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Molecular Genetic Characterization of *ispD*, a Gene Coding for a Novel Immunogenic Surface Protein with a Role in *Listeria monocytogenes* Virulence

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Molecular Genetic Characterization of *ispD*, a Gene Coding for a Novel Immunogenic Surface Protein with a Role in *Listeria monocytogenes* Virulence

By

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Thesis submitted to the
Faculty of Graduate and Postdoctoral Studies
In partial fulfillment of the requirements
For the MSc degree in Microbiology and Immunology

Department of Biochemistry, Microbiology and Immunology

Faculty of Medicine

University of Ottawa

Your file Votre référence
ISBN: 978-0-494-48430-2
Our file Notre référence
ISBN: 978-0-494-48430-2

ABSTRACT

Listeria monocytogenes is a Gram-positive intracellular bacterial pathogen that causes severe invasive infections (listeriosis) in humans. A novel protein of unknown function from *L. monocytogenes* serotype 4b, designated IspD, was previously identified as an immunogenic target that reacts with antiserum (RαL) from rabbits infected with live *L. monocytogenes* but not with antiserum (RαK) from animals immunized with heat-killed bacteria (Yu, MSc. thesis, 2004). This findings suggests that IspD is induced specifically or significantly upregulated *in vivo* during *L. monocytogenes* infection and thus may be important in bacterial pathogenesis. The open reading frame (ORF) of *ispD*, which had been partially determined in the previous study, was further sequenced to obtain a complete ORF of 3,222 bp coding for a 116 kDa protein of 1073 amino acids. Bioinformatics analysis of the deduced amino acid sequence of IspD revealed that this protein contained an N-terminal signaling peptide, leucine rich repeats (LRRs), and a C-terminal cell wall anchoring motif LPXTG, characteristic of a surface protein of the internalin family. The recombinant form of putatively mature IspD, expressed in *Escherichia coli*, was purified and used to raise specific polyclonal antibodies in rabbits for protein characterization. Immunofluorescence and immunogold labeling using anti-IspD antibodies showed that IspD was localized on the bacterial surface, consistent with the presence of a LPXTG motif within IspD. A low level of the native IspD, expressed by *L. monocytogenes*, was detected in all phases during a 48-h *in vitro* growth, although the gene was transcribed only in the early to exponential growth phases. This, together with the previous findings, suggests that IspD is upregulated during *in vivo* infection. Regulated expression of IspD is demonstrated by an increased expression of

IspD following recovery from oxidative stress in brain heart infusion and buffered *Listeria* enrichment broths. Role of IspD in *L. monocytogenes* virulence was investigated by in-frame deletion of the *ispD* gene from the bacterial chromosome to create a Δ*ispD* mutant strain. This mutant exhibited complete abrogation of expression of IspD and showed no defects in *in vitro* growth, colony and microscopic morphologies, or biochemical properties. The cell culture infection assays using number of mammalian cell lines for adhesion and invasion demonstrated that the Δ*ispD* mutant is impaired in adhesion to and invasion of Hep-G2 and L132 cells only compared to the wild-type. This result indicates an involvement of IspD in bacterial adhesion and invasion in a cell-type dependent manner. Furthermore, intracellular growth of the Δ*ispD* mutant in a selected epithelial cell line Vero but not in the murine phagocytic cell line J774 was attenuated at a late infection stage. Altogether, evidence from this study strongly suggests that IspD, an immunogenic surface stress response internalin-like protein, plays a role in *L. monocytogenes* virulence.

To my parents,

wife, family and friends.

ACKNOWLEDGMENTS

First of all, I am grateful to my supervisor, Dr. Min Lin, for his guidance and advice throughout my research. I gained a lot of knowledge and learned a lot about molecular biology and biochemistry throughout this work. I would like to thank him for the patience that he has generously given me toward finishing my thesis and for his support in various ways. Also, it is a pleasure to convey my gratitude to my thesis committee members, Dr. Susan Nadine-Davis and Dr. Franco Pagotto, for their valuable and constructive comments and advice. My special thanks to the Canadian Food Inspection Agency for supporting me in pursuing my project.

I am thankfully acknowledging, Leith Kealy, Bill Trippe, Bob Davis for their generous contribution in the animal study. Many thanks go in particular to Beverly Phipps-Todd for her technical support with electron microscope. It is a pleasure to pay tribute to my lab mates for what I gained while working with them. Special thanks go to Heather McRae, Linru Wang and Hanhong Dan for their support and contributions. As well, I would like to thank Marie-Eve Auclair, Dione Ng, Kelly Coulombe and Steven Luo for their help and assistance.

I was extraordinarily fortunate in having Anwar Hashem as a supportive friend who has inspired me a lot with his scientific discussions and inputs. I am thankful to Dr. Essam Al-Yamani for reading my thesis and for his valuable comments.

From my all heart, a special thanks to my parents. To my father for his encouragement and guidance, so that I became who I am in life. To my sweet-hearted mother, who has been always for me with her continuous support and prayers. I cannot find words that can describe my gratitude to them.

To my wife, I cannot express enough my appreciation and thankfulness for her encouragement, patience, and support. I thank her for being always with me and having confidence in me. Last but not least, I would like to thank all my brothers and sisters, and friends for their support and help. I would like to thank everyone who supported and contributed in making my project a successful one.

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LIST OF ABBREVIATIONS

β-ME, β-mercaptoethanol
aa, amino acid
bp, base pair
BHI, brain heart infusion
BLEB, buffered *Listeria* enrichment broth
BPW, buffered peptone water
BSA, bovine serum albumin
CFU, colony forming unite
DEPC, diethyl pyrocarbonate
DMEM, Dulbecco's Modified Eagle Medium
DTT, dithiothreitol
FBS, fetal bovine serum
HBMEC, human brain microvascular endothelial cell line
Km, kanamycin
IMAC, immobilized metal ion affinity chromatography
IPTG, isopropyl β-D-thiogalactopyranoside
LB, Luria-Bertani broth
LBMOPS, Luria-Bertani broth with morpholinepropanesulfonic acid
LLO, listeriolysin O
LRB, *Listeria* repair broth
LRR, leucin-rich repeats
MOI, multiplicity of infection
NEAA, non-essential amino acids
Ni-NTA, nickel-nitrilotriacetic
NMWL, nominal molecular weight limit
nt, nucleotide
ORF, open reading frame
PBS, phosphate buffered saline
PBS-TT, phosphate buffered saline with tween 20 and triton X-100
PC, phosphatidylcholine
PCR, polymerase chain reaction
PI, phosphatidylinositol
PLC, phospholipase C
SCP, sheep choroid plexus
SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis
SLO, streptolysin O
TCA, trichloroacetic acid
TE, Tris-EDTA
TEM, transmission electron microscopy
TSBA, trypticase soy blood agar
WB, Western blot
WT, wild-type

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CHAPTER ONE

INTRODUCTION

1.1 History of *Listeria monocytogenes*

It was in Cambridge (England) where *Listeria monocytogenes*, which caused a disease characterized by mononuclear leucocytosis, was first isolated from rabbits and guinea pigs in 1924 by E.G.D. Murray (Murray *et al.*, 1926). Due to mononuclear leucocytosis characteristic of the infection, the bacterium was named *Bacterium monocytogenes*. However, it is believed that *Listeria* infection was observed earlier than that date (Gray and Killinger, 1966); bacteria with characteristics similar to *Listeria monocytogenes* was isolated from tissues of dead patients in France and Germany in 1891 and 1893, respectively. Furthermore, Hülphers had encountered similar bacteria in necrotic livers of rabbits in Sweden in 1911 (Hülphers, 1911); due to the bacterial affinity to colonize the liver, it was given the name *Bacillus hepatis*. One year following Murray's description of the bacterium, the exact same microorganism was isolated by Pirie from livers of gerbils known as African jumping mouse (Pirie, 1927) and named *Listerella hepatolytica*. Then, the bacterium was given its current name, *Listeria monocytogenes*, by Pirie in 1940 (Pirie, 1940).

The bacterium showed its first emergence as human pathogen (the agent of listeriosis), not too long after its first isolation. In 1929, Nyfeldt isolated *Listeria* from patients with infectious mononucleosis-like infection in Denmark (Nyfeldt, 1929). Few years later, a second incidence of human listeriosis was reported in the United States in 1933 by

Burn (Burn, 1936). Afterward, *Listeria* continued to cause sporadic infections (Fischer, 1941; (Gray and Killinger, 1966; Hof, 2003; Kapsenberg, 1941) until 1949 when the first report of *Listeria* outbreak among newborns was recorded in Germany (Reiss *et al.*, 1951; Seeliger, 1961), and it was in Canada where *Listeria* was first identified as foodborne pathogen (Schlech *et al.*, 1983) during an outbreak due to consumption of contaminated coleslaw. Then in 1980s, several other outbreaks were reported in North America, Europe, Australia, and New Zealand (Bula *et al.*, 1995; Lennon *et al.*, 1984; McLauchlin, 1990a; McLauchlin, 1990b; Schlech *et al.*, 1983) with about 15 outbreaks of human listeriosis reported in the United States from 1980 to 2000 (Olsen *et al.*, 2005) with a mortality rate of about 28% (Donnelly, 2001; Mead *et al.*, 1999). A recent example is the listeriosis outbreak in Canada in August, 2008. According to the Public Health Agency of Canada (http://www.phac-aspc.gc.ca/alert-alerte/listeria/listeria_2008-eng.php, accessed on September 19th, 2008), there is a total of 48 confirmed listeriosis cases all over the country with 18 confirmed death. Thus, *L. monocytogenes* is of a great importance to public health, which has drawn considerable attention from many researchers worldwide.

Listeria also aroused the interest of immunologists around the world. In 1962, Mackaness and his group demonstrated the ability of the bacteria to evade macrophage killing in mice and its capability of intracellular parasitism (Mackaness, 1962) . As an intracellular pathogen, the bacterium triggers cellular immune response. This amazing discovery made *Listeria* the central focus of immunologist as a model microorganism to study not only cell-mediated immunity but also vaccine efficacy for over 40 years (Cossart, 2007; Zenewicz and Shen, 2007; Cossart and Mengaud, 1989).

The key gene responsible for intracellular growth of *L. monocytogenes* was unknown until 1987 when a group of German scientists inactivated the hemolysin property of the bacteria by transposon Tn916 insertion (Kathariou *et al.*, 1987). Later on, the role of the hemolysin gene (*hly*) product, listeriolysin O (LLO), was determined to be crucial for intracellular growth of the bacterium (Portnoy *et al.*, 1988; Vazquez-Boland *et al.*, 2001b), and the nucleotide sequence of *hly* was determined in 1988 (Mengaud *et al.*, 1988).

In 2001, the entire genome sequence of *L. monocytogenes* strain EGD-e (serovar 1/2a), together with its nonpathogenic counterpart, *L. innocua* strain CLIP 11262 (serovar 6a), was reported (Glaser *et al.*, 2001). This has opened many doors for bioinformatics and experimental studies that led to identification of more potential virulence and biological factors. For example, the genome sequences of *L. monocytogenes* strains F2365 (serotype 4b), EGD-e (serotype 1/2a), H7858 (serotype 4b), and F6854 (serotype 1/2a) revealed the presence of 25, 24, 25, and 26 internalin coding genes, respectively (Nelson *et al.*, 2004); the products of these genes belong to the largest family of proteins in *L. monocytogenes*, which are predicted to be either cell wall-anchored or secreted proteins (Bierne *et al.*, 2007). Among this family of proteins, only internlain A (InlA) and internalin B (InlB) are two well characterized virulence factors required for invading host cells by *L. monocytogenes* (Vazquez-Boland *et al.*, 2001b); the function of most of the remaining proteins remains largely unknown. Much more effort is required to investigate the proteins of unknown function encoded by the genome of *L. monocytogenes* in the future in order to fully understand the biology, pathogenesis of, and immunity to *Listeria*.

1.2 Microbiology of *L. monocytogenes*

Morphology. *L. monocytogenes* is a short, nonspore-forming, Gram-positive rod measuring 1.0 - 2.0 μm in length and 0.5 μm in width with round ends (Gray and Killinger, 1966). On a blood agar plate, the bacterium produces an incomplete β-hemolysis zone, whereas it produces bright blue-green colonies on a blood-free agar plate (Farber and Peterkin, 1991).

Biochemical characteristics. *L. monocytogenes* is facultative anaerobe that is able to grow over a wide range of temperature (-1.5 – 50 °C) and pH (4.3 – 9.6) with an incredible ability to resist high salt challenges (up to 25.5% NaCl), freezing, and drying (Donnelly, 2001). It is catalase positive and oxidase negative organism, and its β-hemolysin works synergistically with that of *Staphylococcus aureus* on blood agar plate generating a large zone of β-hemolysis (CAMP test positive). The bacterium possesses polar flagella that allow the organism to be motile at 20 – 25 °C with a characteristic tumbling motility. This motility vanishes at 37 °C (Farber and Peterkin, 1991; Peel *et al.*, 1988). Furthermore, *L. monocytogenes* ferments L-rhamanose and α-methyl D-mannoside but not D-xylose nor D-mannitol (Bille *et al.*, 1992; McLauchlin, 1997).

Species of *Listeria*. The *Listeria* genus contains six species (*L. monocytogenes*, *L. seeligeri*, *L. welshimeri*, *L. innocua*, *L. ivanovii*, and *L. grayi*). Of these six species, *L. monocytogenes* is the only human pathogen (Donnelly, 2001). *L. monocytogenes* and *L. innocua* are the only species that can ferment L-rhamanose and α-methyl D-mannoside. On the other hand, *L. monocytogenes*, *L. seeligeri*, and *L. ivanovii* are the only ones which give a positive CAMP test result. Therefore, one can easily differentiate *L. monocytogenes* from

other nonpathogenic *Listeria* species by L-rhamanose and α-methyl D-mannoside fermentation, together with the CAMP test (Bille *et al.*, 1992; McLauchlin, 1997).

Serology. Based on somatic (O) and flagella (H) antigens, *L. monocytogenes* is divided into 13 serotypes, three of which (1/2a, 1/2b, and 4b) accounts for almost all human infections (Donnelly, 2001; Lorber, 1997) with serotype 4b strains causing more than half of human listeriosis cases worldwide (Vazquez-Boland *et al.*, 2001b).

1.3 Listeriosis

Epidemiology. *Listeria* species are widely distributed in the environment and frequently isolated from soil, water, effluents, vegetation, wild and domestic animals, dairy products, and human and animal feces. It is also present in the gut of more than 5% of healthy individuals (Ramaswamy *et al.*, 2007).

Human listeriosis is manly acquired through ingestion of contaminated food. This includes raw vegetables, dairy goods, fresh or processed meat and poultry products, fish, and caned foods (Farber and Peterkin, 1991). Although consumption of contaminated food is the main cause of human listeriosis, other modes of transmissions of human listeriosis have been described. *L. monocytogenes* can be transmitted from mothers to their fetuses vertically. Also, cross infections between nurses and neonates and from use of contaminated mineral oil have been documented (Colodner *et al.*, 2003; Farber and Peterkin, 1991; Ramaswamy *et al.*, 2007; Schuchat *et al.*, 1991).

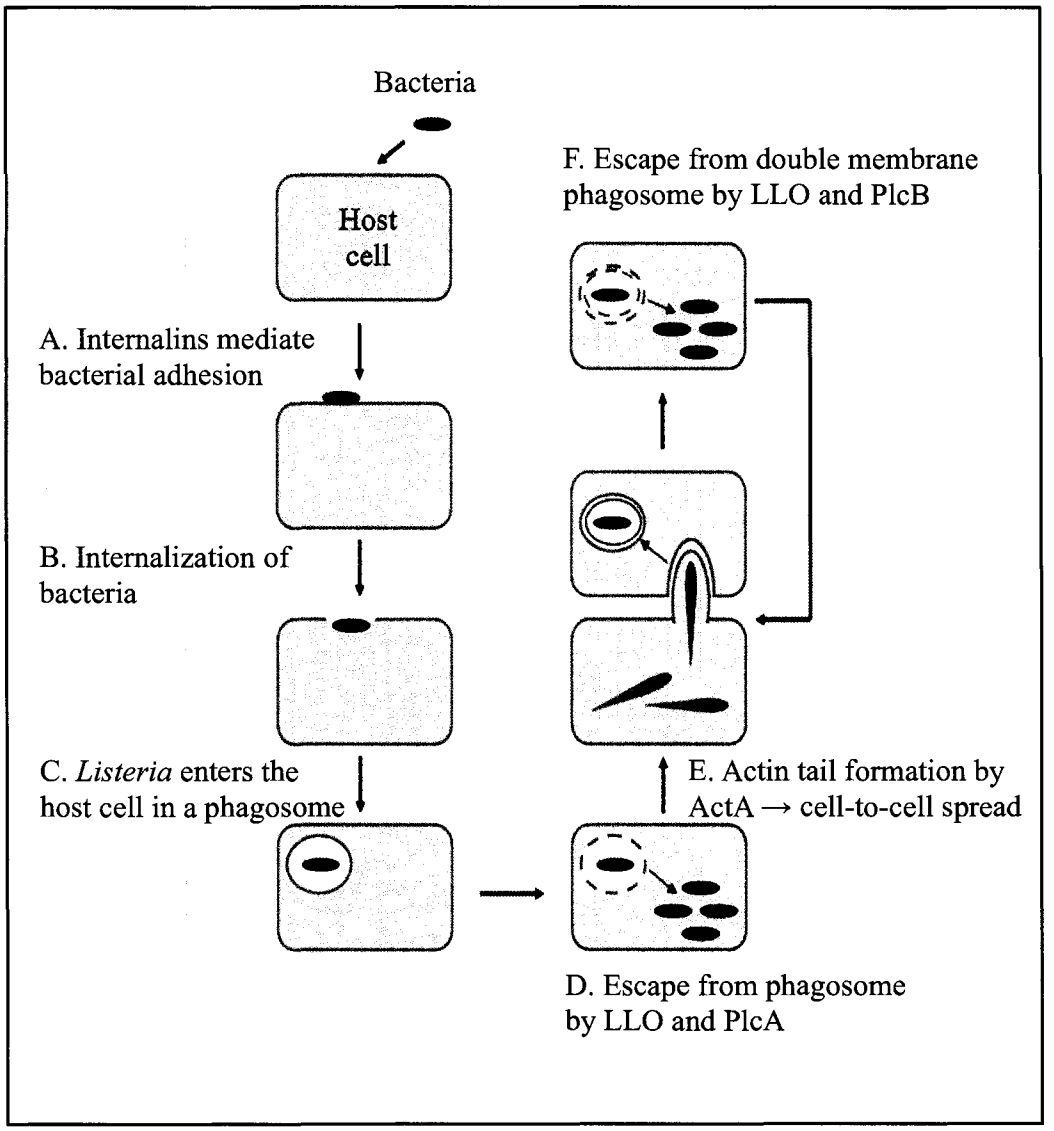
Normally, exposure of healthy immunocompetent individuals to *L. monocytogenes* will result in asymptomatic infection or self-limiting gastroenteritis. However, invasive and disseminated listeriosis mostly affects infants less than one month of age and people over 60 years. Pregnant women, immune suppressed patients such as organ and bone marrow transplant patients, cancer patients, alcoholics, and individuals with acquired immunodeficiency syndrome (AIDS) are also at high risk of developing invasive and disseminated listeriosis (Farber and Peterkin, 1991; Hof, 2003; Vazquez-Boland *et al.*, 2001b).

Pathogenesis. The life cycle of *L. monocytogenes* starts when the bacterium enters the body mainly by ingestion of contaminated food. The oral load of bacteria that is associated with clinical symptoms in human is still unknown. However, as low as 10^2 to 10^4 bacteria per gram of food has been seen to cause human listeriosis. The incubation period in human is about 20 hrs in the case of gastroenteritis following ingestion of food contaminated with a high number of bacteria, and about 20-30 days in the case of disseminated listeriosis. When *L. monocytogenes* reaches the small intestine, it induces its uptake into intestinal and phagocytic cells through the bacterial surface proteins such as InlA, a phenomenon known as "parasite-directed endocytosis" (Figure 1). Then, the bacterium enters the cytoplasm in a phagosome that is then disrupted by the action of listeriolysin (LLO) and phospholipase C (PlcA). Free in the cytoplasm, *L. monocytogenes* multiplicates and initiates intracytoplasmic movement by the bacterial surface protein ActA to recruit host cell actin molecules to form actin tails (actin polymers). Intracellular movement allows the bacterium to reach the cytoplasmic membrane and invade the adjacent cells to form a double membrane phagosome, which is then disrupted by the action of LLO and another phospholipase C

(PlcB); and the cycle repeats. Following crossing the intestinal barrier, the bacterium reaches blood and lymph streams and carried to mesenteric lymph nodes, liver, and spleen. From there, *Listeria* disseminates further and breaches two additional barriers, blood-brain and feto-maternal barriers to infect the central nervous system (CNS) and the fetus, respectively (Farber and Peterkin, 1991; Hamon *et al.*, 2006; Vazquez-Boland *et al.*, 2001b).

A number of important virulence factors involved in the pathogenesis of *L. monocytogenes* are discussed in details in Section 1.4.

Figure 1. The intracellular life cycle of *L. monocytogenes*. Attachment of the bacterium to host cells is mediated by internalins (**A**). Adhesion to host cells promotes bacterial internalization (**B**), leading to the formation of a phagosome containing the bacterium in the cytoplasm (**C**). Then, the bacterium escapes from the phagosome and replicates in the cytoplasm after phagosomal lysis through the action of LLO and PlcA (**D**). The bacterium in the cytoplasm recruits, via the bacterial surface protein ActA, the host cell actin molecules to form actin tails, which facilitates the entry of the bacterium into the cytoplasm of the neighboring cells contained in a double-membrane phagosome (**E**). Lysis of the newly formed phagosome by the action of LLO and PlcB releases the bacterium into the cytoplasm (**F**), and the cycle repeats.


Bacteria
Host cell
A. Internalins mediate bacterial adhesion
B. Internalization of bacteria
C. *Listeria* enters the host cell in a phagosome
D. Escape from phagosome by LLO and PlcA
E. Actin tail formation by ActA → cell-to-cell spread
F. Escape from double membrane phagosome by LLO and PlcB

Clinical manifestations and treatment. Severity of listeriosis mainly depends on the virulence of infecting strain, bacterial load, and host susceptibility to listerial infection. About 98% of human listeriosis cases is attributed to serotypes 1/2a, 1/2b, and 4b (Buchrieser, 2007; Donnelly, 2001; Lorber, 1997) with serotype 4b strains accounting for almost all the major outbreaks and a large portion of sporadic infections worldwide (Buchrieser, 2007; Vazquez-Boland *et al.*, 2001b). In immune competent individuals, ingestion of a low number of bacteria results in asymptomatic illness. However, intake of food heavily contaminated with *L. monocytogenes* cause self-limiting gastroenteritis that might be accompanied by nausea, abdominal pain, diarrhea, and fever (Lecuit, 2007); most of the bacteria that are capable of crossing intestinal barrier to reach liver and spleen are cleared by the immune system of healthy persons (Zenewicz and Shen, 2007). In high-risk individuals, uncleared bacteria in the blood usually cause prolonged bacteremia that is accompanied by fever and myalgias. These bacteria may cross blood-brain barrier causing meningitis, encephalitis, and brain abscess or reach the heart causing endocarditis. In pregnant women, *L. monocytogenes* can breach the feto-maternal barrier causing premature labor, abortion, or generalized fetus infections known as "granulomatosis infantiseptica" (Lecuit, 2007). Other complications of disseminated listeriosis include pneumonia, adult respiratory distress syndrome, acute hepatitis, peritonitis, septicemia, disseminated intravascular coagulation, and acute renal failure (Lamont *et al.*, 1988).

The antibiotic of choice for treating *L. monocytogenes* infection is ampicillin. However, penicillin, amoxicillin, and erythromycin are also used. Combined antibiotic treatment, such as administering amoxicillin together with gentamicin, has shown to give better treatment from listeriosis in some cases (Meier and Lopez, 2001).

1.4.1 Virulence factors involved in adhesion and invasion of host cells

Internalin A (InlA). Bacterial internalization to nonphagocytic cells is an important step in *L. monocytogenes* pathogenesis. The virulence determinants responsible for this process was unknown until 1991 when Gaillarda *et al.* screened for transposon mutants impaired in the ability to adhere to the epithelial cell line Caco-2 *in vitro*. In that study, they have identified the first internalin, designated internalin A (InlA) (Gaillard *et al.*, 1991). InlA is a member of the internalin family, the largest group of surface proteins in *L. monocytogenes*, characterized by the presence of tandem leucin-rich repeats (LRRs) that are mainly engaged in protein-protein interactions (Bierne *et al.*, 2007; Enkhbayar *et al.*, 2004; Gaillard *et al.*, 1991). InlA is a 80-kDa protein of 744 amino acids (aa) with an N-terminal region (region A) consisting of an N-terminal signaling peptide and a conserved internalin cap in addition to 15 LRRs. An inter-repeat region (IR) separates region A from the C-terminal region (region B) that consists of 3 longer LRRs followed by a cell wall sorting signal composed of a LPXTG motif and a hydrophobic tail of about 20 aa; this sorting signal is required for the covalent attachment of the protein onto the bacterial cell wall by the enzyme sortase A (Dussurget *et al.*, 2004; Gaillard *et al.*, 1991; Vazquez-Boland *et al.*, 2001b).

The role of inlA in mediating bacterial invasion of non-phagocytic cells was demonstrated in several *in vitro* and *in vivo* studies. Introduction of *inlA* into the non-invasive strains *L. innocua* and *Enterococcus faecalis* rendered these bacteria the ability to invade Caco-2 epithelial cells (Gaillard *et al.*, 1991; Lecuit *et al.*, 1997), and coating latex beads with recombinant InlA was sufficient to induce its internalization into LE6 cells (Lecuit *et al.*, 1997). There is evidence that InlA contributes to crossing intestinal and feto-

maternal barriers by *L. monocytogenes* through internalin-E-cadherin interaction (Dramsi *et al.*, 1997; Gaillard and Finlay, 1996; Lecuit *et al.*, 2001; Lecuit *et al.*, 2004; Lingnau *et al.*, 1995). E-cadherin (an epithelial surface molecule), which was first identified as the cellular receptor for inlA by InlA-column affinity chromatography (Mengaud *et al.*, 1996), is a calcium-dependent intercellular adhesion glycoprotein that binds cells together to maintain the integrity of tissues (Mengaud *et al.*, 1996; Pentecost *et al.*, 2006). Normally, E-cadherin is found hidden in the basolateral membrane junction of many polarized epithelium tissues, such as intestine and feto-placental barriers. However, the InlA protein gets access to the transiently exposed E-cadherins at the apical sides of extruded senescent cells (Pentecost *et al.*, 2006). A single E-cadherin molecule consists of seven domains: five immunoglobuline-like extracellular domains (named EC1 to EC5), one α-helix transmembrane domain, and one intracellular domain. InlA interacts with E-cadherin through its N-terminal LRR repeats. These repeats are composed of 16 β-sheets that are curved, forming a hydrophobic pocket. Upon binding of InlA to E-cadherin, the LRR pocket is filled with the first extraceluller domain of E-cadherin (EC1) (Schubert *et al.*, 2002); the last 35 aa of the cytoplasmic (intracellular) domain of E-cadherin interacts with β-catenin that recruits α-catenin. Binding of α-catenin to the host cell actin leads to actin cytoskeleton rearrangement and ultimately to the bacterial internalization (Schubert *et al.*, 2002). Interestingly, the proline residue at position 16 in EC1 domain plays a crucial role in this interaction; a single aa difference between human and murine E-cadherin at that position (proline in human E-cadherin, glutamate in murine counterpart) ceases the binding of InlA to murine E-cadherin, which renders a murine model unsuitable for oral infection with *L. monocytogenes*. Indeed, the importance of the proline residue at this particular position for InlA binding to E-cadherin

was highlighted by trangeneic mice that express human E-cadherin, which become susceptible to oral infection with *L. monocytogenes* (Lecuit *et al.*, 2001).

Internalin B (InlB). InlB is a 67 kDa protein of 630 aa. Similar to InlA, the N-terminal region of InlB has an N-terminal signaling peptide, a short conserved internalin cap, seven LRRs, an IR, and one B repeat (Dramsi *et al.*, 1995; Gaillard *et al.*, 1991). On the other hand, the 232 aa C-terminal region of the protein is different from that of InlA and has 3 C-repeats, each composed of 80 aa that starts with a glycine/tryptophane (GW) dipeptide known as the GW modules. InlB is non-covalently attached to the bacterial cell wall by its C-terminal GW modules by interacting with the cell wall component lipoteichoic acids (Braun *et al.*, 1997; Jonquieres *et al.*, 1999).

Several lines of evidence indicate that InlB is important in *L. monocytogenes* adhesion to and invasion of host cells. This protein is required for invading murine (ATCC TIB73) and human (HepG-2) hepatocytes, human brain microvascular endothelial cells (HBMEC), and epithelia cell lines (Vero and Hela) by *L. monocytogenes* (Dramsi *et al.*, 1995; Greiffenberg *et al.*, 1998; Ireton *et al.*, 1996). Expression of InlB in the non-invasive and nonpathogenic *Staphylococcus carnosus* and *L. innocua* enables these bacteria to invade Vero cells. Also, coating latex beads with recombinant InlB has resulted in internalization of the beads into Vero cells (Braun *et al.*, 1998).

The first receptor for InlB was identified, by affinity chromatography, to be the receptor of the globular part of the complement component C1q (gC1q-R) or p32 (Braun *et al.*, 2000). It is a trimeric doughnut-shaped protein with asymmetric charge distribution on its surface with multi-ligand binding ability (Jiang *et al.*, 1999). In order to interact with gC1q-

R, InlB dissociates from the bacterial cell wall and binds to the receptor through its GW modules. However, the exact mechanism by which gC1q-R is anchored on the cell membrane and its intracellular signal transduction pathway upon activation is still unknown (Marino *et al.*, 2002). The second and main receptor for inlB is the hepatocyte growth factor receptor (HGFR) Met (Shen *et al.*, 2000). It is a transmembrane signaling receptor, a member of tyrosine kinase receptor family (Hubbard and Till, 2000). Binding of the concave shaped LRRs of InlB to the mature Met on the host cell surface activates Met, leading to protein tyrosine phosphorylation at multiple docking sites and activation of phosphoinositide (PI) 3-kinase. Ultimately, activation of PI 3-kinase results in actin cytoskeleton rearrangement and internalization of *L. monocytogenes* (Hubbard and Till, 2000; Ireton *et al.*, 1996; Shen *et al.*, 2000). The third identified receptor to InlB is glycosaminoglycans (GAGs) that is found in the proteoglycan layer on the surface of mammalian cells. Similar to InlB-gC1q-R interaction, binding of InlB to GAGs occurs through the GW modules. This interaction was found to augment the activation of Met, and the absence of GAGs from non-phagocytic cells led to 10-folds decrease in InlB-dependent invasion (Jonquieres *et al.*, 2001).

Other internalins. *L. monocytogenes* mutant stains with deficiency in expression of both *inlA* and *inlB* exhibited attenuated invasion of murine and most mammalian cell lines compared to the wild-type but did not completely abolish the bacterial internalization, with 20 times more efficient adhesion to host cells than the nonpathogenic *L. innocua* (Cossart *et al.*, 2003; Dramsi *et al.*, 1997; Milohanic *et al.*, 2000). This observation may highlight the importance of other internalins in adhesion and invasion or their possible synergism with InlA and InlB. In fact, other internalins have been also characterized, and some has been

demonstrated to be important for *L. monocytogenes* pathogenesis. One example is InlC (or IrpA), a 30 kDa protein of 263 aa secreted by *L. monocytogenes*. This protein has the internalin sequence characteristics such as an internalin cap, LRRs, and IR regions. The involvement of InlC in *L. monocytogenes* virulence was revealed by a marked increase in mice LD_{50} with an *inlC* deletion mutant strain of *L. monocytogenes* (Engelbrecht *et al.*, 1996; Ooi *et al.*, 2006). Also, secretion of InlC has been shown to be increased in the cytoplasm of J774 phagocytic cells at late stage of infection, in which it might play a role in bacterial virulence (Engelbrecht *et al.*, 1996). The precise role of InlC in *L. monocytogenes* pathogenesis has not yet been clearly identified since *inlC* gene deletion mutant has no defect in bacterial entry to host cells, intracellular replication, and cell-to-cell spread *in vitro*. It has been postulated that InlC seems to work synergistically with InlA in order to invade non-phagocytic cells (Bergmann *et al.*, 2002). Another example of internalins is InlH, a protein of 548 aa with a cell wall anchor motif LPXTG and some degree of structural similarity to InlB (Raffelsbauer *et al.*, 1998; Schubert *et al.*, 2001). The InlH gene is transcribed from the *inlGHE* gene cluster that codes for two additional internalins, InlG and InlE, in *L. monocyto*genes EGD. In-frame deletion of the operon *inlGHE* or *inlH* resulted in mutant strains that were able to invade Caco-2 cells but showed reduced virulence in mouse models oral and intravenous infections compared to the wild-type strain (Raffelsbauer *et al.*, 1998; Schubert *et al.*, 2001). Similar to InlC, the exact role of InlH in *L. monocytogenes* virulence is still unknown. Recently, InlJ, a newly identified internalin of 851 aa with a LPXTG cell wall anchor motif in *L. monocytogenes* EGD-e, was investigated with respect to its role in bacterial virulence. The Δ*inlJ* mutant strain showed no difference in *L. monocytogenes* adherence and internalization to various mammalian cell lines *in vitro* compared to the wild-type. However, the mutant showed a 10-fold decrease in tissue colonization in mouse model

of infection, indicating the importance of this protein in bacterial virulence (Sabet *et al.*, 2005). Evidence from a recent study strongly suggests that InlJ is a novel adhesin specifically expressed during infection *in vivo* (Sabet *et al.*, 2008). However, the molecular mechanism by which InlJ contributes to *L. monocytogenes* virulence is largely unknown.

Ami and Auto. Ami is an autolytic amidase (a peptidoglycan hydrolase, also known as autolysin) of 917 aa with a molecular mass of 102 kDa. It has an N-terminal catalytic domain similar to the Atl amidase of *Staphylococcus aureus* and eight C-terminal GW modules similar to those of InlB, which anchor Ami noncovalently to the bacterial cell wall (Braun *et al.*, 1997; McLaughlan and Foster, 1998). Ami plays an important role in adhesion of *L. monocytogenes* to host cells. This was demonstrated by a marked reduction in bacterial adhesion with an *ami* null mutant constructed in *L. monocytogenes EGDΔinlA–F* background (Milohanic *et al.*, 2000). In another report, adhesion of *ami* null mutant strains, derived from *L. monocytogenes EGDΔinlA*, *EGDΔinlB* and *EGDΔinlAB* backgrounds, to Caco-2, SK-MEL 28, and Hep-G2 cells was 5 to 10 times less than the parental strains (Milohanic *et al.*, 2001). The Δ*ami* EGD strain showed a reduced colonization in mouse livers following intravenous administration of the mutant (Milohanic *et al.*, 2001). Another autolysin discovered in *L. monocytogenes* is Auto. The gene *Aut* coding for Auto was first identified by comparative genomics of the genome of *L. monocytogenes* EGD-e as a gene encoding a putative autolysin and is absent in the genome of the nonpathogenic strain *L. innocua*. Auto is a 64 kDa protein of 572 aa with an N-terminal signal peptide, an N-terminal autolysin catalytic domain, and four C-terminal GW modules similar to those of InlB and Ami (Cabanes *et al.*, 2004). Unlike Ami, which plays a role in bacterial adhesion, Auto was shown to be a virulence factor involved in invasion by *in vitro* and *in vivo* studies; the Δ*aut*

mutant showed 5 to 50-folds less invasion of different mammalian cell lines compared to the wild-type strain. Multiplication of the Δ*aut* strain in guinea pig organs was severely impaired following oral administration of the mutant at early stages of infection. Similar observation was made in murine model of intravenous infection with the mutant at later stages of infection (Cabanes *et al.*, 2004). However, the molecular mechanisms underlying the role of Auto in *L. monocytogenes* virulence remains to be elucidated.

p60. The p60 protein is the first autolysin identified in *L. monocytogenes* in 1988 by Goebel *et al.* during the generation of a transposon Tn916 insertion mutant that showed an impaired invasion of mouse embryo fibroblast (3T6) cell line (Goebel *et al.*, 1988). This 60-kDa protein of 484 aa, encoded by the *iap* gene, has an N-terminal signal peptide, threonine-asparagine repeats, and a C-terminal murein (peptidoglycan) hydrolase domain (Bubert *et al.*, 1992; Kuhn and Goebel, 1989; Lenz *et al.*, 2003; Wuenscher *et al.*, 1993). Deletion of *iap* gene resulted in a mutant strain that showed the formation of an abnormal septum and uneven and nonpolar distribution of ActA that affected actin tail formation. The Δ*iap* mutant strain showed a markedly reduced intracellular movement and cell-to-cell spread in different cell lines compared to the wild-type strain (Pilgrim *et al.*, 2003). The importance of p60 in virulence was further demonstrated *in vivo* in murine model of intravenous injection where Δ*iap* strain was severely attenuated in virulence in comparison with the wild-type strain (Pilgrim *et al.*, 2003).

1.4.2 Virulence factors involved in escape from intracellular vacuoles (phagosomes)

Hemolysin. The first virulence gene identified in *L. monocytogenes* was hemolysin gene (*hly*) (Kathariou *et al.*, 1987; Vazquez-Boland *et al.*, 2001b). The *hly* product, listeriolysin O (LLO), is a soluble protein of 60 kDa and a member of the pore-forming, cholesterol-dependent cytolysin (CDC) protein family (Dussurget *et al.*, 2004). This family contains more than 20 members, including *Streptococcus pyogenes*'s streptolysin O (SLO), *Bacillus anthracis*'s anthrolysin O (ALO), *Clostridium perfringens*'s perfringolysin O (PFO), and *Streptococcus pneumoniae*'s pneumolysin. Members of this family share high degree of homology (40 to 70% sequence identity), suggesting that they are similar in structure and function (Schnupf and Portnoy, 2007). LLO contains an undecapeptide sequence ^{483}ECTGLAWEWWR^{493}, which is a characteristic of CDC proteins, at its C-terminus. This particular sequence is crucial for binding cholesterol, and whether or not it binds cholesterol directly remains to be determined (Schnupf and Portnoy, 2007). Alanine substitution of tryptophan residues at positions 491 and 492 has resulted in loss of 95% and 99.9% of the protein activity, respectively, indicating that these hydrophobic residues are critical for the hemolytic activity of LLO. In contrast, substitution of cysteine for alanine at position 484 has resulted in a loss of 25% of the protein activity (Michel *et al.*, 1990).

LLO exhibits its activity in pH range from 4.5 to 6.5 with an optimal pH of 5.5, which resembles the pH inside acidified phagosomes (Beauregard *et al.*, 1997), and thus facilitates the phagosmal lysis. Substitution of leucine at position 461 for threonine resulted in a dramatic change in the optimum pH for the LLO activity, indicating the importance of this residue for the low optimum pH (Glomski *et al.*, 2003). Interestingly, the activity of LLO is restricted by the neutral pH in the cytosol, protecting host cells from lysis by the hemolystic activity of the protein, and it has been postulated that a specific motif within the

LLO protein sequence might initiate protein degradation in the cytosol (Decatur and Portnoy, 2000; Dussurget *et al.*, 2004).

LLO is essential for *L. monocytogenes* to escape primary (single membrane) and secondary (double membrane) phagosomal vacuoles, respectively (Gedde *et al.*, 2000). The role of LLO in *L. monocytogenes* virulence was demonstrated by the findings that the Δ*hly* mutant strain was trapped inside phagosomes (Gaillard *et al.*, 1987; Gedde *et al.*, 2000). By construction of a strain of *L. monocytogenes* in which expression of LLO was under the control of an isopropyl-beta-D-thiogalactopyranoside (IPTG)-dependent promoter, the escape of that bacterial strain from the phagosome was IPTG dose dependent, and that in the absence of IPTG, the bacterium become trapped in the phagosome (Dancz *et al.*, 2002). Furthermore, expression of *hly* in the extracellular bacterium *Bacillus subtilis* rendered this organism able to escape macrophage vacuoles (Bielecki *et al.*, 1990). The molecular mechanism by which LLO disrupts the phagosome membrane is still unknown. In general, CDC monomers oligmerize to form arc- and ring-shaped structures upon binding cholesterol. These arc- and ring-shaped polymers, composed of 45-50 subunits, are then inserted into the eukaryotic cell membrane forming pores of various sizes ranging from 10-30 nm (Kayal and Charbit, 2006). Pore formation by LLO on erythrocytes' membrane has been demonstrated by Jacobs *et al.* using electron microscopy (Jacobs *et al.*, 1998). They showed that LLO complexed with cholesterol was capable of binding to target membrane but failed to polymerize to form pores; treatment of the LLO-cholesterol complex with cholesterol oxidase restored the hemolytic activity of the protein. These findings suggest that cholesterol is not involved in binding of LLO to the membrane initially but involved in interaction with the membrane at subsequent stage, leading to LLO oligomerization.

Phospholipases. Phospholipase activity in *L. monocytogenes* was not known until first described by Fuzi and Pillis in 1962 (Fuzi and Pillis, 1962). *L. monocytogenes* secretes two forms of single polypeptide phospholpase C (PLC). The first PLC produced by *L. monocytogenes* is phosphatidylinositol phospholipase C (PI-PLC) or PlcA, which is specific for phosphatidylinositol. It is a 34 kDa protein of 317 aa and encoded by the gene *plcA* (Titball, 1993). PlcA works hand in hand with LLO to facilitate the bacterium escape from primary phagosomes. PlcA has a low optimum pH for its enzymatic activity, ranging between 5.5 to 6.5 in Triton X-100 mixed micelles, suggesting that it, together with LLO, is active in acidified phagosomes (Vazquez-Boland *et al.*, 2001b). PI-specific PLC cleaves the membrane lipid PI, releasing inositol phosphate and daiacylglycerol (DAG) in which DAG is a host protein kinase C (PKC) activator (Griffith and Ryan, 1999). Inhibition of host cell PKC β has markedly reduced *L. monocytogenes* escape from phagosomal vacuoles (Poussin and Goldfine, 2005), although the role of PKC β in phagosome permealization is still unknown. The role of PlcA in *L. monocytogenes* virulence was revealed by gene deletion studies. A *plcA* in-frame internal deletion mutant strain of *L. monocytogenes* showed a significant reduction in escape from primary phagosomes in murine macrophages and increased mouse LD_{50} by 2 to 3 folds compared to the wild-type strain (Camilli *et al.*, 1993; Goldfine *et al.*, 1995; Smith *et al.*, 1995). Furthermore, a *plcA* knockout mutant has 65% less permeabilized vacuoles in the murine phagocytic cell line J774 from 30 to 60 min post-infection than the wild-type strain (Poussin and Goldfine, 2005).

The second *L. monocytogenes* PLC is PlcB with 289 aa in length and a molecular mass of 29 kDa. It is a zinc-dependent phosphatidylcholine phospholipase C (PC-PLC) that expresses lecithinase activity. Unlike PlcA, PlcB is a phospholipase with broad substrate

specificity that can hydrolyze phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and sphingomyelin and is active over a broad pH range (5.5 – 8.0) with an optimum pH of 6.0-7.0 (Geoffroy *et al.*, 1991; Goldfine *et al.*, 1993; Vazquez-Boland *et al.*, 1992). PlcB is synthesized as proenzyme that is activated by a secreted zinc-dependent metalloprotease (Mpl), a product encoded by the gene *mpl* in *L. monocytogenes* (Mengaud *et al.*, 1991a; Poyart *et al.*, 1993). In contrast to PlcA, PlcB, together with LLO, promote the bacterium to escape secondary phagosomal vacuoles. The role of PlcB in *L. monocytogenes* virulence was first described by Vazquez-Boland *et al.* in 1992 (Vazquez-Boland *et al.*, 1992), who showed that a *plcB* deletion mutant strain formed smaller plaques on L2 fibroblasts monolayer compared to the wild-type strain. This result was explained by the impaired cell-to-cell spread of the *plcB* deletion mutant. In another report, there was a significant lower number of free bacteria in the cytosol of J774 cells 5.5 hrs post-infection with the Δ*plcB* strain, and the mutant was found trapped in the secondary phagosomes (Smith *et al.*, 1995). Furthermore, the Δ*plcB* strain was 20 times less virulent in mice than the wild-type strain and showed an impaired cell-to-cell spread (Smith *et al.*, 1995). In one study, it has been demonstrated that PlcB is not only required for *L. monocytogenes* escape from secondary phagosomal vacuoles but also involved in the dissolution of the primary vacuoles (Grundling *et al.*, 2003). This study also showed that PlcB was enough for the bacterium to escape primary vacuoles in the absence of LLO. Recently, PlcB together with PlcA are suggested to be involved in disrupting the inner membrane of secondary vacuoles since an uninduced IPTG-inducible LLO strain was trapped mainly in single membrane secondary phagosomes. On the other hand, the majority of PlcA and PlcB deletion mutant

strains were found trapped in double membrane secondary phagosomes (Alberti-Segui *et al.*, 2007).

1.4.3 Other virulence factors

ActA. ActA is another *L. monocytogenes* virulence determinant that has been identified by transposon insertion mutagenesis (Kocks *et al.*, 1992). A mutant with the transposon inserted in *actA* gene was found incapable of polymerizing host cell actin molecules to form actin tails that are required for the bacterial intracellular movement and cell-to-cell spread. Recruitment of host cell actin and actin tail formation is ActA-dependent, as ActA coated polystyrene beads have the ability to polymerize actin from an actin-rich cytoplasmic extract and exhibit actin-based motility (Cameron *et al.*, 1999). Also, ActA expressing *L. innocua* display actin-based motility in Xenopus eggs cytoplasmic extracts (Kocks *et al.*, 1995). On the other hand, a *L. monocytogenes* mutant deficient in ActA is impaired in cell-to-cell spread and grows as microcolonies in the cytoplasm of infected cells due to the failure of actin tails formtion (Domann *et al.*, 1992).

ActA is a 639 aa surface protein with a molecular mass of 90 kDa. This protein has an N-terminal signal peptide and a C-terminal hydrophobic transmembrane region followed by positively charged residues, which probably anchors the protein to the cell membrane (the bacterial surface) (Domann *et al.*, 1992; Kocks *et al.*, 1992). The N-terminal domain (aa 31-263) of ActA binds the host Arp2/3 complex to activate the actin polymerization function of the complex, similar to the action of Wiskott-Aldrich syndrome protein (WASP)-family proteins leading to the formation of branched actin tails (Boujemaa-Paterski *et al.*, 2001; Gouin *et al.*, 1999; Skoble *et al.*, 2000). The central region of the ActA protein contains four

proline-rich repeats that bind the protein members of the enabled/vasodilator-stimulated phosphoprotein (Ena/VASP) family, components of the host cytoskeleton; this stimulates the bacterial actin based motility and controls the speed and direction of the movement (Auerbuch *et al.*, 2003; Chakraborty *et al.*, 1995; Geese *et al.*, 2002; Laurent *et al.*, 1999; Niebuhr *et al.*, 1997).

ActA not only plays a significant role in bacterial intracellular movement and cell-to-cell spread but also contributes to bacterial adhesion to and invasion of host cells. A Δ*actA* strain of *L. monocytogenes* was impaired in adhesion to and invasion of IC-21 murine macrophages and Chinese hamster ovary (CHO) epithelial-like cell lines (Alvarez-Dominguez *et al.*, 1997). Deletion of *actA* from the genome of an *L. monocytogenes* strain that highly expresses PrfA-dependent virulence regulon *in vitro* resulted in reduced entry of the mutant strain into Caco-2, HeLa, MDCK and Vero epithelial cells (Suarez *et al.*, 2001). The role of ActA in bacterial invasion was further revealed by the expression of ActA in the non-invasive *L. innocua*, which rendered the bacterium invasive to various epithelial cell lines (Suarez *et al.*, 2001).

Hpt. Multiplication of an intracellular bacterial pathogen in the host cytosol must have evolved a mechanism by which the bacterium can acquire energy from the intracellular environment. This task is achieved in part by the *hpt* gene product hexose phosphate transporter (Hpt) in *L. monocytogenes*, which is absent in the nonpathogenic *L. innocua* (Chico-Calero *et al.*, 2002). Hpt shares structural and functional properties with the eukaryotic microsomal glucose-6-phosphate translocase (G6PT), which transfers cytoplasmic glucose-6-phosphate into the endoplasmic reticulum for the generation of glucose (Pan *et al.*, 1999). Hpt allows *L. monocytogenes* to utilize cytosolic glucose-1-

phosphate in host cells for energy to support the rapid bacterial intracellular replication. In fact, the impaired intracellular growth in different mammalian cell lines and attenuated virulence in mouse model seen with the Δ*htp* mutant strain suggests that Htp has a role in *L. monocytogenes* virulence (Chico-Calero *et al.*, 2002).

1.5 Regulation of virulence genes expression in *L. monocytogenes*

L. monocytogenes is a remarkably tough microorganism that is resistant to adverse environmental conditions such as starvation, freezing, high temperature, drying, osmotic stress, oxidative stress, high alcohol concentration, disinfection, and antimicrobial agents. Therefore, this organism is widely spread in the environment. To survive harsh conditions *in vitro* and *in vivo*, *L. monocytogenes* has adopted different regulatory mechanisms for the control of genes expression including those genes required for *L. monocytogenes* virulence (Farber and Peterkin, 1991; Vazquez-Boland *et al.*, 2001b).

1.5.1 PrfA regulation of virulence genes expression

Expression of the most important virulence genes in *L. monocytogenes* including *inlA*, *inlB*, *inlC*, *hly*, *plcA*, *plcB*, *mpl*, *actA*, and *hpt* is under the control of the positive regulatory factor A (PrfA) (Chakraborty *et al.*, 1992; Dussurget *et al.*, 2004; Vazquez-Boland *et al.*, 2001b), a pleiotropic transcriptional activator belonging to the Crp (cAMP receptor protein)/Cap (catabolite gene activator protein)-Fnr (fumarate and nitrate reductase regulator) family of bacterial transcription factors (Milohanic et al., 2003; Sheehan et al., 1995). PrfA is a 27-kDa protein of 237 aa composed of an N-terminal domain with β-roll and α-helix that provides most of the PrfA dimerization interface, a hinge/interdomain region, a

C-terminal helix-turn-helix (HTH) DNA-binding motif, and a C-terminal extension that the stabilize the HTH motif (Scortti *et al.*, 2007; Vazquez-Boland *et al.*, 2001a). PrfA is absolutely required for *L. monocytogenes* virulence since a Δ*prfA* mutant strain is completely avirulent (Leimeister-Wachter *et al.*, 1990; Sheehan *et al.*, 1996).

L. monocytogenes genes and operons, transcription of which are activated by PrfA, contain a palindromic 14-bp consensus sequence tTAACannTGTTAa (known as PrfA box) centered near position -41 upstream of the transcription start sites (Scortti *et al.*, 2007). This consensus sequence has 7 invariant nucleotides (uppercases) and two-mismatch tolerance (n, lowercases) (Bockmann *et al.*, 2000; Bubert *et al.*, 1999; Scortti *et al.*, 2007; Sheehan *et al.*, 1995; Vazquez-Boland *et al.*, 2001a). Transcription activation of these genes and operons is achieved by binding of the HTH DNA-binding motif in PrfA homodimer to the PrfA box. The degree of transcription activation by PrfA is apparently controlled by the PrfA activity, the PrfA concentration, and the promoter configuration (i.e. the PrfA box) (Scortti *et al.*, 2007).

Studies with the PrfA* mutants provide strong evidence suggesting that upon interaction with a putative cofactor, PrfA undergoes an allosteric shift from weakly active to highly active states (conformations) (Vega *et al.*, 1998; Vega *et al.*, 2004). PrfA-dependent transcription activation is also controlled by the concentration of PrfA, which is regulated at both transcriptional and translational levels. Synthesis of PrfA is achieved by three different promoters: Two promoters (P1*prfA* and P2*prfA*) located in the intergenic region immediately upstream of the *pfrA* ORF, which promote the synthesis of the monocistronic transcript, and a third promoter (P*plcA*) situated upstream of the *plcA* gene, which directs the generation of a bicistronic *plcA-prfA* transcript (Scortti *et al.*, 2007). The promoters P1*prfA* and P2*prfA* play a role in maintaining basal levels of *prfA* transcripts and thus a basal amount of the PrfA

protein during normal bacterial growth. Synthesis of the PrfA protein from the P1*prfA*-directed *prfA* mRNA is thermoregulated through the "thermosensor" secondary structure present at 5'untranslated region (5'-UTR) of the transcript that masks the ribosome binding site at low temperature (30 °C) and prevents PrfA translation; this thermosensor structure is destabilized (melts down) at high temperature (37 °C), allowing protein translation (Johansson *et al.*, 2002). This is consistent with thermoregulated expression of PrfA-dependent virulence gene in *L. monocytogenes* (Leimeister-Wachter *et al.*, 1992). The P*plcA* promoter is dependent on PrfA for generation of the bicistronic *plcA-prfA* transcript through which PrfA can stimulate its own synthesis by positive feedback (Chakraborty *et al.*, 1992; Mengaud *et al.*, 1991a). This suggests that any alteration in PrfA activity may lead to a change in the level of *prfA* transcription.

The efficiency of transcriptional activation is greatly influenced by the degree of symmetry of the PrfA boxes, i.e., PrfA binds more strongly to the promoters with perfectly symmetrical palindromes (e.g., P*hly* and P*plcA*) than those having imperfect palindromic PrfA-binding sites (e.g., P*mpl*, P*actA*, and P*inlA*), thus allowing differential regulation of PrfA-dependent gene expression (Vega *et al.*, 2004). In addition to that, the interspace sequences between or flanking sequences adjacent to the PrfA-box and the -10 Pribnow box (an essential consensus site for transcription to occur), two critical elements of PrfA-dependent promoters, have an effect on the functionality and efficiency of PrfA-dependent transcription (Luo *et al.*, 2004; Luo *et al.*, 2005; Mauder *et al.*, 2006).

1.5.2 Control of virulence gene expression by Sigma B (σ^B)

Bacterial core RNA polymerase (RNAP) composed of five subunits ($\alpha_2\beta\beta'\omega$). Although it has enzymatic activity, it must bind a dissociable transcription initiation factor known as sigma factor (σ) to form a holoenzyme that is required for binding specific DNA sequences (promoters) to initiate the transcription process. One of the sigma factors identified in prokaryotes is the alternative sigma B factor (σ^B) that controls the general stress response in Gram-positive bacteria including *L. monocytogenes* (van Schaik and Abee, 2005). In *L. monocytogenes*, σ^B is involved in the bacterial resistance to low temperature, low pH, osmotic pressure, oxidative stress, and high alcohol concentration (Becker *et al.*, 1998; Freitag and Portnoy, 1994; Freitag *et al.*, 1993; Wemekamp-Kamphuis *et al.*, 2004). A wild-type strain of *L. monocytogenes* was shown to be 10,000-fold more resistant to lethal acid stress, 100-fold resistant to 13.8 mM cumene hydroperoxide, and has longer viability following glucose depletion than the Δ*sigB* mutant strain (Ferreira *et al.*, 2001). This highlights the importance of σ^B factor in controlling the bacterial resistance to adverse environments. In general, the σ^B factor in *L. monocytogenes* is associated with seven different regulators of sigma B (Rsbs), which sense environmental stress stimuli, leading to the formation of an active σ^B isoform that binds the core RNAP to form a holoenzyme. The holoenzyme then either positively or negatively controls the σ^B regulon (Hecker and Volker, 2001).

Several lines of evidence indicate that σ^B affect the expression of several key virulence genes in *L. monocytogenes*. The level of *inlA* and *inlB* transcripts was markedly reduced in the Δ*sigB* mutant strain compared to the wild-type strain (Kim *et al.*, 2004); the mutant strain also showed an attenuated virulence in guinea pig infection model (Garner *et al.*, 2006). In addition, expression of four other internalins (InlC2, InlD, lmo0331, and

lmo0610) was completely dependent on the σ^B factor at low temperature (McGann *et al.*, 2007a; McGann *et al.*, 2007b). In addition, a comparative microarray study has identified 55 genes of *L. monocytogenes* EGD-e that were expressed in the wild-type at a level of at least 1.5-fold higher than that in the Δ*sigB* mutant strain (Kazmierczak *et al.*, 2003). On the other hand, the Δ*sigB* mutant showed an increase in hemolysis of sheep red blood cells, i.e. an increased expression of LLO, which was explained by the negative regulatory control of *hly* expression by σ^B under normal growth conditions (Chaturongakul and Boor, 2004). It was shown that the expression of both PrfA and LLO in *L. monocytogenes* was upregulated following exposure to H_2O_2 *in vitro* (Makino *et al.*, 2005). This could be attributed to σ^B regulation since the function of P2*prfA* promoter was shown to be σ^B dependent (Nadon *et al.*, 2002; Schwab *et al.*, 2005). Taking altogether, the previous findings indicate a dynamic involvement of the σ^B factor in the regulation of gene expression including virulence gene expression in *L. monocytogenes*.

1.5.3 Regulation of gene expression by two-component systems

The bacterial two-component systems are regulatory signal transduction systems that enable bacteria to respond to environmental stimuli often via regulation of gene expression (Stock *et al.*, 2000). A typical two-component system is composed of a membrane-bound histidine kinase sensor and a conserved cytoplasmic response protein regulator. Upon sensing external stimuli, the histidine kinase functions to autophosphorylate its histidine residue, to transfer the gained phosphate group to the conserved aspartate residue of the response regulator, and dephosphorylate the phosphorylated response regulator. Response regulators are mostly DNA-binding transcription factors whose affinities for their specific promoters are modulated by phosphorylation (Eguchi and Utsumi, 2005). Thus, alteration of

the phosphorylated state of the cytoplasmic response protein regulator leads to upregulation or downregulation of target gene expression (Stock *et al.*, 2000). The two-component regulatory systems are widely spread in prokaryotes (both Gram-positive and Gram-negative bacteria) (Eguchi and Utsumi, 2005). In *L. monocytogenes* alone, 16 two-component regulatory systems have been identified by comparative genomics (Glaser *et al.*, 2001). These systems provide the genetic basis for the ability of *L. monocytogenes*, as a pathogen, to respond successfully to its environment.

Examples of two component systems that regulate virulence factor expression in response to specific environmental signals have been demonstrated in *L. monocytogenes*. A mutant of *L. monocytogenes* LO28 unable to induce an acid tolerance response exhibited attenuated virulence in a mouse model (Marron *et al.*, 1997). The *lisRK* operon was identified as the genes encoding a two-component regulatory system in *L. monocytogenes* through screening of a Tn917 library for acid-tolerant mutants. A Δ*lisK* mutant strain of *L. monocytogenes* LO28, although it was more resistant to low pH *in vitro*, was severely impaired in virulence in a murine model compared to the parental strain (Cotter *et al.*, 1999). Thus, the genes coding for LisR and LisK, identified as having a role in stress response to acid, hydrogen peroxide, and antimicrobial agents, also play an important role in *L. monocytogenes* virulence (Cotter *et al.*, 1999; Cotter *et al.*, 2002; Gandhi and Chikindas, 2007).

1.6 Internalins in *L. monocytogenes*

InlA and InlB, encoded by the operon *inlAB*, are the first two members in the *L. monocytogenes* internalin family, which were characterized in 1991 as the virulence factors involved in bacterial adhesion and invasion. These two proteins contain N-terminal leucine-

rich repeat (LRR) domains, which are characteristic of internalin molecules (Gaillard *et al.*, 1991). The *L. monocytogenes* genome encodes many proteins with internalin-like sequence characteristics, which are either present or absent in the genome of the nonpathogenic *L. innocua* (Cabanes *et al.*, 2002). The genome of *L. monocytogenes* strains F2365 (serotype 4b), EGD-e (serotype 1/2a), H7858 (serotype 4b), and F6854 (serotype 1/2a) have been sequenced and revealed the presence of 25, 24, 25, and 26 internalin coding genes, respectively (Nelson *et al.*, 2004). Among these internalin genes, three (LMOf6854_0338, LMOf6854_0364, and LMOf6854_0365) are specific for serotype 1/2a F6854 and two (lmo2026 and lmo2027) are specific for serotype 1/2a EGD-e (Nelson *et al.*, 2004). LRRs containing proteins are not just present in *Listeria* species. Many proteins of this type have been identified in Gram-negative bacteria (Bierne *et al.*, 2007) and also in several Gram-positive pathogenic bacteria such as *Streptococcus pyogenes*, *Streptococcus suis*, *Streptococcus agalactiae*, *Clostridium tetani*, *Bacillus anthracis*, *Bacillus cereus*, *Bacillus thuringensis*, and *Enterococcus faecalis* (Bierne *et al.*, 2007).

1.6.1 Domain organization of internalins

Internalin domain. The sequences of *L. monocytogenes* internalins are typically organized into four main modular units: an N-terminal signal peptide, an N-terminal cap domain, leucin rich repeats (LRRs), and a conserved inter-repeat region (IR) (Vazquez-Boland *et al.*, 2001b). The presence of an N-terminal signal peptide (about 15-30 amino acids) implies that the internalins synthesized in the cytosol is translocated, as directed by the signal peptide, across the cytoplasmic membrane through the Sec pathway. Cleavage of the signal peptide by a signal peptidase presumably release the internalin proteins from the extracytoplasmic side of the membrane, leading to the display of the mature proteins on the

bacterial outer surface using the surface anchoring mechanisms discussed in Section 1.6.2 (Trost *et al.*, 2005; Tuteja, 2005; van Roosmalen *et al.*, 2004).

A mature internalin starts with a short α-helix cap followed by LRRs, which are tandem repeats with leucine at fixed position in the sequence arrangement LXLXX, in which X can be any amino acid. A typical single LRR consists of 22 amino acids with leucine or isoleucine occupying the particular positions of β-sheets and 3_{10}-helix that are curved and twisted around the central axis, which favors ligand binding (Cabanes *et al.*, 2002; Freiberg *et al.*, 2004). LRRs have been documented to be involved in variety of protein-protein interactions including cell-adhesion, receptor-ligand interaction, and signaling (Cabanes *et al.*, 2002). The IR domain is structurally related to immunoglobulin (Ig)-like domains found in antibodies and some surface proteins of eukaryotic cells. Together, the cap/LRRs/IR region forms a stable and correctly folded internalin domain, which is attributed to the shared hydrophobic core between the three units (Bierne *et al.*, 2007; Freiberg *et al.*, 2004). In fact, the cap/LRRs/IR segment has been shown to be crucial and sufficient for internalin-mediated bacterial entry into host cells (Lecuit *et al.*, 1997).

Other modular units. In addition to the internalin domain, many *L. monocytogenes* internalins contains other modules. B-repeats, found in InlA, InlB, and InlF, are composed of about 70 residues (Bierne *et al.*, 2007), which were demonstrated to be necessary for maximum activation of Ras-MAP kinase signalng pathway by InlB (Copp *et al.*, 2003), although the exact function of these repeats remains to be elucidated. On the other hand, several other internalins such as InlI (Lmo0331) and Lmo0333 (Cabanes *et al.*, 2002; Sabet *et al.*, 2005), contains PKD domains, which were initially discovered in Polycystin-1 Kidney Disease and are about 80 amino acids in length with an immunoglobulin like configuration.

Generally, they are involved in protein-protein interactions (Bycroft *et al.*, 1999; Cabanes *et al.*, 2002). However, the function of the internalin PDK domains is unknown (Bierne *et al.*, 2007). In addition, some *L. monocytogenes* internalins contain MUCin-Binding Protein (MucBP) repeats of unknown function, which are arranged in 1 to 14 tandem repeats, each repeat is made up of 60-200 aa (Bierne *et al.*, 2007; Boekhorst *et al.*, 2006).

1.6.2 Attachment of *L. monocytogenes* internalins to the surface

L. monocytogenes internalins are covalently or non-covalently immobilized on the bacterial cell surface or totally secreted into the medium (Bierne *et al.*, 2007). These properties can be predicted by the C-terminal sequence of an internalin. The well studied mechanism responsible for covalent attachment of Gram-positive bacterial surface proteins to the cell wall peptidoglycan requires a C-terminal sorting signal originally characterized in *Staphylococcus aureus* protein A (Navarre and Schneewind, 1999). The characteristics of the sorting signal include a sortase-recognition sequence motif LPXTG (X, any amino acid) followed by a hydrophobic domain and a short tail of positively charged amino acids. *L. monocytogenes* InlA has been demonstrated to be covalently attached by the sortase SrtA to the surface through an amide bond between the threonine of the cleaved LPXTG and the amino group of *meso*-diaminopimelic acid (*m*-Dpm) crossbridge within the peptidoglycan (Bierne and Cossart, 2007; Bierne *et al.*, 2002; Dhar *et al.*, 2000; Garandeau *et al.*, 2002). Surface localization of Vip, a LPXTG protein involved in *L. monocytogenes* virulence, is also SrtA-dependent. The genome of *L. monocytogenes* EGD-e (serotype 1/2a) encodes 41 LPXTG containing proteins, 19 of which belong to the internalin family (Glaser *et al.*, 2001); 10 of these 19 internalin members are absent from the genome of *L. innocua* (Glaser *et al.*, 2001). Thirteen proteins from *L. monocytogenes*, including InlA, have been identified to be the substrates of SrtA by Pucciarelli *et al.* using a novel non-gel proteomic approach

(Pucciarelli *et al.*, 2005). To date, 9 of the LPXTG containing internalins in *L. monocytogenes* (InlA, InlC2, InlD, InlE, InlF, InlG, InlH, InlJ, and InlI) have been experimentally demonstrated to be localized on the cell surface (Braun *et al.*, 1997; Bycroft *et al.*, 1999; Dramsi *et al.*, 1997; Gaillard *et al.*, 1991; Ooi *et al.*, 2006; Raffelsbauer *et al.*, 1998).

Another motif involved in attachment of internalins to the cell wall is the Gly-Trp (GW) motif, which are tandem repeats with a conserved Gly-Trp dipeptide responsible for noncovalent attachment of a number of GW module-containing proteins to the surface of Gram-positive bacteria (Bierne and Cossart, 2007; Braun *et al.*, 1997; Cabanes *et al.*, 2002; Tsai *et al.*, 2006). The C-terminal 232-amino acids region of *L. monocytogenes* InlB is composed of three tandem repeats (GW modules) of about 80 amino acids, which are necessary and sufficient for anchoring the protein to the bacterial cell surface via the interaction with the bacterial cell wall component lipoteichoic acids (LTA) (Braun *et al.*, 1997; Jonquieres *et al.*, 1999). On the other hand, some *L. monocytogenes* internalins, such as InlC, lacking the C-terminal cell wall anchoring motifs or domain were found completely secreted (Bierne *et al.*, 2007).

1.7 Infection studies with *L. monocytogenes*

Infection studies with *L. monocytogenes* have been conducted with various mammalian cell lines or animal models of infection. These studies have greatly helped in understanding the molecular mechanisms underlying the pathogenesis of *L. monocytogenes* and provide considerable knowledge about the various aspects of the immune system including host immune defense against intercellular pathogens.

1.7.1 Animal Model

Mouse. Murine model, which was initially used for the study of *L. monocytogenes* infection in 1960s (Mackaness, 1962), is one of the first animal models used for characterizing *L. monocytogenes* pathogenesis. This model is frequently used in *in vivo* infection with *L. monocytogenes* and mostly preferred over other animal models because of the relatively lower cost and easy maintenance and handling. Murine model has contributed a great deal to the current understanding of the concept of T-cell mediated immunity and virulence determinants including LLO, PrfA, ActA, PlcA, and PlcB in *L. monocytogenes* (Bouwer *et al.*, 1997; Czuprynski and Brown, 1987; Finelli *et al.*, 1999; Gaillard and Finlay, 1996; Garifulin and Boyartchuk, 2005; Kathariou *et al.*, 1987; Mackaness, 1962; Pamer, 2004).

In mouse model of infection, animals are infected with various doses of *L. monocytogenes* inoculums via oral or intragastric, intranasal, intraperitoneal, intravenous, or subcutaneous routes (Liu, 2006; Liu *et al.*, 2007). Among these routes, intravenous (iv) route of inoculation is most commonly used, which requires a lower dose of inoculum compared to oral inoculation, and can induce reproducible, dose-dependent lethality in mice (Lecuit, 2005; Lecuit and Cossart, 2002). In contrast, oral or intragastric rotes often require the use of high inoculums (commonly 10^9 to 10^{10} cells) to cause infection (Pine *et al.*, 1990). Furthermore, many studies using the oral route yield inconsistent and even controversial results (Marco *et al.*, 1992; Pine *et al.*, 1990). The translocation efficiency of *L. monocytogenes* across the murine intestinal barrier is very low at a level similar to that of the non-pathogenic *L. innocua* (Lecuit, 2005; Marco *et al.*, 1992; Pron *et al.*, 1998). This relative resistance to oral infection in mice is attributed to the inability of murine E-cadherin receptor to bind InlA due to a specific residue substitution from Pro^{16} to Glu^{16} in the receptor (Lecuit

and Cossart, 2002; Lecuit *et al.*, 2001). This indicates that mouse model of oral infection is inappropriate for investigating the role of inlA in *L. monocytogenes* virulence. To overcome this problem, Lecuit *et al.* generated transgenic mice expressing human E-cadherin in their enterocytes (Lecuit *et al.*, 2001), thus allowing the study of the *in vivo* function of InlA in crossing the intestinal barrier. However, these transgenic mice are not suitable for investigating the role of InlA in crossing deeper tissues, which does not express human E-cadherin (such as feto-maternal and blood-brain barriers, and liver) (Hamon *et al.*, 2006; Lecuit *et al.*, 2001; Lecuit, 2007).

Guinea pig. Guinea pig is another animal model appropriate for studying human listeriosis. Oral inoculation of *L. monocytogenes* led to the development of gastroenteritis resembling that observed in humans and was capable of inducing dose-dependent lethality in guinea pigs following systemic dissemination (Lecuit and Cossart, 2002). Unlike mice, guinea pigs express a functional E-cadherin identical to that of human and capable of interacting with InlA (Lecuit *et al.*, 2001). Expression of human equivalent E-cadherin in guinea pigs makes this model not only suitable for studying the role of InlA in crossing the intestinal barrier but also in crossing the feto-maternal barrier (Bakardjiev *et al.*, 2004; Williams *et al.*, 2007). However, this animal model is not appropriate for studying the *in vivo* function of InlB during infection, because the Met receptor from guinea pigs does not recognize InlB (Khelef *et al.*, 2006).

1.7.2 Cell culture infection

In vitro cell culture techniques were originally established to study *L. monocytogenes* infection based on the ability of the bacterium to adhere to, invade, multiplicate in, and spread to both phagocytic and non-phagocytic eukaryotic cells. Cultured mammalian cells

have many advantages over animal models in the study of *L. monocytogenes* pathogenesis, including low cost, ease of establishment and maintenance, simplicity of infection experiments, and elimination of live animal use.

In vitro cell culture model of infection has allowed defining some key virulence determinants involved in the infectious life cycle of *L. monocytogenes*. For example, the two well-studied internalins, InlA and InlB, were initially identified based on the inability of transposon insertion mutants to invade epithelial cells (Dramsi *et al.*, 1995; Gaillard *et al.*, 1991). The eukaryotic receptors to InlA and InlB were also identified later using cultured mammalian cells (Braun *et al.*, 2000; Jonquieres *et al.*, 2001; Mengaud *et al.*, 1996; Shen *et al.*, 2000). Furthermore, roles of LLO, PlcA and PlcB in escaping phagosomes by *L. monocytogenes* were elucidated extensively using various mammalian cell lines (Alberti-Segui *et al.*, 2007; Bielecki *et al.*, 1990; Camilli *et al.*, 1993; Cossart and Mengaud, 1989; Dancz *et al.*, 2002; Gaillard *et al.*, 1987; Gedde *et al.*, 2000; Goldfine *et al.*, 1995; Grundling *et al.*, 2003; Poussin and Goldfine, 2005; Vazquez-Boland *et al.*, 1992). Also, using cell culture infection model, it has been demonstrated that ActA plays a key role in bacterial intracellular movement and cell-to-cell spread in the infectious life cycle of *L. monocytogenes* (Alvarez-Dominguez *et al.*, 1997; Cameron *et al.*, 1999; Domann *et al.*, 1992; Kocks *et al.*, 1992; Kocks *et al.*, 1995). In addition, *in vitro* cell culture infection with *L. monocytogenes* has allowed to gain knowledge about T-cell mediated immunity and antigen presentation and to dissect the cellular and humoral immune responses to intracellular pathogens (Kaufmann, 1993; Kuhn and Goebel, 1994; Kuhn and Goebel, 1997; Kuhn and Goebel, 1998; Unanue, 1984).

A broad range of eukaryotic cells that the bacterium may encounters during *in vivo* infection have been used in cell culture infection experiments, including Human U937

macrophage cell line (Alberti-Segui *et al.*, 2007), murine macrophage cell line J774 (Inoue *et al.*, 1995), African green monkey kidney cell line Vero (Braun *et al.*, 1998), human colon carcinoma enterocyte-like epithelial cell line Caco-2 (Gaillard *et al.*, 1987), human choriocarcinoma cell line JEG-3 (Sabet *et al.*, 2005), human brain microvascular endothelial cell line (HBMEC) (Greiffenberg *et al.*, 1998), mouse L2 fibroblasts cell line (Jones and Portnoy, 1994), human cervical epithelial cell line Hela (Welch *et al.*, 1997), and human hepatocellular carcinoma cell line Hep-G2 (Milohanic *et al.*, 2004).

CHAPTER TWO

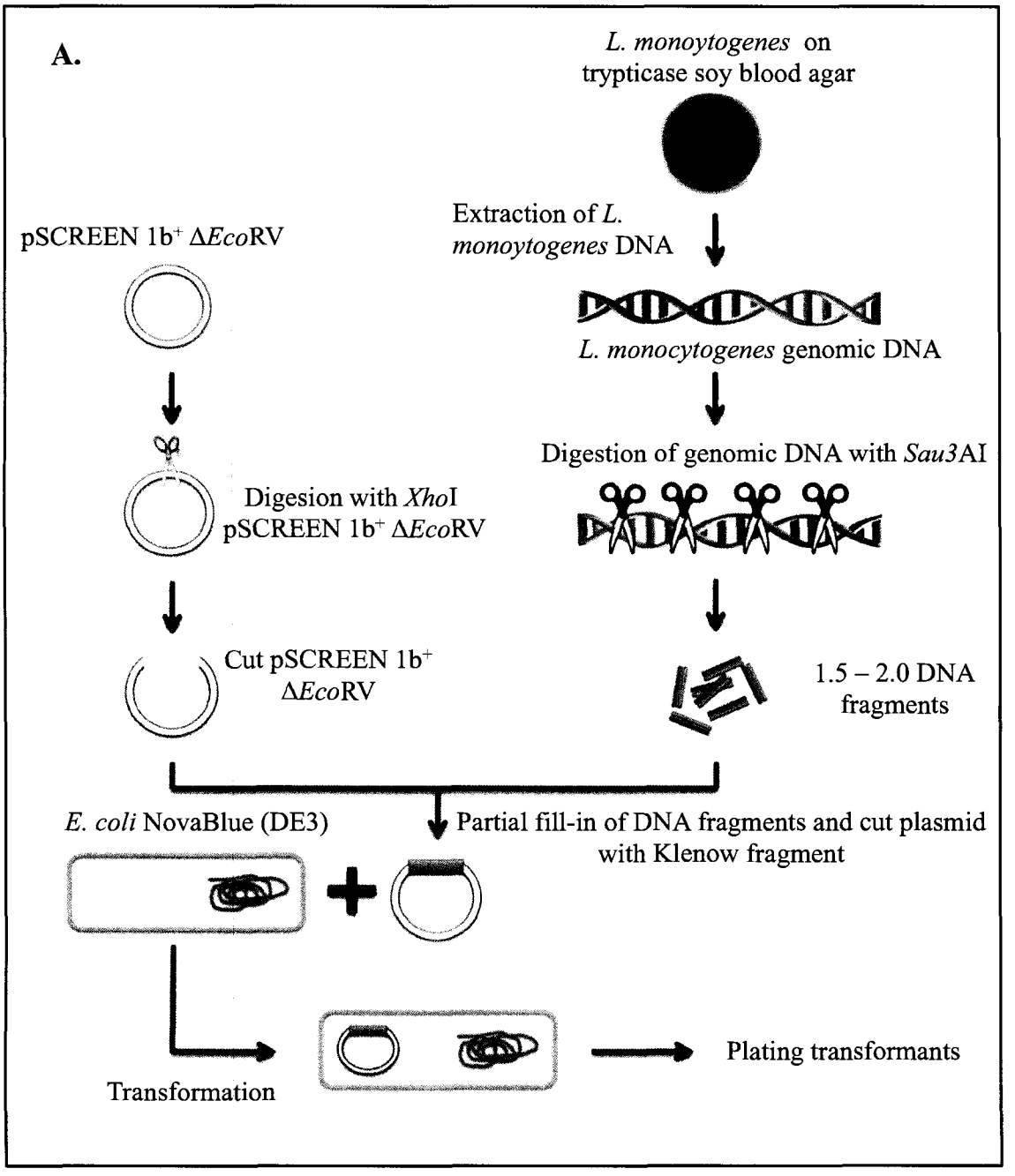
RESEARCH RATIONALES, HYPOTHESES, AND OBJECTIVES

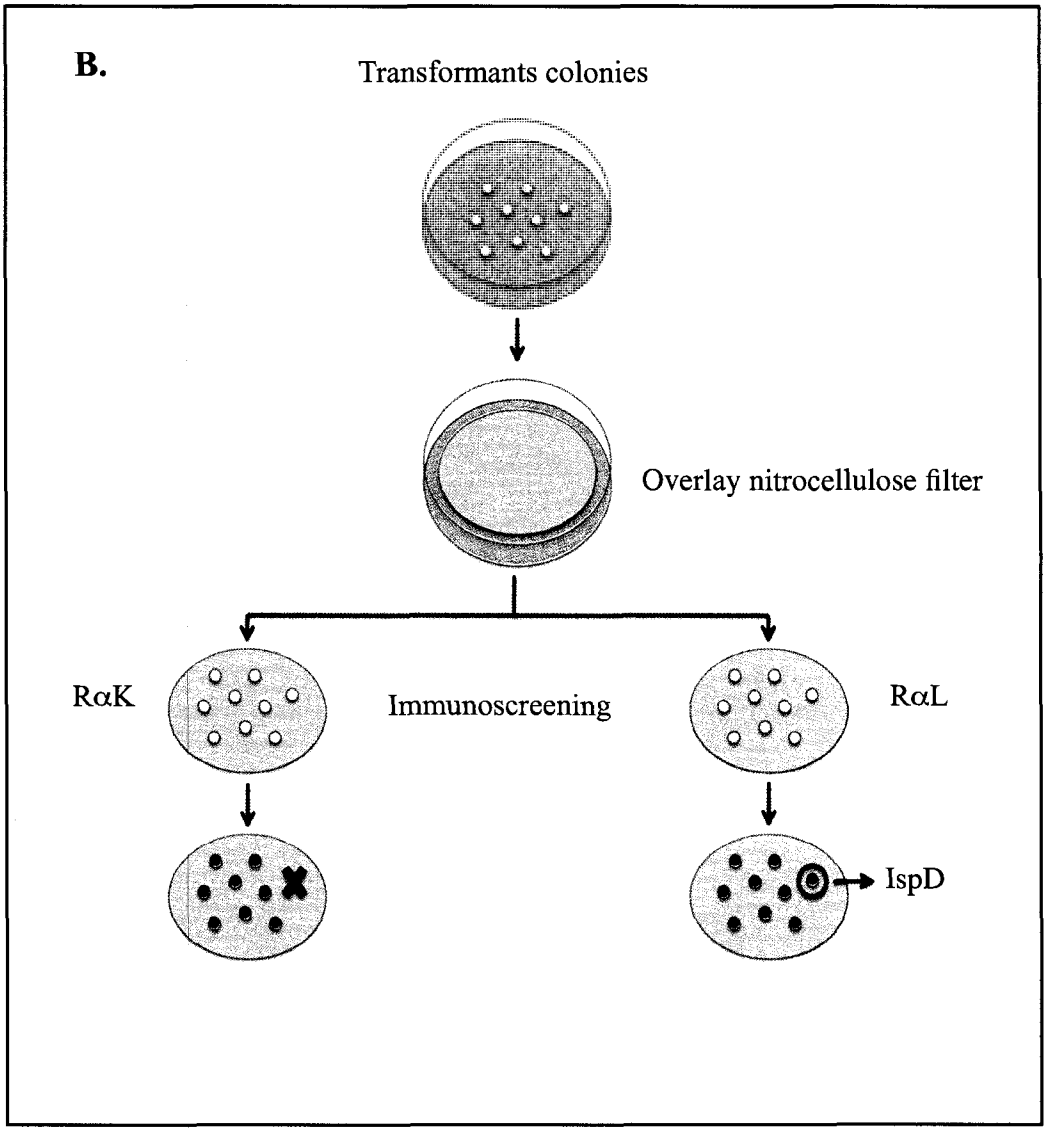
2.1 Previous work

The immunogenic surface proteins (Isp) are less characterized and understood in *L. monocytogenes*, although a number of surface proteins have been predicted from the ORFs in the bacterial genome sequenced earlier (Glaser *et al.*, 2001; Nelson *et al.*, 2004). In fact, about 40% of the predicted coding regions in the genome of *L. monocytogenes* has unknown functions and many of the remaining 60% of ORFs have assigned putative functions that have not been characterized experimentally (Nelson *et al.*, 2004). Previously Dr. Lin's laboratory has initiated studies aiming to identify *L. monocytogenes* proteins that trigger humoral immune response in rabbits following infection with the bacterium. By differential screening of a *L. monocytogenes* genomic expression library (Figure 2.1), this group has identified genes coding for proteins reactive to antiserum (RαL) from rabbits infected with live bacteria but not to antiserum (RαK) from animals receiving heat-killed bacteria respectively (Yu *et al.*, 2007). These genes products that react exclusively with RαL are three members of the internalin family (inlA, inlC2, and inlD) and five other novel proteins of unknown function (designated IspA, IspB, IspC, IspD and IspE, respectively). While the only well characterized protein among the eight proteins is inlA with a critical role in *Listeria* virulence (Gaillard *et al.*, 1991), the other proteins have unclear or uncharacterized function in *L. monocytogenes* and require experimental studies. One of the five novel

immunogenic proteins of unknown function identified earlier in *L monocytogenes* (Yu *et al.*, 2007), IspD, was therefore targeted for my thesis research.

Figure 2.1. Schematic representation of the construction and differential immunoscreening of a *L. monocytogenes* serotype 4b expression library. (**A**) Genomic DNA extracted from *L. monocytogenes* serotype 4b was digested with *Sau3*AI to generate DNA fragments of 1.5 – 2.0 kb. Agarose gel purified genomic DNA fragments were partially filled-in with dGTP and dATP using Klenow fragment. In parallel, the plasmid vector pSCREEN $1b^{+}$ Δ*Eco*RV was digested with *XhoI* and partially filled-in with dCTP and dTTP using Klenow fragment. Then, the partially filled-in genomic and plasmid DNA fragments were ligated and used to transform *E. coli* NovaBlue (DE3) competent cells. *E. coli* transformants were plated on LB agar plates for expression screening. (**B**) For differential immunoscreening of the expression library, each agar plate containing *E. coli* colonies were overlaid with two nitrocellulose membranes separately. The first one was probed with antiserum (RαL) from rabbits infected with live *L. monocytogenes* serotype 4b, and the second one was probed with antiserum (RαK) from rabbits immunized with heat-killed bacteria. Colonies that expressed the proteins which reacted only to RαL were selected for the mini-preparation of recombinant plasmids, which were then sequenced to identify the ORFs coding for immunoreactive proteins (Yu *et al.*, 2007).


A.
L. monoytogenes on trypticase soy blood agar
Extraction of L. monoytogenes DNA
L. monocytogenes genomic DNA
Digestion of genomic DNA with Sau3AI
1.5 – 2.0 DNA fragments
pSCREEN 1b+ ΔEcoRV
Digesion with XhoI pSCREEN 1b+ ΔEcoRV
Cut pSCREEN 1b+ ΔEcoRV
E. coli NovaBlue (DE3)
Partial fill-in of DNA fragments and cut plasmid with Klenow fragment
Transformation
Plating transformants


B.
Transformants colonies
Overlay nitrocellulose filter
RαK
Immunoscreening
RαL
IspD

2.2 Research rationales and hypotheses

The IspD protein of *L. monocytogenes* was chosen for my thesis research for several reasons. Except for the knowledge that IspD is one of the five novel proteins targets of humoral immune response to *L. monocytogenes* infection in rabbits (Yu *et al.*, 2007), very little is known about this protein. Comparison of the *ispD* ORF determined initially from *L. monocytogenes* serotype 4b strain LI0521 (Yu, MSc. thesis, 2004) with the gene homologue *LMOf2365_0349* (GenBank accession number YP_012958) from *L. monocytogenes* strain F2365 (serotype 4b) revealed a missing 510 bp sequence in the ORF of *ispD*. Further sequence determination is proposed to verify the completeness of the *ispD* ORF. This would allow for the identification of potential domains or motifs within the deduced amino acid sequence from the complete *ispD* ORF to provide functional clues for the protein and may be useful in designing experiments for functional study of the protein.

The presence of antibodies to IspD in RαL antiserum and lack of reactivity of IspD with RαK antiserum, as described earlier (Yu *et al.*, 2007), led to the hypothesis that expression of IspD is subject to regulation by environmental factors and suggested that IspD is not expressed or expressed at a minimal level in *in vitro* cultured *L. monocytogenes* and induced specifically or upregulated *in vivo* during infection. To demonstrate if IspD is expressed in *L. monocytogenes* under normal *in vitro* growth conditions, experiments are proposed to analyze the expression of IspD at both the transcriptional and translational levels under normal culture conditions. Also, experiments are proposed to determine the effect of stressed growth conditions on IspD expression. The results obtained from these experiments would provide information about regulated expression of IspD *in vitro* and may provide an insight into the regulated expression of this protein *in vivo* during infection.

Knowledge of cellular localization of the IspD protein remains unknown and is necessary to elucidate the function of this protein. Surface proteins of pathogenic bacteria are most likely to be targeted by the host immune response during infection. Given the facts that IspD is the target of antibody response to listerial infection (Yu *et al.*, 2007) and that a cell-wall anchoring motif LPXTG is present at the C-terminus of the deduced amino acid sequence for the partial *ispD* ORF (Cabanes *et al.*, 2002; Yu *et al.*, 2007), it is hypothesized that IspD is localized on the bacterial surface. To test this hypothesis, polyclonal antibodies are to be raised against a recombinant form of IspD and used to detect the surface localization of IspD using immuofluorescence and imunogold labeling techniques.

Analysis of the data obtained previously led to the development of a hypothesis that IspD is a virulence factor in *L. monocytogenes*. IspD contains some sequence characteristics such as LRR repeats, which are present in the two well characterized virulence factors InlA and InlB in *L. monocytogenes* (Gaillard *et al.*, 1991), suggesting that IspD is an internalin-like protein. The immunogenicity of and the presence of a cell wall anchoring motif LPXTG in IspD suggest that this protein is surface localized and thus accessible for interaction with and/or invasion of host cells in pathogenesis. To elucidate the role of IspD in *L. monocytogenes* virlence, experiments are proposed to create an *ispD* knockout mutant strain and assess the deficiency of IspD on the virulence of *L. monocytogenes* in a cell culture model. The results obtained would lead to a better understanding of how IspD is contributing to the pathogencity of *L. monocytogenes*.

2.3 Research Objectives

My thesis research will focus on experimental characterization of the IspD protein from *L. monocytogenes* strain LI0521 (serotype 4b) using molecular, immunological and genetic techniques, with an emphasis on the role of this protein in virulence. Specific aims are (i) to determine the complete ORF of *ispD* and analyze the characteristics of the deduced amino acid sequence of the protein using bioinformatics tools; (ii) to generate a protein expression construct by cloning the *ispD* ORF into an *E. coli* expression vector; (iii) to produce and purify the recombinant IspD protein for raising polycolonal antibodies in rabbits; (iv) to localize the IspD protein using immunofluorescence microscopy and immunogold labeling transmission electron microscopy (TEM); (v) to examine the expression of IspD in *in vitro* cultured *L. monocytogenes* at both the transcriptional (using RT-PCR) and translational (using Western blots) level; (vi) to analyze the expression of IspD in *L. monocytogenes* grown under various stress conditions; and (vii) to investigate the role of IspD in *L. monocytogenes* virulence (using cell culture model) and the biological function of this protein by creating an *ispD* in-frame deletion mutant strain of *L. monocytogenes*.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Cultivation of *Listeria monocytogenes*

L. monocytogenes strain LI0521 (serotype 4b) was taken from bacterial glycerol stock stored at -80 °C and streaked onto Brain Heart Infusion (BHI) agar plates (Appendix A) and incubated at 37 °C overnight. Colonies on BHI plates were maintained at 4 °C, and a single colony was subsequently used to inoculate a broth culture.

3.2 *L. monocytogenes* genomic DNA extraction

A single well-separated colony of *L. monocytogenes* was picked from a fresh BHI culture plate and inoculated into 3 ml BHI broth (Becton Dickinson) (pH 7.4). Bacteria were grown overnight at 37 °C with shaking at 200 rpm in a MAXQ4000 shaker (VWR International). Cells in 1 ml were spun at 13,000 × g for 2 min and resuspended in 100 µl sterile 1 x Tris-EDTA (TE) buffer (pH 8.0) (Appendix A) containing 3 mg/ml lysozyme (Sigma-Aldrich Co.). Following incubation for 30 min at 37 °C, total DNA was extracted from bacterial cells using DNAzol (Invitrogen Corp.) as per manufacturer's instructions. Briefly, 0.5 ml of DNAzol reagent was added to the cells and mixed thoroughly by repeated pipetting. Then, 0.25 ml of 100% ethanol was used to precipitate DNA by inverting the tube several times which was then collected at 13,000 × g for 1 min. Precipitated DNA was washed twice with 0.75 ml of 75% ethanol, air-dried briefly, dissolved in 0.25 ml deionized H_2O (dH_2O), and stored at -20 °C until used.

3.3 Quantification of *L. monocytogenes* genomic DNA

The concentration of genomic DNA extracted from *L. monocytogenes* was determined by measuring the absorbance at 260 nm, and its purity was checked by determining the 260/280 absorbance ratio using BioMate 3 UV-Vis spectrophotometer (Thermo Fisher Scientific Inc.). Briefly, the spectrophotometer was blanked using dH_2O followed by measuring the absorbance at 260 nm and the 260/280 absorbance ratio of DNA samples that have been diluted 1:100 in dH_2O in a total volume of 500 µl. Then, DNA concentration was calculated using the following formula:

$$\text{DNA concentration (ng/µl)} = OD_{260} \times 50 \times \text{dilution factor}$$

3.4 DNA sequencing of *ispD* open reading frame (ORF)

3.4.1 Polymerase chain reaction (PCR)

The sequence of *ispD* ORF from *L. monocytogenes* strain LI0521 (serotype 4b) has been reported in a previous study (Yu, MSc. thesis, 2004). Sequence alignment of the reported *ispD* ORF sequence with that of the *ispD* gene homologue *LMOf2365_0349* from *L. monocytogenes* strain F2365 (serotype 4b) (GenBank accession number AE017262.2) using the MegAlign module of Lasergene version 5.08 (DNAStar Inc.) revealed a missing 510 bp from the previously determined *ispD* ORF starting from nucleotide 942, in which the first nucleotide of the start codon in the ORF was numbered as 1. In this study, the missing portion from the complete *ispD* ORF sequence was derived from the *L. monocytogenes* genomic DNA by PCR with the primer pairs P696 (5'-CGTTGCGCGATAGAAGAGTT-3') and P697 (5'-CGTTTTCAAACTTGTTGGCAA-3') and sequenced to obtain the full *ispD* ORF (with help from L. Wang). P696 and P697 were designed to have annealing sites 133 bp upstream and 81 bp downstream of the missing sequence, respectively. PCR amplification was done using the Expand Long Template PCR System (Roche Ltd.). A 50 µl reaction

mixture was prepared containing 5 µl 10 x PCR buffer 1 (17.5 mM Mg_2Cl), 1 µl dNTP mix (10 mM), 1 µl P696 (25 mM), 1 µl P697 (25 mM), 1 µl DNA polymerase (3.5 U/µl), and 1 µl of the *L. monocytogenes* genomic DNA template (Section 2.2). PCR was performed in a Mastercycler® ep thermocycler (Eppendorf AG) programmed to 3 min at 94 °C, 35 cycle of 45 sec. at 94 °C, 45 sec. at 55 °C, and 1 min at 72 °C, and a final extension at 72 °C for 10 min. The PCR product (designated MisIspD) was purified from a low-melt agarose gel using Wizard PCR Preps DNA Purification System® (Promega Corp.) according to the manufacturer's instructions, eluted in 50 µl dH_2O, and stored at -20 °C until used.

3.4.2 Cloning of MisIspD into pCR2.1

Following gel purification, the MisIspD DNA fragment was cloned into pCR2.1 using TA Cloning® Kit (Invitrogen Corp.) to create pMisIspD. A ligation mixture (20 µl) containing 2 µl 10 x T4 DNA ligase buffer, 2 µl 10 x ATP (10 mM), 2 µl dH_2O, 8 µl of the purified MisIspD, 4 µl of pCR2.1 (25 ng/µl), and 2 µl T4 DNA ligase (4.0 Weiss units/µl) was prepared and then incubated at 16 °C overnight. Subsequently, the ligated product was used to transform *E. coli* DH5α competent cells (Invitrogen Corp.) utilizing standard procedures (Sambrook and Russel, 2001). Five µl of the ligation product was mixed with 50 µl competent cells with a pipette tip and incubated on ice for 30 min. Then, the cells were heat shocked at 42 °C for 45 sec., incubated on ice for 2 min, supplemented with 250 µl of SOC media (Appinex A), and incubated with 225 rpm constant shaking at 37 °C for 1 hr. Transformed cells were spread onto Luria-Bertani (LB) agar plate containing 50 µg/ml kanamycin and incubated overnight at 37 °C.

3.4.3 PCR screening for pMisIspD positive clones

Ten well-separated *E. coli* DH5α colonies from the LB agar plate above (Section 3.4.2) were grown each in 5 ml LB broth (pH 7.0) containing 50 μg/ml kanamycin at 37 °C overnight. Subsequently, plasmid DNA was miniprepped from the cultures using QIAprep Spin Miniprep Kit (QIAGEN Inc.) according to the manufacturer's instruction and eluted in 50 μl dH_2O.

The purified plasmids were screened for the presence of an insert by PCR with T7 promoter (5'-TAATACGACTCACTATAGGG-3') and M13 reverse (5'-CAGGAAACAGCTATGAC-3') primer pairs using Taq DNA polymerase. One μl of plasmid DNA from each colony was mixed with 10 μl of 10 x PCR buffer, 6 μl $MgCl_2$ (50 mM), 2 μl dNTP mix (10 mM), 1 μl of T7 promoter forward primer (25 mM), 1 μl of M13 reverse primer (25 mM), 1 μl of Taq polymerase (1 U/μl) in a total volume of 100 μl. Then, the thermocycling reaction was performed in Mastercycler® ep thermocycler (Eppendorf AG) using the following parameters: 3 min at 94 °C, 35 cycle of 45 sec. at 94 °C, 45 sec. at 55 °C, and 1 min at 72 °C, and 10 min final extension at 72 °C. The PCR products were analyzed by electrophoresis in 1% agarose gel.

3.4.4 Sequencing of pMisIspD

The insert of recombinant pMisIspD was sequenced using the T7 promoter and M13 reverse primer pair and additional primers using automatic sequencing services provided by Ottawa Health Research Institute, Ontario Genomics Innovation Centre, DNA Sequencing Facility.

3.5 Generation of IspD Expression Constructs

Two IspD expression constructs were generated as described below and used in this study.

3.5.1 PCR amplification of truncated or full-length *ispD* ORF

A truncated *ispD* ORF coding for the predicted mature protein (aa 29-1042) (i.e. 3045 bp out of the 3222 bp complete ORF), was amplified by PCR from *L. monocytogenes* 4b genomic DNA (Section 3.2) with a forward primer (P782, 5'-ACAGCTAGCGAAGAAAATGAATCGAAATCAGTAAATACA-3') and a reverse primer (P783, 5'-ACACTCGAGTGTAGCTGGTAATTTAATATTAGATCTATTTTCCA-3'). P782 starts immediately downstream of the DNA sequence coding for a predicted 28-aa signal peptide; P783 ends immediately uspstream the DNA sequence coding for a glycine residue in a predicted LPXTG motif. P782 and P783 primer pairs were designed to introduce *NheI* and *XhoI* restriction sites (underlined sequences) at their 5' ends, respectively. For amplification of the full-length *ispD* ORF, P716 (5'-ACAGCTAGCATGAAAAGCAAAACAAAACA-3') and P707 (5'-ACACTCGAGTTTAGCACGCTTTTTTTGAGTA-3') primer pairs were designed with the *NheI* and *XhoI* restriction sites (underlined sequences) introduced at their 5' ends respectively. PCRs were performed using PfuUltra® High-Fidelity DNA Polymerase system (Stratagene Corp.) in 50 µl master mixes containing 5 µl 10 x PfuUltra PCR buffer, 1 µl dNTP mix (10 mM), 1 µl forward primer (P782 or P716) (25 mM), 1 µl reverse primer (P783 or P707) (25 mM), 1 µl PfuUltra polymerase (2.5 U/µl), and 1 µl of *L. monocytogenes* 4b genomic DNA. The PCR reaction mixtures were incubated in a Mastercycler® ep thermocycler (Eppendorf AG) for 3 min at 94 °C, followed by 35 cycles of 45 sec. at 94 °C, 45 sec. at 55 °C, 3.5 min at 72 °C, and final extension for 10 min at 72 °C.

Following amplification, PCR products (truncated *ispD* ORF and full-length *ispD* ORF) were purified from a low-melt agarose gel using Wizard PCR Preps DNA Purification System® (Promega Corp.) as per manufacturer's instruction and eluted in 50 µl dH_2O and stored at -20 °C until used.

3.5.2 pET24a plasmid preparation

The pET24a plasmid vector (Novagen Inc.) was propagated in *E. coli* DH5α (Invitrogen Corp.) in 5 ml LB broth containing 50 µg/ml kanamycin at 37 °C overnight. Subsequently, pET24a was miniprepped from the culture using QIAprep Spin Miniprep Kit (QIAGEN Inc.) according to the manufacturer's instruction and eluted in 50 µl dH_2O and stored at -20 °C.

3.5.3 Digestion

The purified DNA fragments of truncated *ispD* and full-length *ispD* and the pET24a plasmid DNA were double digested with *NheI* and *XhoI* (New England Biolabs Ltd.). Briefly, 42 µl of each DNA sample was mixed in separate tubes and incubated overnight at 37 °C with 6 µl 10 x NEBuffer 2, 6 µl 10 x bovine serum albumin (BSA) (1 mg/ml), 3 µl *NheI* (10 U/µl), and 3 µl *XhoI* (20 U/µl). Digested DNA fragments were purified afterward from a low-melt agarose gel using Wizard PCR Preps DNA Purification System® (Promega Corp.).

3.5.4 Cloning of digested *ispD* DNA fragments into pET24a

The digested truncated or full-length *ispD* DNA fragments was cloned into the *NheI* and *XhoI* sites of pET24a to generate the expression construct ptIspD or pIspD, respectively.

Ligation reaction followed by transformation of *E. coli* BL21 (DE3) competent cells (Novagen Inc.) with the ligation products were done essentially as described in Section 3.4.2.

3.5.5 Identification of positive clones

For each expression construct, plasmid DNA was miniprepped from the culture of 10 colonies, each in 5 ml LB broth containing 50 µg/ml kanamycin, using QIAprep Spin Miniprep Kit (QIAGEN Inc.) and used to confirm the presence of the correct inserts by PCR, restriction digestion, and DNA sequencing.

3.5.5.1 PCR screening for ptIspD and pIspD positive clones

Recombinant plasmids were screened for the presence of an insert by PCR with Taq DNA polymerase and T7 promoter (5'-TAATACGACTCACTATAGGG-3') and T7 terminator (5'-GCTAGTTATTGCTCAGCGG-3') primer pair under similar conditions as described in Section 3.4.3 with the exception that the extension time was set to 3 min instead of 1 min. The amplified products were analysed afterward by electrophoresis in 1% agarose gel.

3.5.5.2 Restriction analysis of ptIpsD and pIspD positive clones

Recombinant plasmids were digested with *NheI* and *XhoI* in 10 µl reaction volumes containing 7 µl of plasmid DNA, 1 µl 10 x NEBuffer 2, 1 µl 10 x BSA (1 mg/ml), 0.5 µl *NheI* (10 U/µl), and 0.5 µl *XhoI* (20 U/µl). After digestion for 2 hrs at 37 °C, the digested products were analysed by electrophoresis in 1% agarose gel.

3.5.5.3 DNA Sequencing of ptIpsD and pIspD

The recombinant plasmids ptIpsD and pIspD, which had been confirmed to contain the inserts, were further analyzed by DNA sequencing (see Section 3.4.4) to ensure that the inserted DNA has the correct sequence. The primers used for DNA sequencing are T7 promoter primer, T7 terminator primer, and additional gene specific primers.

3.6 Expression of tIspD and IspD in *E. coli*

Recombinant plasmids ptIspD or pIspD were used to transform *E. coli* BL21 (DE3) for production of recombinant truncated IspD (rtIspD) or recombinant full-length IspD (rIspD). Both recombinant proteins are fused with a polyhistidine tag at their C-terminal ends.

One colony of *E. coli* BL21 (DE3)/ptIspD or *E. coli* BL21 (DE3)/prIspD from LB agar plates were inoculated into 24 ml LB broth (pH 7.0) containing 50 μg/ml kanamycin and incubated overnight at 37 °C with constant shaking at 225 rpm in a MAXQ4000 shaker (VWR International). Overnight cultures were diluted 1:100 in 2 L of fresh LB broth (pH7.0) containing 50 μg/ml kanamycin and incubated for 2.5 hrs at 37 °C with shaking at 225 rpm. When the cultures reached the early exponential phase of growth (OD_{590} = ~ 0.5), isopropyl β-D-thiogalactopyranoside (IPTG) (Gold Biotechnology Inc.) was added at 1 mM to induce protein expression for 3 hrs at 37°C and then overnight at 4 °C. Bacterial cells were harvested by centrifugation at 3,500 × g at 4 °C for 30 min and stored at -80 °C until used for protein purification.

3.7 Purification of rtIspD and rIspD

Frozen bacterial cell pellets containing either rtIspD or rIspD obtained above (Section 3.6) were thawed at room temperature for 15 min and suspended in 50 ml of BugBuster

Master Mix® Protein Extraction Reagent (Novagen Inc.) containing 20 mM β-mercaptoethanol (β-ME) (Sigma-Aldrich Co.), 45 μg/ml Protease inhibitor cocktail (Sigma-Aldrich Co.), and 20 mM imidazol (Sigma-Aldrich Co.). The cell suspension was incubated for 25 min at RT with gentle rocking followed by spinning the lysates at 10,000 × g for 20 min at 4 °C. The supernatant was applied to a column (1 × 2 cm) of nickel-nitrilotriacetic acid (Ni-NTA) superflow (QIAGEN Inc.) at 1 ml/min. Then, the column was washed twice with 35 ml of buffer A (50 mM sodium phosphate monobasic, 300 mM sodium chloride) containing 20 mM imidazol. The bound protein was eluted (fraction size, 1 ml) with buffer A containing 250 mM imidazol. The purified recombinant protein was analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)(Appendix A); and Western blots with mouse anti-His antibody (QIAGEN Inc.), antiserum (RαL) from rabbits infected with live *L. monocytogenes* serotype 4b, and antiserum (RαK) from rabbits immunized with heat-killed bacteria (Yu *et al.*, 2007) as described in Section 3.9. Fractions containing the recombinant protein were pooled together and concentrated using Amicon Ultra centrifugal filter 5,000 Nominal Molecular Weight Limit (NMWL) device (Millipore Corp.) according to manufacturer's instructions. The protein concentration was determined by the Bradford method (Bradford, 1976) using BSA as standard.

3.8 Generation of rabbit polyclonal antibodies to rtIspD

Two female New Zealand rabbits were injected subcutaneously at 4-6 sites on day 0, following the collection of preimmune sera, with 100 μg of the purified rtIspD in 0.5 ml phosphate buffer saline (PBS) (pH 7.2) (Appendix A) emulsified with an equal volume of Freund's adjuvant (Sigma-Aldrich Co.). Then, on day 14 and 28, each rabbit received booster shots with 50 μg of the protein in 0.5 ml PBS emulsified with an equal volume of

incomplete Freund's adjuvant (Sigma-Aldrich Co.). On day 42, the animals were exsanguinated via cardiac puncture in order to obtain a maximal volume of blood. The clotted blood cells was then spun down at 2,850 × g for 10 min at 4 °C to collect sera, which were stored at -20 °C until used.

3.9 SDS-PAGE and Western blots

3.9.1 Preparation of protein samples

E. coli cells collected from broth culture were adjusted to OD_{590} of 20 with PBS (pH 7.2) and lysed by adding an equal volume of 2 × SDS-PAGE sample buffer (Appendix A) and 1% (w/v) SDS followed by incubation in boiling water for 10 min. The total protein lysate was stored at -20 °C until used for SDS-PAGE analysis.

3.9.2 SDS-PAGE

SDS-PAGE was carried out as described by Laemmli (Laemmli, 1970), using 4% stacking gel and 12% separating gel in Bio-Rad mini-gel casting apparatus (Appendix A). 20 µl of the protein lysate (prepared as in Section 3.9.1) was loaded into each gel well and run at 200 V for approximately 45 min. Following electrophoresis, a separating gel is either stained with Coomassie blue (Appendix A) or processed for Western blotting.

3.9.3 Western blots

Proteins separated by SDS-PAGE were equilibrated in protein transfer buffer (Appendix A) for 5 min at room temperature and blotted onto nitrocellulose membranes at 15 V for 30 min using a Trans-Blot SD semi-dry transfer cell (Bio-Rad Laboratories Inc.) according to manufacturer's instructions. Then, the membranes were blocked with 3% (w/v)

skim milk in PBS containing 0.05% (v/v) Tween 20 and 0.2% (v/v) Triton X-100 (PBS-TT) (Appendix A) for 1 hr at room temperature and washed with PBS-TT for 3 min. Subsequently, the membranes were treated for 1 hr with rabbit anti-IspD antisera or anti-His antibody (QIAGEN Inc.) diluted 1:1000 in 3% (w/v) BSA in PBS-TT. After washing 5 times with PBS-TT for 3 min each, the membranes were incubated with Alkaline Phosphotase-conjugated AffiniPure Goat Anti-Rabbit IgG (H+L) or Alkaline Phosphotase-conjugated AffiniPure Goat Anti-Mouse IgG (H+L) (Jackson ImmunoResearch Laboratories Inc.) diluted 1:1000 in 3% (w/v) BSA in PBS-TT for 1 hr. Following the final washing for 5 × 3 min with PBS-TT, the membranes were immunostained with HRP Conjugate Substrate Kit (Bio-Rad Laboratories Inc.) for 10 min according to the manufacturer's instructions.

3.10 IspD Expression level in *in vitro* cultured *L. monocytogenes*

3.10.1 Time course expression of IspD in *in vitro* culture

IspD expression was monitored over a period of 48 hrs at both transcriptional and translational levels by reverse transcription (RT)-PCR and Western blot analysis, respectively. The overnight culture of *L. monocytogenes* was diluted 1:100 in 2 L of fresh LB broth containing 50 mM morpholinepropanesulfonic acid (LBMOPS) (pH 7.2) (Appendix A) and incubated at 37 °C with shaking at 225 rpm. Then, bacteria (1×10^9 cells) were harvested by centrifugation at different time points (i.e different OD_{620}) over a period of 48 hrs for total RNA extraction. Immediately following harvesting, the cell pellets were snap frozen in liquid nitrogen for 15 min then stored at -80 °C until used. Simultaneously, a culture volume containing cells equivalent to that of 50 ml culture with an OD_{620} of 0.5 were harvested at each time point by centrifugation and stored at -80 °C until used for total protein preparation.

3.10.1.1 Total RNA extraction

Bacterial pellets (1×10^9 cells) harvested at different time points (Section 3.10.1), were removed from a -80 °C freezer and thawed on ice for 10 min. Then, the pellets were resuspended in 100 µl TE buffer (pH 8.0) (Appendix A) containing 5 µg/µl lysozyme (Sigma-Aldrich Co.) and 10 µl RNAlater® (QIAGEN Inc.) followed by incubating the suspension at room temperature for 20 min. RNA extraction was carried out afterward using a RNeasy® Mini kit (QIAGEN Inc.) as per manufacturer's instruction. Finally, total RNA was eluted with 30 µl of 0.1% (w/v) diethyl pyrocarbonate (DEPC)-treated H_2O (Appendix A) containing 1 µl RNAlater®.

3.10.1.2 DNase treatment

Total RNA was treated with RQ1 RNase-Free DNase (Promega Corp.), according to manufacturer's instruction, followed by RNA cleanup using a RNeasy® Mini kit (QIAGEN Inc.). RNA was eluted as above (Section 3.101.1) and stored at -20 °C until used.

3.10.1.3 RT-PCR

Reverse transcription were performed from the extracted RNA using ThermoScript™ RT-PCR System (Invitrogen Corp.). Briefly, 100 ng of the extracted RNA from each time point was mixed in a final volume of 20 µl with 1 µl random primer (50 ng/µl), 2 µl dNTP mix (10 mM), 4 µl 5 x cDNA Synthesis Buffer, 1 µl dithiothreitol (DTT) (0.1 M), 1 µl RNaseOUT (40 U/µl), and 1 µl ThermoScript RT (15 U/µl). The mixture was incubated for 10 min at 25°C, 30 min at 55°C, and 5 min at 85°C. The cDNA thus obtained was stored at -20 °C until used for PCR.

Two sets of PCR reactions were conducted with the cDNA template obtained at each time point. The first set was performed as internal control using the primer pair P394 (5'-TTAGCTAGTTGGTAGGGT-3') and P395 (5'-AATCCGGACAACGCTTGC-3'), targeting 16S rRNA. The second PCR set was done using an *ispD* gene specific primer pair P696 and P697 (Table 3.1). Both sets of PCR reactions were carried out using Taq DNA polymerase (as described in Section 3.4.3) using 2 µl of the cDNA template. The PCR products (10 µl) were analyzed by electrophoresis in 1% agarose gel.

3.10.1.4 Preparation of *L. monocytogenes* protein lysate

Frozen *L. monocytogenes* cells harvested at each time point (Section 3.10.1) were thawed briefly and resuspended in 250 µl of PBS and 250 µl of 2 x SDS-PAGE sample buffer (Appendix A). The mixture was transferred into a Lysing Matrix B tube (Qbiogene Inc.), and bacterial cells were lysed using a FastPrep Homogenizer (Qbiogene Inc.) in 5 cycles of homogenization at speed 6.5 for 45 sec. and incubation on ice for 2 min. The *Listeria* protein lysate was then boiled for 10 min and analyzed by using SDS-PAGE and Western blots (see Section 3.9).

3.10.2 IspD expression following stress recovery in enrichment broths

To investigate the effect of various environmental stresses on IspD expression, the wild-type *L. monocytogenes* serotype 4b strain was stressed in different stress environments in BHI broth (Becton Dickinson) and then recovered in diverse enrichments broths as described previously (Geng, 2006; Hahm, 2006). Briefly, 5 ml of the overnight culture grown in BHI (Becton Dickinson) (pH 7.4) was diluted 1:100 in 50 ml BHI subjected to the following stress conditions: pH 5.0, pH 3.0, 45 °C, 4 °C, 5.5% NaCl, 5% ethanol, or 15 mmol

H_2O_2 for 3 hrs. Then, stressed bacteria were recovered overnight at 37 °C in BHI (pH 7.4), LBMOPS (pH 7.0), *Listeria* repair broth (LRB) (pH 7.0) (Appendix A) (Busch, 1992), or buffered *Listeria* enrichment broth (BLEB) (Becton Dickinson) (pH 7.1); environmental stress was done in triplicate for each condition. Following stress recovery, total proteins were extracted from the culture containing bacterial cells equivalent to that of 50 ml culture with an OD_{620} of 0.5 as described (Section 3.10.1.4) and analyzed by SDS-PAGE and Western blots using rabbit anti-IspD antisera as described before (Section 3.9.2 and 3.9.3).

3.11 IspD Localization

3.11.1 Immunofluorescent labeling of *L. monocytogenes*

Live *L. monocytogenes* serotype 4b strain (3×10^8 cells), harvested from the overnight culture in LBMOPS broth, was resuspended in 500 µl PBS containing 5% (w/v) BSA (PBS-5% BSA) and rocked for 1 hr at room temperature. Then, bacteria were probed with rabbit anti-IspD antiserum or preimmune serum (negative control) at 1:50 dilution in 500 µl PBS-5% BSA for 1 hr at room temperature with gentle rocking, washed 6 times each with 500 µl PBS, incubated for 1 hr at room temperature with gentle rocking with ZyMax Goat Anti-Rabbit IgG H+L Fluorescein Isothiocyanate (FITC) Conjugate (Invitrogen Corp.) at 1:50 dilution in 250 µl PBS-5% BSA for 1 hr, washed 3 times each with 500 µl PBS, and then resuspended in 50 µl PBS. Bacterial suspension (10 µl) was placed on a glass slide, covered with a cover slip, and sealed with Cytoseal 60 (Richard-Allan Scientific). Bacteria on a slide were examined under an Olympus BX61 fluorescence microscope (Olympus Inc.).

3.11.2 Immunogold labeling of *L. monocytogenes*

Live *L. monocytogenes* (3×10^8 cells) obtained as above (Section 3.11.1) were washed once with 500 µl PBS followed by incubation at room temperature for 30 min with gentle rocking in 500 µl PBS containing 3% (w/v) BSA (PBS-3% BSA). After washing once with 500 µl PBS, bacteria were probed with rabbit anti-IspD antiserum or preimmune serum (negative control) at a dilution of 1:50 (v/v) in 500 µl PBS-3% BSA for 1 hr at room temperature with gentle rocking. Subsequently, bacteria were washed 6 times each with 500 µl PBS, incubated with 12nm Colloidal Gold-AffiniPure Goat Anti-Rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories Inc.) at a dilution of 1:30 (v/v) in 300 µl PBS-3% BSA for 30 min at room temperature with gentle rocking, washed three times each with 500 µl PBS followed by washing once with 500 µl dH_2O, and resuspended in 50 µl dH_2O. Then, a drop of bacterial suspension (about 10 µl) was placed on a groove made on a piece of parafilm, adsorbed onto a carbon-coated nickel grid for 10 min. Bacteria adsorbed onto the grid were stained with 0.25% (w/v) uranyl acetate for 3 sec., air dried, and examined with a Hitachi H-7000 transmission electron microscope (Hitachi High Technologies Inc.).

3.12 Generation of an *L. monocytogenes ispD* in-frame deletion mutant strain

Generation of an *ispD* in-frame deletion mutant strain (designed Lm4bΔispD) was carried out using the homologous recombination procedure summarized in Figure 3.1.

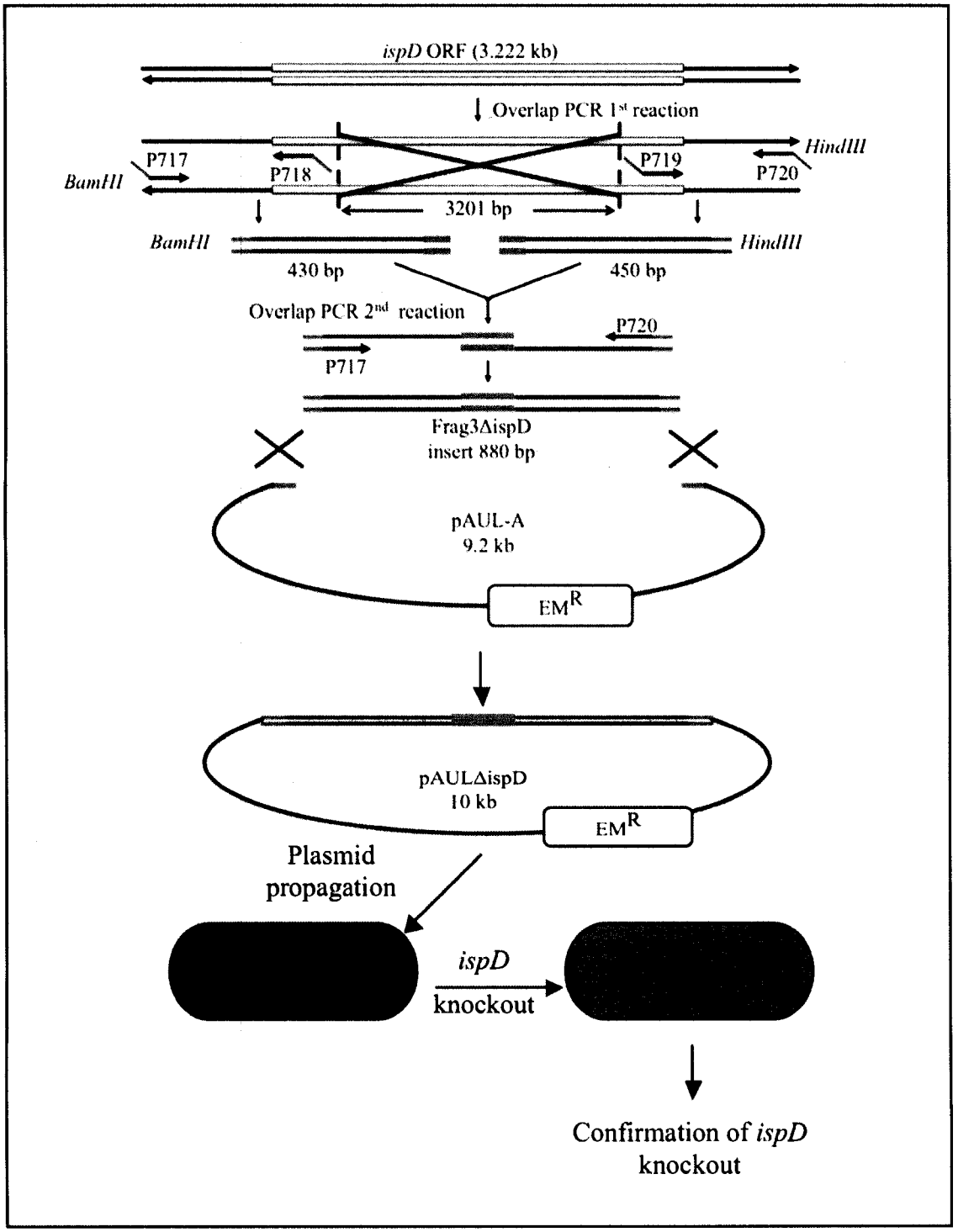
3.12.1 Internal deletion within the *ispD* ORF by PCR

Overlap extension PCR strategy was employed to create an internal deletion, as described by Wang *et al.* (Wang and Lin, 2007), of the nucleotide sequence (nt 10-3210) within the complete *ispD* ORF of 3,222 bp (Figure 3.1). Two sets of PCR reactions were first conducted using *L. monocytogenes* strain LI0521 (serotype 4b) genomic DNA in order to

generate two DNA fragments: (i) a DNA fragment (of size 488 bp) composed of 430 bp flanking sequence upstream of the *ispD* ORF together with the first three codons using the primer pair P717 (5'-ACAGGATCCGTTACGAATTGCGGTCGATT-3') and p718 (5'-TTTATTTAGCACGGCTTTTCATAATCTATCTCCTTC-3'), and (ii) a second DNA fragment (of size 471 bp) consisting of 450 bp flanking sequence downstream of the *ispD* ORF together with the last four codons using the primer pairs P719 (5'-GATTATGAAAAGCCGTGCTAAATAAAAGCTCATAG-3') and P720 (5'-ACAAAGCTTTCAAAAGGCGCTAAATAACCA-3'). The amplicons from the first and second PCR reactions were gel purified (Section 3.4.1) and mixed (2 µl each) for use as a template to create a DNA fragment containing an internally deleted *ispD* in the third set of PCR with the primer pair P717 and P720. P718 (the reverse primer in the first PCR) and P719 (the forward primer in the second PCR) were designed to contain a 26 bp sequence complementary to each other at their 5' ends (highlighted sequences). The primers P717 and P720 were designed to introduce *BamHI* and *HindIII* sites at their 5' ends for directional cloning (underlined sequences), respectively. The first and second PCRs were conducted using PfuUltra® High-Fidelity DNA Polymerase system (Stratagene Corp.) as described earlier (Section 3.5.1). The third PCR reaction was carried out as follows. A reaction mixture (48 µl) containing 5 µl 10 × PfuUltra PCR buffer, 1 µl dNTP mix (10 mM), 2 µl each of the first and second PCR products and 1 µl PfuUltra polymerase (2.5 U/µl) was incubated at 94 °C for 3 min; followed by 10 cycles of 45 sec. at 94 °C, 45 sec. at 55 °C, and 1 min at 72 °C. Immediately after the completion of the 10th cycle, 1 µl of P717 (25 mM) and 1 µl of P720 (25 mM) were added to the mixture, and the reaction continued for 35 cycles at 45 sec. at 94 °C, 45 sec. at 55 °C, and 1 min at 72 °C with a final extension at 72 °C for 10 min. The

resulting 880 bp amplicon (designated Frag3ΔispD) was purified from low-melt agarose gel (Section 3.4.1) and stored at -20 °C until used.

Figure 3.1. Generation of an *ispD* in-frame deletion mutant strain Lm4bΔispD from *L. monocytogenes* (serotype 4b) strain LI0521. A 880-bp DNA fragment (designated Frag3ΔispD insert) containing an internal in-frame deletion of 3201 bp (crossed region between two dashed lines) within the 3.222 kb *ispD* ORF was derived from the genomic DNA of the parent strain using the overlap PCR strategy. The primer pairs P717/P718 and P719/720 were used in the primary PCR reaction, and the primer pair P717/P720 was used the secondary PCR reaction. The Frag3ΔispD insert was cloned into the *BamHI* and *HindIII* sites of the pAUL-A shuttle vector to create pAULΔispD. The recombinant plasmid thus constructed was used to transform electrocompetent cells of *L. monocytogenes* serotype 4b strain LI0521 (Lm4b wild-type) for the generation and screening of an Lm4bΔispD mutant strain as described in the text (Sections 3.12.4 and 3.12.5).


ispD ORF (3.222 kb)
Overlap PCR 1st reaction
BamHI
P717
P718
P719
P720
HindIII
3201 bp
BamHI
HindIII
430 bp
450 bp
Overlap PCR 2nd reaction
P720
P717
Frag3ΔispD
insert 880 bp
pAUL-A
9.2 kb
EM^R
pAULΔispD
10 kb
EM^R
Plasmid
propagation
ispD
knockout
Confirmation of *ispD*
knockout

3.12.2 Digestion of Frag3ΔispD and pAUL-A

The temperature sensitive shuttle vector pAUL-A (kindly provided by Prof. T. Chakraborty, Institute of Medical Microbiology, Justus-Liebig University, Germany; minipreped from *E. coli* DH5α as described in Section 3.5.2) and the PCR product Frag3ΔispD were double digested with *BamHI* and *HindIII* (New England Biolabs Ltd.). A digestion reaction mixture containing 42 µl of Fra3ΔIspD or pAUL-A DNA, 6 µl 10 x NEBuffer 2, 6 µl 10 x BSA (1 mg/ml), 3 µl *BamHI* (20 U/µl), and 3 µl *HindIII* (20 U/µl) were prepared and incubated overnight at 37 °C. The *BamHI-HindIII* cut DNA fragments were purified from low-melt agarose gel as described (Section 3.4.1).

3.12.3 Cloning of Frag3ΔispD into the *BamHI* and *HindIII* sites of pAUL-A

Following digestion, a ligation reaction mixture was prepared containing 8 µl of the digested Frag3ΔispD, 4 µl of precut pAUL-A, 2 µl 10 x T4 DNA ligase buffer, 2 µl 10 x ATP (10 mM), 2 µl T4 DNA ligase (400 U/ µl) (New England Biolabs Ltd.), and 2 µl dH_2O; this mixture was then incubated overnight at 16 °C to generate the recombinant plasmid pAULΔispD. pAULΔispD were propagated in and miniprepared from *E. coli* DH5α as described in Section 3.5.2. Transformation of *L. monocytogenes* strain LI0521 (serotype 4b) were started by adding 1 µl of pAULΔispD to 50 µl of electrocompetent cells (1×10^{11} cell/ml) on ice, which were prepared as described by Park (Park, 1990). *Listeria* cells were then subjected to electoporation using *E. coli* Pulser™ Transformation Apparatus (Bio-Rad Laboratories, Inc.) set to 2.5 kV. Immediately after electroporation, 1 ml of SOC (Appendix A) was added to the cells which were then incubated at 30 °C for 3 hrs with constant shaking at 225 rpm followed by spreading the transformants onto LB agar plates containing 5 µg/ml erythromycin. The plates were incubated at 30 °C for 3-6 days. Three colonies were picked

from the plates and cultured individually at 30 °C overnight in 5 ml LBMOPS broth containing 5 μg/ml erythromycin; plasmid DNA minipreped from each overnight culture was sequenced with M13 forward (5'-CGCCAGGGTTTTCCCAGTCACGAC-3') and M13 reverse primers (5'-GAGCGGATAACAATTTCACACAGG-3') to verify the presence of pAULΔispD in *L. monocytogenes*.

3.12.4 Integration of pAULΔispD into *L. monocytogenes* genome

Integration of the recombinant plasmid pAULΔispD into *L. monocytogenes* genome was carried out as described previously (Schaferkordt *et al.*, 1998). Briefly, one colony of pAULΔispD^{+} *L. monocytogenes* was streaked on a LB agar plate containing 5 μg/ml erythromycin and incubated at 42°C (non-permissive temperature) overnight. This step was repeated twice with a single colony from the plate incubated at 42°C. Then, a single colony from the last plate was inoculated into 20 ml LBMOPS broth (without erythromycin) and incubated at 30°C overnight with gentle shaking at 100 rpm; this culture was further diluted 1:2000 in LBMOPS without erythromycin and incubated at 30°C overnight with shaking at 100 rpm. The broth culture was then diluted 1:100 in fresh LBMOPS without antibiotic and incubated at 42°C overnight with shaking at 100 rpm. The culture was serially diluted with LBMOPS to adjust the cell number to 500-600 cells/100 μl based on the OD_{620} of the undiluted culture (an OD_{620} of 0.61 is equivalent to 1×10^{9} cells/ml) (Yu *et al.*, 2007); 100 μl of the diluted culture (500-600 cells) was spread on a LB agar plate (total 10 plates) and incubated at 37 °C overnight. To identify erythromycin-sensitive colonies, each individual colony from the plates was spotted onto two different LB agar plates (one containing no antibiotic and the other containing 5 μg/ml erythromycin) and incubated at 30°C overnight.

3.12.5 Identification of the *ispD* in-frame deletion mutant strain

Erythromycin-sensitive colonies were screened to identify an *ispD* in-frame deletion mutant strain (Lm4bΔispD) of *L. monocytogenes* by PCR analysis of the genomic DNA followed by DNA sequencing of the PCR products. Lack of IspD expression in the Lm4bΔispD strain was further confirmed at the translational level using Western blotting and immunofluorescent labelling techniques.

3.12.5.1 PCR analysis of the Lm4bΔispD genomic DNA

The genomic DNA was extracted from the cultures of 20 erythromycin-sensitive colonies as described in Section 3.2 and analyzed by PCR with two sets of primer pairs: The first pair, P696 and P697, was designed to hybridize to the deleted region within the *ispD* gene; the second pair, P780 (5'-TGCTCCTTCAGTTGCTGTTG-3') and 781 (5'-TGGCTCTTCTGCCTTTTCAT-3'), was designed to anneal to the sites external to the deleted portion within the gene. PCR conditions and agarose gel electrophoresis analysis of the PCR products obtained were described in Section 3.4.3. As a control, PCR analysis of the genomic DNA from the wild-type strain was similarly performed.

3.12.5.2 Confirmation of the Lm4bΔispD mutant by DNA sequencing

The genomic DNA from two Lm4bΔispD mutants identified above by PCR was used as a template to amply a DNA segment bearing the deleted *ispD* using the Expand Long Template PCR System (Roche Ltd.). A PCR reaction mixture was prepared in a 50 µl-volume containing 5 µl 10 × PCR buffer 1 (17.5 mM Mg_2Cl), 1 µl dNTP (10 mM), 1 µl P780 (25 mM), 1 µl P781 (25 mM), 1 µl DNA polymerase mix (3.5 U/µl), and 1 µl of the extracted genomic DNA. The PCR parameters defined in Section 3.4.3 were used. The PCR

products were gel purified (Section 3.4.1) and cloned into pCR2.1 (Invitrogen Corp.) using the same procedure as described earlier (Sections 3.4.2 and 3.4.3). The resulting recombinant plasmid, designated pCR2.1sΔIspD, was sequenced using the T7 promoter and M13 reverse primer pair (Section 3.4.4) to ensure the correct deletion of *ispD* from the chromosome.

3.12.5.3 Conformation of *ispD* knockout mutant at the translational level

Expression of IspD in the wild-type and the Lm4bΔispD mutant strain was investigated in *in vitro* culture. One fresh colony from each strain was inoculated into 50 ml LBMOPS broth (pH 7.2) and incubated at 37 °C with shaking at 225 rpm overnight. Bacteria in a culture volume equivalent to 50 ml at OD_{620} of 0.5 was harvested by centrifugation at 3,500 × g for 30 min and used to extract total cellular proteins with a FastPrep Homogenizer (Section 3.10.1.4). The secreted proteins in the culture supernatant were precipitated with trichloroacetic acid (TCA) (Section 3.12.5.4). Western blot analysis of both total cellular and secreted proteins were carried out using rabbit anti-IspD antiserum (Section 3.9.3).

Furthermore, absence of expression of the IspD protein in the Lm4bΔispD mutant was further confirmed using immunofluorescent microscopy and immunegold labelling electron microscopy as described earlier (Sections 3.11.1 and 3.11.2).

3.12.5.4 Precipitation of secreted proteins from *L. monocytogenes* culture supernatant

The culture supernatant from the wild-type or the Lm4bΔispD mutant (Section 3.10.1) was adjusted with 100% TCA (Sigma-Aldrich Co.) to a final concentration of 10% TCA, incubated on ice for 30 min, and then centrifuged at 12,000 × g for 5 min. The precipitated proteins were washed once with ice-cold ethanol and dissolved in 100 µl 1 x

SDS-PAGE sample buffer. After incubation for 5 min in boiling water bath, the proteins samples were used for Western blot analysis (Section 3.9.3).

3.13 Phenotypic characterization of the Lm4bΔispD strain

Phenotypic comparison between Lm4bΔispD and wild-type strains was made in terms of their *in vitro* growth, morphology, and biochemical characteristics.

3.13.1 Analysis of bacterial growth curve

The overnight culture of each strain was diluted into fresh BHI broth (final volume = 100 ml) to obtain an OD_{620} of 0.01 and incubated at 37 °C with shaking at 225 rpm. The bacterial growth for both strains was monitored (i.e., OD_{620}) every 30 min over a period of 8 hrs. The experiment was done in triplicate for each strain.

3.13.2 Morphology

Both macroscopic and microscopic aspects of morphology were examined for both strains. Colony morphologies of the Lm4bΔispD and wild-type strains were examined by culturing bacteria on BHI agar and tryptic soy blood agar (TSBA) (Appendix A) plates at 37 °C overnight. Bacterial morphology at different growth phases (early-log, mid-log, late-log, and stationary phase) was inspected using phase contrast light microscopy and at mid-log phase using transmission electron microscopy (TEM). The microscopy procedures described earlier in Sections 3.11.1 and 3.11.2 were used.

3.13.3 Biochemical tests

Biochemical characteristics of the wild-type and Lm4bΔispD mutant strains were compared by performing catalase test, oxidase test, nitrite reduction test, H_2S test, CAMP test (*Saphylococcus aureus* and *Rhodococcus equi*), and motility test at 25 °C and 35 °C according to established procedures (MacFaddin, 2000; Wang and Lin, 2008). Furthermore, 49 carbohydrates oxidation and fermentation pattern was investigated in triplicate for each strain using API 50 CH strips (BioMérieus) and CHB/E medium according to the manufacturer's instructions.

3.14 Virulence study in mammalian cell culture model

Cell culture models of infection with the wild-type and Lm4bΔispD mutant strains were employed to assess the role of IspD in *L. monocytogenes* virulence.

3.14.1 Mammalian cell lines

Cell lines of one professional phagocytic murine macrophage cell line J774 and eight non-professional phagocytes [African green monkey kidney cell line Vero, sheep choroid plexus (SCP) epithelial cell line, human colon carcinoma enterocyte-like epithelial cell line Caco-2, human choriocarcinoma cell line JEG-3, human brain microvascular endothelial cell line (HBMEC), human embryonic lung fibroblast L132 cell line, human cervical epithelial cell line Hela, and human hepatocellular carcinoma cell line Hep-G2] were used in the virulence study. All the cell lines were used in adhesion and invasion assay except that J774 cells were not used in invasion assay. Vero and J774 representative of non-professional and professional phagocytes respectively were used in intracellular growth assay.

3.14.2.1 Mammalian cell culture

Mammalian cell lines (1 ml in a vial), kept frozen in a liquid nitrogen tank, were removed and immediately thawed at 37 °C in water bath. Cells were transferred into a falcon tube (sterile) containing 10 volumes of pre-warmed complete media [CS-C complete medium (Cell Systems Corp.) for HBMEC, MEM complete medium (1 x Minimal Essential Medium (Invitrogen Corp.) supplemented with 0.15% $NaHCO_3$ (Invitrogen Corp.), 10% Heat-inactivated horse serum, 0.1 mM non-essential amino acids (NEAA) (Invitrogen Corp.), and 2 mM L-glutamine (Invitrogen Corp.) for SCP cells; MEM complete medium supplemented with 10% fetal bovine serum (FBS) instead of horse serum for other cell lines. Sodium pyruvate (Invitrogen Corp.) was added at 1 mM to MEM complete medium for Hep-G2 cells. Cells were then spun down for 5 min at 150 × g for HBMEC and 1000 × g for other cell lines to discard the supernatants, resuspended in 5 ml of pre-warmed appropriate complete media, and transferred into T25 cell culture flasks. For HBMEC, cell culture flasks were pre-coated with pre-warmed attachment factor (Cell Systems Corp.). Cells are incubated at 37 °C in humid air containing 5% CO_2 in a CO_2 incubator (Sanyo Electric Biomedical Co., Ltd.) until they reached 80-90 % confluence and then split in appropriate number of T75 cell culture flasks with a final volume of 15 ml appropriate complete medium per flask. Cells are inspected daily with an Olympus CKX41 inverted microscope (Olympus Inc.) and fed as necessary (2-3 days) with fresh complete medium.

3.14.2.2 Adhesion, invasion, and intracellular growth assays

Mammalian cells (2×10^5 cells, or 1×10^5 cells in the case of HBMEC, in 1 ml of appropriate complete medium) were seeded into one well of 24-well tissue culture plates (VWR International) and incubated in a CO_2 incubator (Section 3.41.2.1) for 48 hrs. After replacement with 1 ml of fresh MEM complete medium (for HBMEC, MEM complete

medium was supplemented with 1 mM sodium pyruvate and 25 mM HEPES), mammalian cells were infected with 0.5 ml of MEM complete media containing bacteria at a multiplicity of infection (MOI) of 50 bacteria per eukaryotic cell. Infection was carried out in triplicate for each bacterial strain (i.e. wild-type and Lm4bΔispD). For adhesion assay, cells were incubated for 1 hr following infection and washed with 5 × 3 ml of sterile PBS [pre-warmed Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen Corp.) in the case of HBMEC] to remove free bacteria. Then, mammalian cells in each well were lysed with 1 ml of 1% (v/v) Triton X-100 (sterile) at 37 °C for 5 min, 10-fold serially diluted in sterile PBS, and spread on BHI agar plates with 100 µl of cell lysate from each dilution. The number of colony forming unites (CFU) was determined on the plates after incubation at 37 °C overnight. For bacterial invasion assay, cells were incubated for 1.5 hrs after infection, washed with 3 × 3 ml of sterile PBS (pre-warmed DMEM for HBMEC), and incubated with 1 ml of MEM complete media containing 100 µg/ml gentamycin (Invitrogen Corp.) for 1 hr to kill extracellular bacteria. After washing with 2 × 3 ml of sterile PBS (pre-warmed DMEM for HBMEC), surviving intracellular bacteria were quantified as above. For intracellular growth assay, the incubation in the media containing 100 µg/ml gentamycin g was extended to 2, 4, 6, and 8 hrs prior to determining the CFUs of surviving intracellular bacteria as above.

3.16 Sequence analysis of the IspD protein

The deduced amino acid sequence of IspD was analyzed for protein sequence similarities using the NCBI BLAST search tool (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi), predicted N-terminal signaling peptide using SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/), predicted domains and motifs using

the Swiss-Prot database through InterProScan (http://www.ebi.ac.uk/InterProScan/), and protein statistics using the Protean module (version 5.08) of Lasergene (DNAStar Inc.).

Table 3.1. Primers used in this study

Primer Name	Sequence 5' to 3'	Annealing Target
P696* (F) P697* (R)	5'-CGTTGCGCGATAGAAGAGTT-3' 5'-CGTTTTCAAACTTGTTGGCAA-3'	Within *ispD* ORF
T7 promoter (F)	5'-TAATACGACTCACTATAGGG-3'	pET24, pCR2.1, and their derivatives
T7 terminator (R)	5'-GCTAGTTATTGCTCAGCGG-3'	pET24 and its derivatives
M13 (F)	5'-CGCCAGGGTTTTCCCAGTCACGAC-3'	pAUL-A and its derivatives
M13 (R)	5'-CAGGAAACAGCTATGAC-3'	pCR2.1, pAUL-A, and their derivatives
P780 (F) P781 (R)	5'-TGCTCCTTCAGTTGCTGTTG-3' 5'-TGGCTCTTCTGCCTTTTCAT-3'	External to the deleted portion of *ispD*
P782 (F) P783 (R)	5'-ACAGCTAGCGAAGAAAATGAATCGAAATCAGTAAATACA-3' 5'-ACACTCGAGTGTAGCTGGTAATTTAATATTAGATCTATTTTCCA-3'	Within *ispD* ORF (trancated *ispd* ORF)
P716 (F) P707 (R)	5'-ACAGCTAGCATGAAAAGCAAAACAAAACA-3' 5'-ACACTCGAGTTTAGCACGCTTTTTTTGAGTA-3'	*ispD* ORF
P717 (F) P718 (R)	5'-ACAGGATCCGTTACGAATTGCGGTCGATT-3' 5'-TTTATTTAGCACGGCTTTTCATAATCTATCTCCTTC-3'	Flanking sequence upstream of *ispD* ORF
P719 (F) P720 (R)	5'-GATTATGAAAAGCCGTGCTAAATAAAAGCTCATAG-3' 5'-ACAAAGCTTTCAAAAGGCGCTAAATAACCA-3'	Flanking sequence downstream of *ispD* ORF
P394 (F) P395 (R)	5'-TTAGCTAGTTGGTAGGGT-3' 5'-AATCCGGACAACGCTTGC-3'	16S rRNA

F, forward primer; R, reverse primer; *, used also for detection of the *ispD* transcript by RT-PCR and PCR screening of the Lm4bΔispD mutant; sequences highlighted are complementary to each other; underlined sequences are restriction sites.

CHAPTER FOUR

RESULTS

4.1 The complete nucleotide sequence of *ispD* ORF

The sequence of *ispD* ORF from *L. monocytogenes* serotype 4b strain LI0521 has been reported in a previous study (Yu, MSc. thesis, 2004) and was found in this study to be incomplete because sequence alignment of the previously determined *ispD* ORF with its gene homologue, *LMOf2365_0349* (GenBank accession number YP_012958), from *L. monocytogenes* strain F2365 (serotype 4b) revealed a missing 510 bp sequence starting from nt 942 in the incomplete *ispD* ORF. The presence of repeated sequence, as observed in *LMOf2365_0349*, may have resulted in an internal missing portion of the *ispD* during the previous nucleotide sequence assembly (contig alignment). The nucleotide sequence of the missing part was successfully determined here; this, together with the incomplete ORF of *ispD* reported earlier (Yu, MSc. thesis, 2004), gave a complete *ispD* ORF of 3,222 bp in length (GenBank accession number EF409983), coding for a protein of 1073 amino acids (Appendix B) with a predicted molecular weight of ~116 kDa and a theoretical isoelectric point of 4.60. NCBI BLAST similarity search shows that the IspD protein is highly homologous (96 % identity) to the putative cell wall-anchored protein LMOf2365_0349 (GenBank accession number YP_012958) from *L. monocytogenes* serotype 4b F2365. Also, the protein has 58-23 % homology (identity) to other internalin proteins from *L. monocytogenes* (Table 4.1).

Sequence analysis using bioinformatics tools revealed that the IspD protein contains a number of structural or functional domains and motifs. IspD holds modular structure (domain organization) common to the internalin family of proteins (Cabanes, 2002), being composed of a predicted N-terminal signal peptide, three leucine rich repeats (LRRs), and a C-terminal cell wall anchoring motif (LPXTG) (Table 4.2). In addition to having an internalin like structure, IspD has other sequence characteristics including four Polycystic kidney disease (PKD) domains (Bycroft, 1999; Cabanes, 2002) and a two-component regulator (Stock *et al.*, 2000) (Table 4.2).

Table 4.1. Sequence similarity of IspD to several selected proteins found during BLSAT similarity search

Protein Name	***L. monocytogenes* Strain**	**Genbank Accession No.**	**Percent Homology**
Putative cell wall-anchored protein, LMOf2365_0349	4b F2365	YP_012958	96
Putative internalin protein, lin1204	1/2a F6854	ZP_00234781	58
Internalin D	4b F2365	YP_012892	48
Internalin E	HPB2262	ZP_01942811	43
Internalin A	EGD-e	NP_463962	23

Table 4.2. Predicted IspD Domains and Motifs

Predicted Domains and Motifs	Amino Acid Position
N-terminal signal peptide	aa 1-28
Lucine Rich Repeats (LRRs)	LRR1 aa 131-151 LRR2 aa 151-164 LRR3 aa 447-467
LPXTG motif	aa 1039-1073
Polycystic kidney disease (PKD) domains	PKD1 aa 642-692 PKD2 aa 721-772 PKD3 aa 802-852 PKD4 aa 882-932
Two component regulator	aa 738-765
Leucine-rich repeat adjacent	aa 547-606

4.2 Expression of IspD in *E. coli*

The IspD was expressed from both pIspD and ptIspD constructs. Upon IPTG induction in *E. coli* BL21 (DE3) containing pIspD, a protein band migrating to the position corresponding to the predicted size (117 kDa) of the IspD protein was detected by anti-His mAb on Western blot (Figure 4.1B, lane 3), indicating that the full length recombinant IspD (rIspD) is expressed. Similarly, Western blot using anti-His mAb revealed that the truncated recombinant IspD (rtIspD) with a predicted molecular weight of 111 kDa was expressed in *E. coli* BL21 (DE3) from the construct ptIspD (Figure 4.1D, lane 3). The Western blot results showed a higher level of protein expression for rtIspD compared to rIspD. Alteration in expression conditions such as the induction time, temperature, the concentration of IPTG,

and use of other *E. coli* expression hosts [BL21 (DE3)/pLysS, Rosetta-(DE3)/pLysS, and BL21-CodonPlus-(DE3)-RIPL] failed to improve the level of IspD expression (data not shown). Thus, the expression construct ptIspD was used in this study for the production of rtIspD, with which rabbits were immunized to raise polyclonal antibodies against IspD.

4.3 Purification of rtIspD

Fractions of the protein peak resolved by Ni-NTA agarose affinity chromotagraphy were shown to contain the rtIspD protein by SDS-PGAE (stained with Coomassie blue) (Figure 4.2 A) and Western blot (probed with mouse anti-His mAb) (Figure 4.2 B). Peak fractions containing rtIspD were pooled together and concentrated to about 550 μg/ml. The rtIspD protein in the final preparation was estimated to be at least 80% pure and used for the production of polyclonal antibodies to IspD in rabbits.

Figure 4.1. SDS-PAGE and Western blot analysis of IspD expression from the constructs pIspD and ptIspD. *E. coli* BL21 (DE3)/pIspD, following induction with 1 mM IPTG, was analyzed by SDS-PAGE (**A,** Coomassie blue staining) and Western blot (**B,** probed with mouse anti-His mAb) for the presence of rIspD. Similarly, *E. coli* BL21 (DE3)/ptIspD was analyzed for the presence of rtIspD by SDS-PAGE (**C**) and Western blot (**D**). Lane 1, molecular weight markers labeled in kDa; Lane 2, total cell lystes from non-induced *E. coli*; Lane 3, total cell lystes from IPTG-induced *E. coli*. Each lane contains total proteins from *E. coli* cells equivalent to 1 ml of culture with OD_{590} to 0.2.

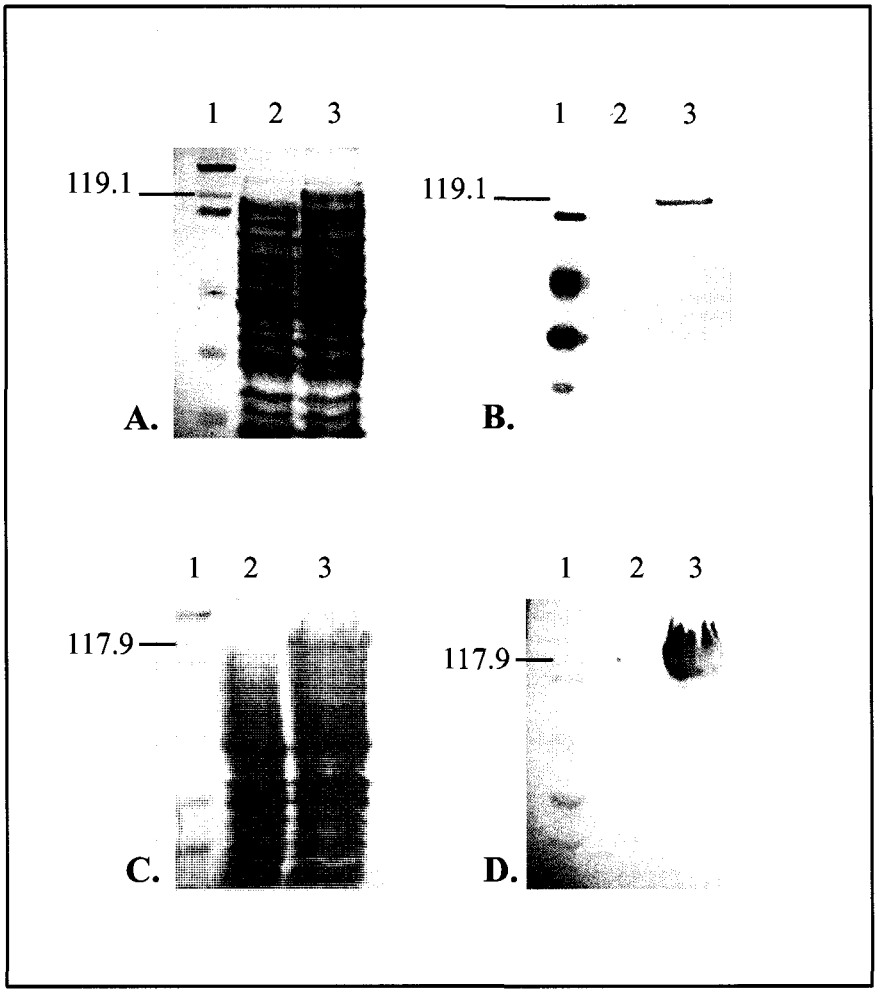
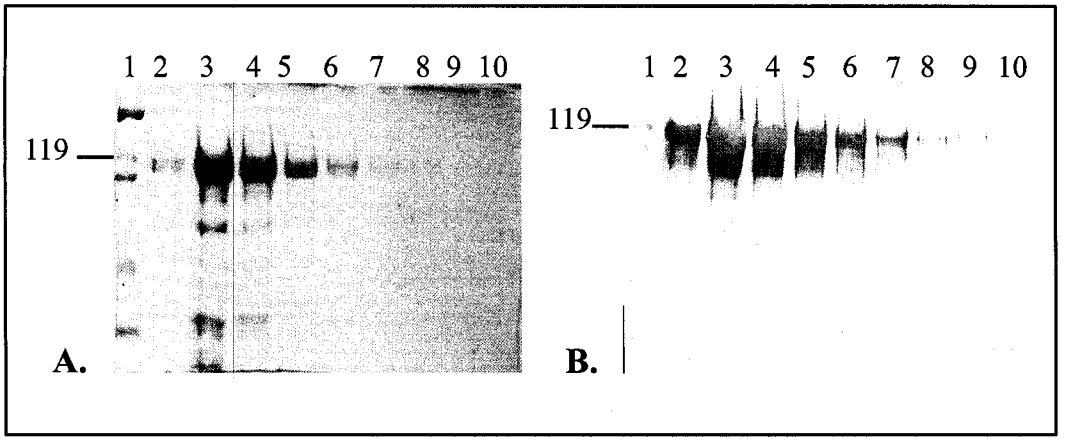

1 2 3
119.1
A.
1 2 3
119.1
B.
1 2 3
117.9
C.
1 2 3
117.9
D.

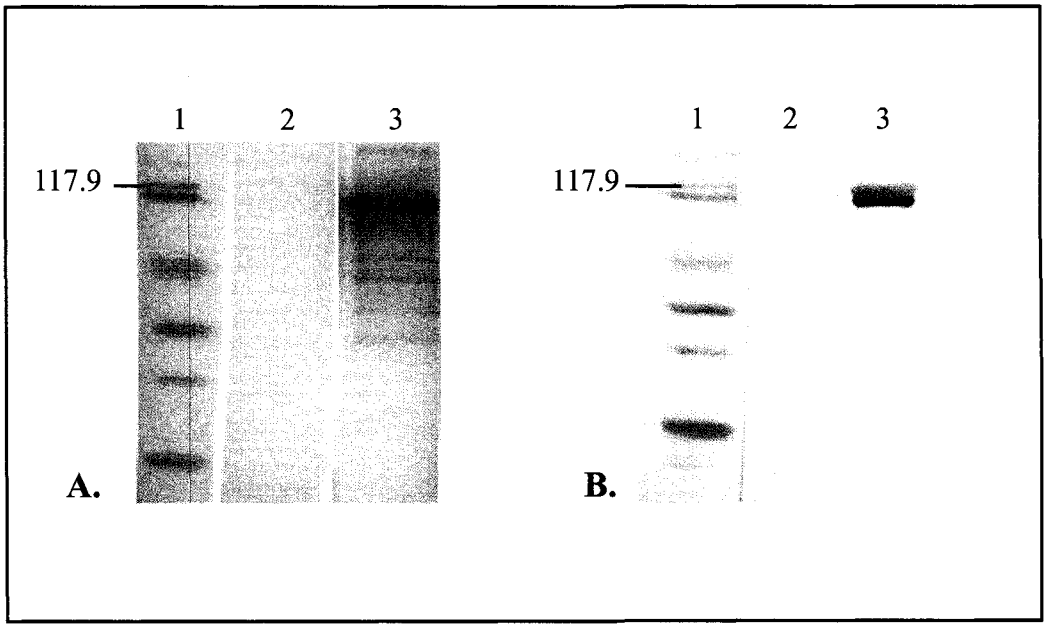
Figure 4.2. SDS-PAGE and Western blot analysis of affinity purified rtIspD. The protein peak fractions (10 µl each) from Ni-NTA agarose affinity chromatography were analyzed by SDS-PAGE (**A,** Coomassie blue staining) and Western blot (**B**, probed with mouse anti-His antibody). Lane 1, molecular weight markers labeled in kDa; Lanes 2 to 10, individual protein samples from 9 fractions of the protein peak.


1 2 3 4 5 6 7 8 9 10
119
A.
1 2 3 4 5 6 7 8 9 10
119
B.

4.4 Immunogenic characteristics of IspD

Purified rtIspD was shown to react with RαL antiserum from rabbits infected with live *L. monocytogenes* but not with RαK antiserum from rabbits immunized with heat-killed bacteria (Figure 4.3 B). This result is consistent with how IspD was identified previously as a protein target reactive exclusively to RαL by differential immunoscreening of a *L. monocytogenes* genomic expression library (Yu *et al.*, 2007). With the availability of purified rtIspD as an immunogen, polyclonal antibodies to IspD was successfully raised in rabbits. Figure 4.3 A shows that purified rtIspD reacted with rabbit anti-IspD sera (RαIspD) but not pre-immune sera on Western blot. RαIspD was useful for the detection of IspD expression in *L. monocytogenes* and for the localization of IspD in this bacterium.

Figure 4.3. Western blot analysis of purified rtIspD with rabbit antisera. The purified rtIspD (1.5 μg per lane) was analyzed by Western blotting, probed with RαK antiserum (**B**: lane 2), RαL (**B**: lane 3), preimmune serum (**A**: lane 2), and RαIspD antiserum (**A**: lane 3). Lane 1, molecular weight markers labeled in kDa. All rabbit antisera were used at a dilution of 1:1,000.


1
2
3
117.9
A.
1
2
3
117.9
B.

4.5 Expression of IspD in *in vitro* cultured *L. monocytogenes* under various growth conditions

The immunological reaction of IspD with RαL but not with RαK, as demonstrated in the previous study (Yu *et al.*, 2007) and here, suggested that the IspD protein is expressed only *in vivo* or expressed at a low level in *in vitro* culture. In order to clarify this, IspD expression was monitored in *in vitro* cultured *L. monocytogenes* over a period of 48 hrs at both the transcriptional (using RT-PCR) and translational levels (using Western blot). Figure 4.4 D shows that IspD was expressed at a low level over the entire 48 hrs growth time with a peak expression level at 5.3 hrs in the early growth phase (i.e. OD_{620} of 0.3). The level of IspD expression correlates well with the result of RT-PCR which shows a transient transcription of *ispD* gene in the early log phase (Figure 4.4 C). Interestingly, no transcripts were detected beyond 7.5 hrs of growth while the protein continued to show a relatively constant low level of expression over the 48 hrs growth period (Figure 4.4 C and D).

To determine if the expression of IspD is influenced by environmental factors, *L. monocytogenes* was exposed to various stress conditions for a short period of time followed by recovering bacteria overnight in different enrichment media. This strategy has been similarly applied to the study of the effect of environmental conditions on the expression of some surface proteins in *L. monocytogenes* in previous studies (Geng, 2006; Hahm, 2006). After being stressed for 3 hrs in BHI broth with acidic pH (pH 5.0 and pH 3.0), cold temperature (4 °C), heat (45 °C), osmotic pressure (5.5% NaCl), oxidative stress (15 mmol H_2O_2), and alcohol (5% ethanol) followed by recovery from each stress condition overnight at 37 °C in four different enrichment broths (BHI, BLEB, LBMOPS, and LRB) with near-neutral pH values (pH 7.0-7.4), bacterial cells showed no alteration in IspD expression with

injury recovery in LBMOPS and LRB enrichment media compared to the unstressed cells (data no shown). However, a marked increase in IspD expression was observed on Western blot analysis using RαIspD of total proteins from bacteria recovered in BHI and BLEB enrichment media following H_2O_2 stress (Figure 4.5 A and B). In contrast, other stress conditions showed suppression of the IspD expression in bacteria recovered in BHI and BLEB.

Figure 4.4. Expression of the *ispD* gene and IspD protein in *in vitro* cultured *L. monocytogenes*. IspD expression was monitored over a period of 48 hrs (i.e. different OD_{620}) at both the transcriptional (using RT-PCR) and translational levels (using Western blot probed with RαIspD). For RT-PCR, about 100 ng of total RNA was used at each time point; for Western blot analysis, total proteins from the culture equivalent to 2 ml at OD_{620} of 0.5 at each time point was probed with RαIspD at a dilution of 1:1000. Samples taken at various time points are indicated by time (bottom of the figure) and corresponding OD_{620} readings (top). (**A**) PCR analysis of the 16S rRNA gene without the reverse transcriptase step showing the absence of any amplification products (i.e. no DNA contamination); (**B**) RT-PCR detection of 16S rRNA gene transcript serving as an internal control; (**C**) RT-PCR detection of the *ispD* gene transcripts. (**D**) Western blot analysis of total *L. monocytogenes* proteins using RαIspD.

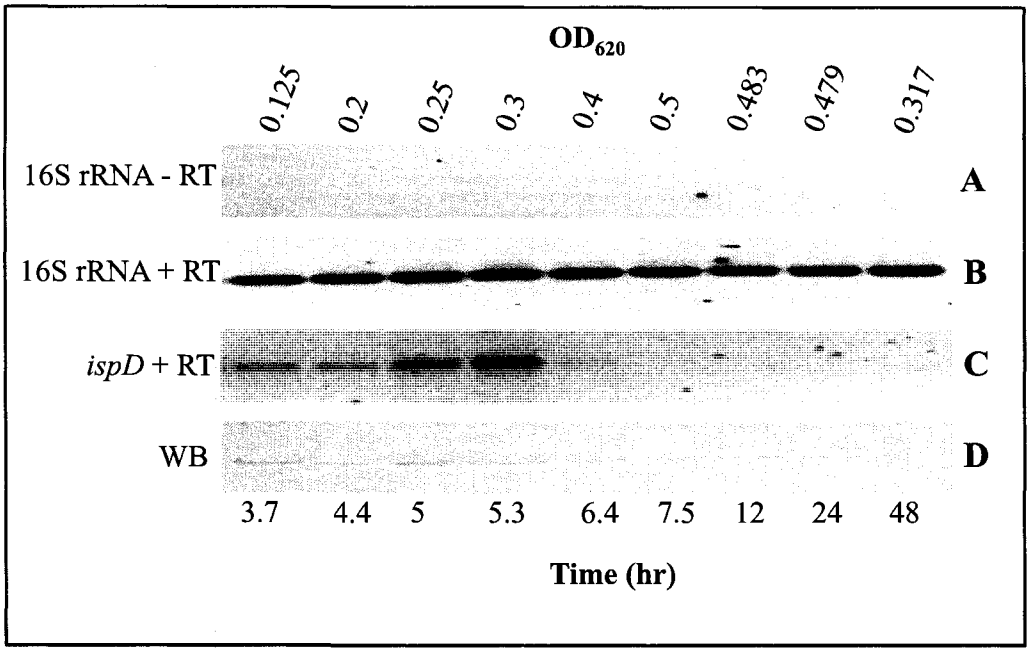
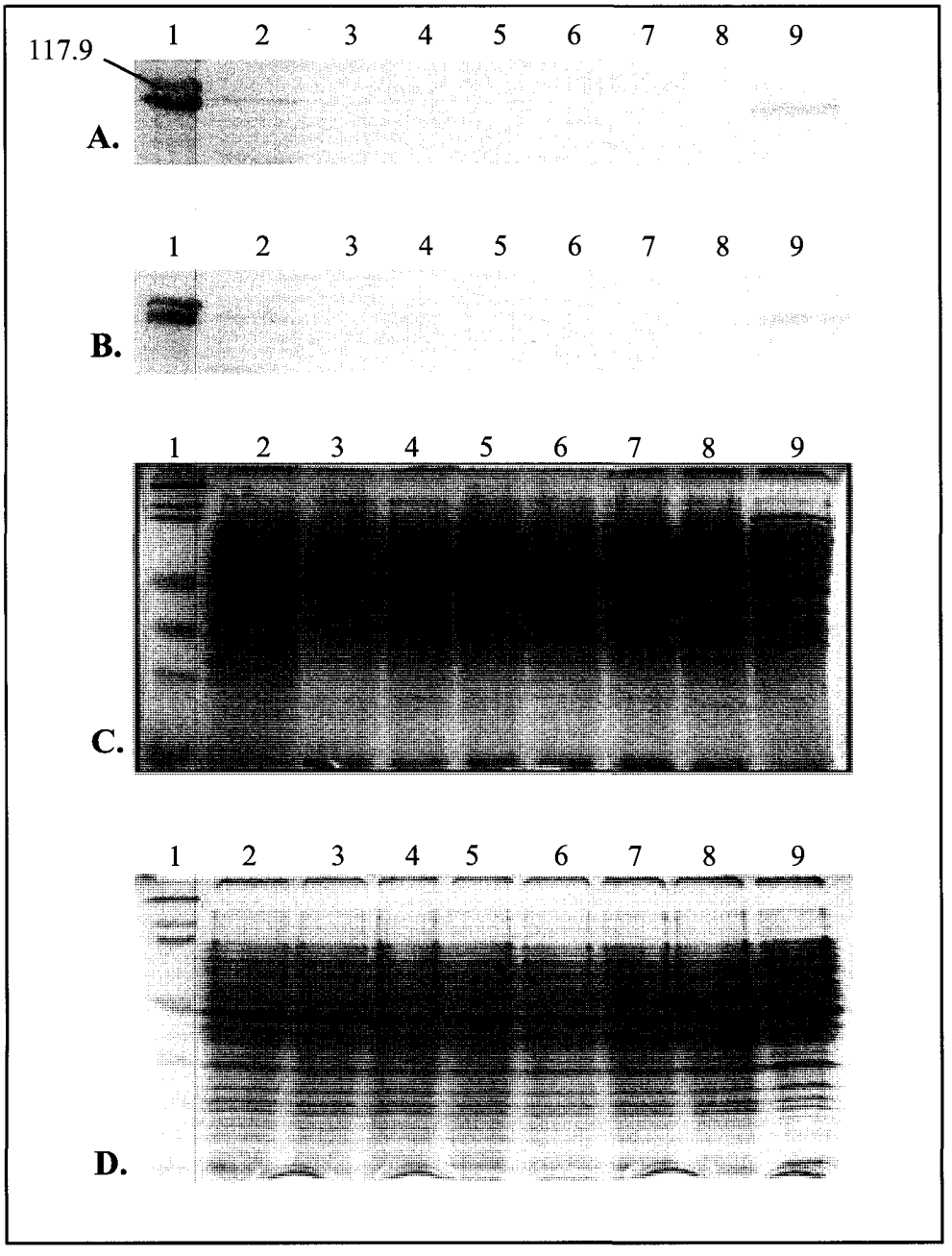

OD620
0.125
0.2
0.25
0.3
0.4
0.5
0.483
0.479
0.317
16S rRNA - RT
A
16S rRNA + RT
B
ispD + RT
C
WB
D
3.7
4.4
5
5.3
6.4
7.5
12
24
48
Time (hr)

Figure 4.5. Western blot and SDS-PAGE analysis of total cellular protein extracts from stress recovered bacteria in BHI and BLEB. Bacteria were stressed for 3 hrs in brain heart infusion (BHI) broth subjected to acid, cold temperature, heat, osmotic pressure, oxidative stress, and high alcohol concentration followed by recovering the bacteria in BHI or buffered *Listeria* enrichment broth (BLEB). Lane 1, molecular weight marker in kDa; Lane 2, total protein extract from control (i.e. no stress); Lane 3, total protein extract from bacteria recovered from pH 5.0 stress; Lane 4, total protein extract from bacteria recovered from pH 3.0 stress; Lane 5, total protein extract from bacteria recovered from 45 ^{o}C stress; Lane 6, total protein extract from bacteria recovered from 4 ^{o}C stress; Lane 7, total protein extract from bacteria recovered from 5.5% NaCl stress; Lane 8, total protein extract from bacteria recovered from 5% ethanol stress; Lane 9, total protein extract from bacteria recovered from 15 mmol H_2O_2 stress. (**A**) and (**B**) represent Western blot membranes of total protein extract from stress recovery in BHI and BLEB broths respectively probed with RαIspD serum, and (**C**) and (**D**) represent SDS-PAGE membranes of total protein extract from stress recovery in BHI and BLEB broths respectively stained with Coomassie blue. Each lane was loaded with protein extract from bacterial culture equivalent to 2 ml at OD_{620} of 0.5 and probed with RαIspD at a dilution of 1:1000.


117.9
1 2 3 4 5 6 7 8 9
A.
1 2 3 4 5 6 7 8 9
B.
1 2 3 4 5 6 7 8 9
C.
1 2 3 4 5 6 7 8 9
D.

4.6 Localization of IspD in *L. monocytogenes* serotype 4b

Presence of LPXTG motif in the deduced aa sequence of IspD and the immunogenicity of this protein suggests that it is localized on the bacterial surface. In order to demonstrate this, live *L. monocytogenes* was probed with RαIspD antiserum and then subjected to imunofluorescence staining or immunogold labeling. IspD was detected on the bacterial surface by fluorescence microscopy (Figure 4.6 C). This observation was further supported by immunogold labeling which showed the presence of gold particles on the bacterial surface (Figure 4.7 C). Specificity of binding of RαIspD to surface-localized IspD was revealed by the absence of fluorescence staining (Figure 4.6 A) or gold particles (Figure 4.7 A) on the surface of bacteria probed with preimmune serum.

Figure 4.6. Localization of IspD on the surface of *L. monocytogenes* strain LI0521 (serotype 4b) using immunofluorescent labeling. Live bacteria (3×10^8 cells) were probed with either RαIspD (**B** and **C**) or rabbit preimmune serum (**A**) followed by incubation with FITC-conjugated goat anti-rabbit antibody as described in Materials and Methods. The wild-type strain (**A** and **C**) and the mutant strain Lm4bΔispD (**B**) were used here. Bacteria were examined under a fluorescence microscope equipped with a digital video camera; both fluorescence (right panel) and phase-contrast images (left panel) of bacteria in the same field were captured.

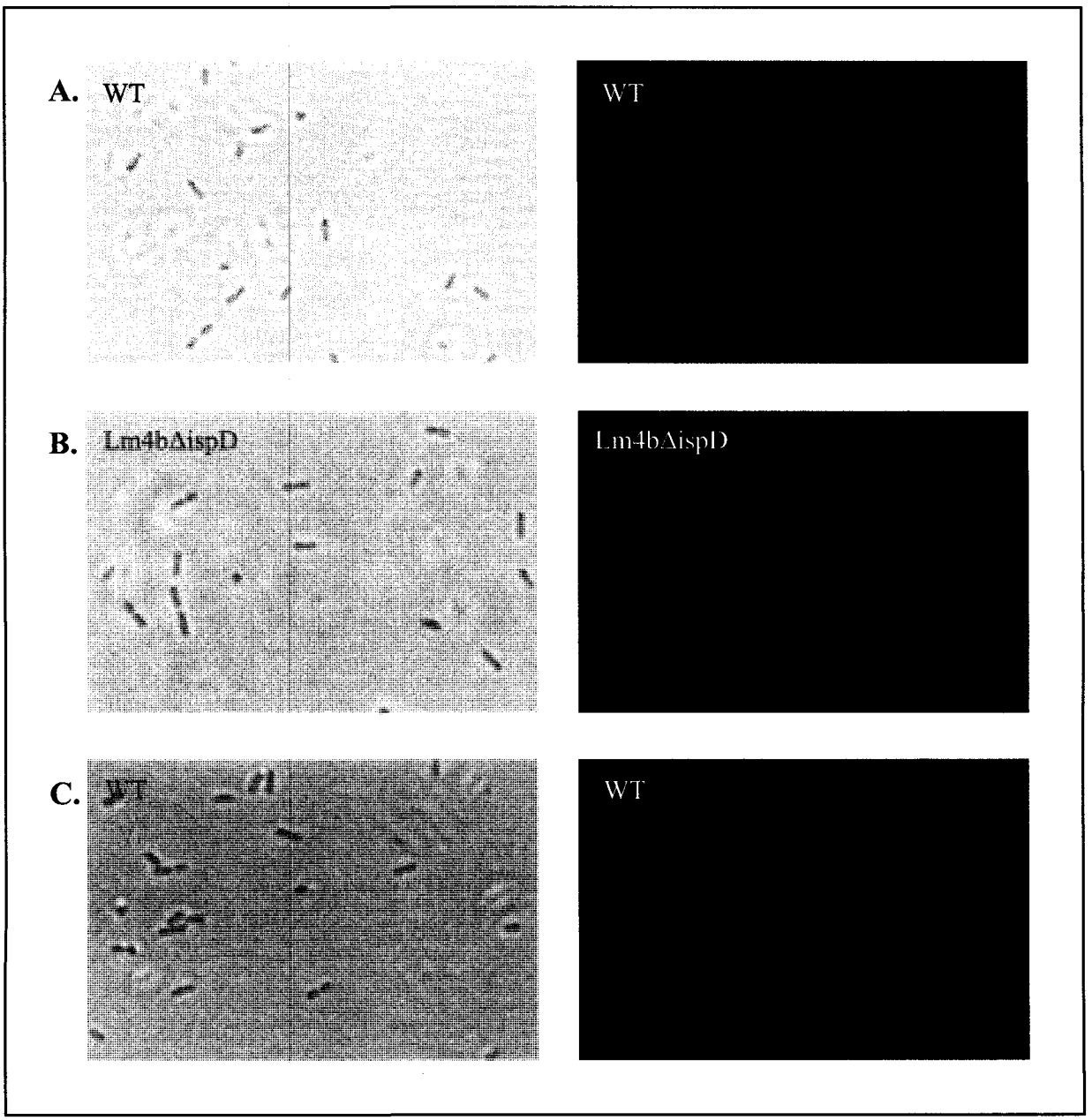
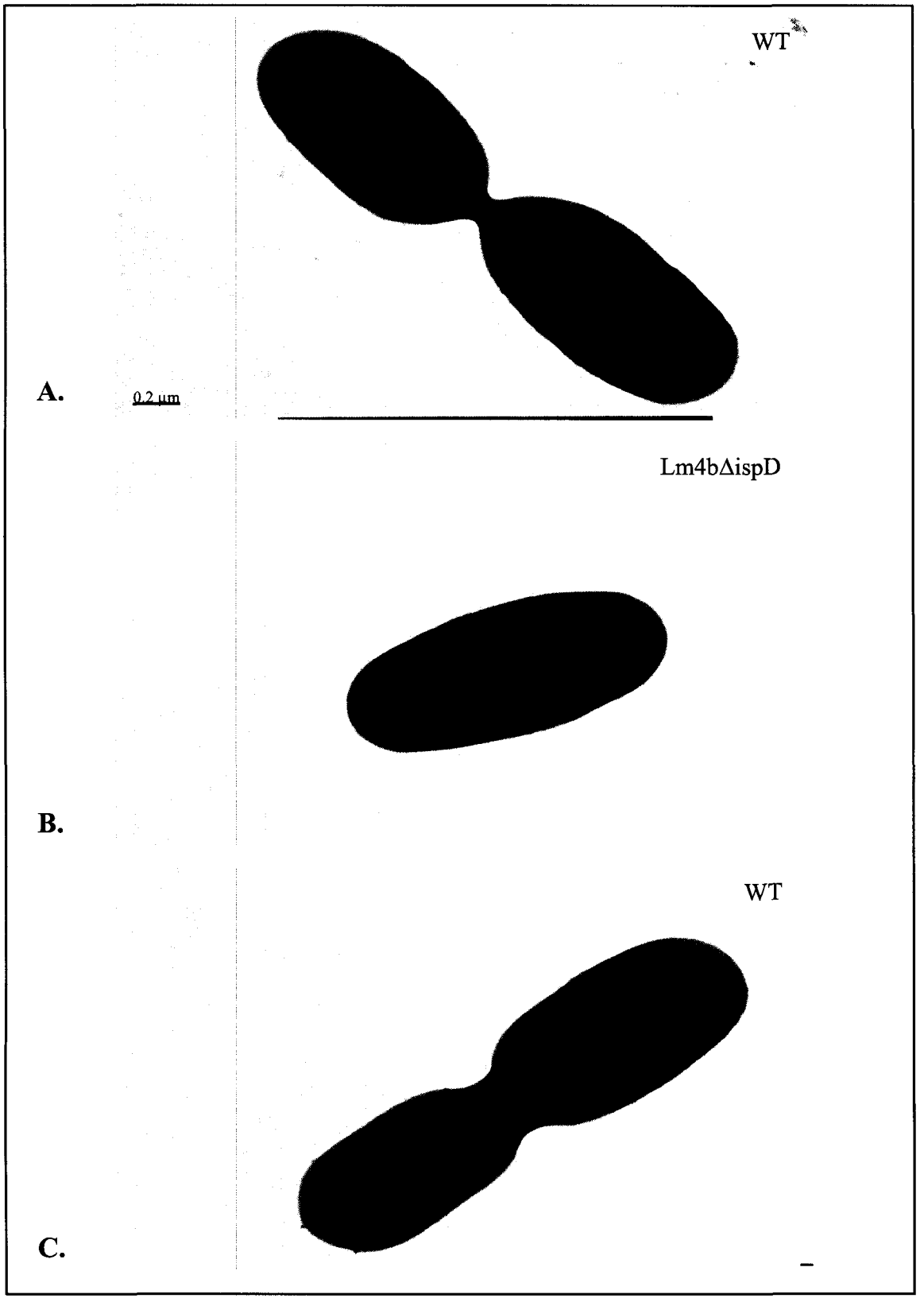

A.
WT
WT
B.
Lm4bΔispD
Lm4bΔispD
C.
WT
WT

Figure 4.7. Localization of IspD on the surface of *L. monocytogenes* strain LI0521 (serotype 4b) using immunogold labeling. Live bacteria (3×10^8 cells) were probed with either RαIspD (**B** and **C**) or rabbit preimmune serum (**A**) followed by reaction with 12 nm colloidal gold-AffiniPure goat anti-rabbit antibody as described in Materials and Methods. The wild-type strain (**A** and **C**) and the mutant strain Lm4bΔispD (**B**) were used here. Bacteria were examined with a transmission electron microscope (TEM) at a magnification of × 30,000. Bar, 0.2 μm.


WT
A.
0.2 µm
Lm4bΔispD
B.
WT
C.

4.7 Generation of *ispD* in-frame deletion mutant strain

A mutant strain (designed Lm4bΔispD) was successfully generated from the parental strain *L. monocytogenes* serotype 4b by in-frame deletion of the *ispD* gene from the chromosome using the homologous recombination technique. This mutant generation, lacking a 3201 bp internal *ispD* sequence (nt 10-3210) coding for almost the entire sequence of the IspD protein (aa 4-1070), was confirmed at the DNA level by PCR and DNA sequencing of the PCR products. As expected, PCR with the P696 and P697 primer pair targeting the deleted region of *ispD* showed no amplified product from the genomic DNA of the Lm4bΔispD strain but gave a product close to the calculated size (724 bp) from the wild-type genomic DNA (Figure 4.8 B). Furthermore, PCR with the primer pair P780 and P781 annealing to the sites outside the deleted part of *ispD* resulted in amplified products close to the predicted sizes of 1.1 kb and 4.2 kb from the genomic DNA of the Lm4bΔispD and wild-type strains, respectively (Figure 4.8 B). In addition, DNA sequencing of the PCR products derived from the Lm4bΔispD genome confirmed an internal deletion of 3201 bp (nt 10-3210) out of the 3,222 bp ORF of the *ispD* gene.

Deletion of the *ispD* gene completely abrogated the expression of the IspD protein, as no protein was detected by Western blot analysis of total proteins from the Lm4bΔispD strain using RαIspD (Figure 4.8 C). Moreover, immunofluorescence and immunogold labeling microscopy revealed the IspD protein, localized on the surface of the wild-type bacterium, was deficient in the Lm4bΔispD mutant (Figures 4.6 B and 4.7 B). Altogether, the *ispD* gene was successfully in-frame deleted from the chromosome to create the mutant strain Lm4bΔispD, which was confirmed at both DNA and protein levels.

4.8 Phenotypic characterization of the mutant strain Lm4bΔispD

L. monocytogenes strain Lm4bΔispD was compared to the wild-type strain phenotypically in order to determine if lack of IspD expression in *L. monocytogenes* displayed any defects in bacterial growth, colony and microscopic morphologies, and biochemical properties. The Lm4bΔispD mutant and its parental strain showed a similar colony morphology on BHI and TSBA agar plates. Also, similar morphologies were observed for both strains at different growth phases under light microscope and at mid-log phase under transmission electron microscope (Figures 4.9 B and 4.9 C). The *in vitro* growth of Lm4bΔispD at 37 °C was undistinguished from that of the wild-type over a period of 8 hrs (Figure 4.9 A). Biochemical tests reveled that the Lm4bΔispD mutant was similar to the wild-type with respect to motility at 25 °C and 35 °C, hemolysis on sheep blood agar plates, carbohydrates oxidation and fermentation on API 50 CH strips, catalase test, oxidase test, CAMP test (*S. aureus* and *Rhodococcus equi*), H_2S test, and nitrate reduction test.

Figure 4.8. Generation and confirmation of *ispD* ORF knockout. (**A**) DNA electrophoresis of overlap PCR products using wild-type genomic DNA as template. Lane 1, molecular weight marker labelled in kb; Lane 2, product from P717/P718 primer pair amplification (Fragment 1 as described in Section 3.12.1); Lane 3, product from P719/720 primer pairs amplification (Fragment 2 as described in Section 3.12.1); Lane 4, product from P717/P720 primer pairs amplification (Frag3ΔispD as described in Section 3.12.1). (**B**) Confirmation of *ispD* knockout with PCR using genomic DNA extracted from wild-type and Lm4bΔispD strains. Left gel shows products from P780/P781 primer amplification (external primers to the deleted part), and right gel shows products from P696/P697 primer pairs amplification (internal primers to the deleted region of *ispD* ORF). Lane 1, molecular weight marker labeled in kb; Lane 2, products from wild-type genomic DNA; Lane 3, products from Lm4bΔispD genomic DNA. (**C**) Western blot analysis of total protein extract from wild-type strain (lane 2) and Lm4bΔispD strain (lane 3) probed with RαIspD at a dilution of 1:1000. Each lane contains total cellular protein from culture volume equivalent to 2 ml at OD_{620} of 0.5. Lane 1, molecular weight marker labeled in kDa.

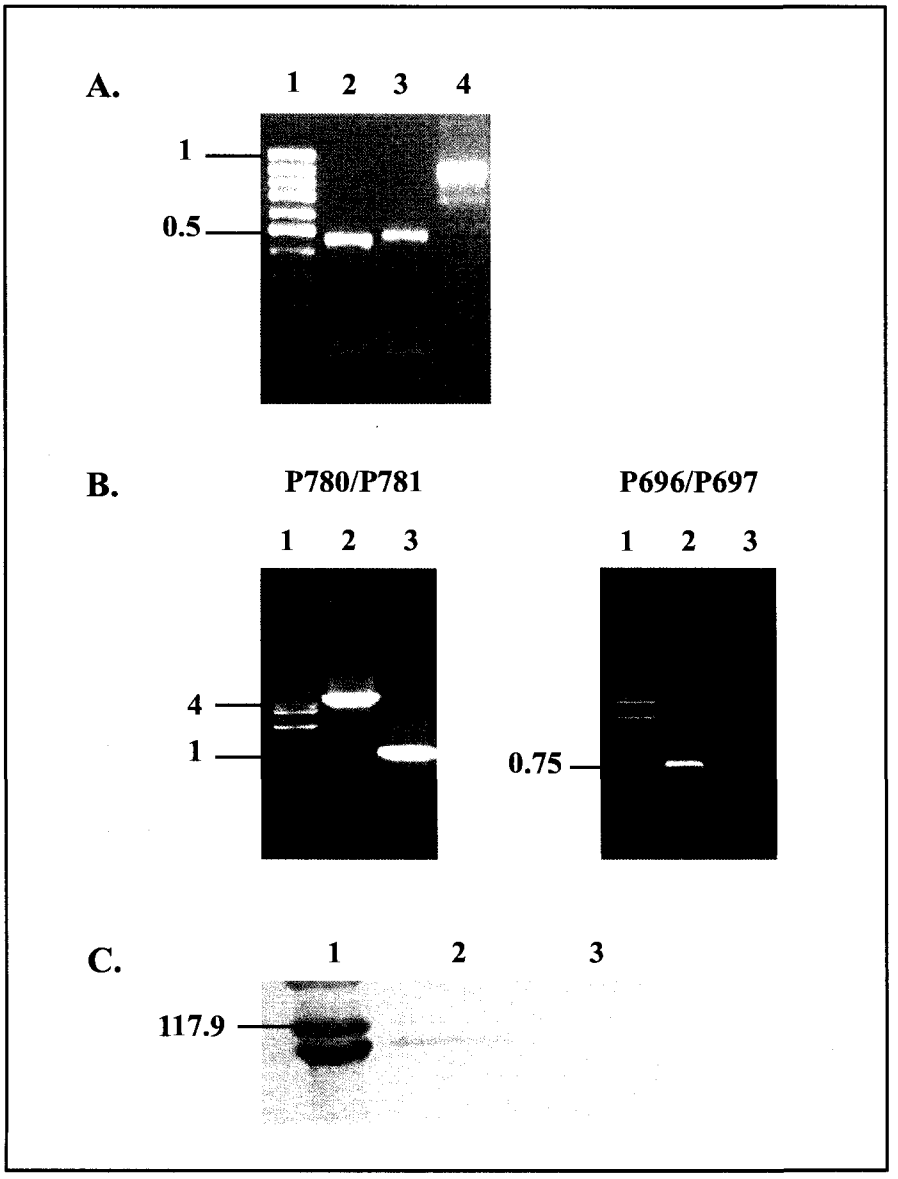
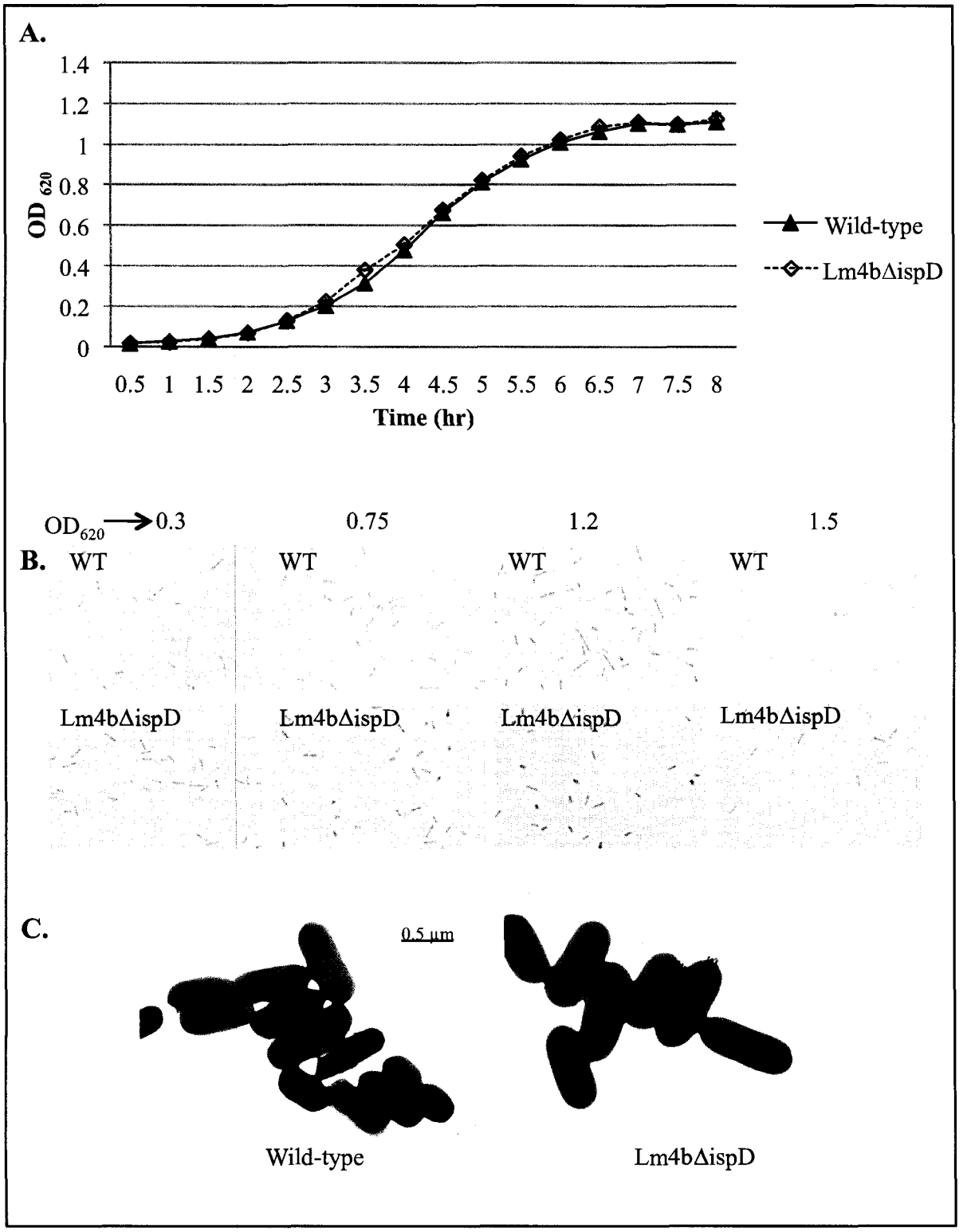

A.
1 2 3 4
1
0.5
B.
P780/P781
P696/P697
1 2 3
1 2 3
4
1
0.75
C.
1 2 3
117.9

Figure 4.9. Comparison of the mutant strain Lm4bΔispD with the wild-type *L. monocytogenes* serotype 4b in *in vitro* growth and microscopic morphology. (**A**) The *in vitro* growth of both strains, started with the cultures in triplicate (n = 3) with the same OD at 620 nm, were monitored over a period of 8 hrs. The OD_{620} value for each culture was measured every 30 min. (**B**) Both strains were grown in BHI broth at 37 °C. Bacteria (3×10^8) of each strain were harvested at early-log (OD_{620} of 0.3), mid-log (OD_{620} of 0.75), late-log (OD_{620} of 1.2), and stationary (OD_{620} of 1.5) phases and then examined under a phase contrast microscope. (**C**) Both strains were grown in BHI broth at 37 °C. Bacteria (3×10^8) of each strain were harvested at mid-log (OD_{620} of 0.75) phase, stained with 0.25% (w/v) uranyl acetate, and examined with TEM at a magnification of × 15,000. Bar, 0.5 μm.


A.
1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD 620
0.5 1 1.5 2 2.5 3 3.5 4 4.5 5 5.5 6 6.5 7 7.5 8
Time (hr)
Wild-type
Lm4bΔispD
OD620 → 0.3
0.75
1.2
1.5
B.
WT
WT
WT
WT
Lm4bΔispD
Lm4bΔispD
Lm4bΔispD
Lm4bΔispD
C.
0.5 μm
Wild-type
Lm4bΔispD

4.9 Role of IspD in bacterial adhesion to mammalian cell lines

The ability of IspD in promoting bacterial adhesion to various mammalian cell lines was investigated with the Lm4bΔispD mutant strain and the wild-type strain. Of the nine eukaryotic cell lines used in the adhesion assay (Vero, J774, SCP, CaCo-2, Hep-G2, JEG-3, L132, Hela, and HBMEC), Lm4bΔispD exhibited a reduction in adhesion only to Hep-G2 and L132 cell lines by about 38% and 70%, respectively, compared to the wild-type strain (Figure 4.10). The defect in adhesion to these cell lines was statistically significant with *P*-value < 0.05. This result highlights the importance of IspD in the adhesion of *L. monocytogenes* to such cells.

4.10 Role of IspD in bacterial invasion of mammalian cell lines

The involvement of IspD in the invasion of mammalian cells by *L. monocytogenes* was investigated with the Lm4bΔispD mutant strain and the wild-type strain. The invasion assay using eight non-phagocytic mammalian cell lines (Vero, SCP, CaCo-2, Hep-G2, JEG-3, L132, Hela, and HBMEC) revealed that the *ispD* knockout strain was impaired in the ability to invade Hep-G2 and L132 with a reduction in internalization into the two cell lines by about 57% and 69%, respectively, compared to the wild-type strain. The defect in entry into Hep-G2 and L132 by the Lm4bΔispD mutant was statistically significant with *P*-value < 0.05 (Figure 4.11).

Figure 4.10. Adhesion of the Lm4bΔispD mutant to mammalian cell lines in comparison with the wild-type *L. monocytogenes*. Each mammalian cell line was infected in triplicate with either the mutant or the wild-type at a MOI of 50 for 1 hr. After washing, bacteria associated with the mammalian cells were quantified by plating the cell lysates as described in Materials and Methods. The quantity of the mutant is calculated relative to that of the wild-type strain that was set to 100%. The results are expressed as mean ± SD (n=3). An asterisk indicates a statistically significant difference (P value < 0.05) between the mutant and the wild-type.

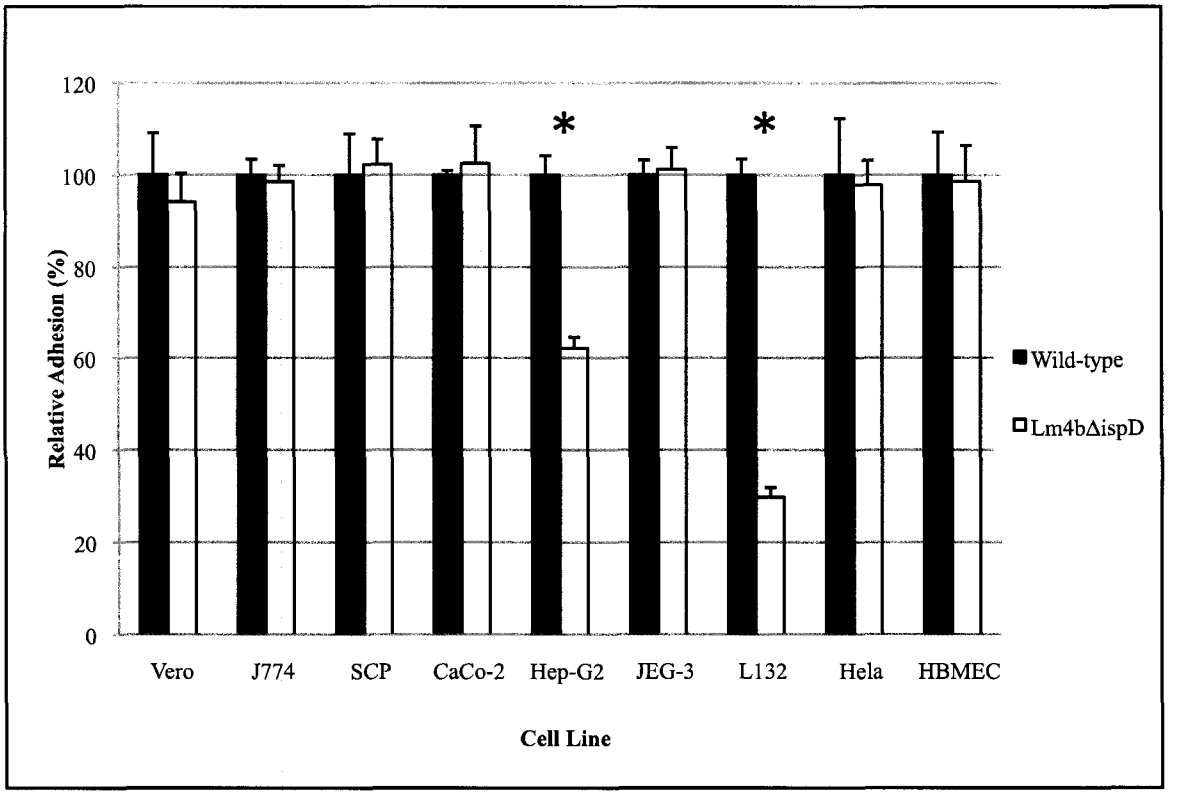
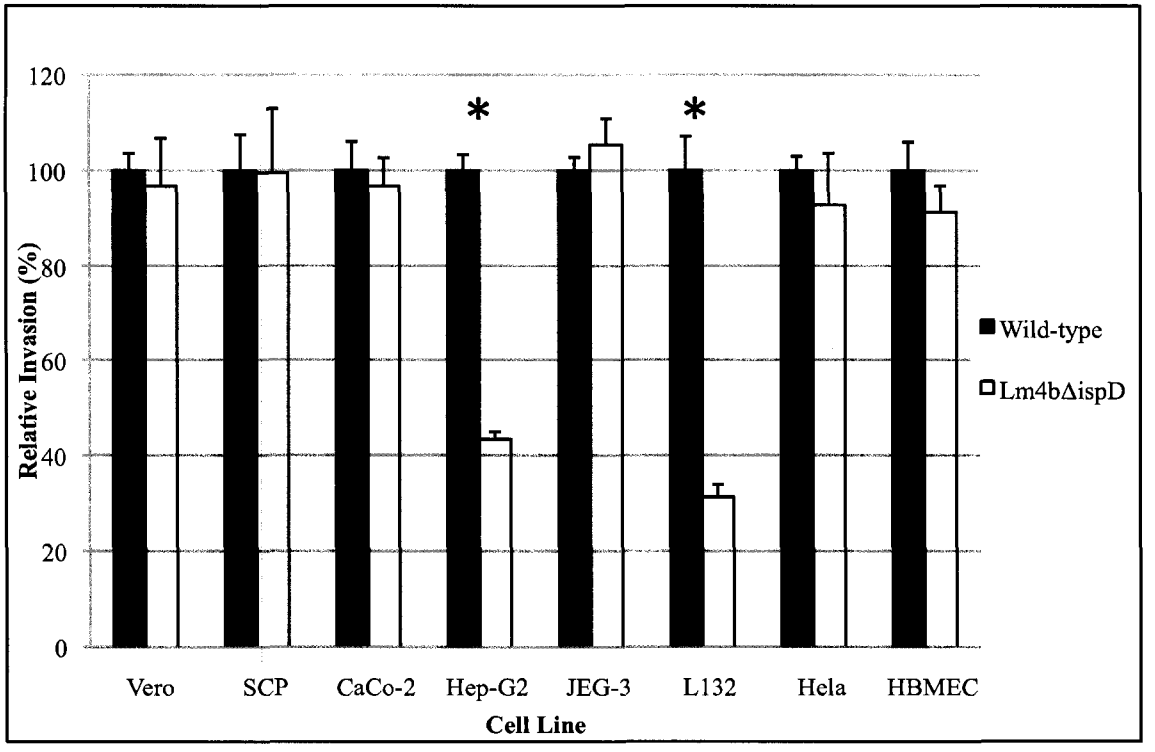

120
100
80
60
40
20
0
Relative Adhesion (%)
*
*
Vero
J774
SCP
CaCo-2
Hep-G2
JEG-3
L132
Hela
HBMEC
Cell Line
Wild-type
Lm4bΔispD

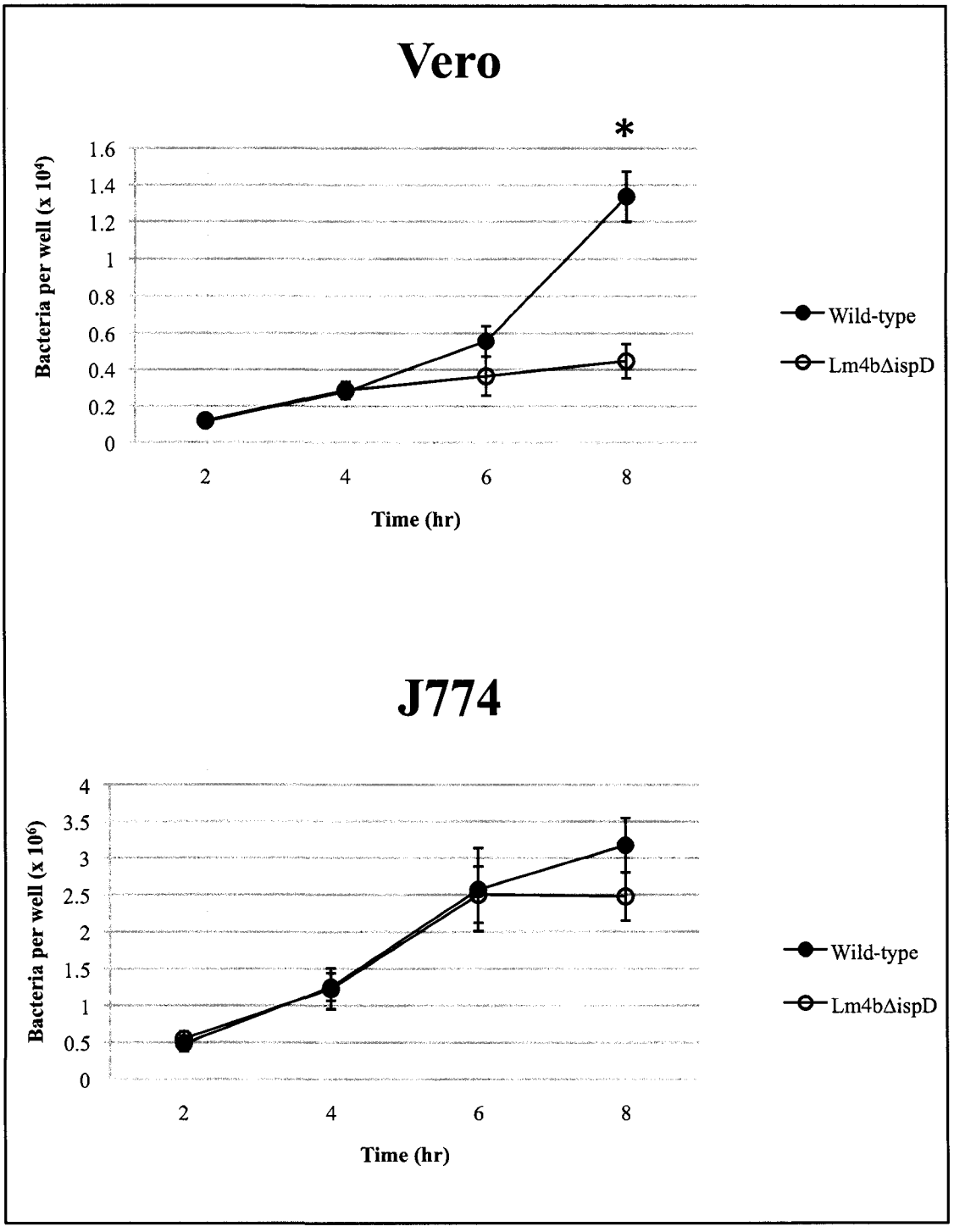
Figure 4.11. Entry of the Lm4bΔispD mutant into mammalian cell lines in comparison with the wild-type *L. monocytogenes*. Each mammalian cell line was infected in triplicate with either the mutant or the wild-type at a MOI of 50 for 1.5 hrs and treated with gentamicin (100 μg/ml) for 1 hr. The intracellular bacteria were quantified by plating the cell lysates as described in Materials and Methods. The quantity of the mutant is calculated relative to that of the wild-type strain that was set to 100%. The results are expressed as mean ± SD (n=3). An asterisk indicates a statistically significant difference (P value < 0.05) between the mutant and the wild-type.


120
100
80
60
40
20
0
Relative Invasion (%)
*
*
Vero
SCP
CaCo-2
Hep-G2
JEG-3
L132
Hela
HBMEC
Cell Line
Wild-type
Lm4bΔispD

4.11 Role of IspD in bacterial intracellular growth

The contribution of IspD to intracellular growth of *L. monocytogenes* in two representative mammalian cell lines (Vero, a non-phagocytic cell line; J774, a professional phagocytic cell line) was evaluated with the Lm4bΔispD strain and the wild-type strain. The number of the Lm4bΔispD strain within Vero cells was significantly reduced by ~ 3 folds (P-value < 0.05) compared to the wild-type at 8 hrs time point (Figure 4.12). On the other hand, no significant difference was observed between the mutant and the wild-type in the intracellular growth within J774 cells over a period of 8 hrs (Figure 4.12). Taking altogether, IspD plays a role in promoting bacterial intracellular growth in a cell type-dependent manner.

Figure 4.12. The intracellular growth of the Lm4bΔispD mutant and the wild-type in Vero and J774 cell lines. Each cell line was infected in triplicate with either the mutant or the wild-type at a MOI of 50 for 1 hr followed by treatment with gentamicin (100 μg/ml) over an 8 hrs time course. The numbers of intracellular bacteria were determined 2, 4, 6, 8 hrs after addition of gentamicin as described in Materials and Methods. The results are expressed as mean ± SD (n=3). An asterisk indicates a statistically significant difference (*P*-value < 0.05) between the mutant and the wild-type.


Vero
*
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Bacteria per well (x 10⁴)
2
4
6
8
Time (hr)
Wild-type
Lm4bΔispD
J774
4
3.5
3
2.5
2
1.5
1
0.5
0
Bacteria per well (x 10⁶)
2
4
6
8
Time (hr)
Wild-type
Lm4bΔispD

CHAPTER FIVE

DISCUSSION

5.1 Discussion

The IspD protein was originally identified as a novel target of humoral immune response to listerial infection by differential immunoscreening of *L. monocytogenes* serotype 4b genomic expression library with antiserum (RαL) from rabbits infected with live *L. monocytogenes* and antiserum (RαK) from rabbits infected with heat-killed bacteria (Yu *et al.*, 2007). However, very little is known about IspD until the present research is conducted to characterize this novel protein of unknown function using molecular, immunological and genetic techniques. This study provides the first experimental evidence that IspD is a surface-localized protein that is regulated by environmental factors and plays an important role in *L. monocytogenes* virulence. Knowledge gained and materials (i.e. antibodies to IspD and the *ispD* knockout strain) generated from this study should set the stage for future studies on the IspD protein.

The previous study (Yu, MSc. thesis, 2004) defined the *ispD* ORF of *L. monocytogenes* serotype 4b as a 2,712 bp coding sequence, in which a 510 bp internal segment was found missing when aligned with the gene homologue *LMOf2365_0349* (GenBank accession number YP_012958) from *L. monocytogenes* strain F2365 (serotype 4b). Inspection of the *LMOf2365_0349* sequence revealed that there are nucleotide sequence repeats in the ORF. Likely such sequence repeats are present in the *ispD* ORF and may thus result in missing the 510 bp internal segment between the sequence repeats during the contig

alignment. The missing segment in the previously determined *ispD* ORF was demonstrated by PCR and DNA sequencing. With this additional nucleotide sequence, a complete *ispD* ORF of 3,222 bp, similar to the size of the *LMOf2365_0349* ORF with 98 % homology (identity) in nucleotide sequence and encoding a 116 kDa protein of 1073 aa, was obtained. Although an N-terminal signal peptide (aa 1-28) with a signal peptidase cleavage site between alanine at position 28 and glutamic acid at position 29 was predicted using SignalP 3.0, demonstration of removing the signal peptide from the full-length protein expressed in *E. coli* was unsuccessful using N-terminal sequencing in three separate experimental trails, most likely due to N-terminal blockage. In contrast, the immunogenic protein IspC of *L. monocytogenes*, a cell wall-anchored autolysin essential for bacterial virulence (Wang and Lin, 2008), was shown to contain a 23-residue N-terminal signal peptide being processed in *E. coil* (Wang *et al.*, 2007).

Sequence characteristics of the IspD proteins, including a predicted N-terminal signal peptide, LLR repeats, and a cell wall-anchored motif LPXTG, suggests that it is a internalin-like surface protein belonging to the internalin family and may be important in *L. monocytogenes* virulence. Using the immunofluorescence and immunogold labeling techniques, IspD was showed to be localized on the bacterial surface. This result is consistent with the immunogenicity of IspD during rabbit infection (Yu *et al.*, 2007). Uneven distribution of IspD on the surface is in contrast to the relatively polarized distribution seen with InlA, a LPXTG-containing surface internalin responsible for adhesion to and invasion of epithelial cell lines by *L. monocytogenes* (Gaillard *et al.*, 1991; Lebrun *et al.*, 1996). In *S. aureus*, a sortase SrtA (a membrane-bound transpeptidase) (Mazmanian *et al.*, 1999) catalyzes the cleavage between the threonine (T) and the glycine (G) of the LPXTG motif and subsequent formation of the amide linkage between the carboxyl group of threonine and

an amine group of the peptide (pentaglycine) crossbridge on the cell wall peptidoglycan precursor, which is then incorporated into the mature cell wall (Scott and Barnett, 2006). Similarly, the InlA protein of *L. monocytogenes* has been shown to be covalently anchored by SrtA to the surface through an amide bond between the threonine of the cleaved LPXTG and the amino group of *meso*-diaminopimelic acid (*m*-Dpm) crossbridge within the peptidoglycan (Bierne and Cossart, 2007; Bierne *et al.*, 2002; Dhar *et al.*, 2000; Garandeau *et al.*, 2002). It is conceivable that the same SrtA-mediated mechanism is responsible for anchoring IspD on the surface of *L. monocytogenes*. The surface localization makes the IspD protein the potential candidate as a virulence factor accessible for interaction with host cells.

Reaction of several *L. monocytogenes* proteins (i.e. three internalins InlA, InlD and InlC2; five novel proteins of unknown function, designated IspA, IspB, IspC, IspD, and IspE, respectively) with RαL antiserum but not with RαK antiserum was previously interpreted as no or minimal expression of these proteins in *in vitro* cultured *L. monocytogenes* and suggested that they were specifically induced or upregulated *in vivo* during infection (Yu *et al.*, 2007) and may thus be important in *L. monocytogenes* pathogenesis. In fact, InlA is a well-known virulence factor involved in mediating the internalization of *L. monocytogenes* into host cells (Gaillard *et al.*, 1991; Vazquez-Boland *et al.*, 2001b). Furthermore, the IspC protein has recently been shown to be essential for *L. monocytogenes* virulence (Wang and Lin, 2008). Detection of the *ispD* transcript only at early log phase, together with a low level expression of the IspD protein during *in vitro* growth over the 48 hrs period, indicated that the *ispD* gene was not actively expressed outside the host environment. This result, together with the previous findings discussed above (Yu *et al.*, 2007), strongly supports the view that IspD is highly upregulated *in vivo* during infection and, therefore, might be crucial for *L. monocytogenes* virulence. A possible

explanation for the presence of a trace amount of the IspD protein at stationary phase during *in vitro* growth is that this protein is quite stable and less susceptible to proteolytic degradation. A similar finding has been recently reported for expression of the *ispC* gene in *in vitro* cultured *L. monocytogenes* (Wang and Lin, 2007).

The presence of antibodies to IspD in RαL but not in RαK (Yu *et al.*, 2007) gives an indirect indication of upregulation of expression of the IspD protein upon infection of mammalian hosts. Direct evidence for that would be necessarily obtained from the analysis of the *ispD* expression in infected hosts, which is to be addressed in a future study. Nevertheless, regulated expression of the IspD protein by environmental factors was demonstrated by the result that expression of IspD was increased under some certain stress conditions (i. e., growth in the presence of H_2O_2 and recovery in BHI and BLEB but not LBMOPS nor LRB broths). Similarly, expression of other microbial proteins has been shown to be induced under environmental stress conditions in *L. monocytogenes* (Geng *et al.*, 2006; Hahm and Bhunia, 2006) and in other bacteria such as *Enterococcus faecalis* and *Bacillus subtilis* (Bernhardt *et al.*, 1997; Rince *et al.*, 2002; Rouquette *et al.*, 1998). The observation that IspD was stress-induced may indicate that upregulation of this protein is likely triggered by the stress conditions encountered by *L. monocytogenes* inside infected hosts. Failure to demonstrate an increased expression of IspD, following H_2O_2 stress recovery in LBMOPS and LRB broths may be attributed to the nutrient composition in these media (Jaradat and Bhunia, 2002). LRB contains a high concentration of glucose, which is known to suppress the expression of some *L. monocytogenes* proteins (Milenbachs *et al.*, 1997; Park and Kroll, 1993; Ripio *et al.*, 1996). Given that LBMOPS is similar in nutrient composition to LB broth, which was shown to limit the expression of some *L. monocytogenes* proteins such as

ActA (Lathrop *et al.*, 2008), expression of IspD may be inhibited or suppressed following H_2O_2 stress recovery in LBMOPS.

Several members of the internalin family contribute greatly to the virulence of *L. monocytogenes*, including three LPXTG containing internalins (inlA, inlH, and inlJ), inlB (an internalin non-covalently associated with the cell wall via the cell wall binding domain made up of three GW modules), and InlC (a secreted internalin) (Engelbrecht *et al.*, 1996; Gaillard *et al.*, 1991; Ooi *et al.*, 2006; Raffelsbauer *et al.*, 1998; Sabet *et al.*, 2005; Schubert *et al.*, 2001). In this study, deletion of the *ispD* gene from the *L. monocytogenes* chromosome led to an attenuated virulence of the mutant Lm4bΔispD strain in a cell type-dependent manner in a cell culture infection model using various mammalian cell types that the bacterium normally encounters during *in vivo* infection. The impaired ability of the Lm4bΔispD mutant to adhere to Hep-G2 and L132 cells but not to other mammalian cells (Vero, J744, SCP, Caco-2, JEG-3, Hela, and HBMEC) suggests that IspD functions in pathogenesis as an adhesin mediating the attachment of the bacterium to certain mammalian cells which presumably express the unidentified receptor(s) specific for IspD. Consistent with this adhesin nature is the presence of LLR repeats in the IspD protein (see below for more discussion). Also, localization of IspD on the cell surface satisfies the requirement for this protein to act as an adhesin. Correlated well with the results of the adhesion assay is the findings that an attenuated bacterial invasion of Hep-G2 (57% less) and L132 (69% less) but not other mammalian cell types was observed with the Lm4bΔispD mutant compared to the wild-type strain. The reduced adhesion to and invasion of Hep-G2 and L132 by the Lm4bΔispD mutant is not attributed to impaired replication or altered biochemical or morphological properties of the mutant since both the Lm4bΔispD and wild-type strains are

similar in *in vitro* growth, biochemical characteristics, and morphologies. Altogether, these data indicate that IspD is essential for adhesion to and invasion of Hep-G2 and L132 cells by *L. monocytogenes*. Knowing that Hep-G2 and L132 cells are originated from human liver and lung, respectively, it is possible that IspD plays a role in dissemination of the bacterium into such locations during *in vivo* infection. However, identifying the cellular receptor(s) for IspD would be necessary to understand the molecular mechanisms by which IspD protein contributes to the adhesion and invasion processes in *L. monocytogenes* virulence.

In *L. monocytogenes*, the InlA protein is known to interact with the E-cadherin receptor on the epithelial cell surface while InlB plays an essential role in bacterial entry into a much broader spectrum of host cells including hepatocytes, fibroblasts, certain epithelial cells, and endothelial cells and binds to three different receptors: the receptor of the globular part of the complement component C1q (gC1q-R), hepatocyte growth factor receptor (HGFR) Met, and glycosaminoglycans (GAGs) (Braun *et al.*, 2000; Dramsi *et al.*, 1997; Gaillard and Finlay, 1996; Jonquieres *et al.*, 2001; Shen *et al.*, 2000; Vazquez-Boland *et al.* 2001b). Functional studies showed that the N-terminal LRR and IR regions were necessary and sufficient for InA-mediated entry (Lecuit *et al.*, 1997). On the other hand, InlB-Met interaction stimulates protein tyrosine phosphorylation and phosphoinositide (PI) 3-kinase activity and ultimately induces actin cytoskeleton rearrangements required for invasion into target mammalian cells such as Vero, Hep-G2, and Hela by *L. monocytogenes* (Braun *et al.*, 1998; Gaillard *et al.*, 1987; Ireton *et al.*, 1996; Ireton *et al.*, 1999; Tang *et al.*, 1994; Velge *et al.*, 1994). InlB activates the Met receptor through its LRR and IR domains binding to the receptor (Braun *et al.*, 1999; Freiberg *et al.*, 2004). In other bacterial species, LRR containing proteins are involved in host-pathogen interactions. Examples of such proteins are

secreted internalins of *Listeria ivanovii*, Slr from *Streptococcus*, YopM from *Yersinia*, SspH from *Salmonella*, and IpaH from *Shigella* (Engelbrecht *et al.*, 1998; Fernandez-Prada *et al.*, 2000; Leung and Straley, 1989; McDonald *et al.*, 2003; Miao *et al.*, 1999; Reid *et al.*, 2003; Toyotome *et al.*, 2001). Likely, IspD-mediated entry of *L. monocytogenes* into target mammalian cells could be through interaction of the LLR repeats (domain) with the cellular receptor(s) to IspD. In contrast, InlB is able to interact with the receptors GAG and gC1q-R through its C-terminal GW modules, which further facilitating bacterial internalization mediated by InlB-Met interaction (Braun *et al.*, 2000; Jonquieres *et al.*, 2001). Lack of GW modules in IspD suggests that GAG and gC1q-R are not the potential reporters for IspD. It would be interesting to determine in the future if IspD could interact with the Met receptor and stimulate protein tyrosine phosphorylation and phosphoinositide (PI) 3-kinase activity, ultimately leading to actin cytoskeleton rearrangements required for bacterial invasion. In addition to the LRR repeats, the IspD contains four PKD domains, which are shorter in sequence than a typical, PKD domain of 80 aa (Bycroft *et al.*, 1999; Cabanes *et al.*, 2002). Of 41 LPXTG motif-containing proteins in *L. monocytogenes* strain EGD-e (serovar 1/2a), 11 proteins have C-terminal PKD domains, and 2 proteins (inlI and Lmo0333) contain both PKD domains and LRR domains (Cabanes *et al.*, 2002). Given that PKD domains forms complex with E-cadherin and α-, β-, and γ-catenin (Huan and van Adelsberg, 1999), the PDK domains present in the IspD protein may have a similar molecular binding function and thus may play a role in bacterial adhesion to host cells and signaling, which needs to be experimentally examined in a future study.

It is interesting to observe that the ability of *L. monocytogenes* to grow intracellularly was impaired at later stages (6-8 hrs after entry) in Vero cells (non-phagocytic) but not in

J774 (professional phagocytes) by lack of the IspD expression. This result may indicate that IspD plays a role in bacterial survival in the interacellular environment in a cell type dependent manner. The combined results from the virulence study with cultured mammalian cells strongly suggest that the IspD protein plays a role in *L. monocytogenes* virulence. However, infection experiments in animal models with the Lm4bΔispD mutant, in comparison to the wild-type, is necessary to confirm the role of IspD in *L. monocytogenes* pathogenesis *in vivo*.

5.2 Conclusions

Several conclusions can be drawn from the present work. (i) IspD is 116 kDa protein of 1073 aa localized on the bacterial surface with the deduced amino acid sequence characteristic of a protein of the internalin family. (ii) IspD is expressed by *L. monocytogenes* at a low level under normal *in vitro* growth conditions with the gene transcript detected only in the early exponential growth phases. (iii) Expression of IspD is regulated in response to environmental stress such as oxidative (H_2O_2) stress. (iv) Expression of IspD is important in bacterial adhesion to and invasion of host cells in a cell-type dependent manner and thus is a new virulence factor of *L. monocytogenes*. (v) IspD, although not important in *in vitro* growth of *L. monocytogenes,* contributes significantly to bacterial growth and survival intracellularly in a cell-type dependent manner. Future studies could be emphasized on: (i) elucidation of the molecular mechanism underling the regulated expression of IspD in response to environmental stress; (ii) investigation into the role of IspD in *L. monocytogenes* virulence in an animal model using the Δ*ispD* mutant; and (iii)

identification of the potential host cellular receptor(s) to IspD and dissection of the signal pathway(s) triggered by binding of IspD to its specific receptor.

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APPENDIX A

Preparation of Culture Media, Bufferes, and Solutions

0.1% Coomassie Brilliant Blue in 50% methanol Staining Solution

For 50 ml:	
Coomassie Brilliant Blue	50 mg
100% methanol	25 ml
dH_2O	500 ml

Mix to dissolve completely

0.5 M Tris-HCl- pH 6.8

For 500 ml:	
Tris	30.275 g
dH_2O	up to 500 ml

Adjust pH to 6.8 with 6 M HCl.

1 M IPTG

For 10 ml:	
IPTG (Isopropyl β-D-Thiogalactopyranoside)	2.383 g
dH_2O	up to 10 ml

Pass through 0.22 μm filter

1 x PBS

For 100 ml:	
25 x PBS	4 ml
dH_2O	96 ml

1 x PBS-TT

For 10 L:	
25 x PBS	400 ml
Tween 20	5 ml
Triton X-100	20 ml
dH_2O	up to 10 L

Stir overnight to mix completely

1 x SDS-PAGE Running Buffer

For 1 L:	
10 x SDS-PAGE Running Buffer	100 ml
dH_2O	900 ml

1 x TE Buffer

For 100 ml:

10 x TE buffer	10 ml
Sterile dH_2O	90 ml

1% Buffered Peptone Water

For 1 L:	
Peptone	0.1 g
Sodium chloride	0.05 g
Disodium hydrogen phosphate dodecahydrate	0.09 g
Potassium dihydrogen phosphate	0.015 g
dH_2O	up to 1 L
Adjust pH to 7.2	
Sterilize by autoclaving	

1% Triton X-100 in H_2O

For 100 ml:	
Triton X-100	1 ml
dH_2O	99 ml
Stir overnight to mix completely	
Sterilize by autoclaving	

1.5 M Tris-HCl- pH 8.8

For 500 ml:	
Tris	90.825 g
dH_2O	up to 500 ml
Adjust pH to 8.8 with 6 M HCl	

2 x SDS-PAGE Sample Buffer

For 10 ml:	
0.5 M Tris-HCl-pH 6.8	2 ml
Glycerol (100%)	2 ml
ß-mercaptoethanol	2 ml
SDS (lauryl sulphate)	0.4 g
0.5% Bromophenol Blue	400 µl
MQH_2O	3.6 ml

3% BSA-1 x PBS-TT

For 1 L:	
BSA (Bovine serum albumin fraction V)	30 g
1 x PBS-TT	up to 1 L

3% BSA-PBS

For 100 ml:	
1 x PBS	100 ml
BSA	3 g

3% Skim Milk-1 x PBS-TT

For 1 L:	
Skim milk powder	30 g

1 x PBS-TT	up to 1 L

4% Stacking Gel

Acrylamide/Bis	0.65 ml
0.5 M Tris-HCl –pH 6.8	1.25 ml
dH_2O	3.05 ml
10% SDS	50 µl
10% APS	25 µl
TEMED	5 µl

5 x SDS-PAGE Sample Buffer

For 10 ml:	
1 M Tris-HCl-pH 6.8	2 ml
Glycerol (100%)	2 ml
ß-mercaptoethanol	5 ml
SDS (lauryl sulphate)	1 g
0.5 % Bromophenol Blue	1 ml

5% BSA-PBS

For 100 ml:	
1 x PBS	100 ml
BSA	5 g

10 x BSA (10 mg/ml)

For 100 ul:	
100 mg/ml BSA	10 µl
dH_2O	90 µl

10 x SDS-PAGE Running Buffer

For 1 L:	
Tris	30 g
Glycine	144 g
SDS (Lauryl sulphate)	6g
dH_2O	up to 1 L

10 x TE Buffer

For 100 ml:	
Tris	1.21 g
0.5 M EDTA pH 8.0	2 ml
dH_2O	up to 100 ml
Adjust pH to 8.0	
Sterilize by autoclaving	

10% APS

For 10 ml:	
Ammonium Persulfate	1 g
dH_2O	up to 10 ml

10% SDS

For 100 ml:	
SDS (Lauryl Sulfate)	10 g
dH_2O	up to 100 ml

12% Separating Gel

Acrylamide/Bis (30% T, 2.67% C)	4.0 ml
1.5 M Tris-HCl –pH 8.8	2.5 ml
dH_2O	3.35 ml
10% SDS	100 ul
10% APS	50 ul
TEMED	5 ul

25 x PBS – pH 7.4

For 1 litre:	
Sodium Phosphate Dibasic (Na_2HPO_4)	27.5 g
Sodium Phosphate Monobasic (NaH_2PO_4)	7.88 g
Sodium chloride (NaCl)	212.5 g
dH_2O	up to 1 L
Adjust pH to 7.4	
Sterilize by autoclaving	

Acrylamide/Bis

For 500 ml:	
Acrylamide	146 g
PDA	4g
dH_2O	up to 500 ml

BHI (Brain Heart Infusion) Agar

For 1 liter:	
BHI Medium (Becton Dickinson)	1 L
Agar	15 g
Adjust the pH to 7.4	
Sterilized by autoclaving	

Coomassie Blue Stain

For 500 ml:	
Coomassie Brilliant Blue	2.5 g
100% Methanol	200 ml
100% Acetic acid	50 ml
dH_2O	250 ml

LB (Luria-Bertani) Agar:

For 1 liter:	
Tryptone	10 g
Yeast Extract	5 g

NaCl (sodium chloride)	10 g
dH_2O	up to 1 L
Agar	15 g
Adjust the pH to 7.0	
Sterilized by autoclaving	

LBMOPS Medium

For 500 ml	
LB Medium	500 ml
MOPS	5.25 g
Adjust pH to 7.2	
Sterilize by autoclaving	

Listeria Repair Broth (LRB)

For 1 L:	
TSB	30 g
Glucose	5 g
Yeast extract	6 g
Magnesium sulfate	4.94 g
Ferrous sulfate	0.3 g
Pyruvic acid (sodium salt)	10 g
3-N-morpholinepropanesulfonic acid (MOPS)-free acid	8.5 g
MOPS sodium salt	13.7 g
dH_2O	up to 1 L
Adjust pH to 7.0	
Sterilize by autoclaving	

Luria-Bertani (LB) Medium

For 1 L:	
Tryptone	10 g
Yeast Extract	5 g
NaCl (sodium chloride)	10 g
dH_2O	up to 1 L
Adjust the pH to 7.0	
Sterilized by autoclaving	

Protein Transfer Buffer

For 1 L:	
Tris	5.82 g
Glycine	2.93 g
dH_2O	up to 1 L

RNase-Free Water (0.1% DEPC Water)

For 100 ml:	
Diethylpyrocarbonate (DEPC)	100 ul
dH_2O	100 ml
Stir overnight at RT and sterilize by autoclaving	

SOC Medium

Tryptone	20 g
Yeast Extract	5.0 g
NaCl	0.5 g

Add to 980 ml H_2O.
Autoclave and cool to 55 °C.
Add 10 ml of 1 M $MgCl_2$ and 10 ml of 1 M of $MgSO_4$

Trypticase Soy Blood Agar (TSBA) Plates

Per liter:

Trypticase Soy Agar	40 g
Defibrinated Sheep Blood	50 ml

Complete to 1 liter with H_2O
Autoclave

APPENDIX B

Nucleotide Sequence containing *ispD* ORF (nt 335-3556)

(GenBank Accession NO. EF409983)

```
   1 TAAAAACTAT TCTGTGAATT TGTCTGTTTA TAGTATAAAT TTACCGAAGA TAGATAAAAA
  61 TCCCTCTTGA TACTATATGA AAAAGTAATA ATAATAGTAC AAACTCGTTG TGAATTTGAT
 121 ATATTATATA TAAAGCAACT CCATCTCCTC ATAATTTTAC GCGAATTTTA GGGCTTTAAC
 181 GAATTAGATG AAAAAAGACT AATTGTTATG TGTGGAGGAG TTTATGGGAT ATATACTTAA
 241 ATAATACTTT TCAATGAAAT AGTAGGTATT TTTAACTTGC TCATTCATTC TATTATTACT
 301 GAAGATGCTA ACAACAACGA GAAGGAGATA GATTATGAAA AGCAAAACAA AACAGATTAT
 361 CATGATTGGA GTGGTCCTTT TTCAATCACT CTTCGCATAC CCGTTAATCA CCATGGCGGA
 421 AGAAAATGAA TCGAAATCAG TAAATACAGA AACCACGTTA GAGCCTAAAG TAGCTCTCGA
 481 AGAAAAAACG CCTCAGAAAC CTACCCTTAC CAATAATCTG AAGCAAGAAA AAACTGTCCT
 541 TCAAGCAGGC GAAACATATG AAACTGTTTT TCCTGATGCA GCTTTAGCTA CTGTAATTGC
 601 AAAAGCAGCA ACTGGTTCAG AGGATATCAC GCAAGAAGTA TCGCAAACAG ACTTGAATAA
 661 AATCACTTCA CTAGCTGCTA CATCTAAAGG GATAGTTGAT TTAACAGGAA TAGATTTACT
 721 TTCAAAATTA ACCTCTTTAA GTATAAGCGG GAACCAAATC ACTGATATTT CTGCACTCAA
 781 TGGTCTCGTG AATTTGTCCA ATCTAAATGT ATCTAATAAT AAAATAACAA GTTTCAACCT
 841 AAACGCGAAT AGTAATTTAC CTATGTTAAG CGCTGTTGAT ATTCGTAGTA ATAACTTAAA
 901 AAATAGAAAT GTTCAAGACC AACCTAAATT ACGCACCATT GAGTGTGACA CAGGTAGTAG
 961 TTCAGAGTTG ACAGAAGTTA CGCTAAAAAA TCTTCCAACT TTAATAGTTG CAGGTAATGG
1021 CTCTAGTGCT TATCAAAATG ATATTGTTTT TTCTAGTACA CCAGGATTAA GTAAGGTGAT
1081 TCTAGAAAAT TTACCATCAA TAAGCTCTTC AGTACGATTA GATCGTTGCG CGATAGAAGA
1141 GTTAGTAATT AATAACCTTC CAAAAAAATC AATGGTAAAT ATAAGTAACA ACAAAATTAC
1201 TACACTAGAA GGACTTGAAA ATTTATCTGC AGTAAACACT TTATACGTAT CTGAGAATTT
1261 AGTGACTGAA ATAGAAAGTA TGCATGCCTT CCCTAAATTA CAGAATTTAG AGCTGGGATG
1321 GAATGCTCTT ACTAATGTGG TAATGGATCA AGTAACAGCG GAAAAACTCC CGTTATTAAG
1381 AACAATGGAT GTTCGTGGTA ATAATTTAAT TAAGATAAAT ATTCAAGACC AGCCAAAATT
1441 ATGGACCTTT GAGTGCGACA CAGGTAGTAG TTCAGAGTTG ACAGAAGTTA CGCTAAAAAA
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1501 TCTTCCAATT TTGATAGTTG CAGGTAATGG CTCTAGTGCT TATCAAAATG ATATTGTTTT

1561 TTCGAGTACA CCAGGATTAA GTAAGGTGAT TCTAGAAAAT TTACCATCAA TAAGCTCTTC

1621 AGTACGATTA GATCGTTGCG CGATAGAAGA GTTAGTAATT AATAACCTTC CAAAAGTATC

1681 AATGGTACAT ATAAGTTACA ACAAAATTAC TACACTAGAA GGACTTGAAA ATTTATCTGC

1741 AGTAAGCACA CTTTATACGT ACGAGAATTT AGTGACTGAA ATAGAGAACC TACATGCGTT

1801 CCCTAAATTA CAGACACTCA CCGTAGACTA TAACGCTCTC ACTGTAGTGC CAACGGATCT

1861 GAAAACGGAA AATCCCGTAT TAACAACGCT AAGTGCGATG AATCAAACAA TCACTCTAAA

1921 GCAAAAAGTT ATCGTGTCAG ACCTAGTTCT TGATAATGAA GTGAAGAATT TCGGTCAAAT

1981 AACCACTGCC AAATCCATCT CTAATAAGGG AACCTATCAA AATAACCAAA TCAAGTGGCT

2041 TTTTGAAGAT ATAAAAAGCG TGAATGCCGT TGATTATCAA TTTAGTGAAC CTGTTCAAGA

2101 GGCAACTATT CAAGGAACTT TTTCGGGGAA AGTGACACAA CCAATCAAAG CATCTAAAGT

2161 ACCAGTTATT AGCGCAGATG CAGAGATGAA TTACCCGAAA AACGAAACGG TATCAGAAGC

2221 TGCCTTTTTC AAAGATATTT CTGCAAGCGT AACGGATGAT GCAACACTAA CTTCTGATTT

2281 TGAAAGTGTT GTGGACTTTG CAAAAGCGGG AACGTATGAA GTGACATTAA ATGCAGTGAA

2341 TGAGGATGGA GTAAAAGCGA CTTCGGTGAC TGTATTAGTG CATATCGCTA AGTCGCCAGC

2401 GCCAGTAATT ACCGCAGATA AAGAAATCAC ATACACTAAA AACGCGGAAG TCAGCATCAC

2461 GGAATATCTT GCAGCGATTC ATGCTAAAAC GAATGATGGT TCACCAATTG AAAGTGATTT

2521 CGCTACGGCT GTAAATTGGG GCACTGCAGG AGATTATACC GTAACGCTAA GGTCTACAAA

2581 TGAAGATGGA GTTGAAGCAA TCCCTGTAGA AGTAACTGTG CACATCGCCA AGTCGCCAGC

2641 GCCAGTAATT ACCGCAGATA AAGAAATTAC GTACGCTAAA AACGCGGAAG TCAGCATCAC

2701 GGAATATCTT GCAGCGATTC ATGCTAAAAC GAGTGATGGT TCATCAATTG AAGCTGATTT

2761 AGATACGGCT GTAACATGGG GCACTGTAGG AGGTTATACC GTAACGCTAA GGTCTACAAA

2821 TGAAGACGGA GTAGAAGCAA TCCCTGTAGA AGTAACTGTG CACATCGCTA AGTCACCAGC

2881 ACCAGTAATT ACCGCAGATA AAGAAATCAC GTACGCTAAA AACGCGGAAG TCAGCATGAC

2941 GGAATTTCTT GCAGCGATTC ATGCTAAAAC GAGTGATGGT TCACCAATTG AAAGTGATTT

3001 CGCTACGGCT GTAATATGGA GCACTGCAGG AGATTATACC GTAACGTTAA AATCTACAAA

3061 TGAAGATGGA GTAGAAGCAA TCCCTGTAGA AGTAAAGGTG CATATCGTAG AGCCACTAGC

3121 ACCAACGATT TCGAATGTGA CATTTGATGT GGATGATGTA CAAACGACAG AATCTCTTGA

3181 AGCTGGAGAG CTAATTTCTG AACCATTGAG CCCAACAAAA GAAGGCTATA CTTTTATTGG

3241 TTGGTATGAC TCGAAAACTG GTGGTAATAA ATGGGATTTT ACAACAGATA AAATGCCAGC

3301 ATATAATATT ATTCTTTATG CTCAGTTTAG TAAAGATACA AATAAAGCAG AAGCGGCCGG

3361 TGGAGATAAG CCCTCAACAC CCTCTTCTAT AAAAGTAAGT CCAACAGGTC AGTCCGAGAG

3421 TGGAAACTTG GAAAATAGAT CTAATATTAA ATTACCAGCT ACAGGCGATG ATAATGCAAC

3481 TGTTTTATTA GTGGGCTTTG GATTACTAAT GTTGGGGCTT TTCATTCGCC TTACTCAAAA

3541 AAAGCGTGCT AAATAAAAGC TCATAGAAAT TTAAAAAATA GTGTAATTGG TTGCTTCTAG

3601 GAGATAAATA GTTGCAACTC ACAAGGAGAG GAGACAGAGA GCTGTC

Deduced Amino Acid Sequence of IspD (GenBank Protein ID NO. ABN80103)

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   1 MKSKTKQIIM IGVVLFQSLF AYPLITMAEE NESKSVNTET TLEPKVALEE KTPQKPTLTN
  61 NLKQEKTVLQ AGETYETVFP DAALATVIAK AATGSEDITQ EVSQTDLNKI TSLAATSKGI
 121 VDLTGIDLLS KLTSLSISGN QITDISALNG LVNLSNLNVS NNKITSFNLN ANSNLPMLSA
 181 VDIRSNNLKN RNVQDQPKLR TIECDTGSSS ELTEVTLKNL PTLIVAGNGS SAYQNDIVFS
 241 STPGLSKVIL ENLPSISSSV RLDRCAIEEL VINNLPKKSM VNISNNKITT LEGLENLSAV
 301 NTLYVSENLV TEIESMHAFP KLQNLELGWN ALTNVVMDQV TAEKLPLLRT MDVRGNNLIK
 361 INIQDQPKLW TFECDTGSSS ELTEVTLKNL PILIVAGNGS SAYQNDIVFS STPGLSKVIL
 421 ENLPSISSSV RLDRCAIEEL VINNLPKVSM VHISYNKITT LEGLENLSAV STLYTYENLV
 481 TEIENLHAFP KLQTLTVDYN ALTVVPTDLK TENPVLTTLS AMNQTITLKQ KVIVSDLVLD
 541 NEVKNFGQIT TAKSISNKGT YQNNQIKWLF EDIKSVNAVD YQFSEPVQEA TIQGTFSGKV
 601 TQPIKASKVP VISADAEMNY PKNETVSEAA FFKDISASVT DDATLTSDFE SVVDFAKAGT
 661 YEVTLNAVNE DGVKATSVTV LVHIAKSPAP VITADKEITY TKNAEVSITE YLAAIHAKTN
 721 DGSPIESDFA TAVNWGTAGD YTVTLRSTNE DGVEAIPVEV TVHIAKSPAP VITADKEITY
 781 AKNAEVSITE YLAAIHAKTS DGSSIEADLD TAVTWGTVGG YTVTLRSTNE DGVEAIPVEV
 841 TVHIAKSPAP VITADKEITY AKNAEVSMTE FLAAIHAKTS DGSPIESDFA TAVIWSTAGD
 901 YTVTLKSTNE DGVEAIPVEV KVHIVEPLAP TISNVTFDVD DVQTTESLEA GELISEPLSP
 961 TKEGYTFIGW YDSKTGGNKW DFTTDKMPAY NIILYAQFSK DTNKAEAAGG DKPSTPSSIK
1021 VSPTGQSESG NLENRSNIKL PATGDDNATV LLVGFGLLML GLFIRLTQKK RAK
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Curriculum Vitae
Turki S. Abujamel

D.O.B: 6-November-1978
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Marital status: Married with no Children.

Permanent Address:

Canada
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Current Position:
Full time M.Sc student, Department of Biochemistry, Microbiology and Immunology, Faculty of Medicine, University of Ottawa, Ottawa, ON, Canada.

Qualifications:
Apr 2001: B.Sc in Medical Technology (grade A 86.92% with first class honors).

Education:
Sep 2005- Present: M.Sc Study, Department of Biochemistry, Microbiology and Immunology, Faculty of Medicine, University of Ottawa, Ottawa, ON, Canada.

Sep 1996- Apr 2001: B.Sc course in Medical Technology at KAAU, Jeddah, KSA.

Work Experiences:
Sep 2003: Part time lecturer at the Specialized Academy for Medical Training (SAMT), teaching Medical Mycology for Clinical Lab Diploma students, Jeddah, KSA.

May 2004 -Present: Demonstrator in Microbiology (Teaching Assistant) with full scholarship to pursue M.Sc and Ph.D degree in North America, Department of Medical Technology, Faculty of Applied Health Sciences, KAAU, Jeddah, KSA.

Aug 2001- Feb 2002: Medical Laboratory Technologist, Hematology laboratory. King Abdul Aziz University Hospital (KAAUH), Jeddah, KSA.

Jul 2000- Jun 2001: KAAUH, Jeddah, KSA and Al-Hada Armed Forces Hospital, Al-Hada, KSA. Internship Clinical Rotation:
Rotation Major: Blood bank.
Rotation Minors: Hematology, Cytology.

Research Experiences:
Sep 2005- Present: M.Sc research, "Biochemical and Functional Characterization of a Novel Immunogenic Protein, IspD, From *Listeria monocytogenes*", supervised by Dr. Min Lin, Canadian Food Inspection Agency, Ottawa, ON, Canada.

Mar 2002: Participated in research workshop in "Writing Research Proposal". Al-Hada Arme Forces Hospital, Al-Hada, KSA.

Apr 2000: 4th year senior project, "Effect of Academic Examination Stress on Serum Immunoglobulin in Healthy Medical Technology Students", supervised by Dr. Hamed Khouja, Department of Medical Technology, Faculty of Applied Health Sciences, KAAU, Jeddah, KSA.

Seminars, Courses, & Training:
Sep 2004-Apr 2005: ESL course, University of Ottawa, Ottawa, Canada.

Mar 2003: Laboratory training in Molecular Diagnostic Unit and Special Infectious Agents Unit, KAAUH and King Fahd Medical Research Center (for 15 months), KAAU, Jeddah, KSA.

Sep 2002: Laboratory training in Medical Mycology Unit (for 2 months).King Khalid University Hospital, King Saud University, Riyadh, KSA.

Feb 2001: Attended the 3rd International Symposium on Hematology and Blood Transfusion, King Saud University, Riyadh, KSA.

Sep 2000: Operator training on the "AxSym System" (Immunological autoanalyzer), by ABBOT Co. at KAAUH, Jeddah, KSA.

Nov 1999: Senior seminar in "Diagnosis of Malaria by PCR", Supervised by Dr. Hitham Zakai, Department of Medical Technology, Faculty of Applied Health Sciences, KAAU and KAAUH.

Jun 1999: Laboratory training at Saudi German Hospital, Jeddah, KSA.

Languages Spoken and Written:
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References:
Available upon request.