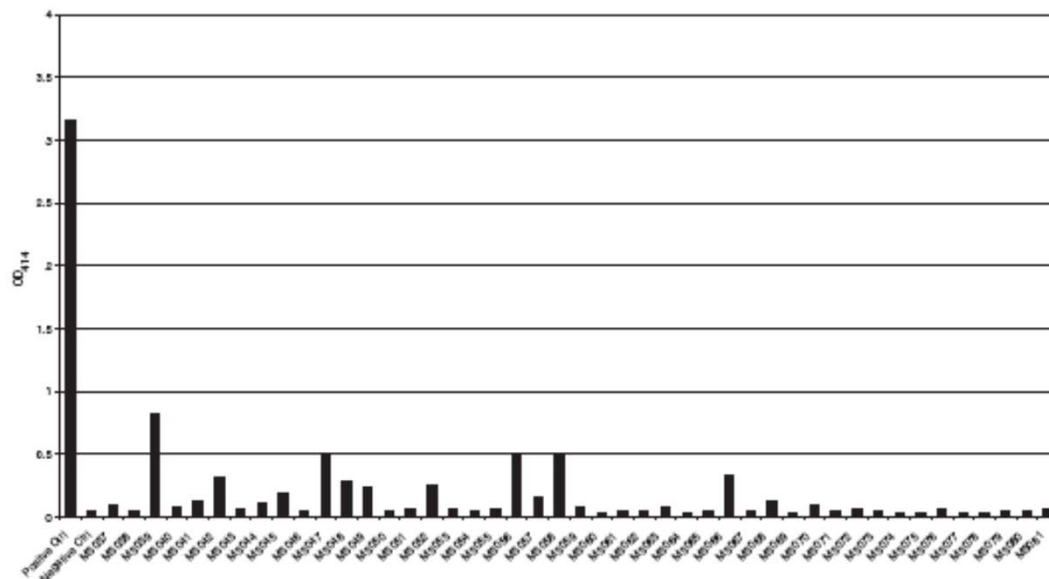


FIG. 4. ELISA analysis of monoclonal antibody interaction with an acetylase negative mutant of *Salmonella* ser. Typhimurium. ELISA plates were coated with formalin-killed cells of *Salmonella* ser. Typhimurium α fa 14027 α fa127::TN 10d-Km⁽¹⁵⁾ at a concentration of approximately 1.0×10^8 cells/well. All ELISA OD values are the average of two determinations.

Salmonella ser. Typhimurium is divided into two named serological variants, non-Copenhagen and Copenhagen based on the presence or absence of the O:5 antigen, respectively. The presence of the O5 antigen requires an LPS acetylase, which is chromosomally encoded, unrelated to phage conversion, and may or may not be present in wild-type

Salmonella isolates.⁽¹²⁾ Other studies have indicated that *Salmonella* ser. Typhimurium variants can differ in the percentage of acetylated abequeose residues they possess.⁽²⁴⁾ It is likely that other group O:4 isolates also vary in the relative abundance of abequeose acetylation. Variation in the percentage of acetylated abequeose residues, along with their absolute

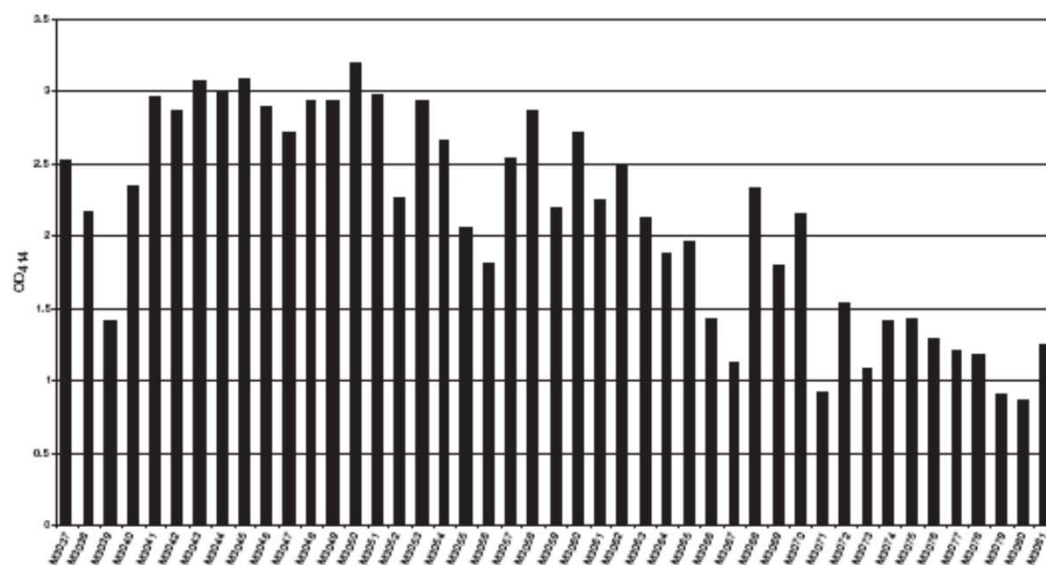


FIG. 5. ELISA analysis of monoclonal antibodies against *Salmonella* ser. Typhimurium DT104. ELISA plates were coated with purified LPS from *Salmonella* ser. Typhimurium DT104 at a concentration of $1 \mu\text{g}/100 \mu\text{L}$. All ELISA OD values are the average of two determinations.

presence or absence is a possible explanation for the variation in reactions between different group O:4 isolates and the panel of 45 MAbs. Evidence for this can be found in the cross-reactivity of the MAbs against *Salmonella* ser. Agona. Although this isolate has the same 1,4,[5],12 serotype as *S. ser. Typhimurium*, it reacted very weakly with most MAbs and not at all with others. This lack of reaction is too pronounced to be explained simply by a difference in the percentage of acetylated residues and is likely the result of the LPS acetylase gene and therefore acetyl group not being present in this particular isolate. This is also the likely cause of the reaction pattern observed for the three *Salmonella* ser. Heidelberg isolates that were examined in this study. One exhibited a strong reaction to the panel of MAbs and the other two did not (Table 2). These observations suggest that other group 4 *Salmonella* such as ser. Heidelberg could be divided into sub-groups based on the presence of the O:5 antigen, similar to *Salmonella* ser. Typhimurium, since they are also serologically distinct.

This study has demonstrated that acetylation of the abequeose residue is a critical factor in determining the binding specificity of the MAbs generated here against the O-antigen of LPS. Other studies have shown similar results as antibodies produced against fully acetylated LPS do not recognize deacetylated LPS.⁽¹³⁾ There are two proposed models to explain this. The first model suggests that the acetylated abequeose residue is the most immunodominant antigen and the majority of the antibodies produced during immunization will be directed against this specific epitope.⁽²⁵⁻²⁷⁾ The second and more recent model suggests that the acetylated abequeose residue alters the conformation of the entire *Salmonella* O-antigen, thereby changing the spectrum of available conformational epitopes.^(15,28) This is reasonable since multiple conformations are known to occur in oligosaccharides due to the fluidity of the glycosidic bonds that connect the sugar residues.⁽²⁹⁾ Our observations support the second model since minor cross-reactions are observed with non-acetylated but closely related isolates (Table 2). If the actual acetylated abequeose residue was the epitope, the reactions would be more binary in contrast to the spectra of cross-reactions that were observed here. It is likely that the spectra of cross-reactions with non-acetylated LPS can be explained by conformations that are slightly less than optimal for antibody binding. This study raises interesting prospects about the humoral immune response to carbohydrate antigens, and more studies are needed to understand what determines the choice of antigens for a humoral response.

Another interesting observation produced from this study is the mild cross-reactions with *Salmonella* ser. Abortus equi^(4,12) and *Salmonella* ser. Paratyphi A.^(1,2,12) The most likely explanation for this fits with the previous explanation in that acetylated abequeose alters the immunogenic epitopes present elsewhere on the LPS structure. The epitope that the MAbs are binding to is likely on the main part of the chain, the O:12, which is composed of repeating mannose, rhamnose, and galactose; however, in *S. ser. Typhimurium*, the stereochemistry of this repeating unit is altered by the acetylated abequeose residue.⁽¹¹⁾ This fits with the *Salmonella* ser. Abortus equi and *Salmonella* ser. Paratyphi A data since the O:12 epitope is still present, but not in optimal conformation since it is lacking the acetylated abequeose residue, leading to a mild cross-reaction. This observation is interesting and should be examined more fully by testing more isolates that lack the O:12 epitope in addition to *S. subsp. arizonae* and *S. ser.*

Thompson (Table 2) for cross-reactivity. Some MAbs with similar properties have been produced, such as 8aC10, which reacts optimally with acetylated group 4 LPS but also cross-reacts with LPS containing the O:8 antigen.⁽³⁰⁾ Interestingly the cross-reactivity of 8aC10 with O:8 containing LPS is observed when using whole cell antigen but is lost when using purified LPS, although it reacts optimally with group 4 LPS regardless of whether whole cell or purified LPS is used. This difference in epitope nature suggests a true cross-reaction where the epitopes are similar but not identical.⁽³⁰⁾ Our observations of cross-reactions with *Salmonella* ser. Abortus equi and *Salmonella* ser. Paratyphi A could also be explained by the presence of epitopes that are similar but not identical to those in group 4 isolates. An alternate possibility is that the structural changes to the oligosaccharide chain are effected by more than the acetylation status of the abequeose residue. Previous work has shown that the conformation of fatty acid chain in glycolipids can regulate the structure of the attached polysaccharide.⁽²⁹⁾ The structure of the lipid A tail anchoring the oligosaccharide to the cell surface may be playing a role in its structure and therefore affecting the presence and absence of conformational epitopes, in addition to acetylation status, although this would have to be confirmed by future work.

Some MAbs produced in this study, such as M3046, M3059, and M3080, are very specific for group 4 and have very low levels of cross-reactions with isolates in other serogroups. These antibodies may be useful in diagnostics. However, it would be important to develop a second MAb that binds to non-acetylated isolates that could work in synergy with these MAbs so that all group 4 *Salmonella*, even non-acetylated variants, would be detected.

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Author Disclosure Statement

The authors have no financial conflicts to declare.

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Jennifer Ronholm Ph.D.

Academic Positions:

Post-Doctoral Fellow - McGill University 2012-present
Supervisor: Dr. Lyle Whyte

Education:

Ph.D. University of Ottawa 2007-2012
Biochemistry, Microbiology and Immunology

B.Sc. University of Waterloo 2003-2007
Biology, Specialization in Microbiology

Scholarships and Awards:

Tuition Fee Scholarship 2013
University of Ottawa (~\$6500) – declined.

Dean's Scholarship 2013
University of Ottawa (\$3000)

Banting and Best Canada Graduate Scholarship - Doctoral Award 2009-2012
Canadian Institutes of Health Research (\$105,000)

National Excellence Scholarship 2009-2012
University of Ottawa (~\$19,000)

Ph.D. Admission Scholarship 2009-2012
University of Ottawa (~\$96,000) –declined.

Canadian College of Microbiology Student Symposium Award 2011
Canadian Society of Microbiologists Annual Meeting 2011 (\$500)

Michael Smith Foreign Study Supplement 2010
Canadian Institutes of Health Research (\$6,000)

Best Research Poster Award 2009
Federal Food Safety Research and Nutrition Meeting

Walkerton Clean Water Scholarship 2008
Walkerton Clean Water Centre (\$10,000)

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Peer-Reviewed Publications:

Ronholm, J., VanFassen, H., McKenzie, R., Zhang, Z., Cao, X, and Lin, M. Monoclonal antibodies recognizing the surface autolysin IspC of *Listeria monocytogenes* serotype 4b: epitope localization, kinetic characterization, and cross-reaction studies (PLoS ONE in press)

Ronholm, J., Cao, X., and Lin, M. Unveiling the expression characteristics of IspC, a cell wall-associated peptidoglycan hydrolase in *Listeria monocytogenes* during growth under stress conditions. 2012. Applied and Environmental Microbiology. 78(22): 7833-7840.

Ronholm, J., Wang, L., Hayashi, I., Sugai, M., Zhang, Z., Cao, X. and Lin, M. The *Listeria monocytogenes* serotype 4b autolysin IspC has *N*-acetylglucosaminidase activity. 2012. Glycobiology. 22(10): 1311-1320.

Ronholm, J., Zhang, Z., Cao, X., and Lin, M. Production and Characterization of monoclonal antibodies to lipopolysaccharide antigens of *Salmonella enterica* serotype Typhimurium DT104. 2011. Hybridoma. 30(1): 43-52.

Lin, M., Armstrong, S., **Ronholm, J.**, Dan, H., Auclair, M.-E., Zhang, Z., and Cao, X. Screening and characterization of monoclonal antibodies to the surface antigens of *Listeria monocytogenes* serotype 4b. 2009. Journal of Applied Microbiology. 106(5): 1705-1714.

Ronholm, J. Cao, X., and Lin, M. The *Listeria monocytogenes* serotype 4b autolysin IspC is SecA2-independent. Submitted to Basic Microbiology. Manuscript ID jobm.201300007.

Theses:

Ronholm, J., Cao, X. and Lin, M. 2012. Evidence for the *N*-acetylglucosaminidase activity of cell wall-associated autolysin IspC and its suitability as a diagnostic marker for *Listeria monocytogenes* serotype 4b. Ph.D. University of Ottawa.

Ronholm, J. and Ma, K. 2007. Metabolic conversion of glycerol to acetate by the hyperthermophilic anaerobe *Thermotoga maritima*. B.Sc. University of Waterloo.

Invited Presentations:

IspC is conserved in *Listeria monocytogenes* serotype 4b and has potential for use in food-processing diagnostics. January 19, 2012. Canadian Food Inspection Agency. Ottawa, ON

IspC is a Novel *N*-acetylglucosaminidase conserved in *Listeria monocytogenes* serotype 4b. December 14, 2011. University of Ottawa. Ottawa, ON

IspC – An Autolysin with potential for use in food microbiology. March 3, 2010. University of Hiroshima. Hiroshima, Japan.

Jennifer Ronholm

Conference Presentations:

The *Listeria monocytogenes* serotype 4b autolysin IspC acts as an *N*-acetylglucosaminidase. June 23, 2011. Canadian Society of Microbiologists. Oral presentation. St. John's, NFLD.

Advanced characterization of Monoclonal Antibodies against *Listeria monocytogenes* serotype 4b. November 10, 2010. Federal Food Safety Research and Nutrition Meeting. Poster presentation. Gatineau, QC.

The Autolysin IspC Acts as an *N*-Acetylglucosaminidase in *Listeria monocytogenes* serotype 4b and has Significantly Reduced Activity on De-Acetylated Peptidoglycan. May 7, 2010. International Symposium on Problems of *Listeriosis*. Porto, Portugal.

IspC – A Protein with Potential to be targeted for Immunological Detection of *Listeria monocytogenes* serotype 4b. Federal Food Safety Research and Nutrition Meeting. Poster presentation. November 4, 2009. Ottawa ON.

General Interest Publications:

Ronholm, J. I still have faith in the meat inspection system. Published September 5, 2008. *Ottawa Citizen*.

Ronholm, J. Northern Lights – Science Education in the Far North. Published Summer 2012. *Science Travels Newsletter*.

Teaching Experience:

Guest Lecturer – Pollution and Bioremediation (December 4)	2012
Teaching Assistant – Biochemistry Honours Project Seminars	2011
Teaching Assistant – Molecular Biology Laboratory	2009-2010
Teaching Assistant - Virology	2007
Teaching Assistant – Cell and Molecular Biology	2005-2006

Professional Memberships:

Quebec Centre for Biodiversity Science	Since 2012
Canadian Society of Microbiologists	Since 2010
International Society for Infectious Diseases	Since 2011

Jennifer Ronholm

Volunteer Work:

Science Travels	2011-2012
Travel to remote First Nation Reserves and provide hands-on science activities and training to students and teachers	
○ Northwest Territories – Yellowknife, Hay River and Fort Resolution (Team Leader)	2012
○ Northern Ontario – Kenora, Dryden and Thunder Bay	2011
Aboriginal Mentorship Program	2011
Provide academic and personal mentorship to marginalized aboriginal youth	
Let's Talk Science	Since 2010
Provide mentorship and hands-on science instruction to elementary and high-school students	