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AND POSTDOCTORAL STUDIES

THE GENE PRODUCTS OF *LISTERIA MONOCYTOGENES* INDUCED SPECIFICALLY DURING RABBIT INFECTION

A thesis submitted to the School of Graduate Studies

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By

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ABSTRACT

Listeria monocytogenes is a Gram-positive, facultative, intracellular bacterium that can cause severe food-borne illness referred to as listeriosis in both humans and animals. This study has developed a rabbit model for the identification of *L. monocytogenes* gene products specifically induced during infection by constructing a *L. monocytogenes* protein expression library followed by differential immunoscreening with two kinds of antisera, one (RaL) from rabbits infected with viable bacteria, and the other (RaK) from animals immunized with heat-killed bacteria. This strategy led to the discovery of several *L. monocytogenes* proteins, including three members (InlA, InlD, and InlC2) of the internalin family, in which InlA and InlB are known bacterial virulence factors, and five novel proteins of unknown function (designated IspWei, IspLing, IspYu, IspMin, and IspLin, respectively). Library screening and Western blots showed that these proteins reacted only with RaL, indicating that they are specifically induced during infection. The five novel proteins were first demonstrated being expressed in *L. monocytogenes*. With the exception of antibodies to InlA, the presence of antibodies to the seven proteins (InlD, InlC2, IspWei, IspLing, IspYu, IspMin, and IspLin) was first shown in infected hosts. Protein sequence analysis and comparison indicated that (i) IspWei is an internalin-like LPXTG protein; (ii) IspLing is a protein similar to hypothetical transmembrane proteins of *L. monocytogences* EGD-e and *L. innocua* Clip11262, containing a sulfatase domain; (iii) IspYu is a protein similar to putative amidase-like proteins of *L. monocytogences* EGD-e and *L. innocua* Clip11262; (iv) IspMin is a protein similar to a putative protein ORFA of *L. seeligeri* and a putative peptidoglycan protein of *L. monocytogenes* EGD-e; and (v) IspLin is a protein similar to a putative peptidoglycan bound protein of *L. monocytogenes* EGD-e, containing a signal peptide (aa 1-47). Sequence characteristics and immunogenicity of the five novel proteins and their co-expression with several internalin family members (known virulence factors) appear to suggest that these novel proteins are involved in *L. monocytogenes* virulence and pathogenesis.

Dedicated to my parents, my family members, especially to my son, Nick.

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LIST OF ABBREVIATIONS

aa, amino acid
BCIP, 5-bromo-4-chloro-3-indolyl phosphate
BHI, brain heart infusion
CDTX, cholesterol-dependent, thiol-activated pore-forming toxin
CR, catabolite repression
2D-PAGE, two dimensional polyacrylamided gel electrophoresis
ELISA, enzyme-linked immunosorbent assay
FACS, fluorescence-activated cell sorting
G1P, glucose 1-phosphate
GFP, green fluorescence protein
HGF, hepatocyte growth factor
HS, heparan sulfate
HSPG, heparan sulfate proteoglycan
HUVEC, human umbilical vein endothelial cells
IEF, isoelectric focusing
IVET, *in vivo* induced expression technology
LBMOPS, Luria-Bertani broth containing MOPS
LLO, listeriolysin O
LRR, leucin-rich repeat
NBT, nito blue tetrazolium
nt, nucelotide
ORF, open reading frame
PBS, phosphate buffered saline
PC, phosphatidylcholine
PE, phosphatidylethanolamine
PFGE, pulsed-field gel electrophoresis
PI, phosphatidylinositol
PLC, phospholipase C
PS, phosphatidylserine
SCID, severe combined immunodeficiency
SCOTS, selective capture of transcriped sequences procedure
SLO, streptolysin O
SOD, superoxide dismutase
SM, sphingomyelin
TSBA, trypticase soy blood agar

VASP, vasodilator-stimulated phosphoprotein

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CHAPTER ONE

INTRODUCTION

1.1 Historical Aspects

Our knowledge and understanding of *listeria monocytogenes* began in 1924 when Murray, Webb and Swann isolated a bacterium, the causative organism of the disease listeriosis, during an epizootic among rabbits and guinea-pigs in the stables of their laboratories in Cambridge (Murray et al., 1926; Seeliger, 1961). This marked the official discovery of *L. monocytogenes*. At that time, however, the name *Bacterium monocytogenes* was given to the organism because a typical monocytosis was frequently observed in infected rabbits. One year later, Pirie isolated from the gorilla a bacterium that caused typical liver lesions in experimentally infected animals (Pirie, 1927). The name *Listerella hepatolytica* was given to this organism in honor of Lord Lister. When *Bacterium monocytogenes* and *Listerella hepatolytica* turned out to be the same organism, a new designation *Listerella monocytogenes* was created (Seeliger, 1961). In 1940, Piris suggested a new name to *Listeria monocytogenes* for the organism (Piris, 1940); this designation has become accepted in the *Listeria* field since than.

The first cases of human listeriosis were reported in 1929 in Denmark (Nyfelt, 1929). In early1980s, a number of large outbreaks of human listeriosis in North America and Europe were linked to the consumption of foods contaminated with *L. monocytogenes* (Schlech et al., 1983; Fleming et al., 1985; Linnan et al., 1988; McLauchlin, 1987; Farber and Peterkin, 1991; Stephens et al., 1991;Rocourt and Bille, 1997; Meier and Lopez, 2001; Donnelly, 2001;Lorber, 1997), with a mortality rate of about 30%; this clearly established the bacterium as an important food-borne pathogen. Foods implicated in transmitting the pathogen include soft cheeses, milk, coleslaw, smoked fish and in general industrially produced, refrigerated ready-to-eat products.

Immunologists were interested in *L. monocytogenes* long before the organism was recognized as a great threat to public health and food safety. The pioneer work of Mackaness (Mackaness, 1962) demonstrated that *L. monocytogenes* is capable of surviving and multiplying inside macrophages and therefore escapes the host defenses in the early phase of bacterial growth in infected tissues. The concept of T-cell dependent macrophage activation and the concept of cellular immunity with the requirement for T lymphocytes and activated macrophages in the acquired immunity to *L. monocytogenes* were established based on the earlier findings of Mackaness and others (MacDonald and Carter, 1980). Subsequently, *L. monocytogenes* has quickly become a model organism used by immunologists to study T-cell mediated immunity against intracellular parasites and also an important model system for study of the molecular mechanisms of intracellular parasitism (Cossart and Mengaud, 1989). The discovery in *L. monocytogenes* of the hemolysin (listeriolysin O) gene, *hly*, and the elucidation of the key role of listeriolysin O (LLO) involved in destruction of phagosomes between 1986 and 1989 (Vazquez-Boland et al., 2001), clearly demonstrated that this protein functions as a virulence factor essential for bacterial intracellular survival. The recent work of Lecuit *et al.* (Lecuit et al., 2001) using the transgenic mouse model and the guinea pig model shows that *L. monocytogenes* invades intestinal epithelial cells through binding of the bacterial surface protein InlA to the epithelial transmembrane protein receptor E-cadherin. A single amino acid Pro^{16} in E-cadherin is responsible for the susceptibility of epithelial cells to *L. monocytogenes* infection. This remarkable study elucidates the molecular mechanism underlying how the bacterium breaches the intestinal barrier.

The year 2001 also sets an important milestone in the *Listeria* field when Glaser et al (Glaser et al., 2001) published the complete genome sequences of *L. monocytogenes* (2944528 bp) and a nonpathogenic species, *L. innocua* (3,011,209 bp). This has led to the identification of

2853 and 2973 protein-coding genes in *L. monocytogenes* and *L. innocua*; respectively. However, it may require years and years of studies before one can understand how the expression of many of these new genes are regulated, and what roles their products have in pathogenesis, virulence, immunity, parasite-host interaction, and bacterial survival. This challenge has formed part of the rationale for conducting the present study described in the thesis.

1.2 Microbiology of *L. monocytogenes*

Microbiological and biochemical characteristics. *L. monocytogenes* is a gram-positive rod (0.4-0.5 × 0.5-2 μm) (Seeliger, 1984) residing within the genus *Listeria* in which seven species have been recognized: *L. monocytogenes*, *L. innocua*, *L. ivanovii*, *L. welshimeri*, *L. seeligeri*, *L. grayi* and *L. murrayi*) (Farber and Peterkin, 1991). Evidence has indicated that *L. grayi* and *L. murrayi* should be considered as a single species, designated *L. grayi* (Rocourt et al., 1992). *L. monocytogenes* is widespread in the environment found in water, soil and vegetation. This bacterium is capable of growing over a wide range of temperatures from -1.5 to 50 °C and within a pH range of 4.3 to 9.6 (Donnelly, 2001) and can survive freezing and drying. These characteristics render *L. monocytogenes* a difficult organism to be eliminated from food chains. *L. monocytogenes* is catalase-positive, oxidase-negative, facultatively anaerobic and non-sporulating, expresses ß-hemolyic activity, and exhibits characteristic motility when cultured at 20 to 25°C but not at 37°C. (Gray and Killinger, 1966; Farber and Peterkin, 1991; Hayes et al., 1991; Low and Donachie, 1997). The hemolytic activity was also observed with *L. ivanovii* and *L. seeligeri*. Other *Listeria* species are non-hemolytic. All strains of *L. monocytogenes* produce acid from L-rhamose and a-methyl-D-manoside but not from D-xylose and a-methyl-D-manoside (Rocourt et al., 1983; Bille et al., 1992).

Antigenic Heterogeneity. Antigenic heterogeneity among the *Listeria* strains or isolates were demonstrated to be associated with somatic O- and flagella H-antigens by the early serological studies of Paterson (Paterson, 1939). This heterogeneity forms the basis of the antigenic scheme for classification of the *Listeria* strains using a serological criterium. The currently accepted antigenic structure scheme was established by Seeliger (Rocourt and Seeliger, 1985; Seeliger and Langer, 1989) and Donker-Voet (Donker-Voet, 1972), which includes 16 serovars of the genus (Table 1). Although sereotyping is widely used in epidemiological studies, it does not correlate with the species distinctions and is therefore of very limited value. In *L. monocytogenes*, there are 13 serovars, of which three serovars, 1/2a, 1/2b and 4b, are responsible for most human infections (Lorber, 1997; Donnelly, 2001).

Genome organization. During the study of DNA polymorphism in various *L. monocytogenes* strains by restriction endonuclease (*Apa*I and *Not*I) digestion followed by pulsed-field gel electrophoresis (PFGE) analysis, Carriere *et al.* (Carriere et al., 1991) estimated the genome sizes of these strains to be from 2340 to 2710 kb. Subsequently, the circular genetic and physical map of *L. monocytogenes* genome was established by using PFGE in strain LO28 (serovar 1/2c) with a genome size of 3150 kb (Michel and Cossart, 1992). Recently P. Glaser *et al.* (Glaser et al., 2001) reported the complete genome sequences of *L. monocytogenes* EGD-e (serovar 1/2a) and *L. innocua* CLIP 11262 (serovar 6a). The *L. monocytogenes* EGD-e contains one circular chromosome of 2,944,528 bp with an average G + C content of 39% (GenBank/EMBL accession number AL591824). The genome of *L. innocua* strain CLIP 11262 has a similar size of 3,011,209 bp and a similar G + C content of 37% (GenBank/EMBL accession number AL582022). The *L. innocua* strain also contains a plasmid of 81,905 bp (GenBank/EMBL accession number AL592102). In the genome of *L. monocytogenes*, 2853 protein-coding genes

Table 1. Serovars of *Listeria* spp.

Listeria	Serovars*	O antigens	H antigens
monocytogenes	1/2a	I, II, (III)[a]	A, B
monocytogenes, seeligeri	1/2b	I, II, (III)	A, B, C
monocytogenes	1/2c	I, II, (III)	B, D
monocytogenes	3a	II, (III), IV	A, B
monocytogenes	3b	(III), IV, (XII, XIII)	A, B, C
monocytogenes	3c	(III), IV, (XII, XIII)	B, D
monocytogenes	4a	(III), (V), VII, IX	A, B, C A,
monocytogenes, innocua	4ab	(III), V, VI, VII, IX, X	B, C
monocytogenes	4b	(III), V, VI	A, B, C
monocytogenes, seeligeri	4c	(III), V, VI	A, B, C
monocytogenes, seeligeri	4d	(III), V, VI, VIII	A, B, C
monocytogenes	4e	(III), V, VI, (VIII, IX)	A, B, C
invanovii	5	(III, V), VI, (VIII), X	A, B, C
monocytogenes	7	(III), XII, XIII	A, B, C
innocua, welshimeri	6a	(III), V, (VI, VII, IX),XV	A, B, C
innocua, welshimeri, selligeri	6b	(III, V, VI, VII), IX, X, XI	
grayi		(III), XII, XIV	E
innocua, seeligeri	undesignated		

*Designation of Seeliger (Rocourt and Seeliger, 1985) and Donker-Voet (Donker-Voet, 1972)
[a] = not always present.

were identified and 2973 in that of *L. innocua*. On the basis of encoded proteins the striking similarity of these genes to those of the soil bacterium *Bacillus subtilis*, Glaser *et al.* (Glaser et al., 2001) classified the *Listeria* genes according to the functional categories defined for *B. substilis*. About one third of *L. monocytogenes* genes (35.3%) and *L. innocua* genes (37%) can not be assigned to any predicted function. Comparative characteristics of the *L. monocytogenes* and *L. innocua* genomes are presented in Table 2.

Proteome. Our knowledge about the complete set of proteins expressed by *L. monocytogenes* is very limited. A total of 2853 protein-coding genes were derived from the genome sequence of *L. monocytogenes* EGD-e (serovar 1/2a) released recently (Glaser et al., 2001); this has provided a theoretical basis for the design of experiments to elucidate the total protein complement of the genome. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) was introduced to study bacterial proteins close to three decades ago (O'Farrell, 1975). With the improvement of the technique over the years, 2D-PAGE now has the potential to resolve thousands of proteins from a complex protein mixture (Klose and Kobalz, 1995). Thus, 2D-PAGE is currently the highest-resolution analytical technique available for the study of global protein expression in cells, tissues or organisms. This technique has been used to study the responses of *L. monocytogenes* to various environmental stresses including acidic pH (Davies, 1983; O'Driscoll et al., 1996; Phan-Thanh et al., 2000; Ravishankar and Harrison, 1999), cold shock (Bayles et al., 1996; Hebraud and Guzzo, 2000; Wemekamp-Kamphuis et al., 2002), the hostile environment within phagocytic cells (Hanawa et al., 1995), exposure to high salts (Esvan et al., 2000) and antimicrobial compounds (Duffes et al., 2000; Gravesen et al., 2002; Ramnath et al., 2000). The 2D-PAGE technique has been also used to profile the proteins of *L. monocytogenes* strains or isolates for identification and classification (Phan-Thanh and Gormon, 1995) or analyses of

Table 2. General features of the two *Listeria* genomes. (Glaser et al., 2001)

	L. monocytogenes	*L. innocua*
Size of the chromosome (bp)	2,944,528	3,011,209
G+C content (%)	39	37
G+C content of protein-coding genes (%)	38	38
Total number of protein-coding genes	2853	2973
Average length (codons) of protein-coding genes	306	299
Number of rRNA operons (16s-23s-5s)	6	6
Number of tRNA genes	67	66
Percentage coding	90.3%	90.3%
Prophages	1 (60 genes, 41.6 kb)	5 (301 genes, 219.4 kb)
Plasmid	0	1 (79 genes, 81.9 kb)
Number of strain-specific genes*	270 (294 kb)	149 (195 kb)
Number of orthologous genes*	2523	2523
Number of transposons	1 (Tn916-like)	-

* Prophage genes excepted.

strains having various origins (Ramnath et al., 2003). All these 2D-PAGE analyses have detected only a few hundred protein species (spots) from the *Listeria* protein extracts although 1357 protein spots can be obtained for a *L. monocytogenes* strain from the compilation of a number of gel images of 2D protein patterns (Gormon and Phan-Thanh, 1995).Thus, there is a significant difference between the coding capabilities and the number of protein species detected in the global analysis of *L. monocytogenes* protein expression. This difference may be attributed to (i) factors associated with the 2D-PAGE technique including the ranges of pI and the concentrations of resolving gels used, the insolubility of some proteins in the isoelectric focusing (IEF) sample buffer, and the sensitivity of staining procedures; (ii) lack of expression or low expression of proteins under growth used in experiments. In conjunction with 2D-PAGE, other techniques for the analysis of proteins in *L. monocytogenes* cultured in various growth conditions may be necessary for the construction of a complete proteome.

1.3 Infection and Pathogenesis

Listeriosis. *L. monocytogenes* can infect both humans and animals, which leads to the development of serious invasive illness referred to as listeriosis (Farber and Peterkin, 1991; Tran and Kathariou, 2002; Low and Donachie, 1997; Suarez et al., 2001; Schuchat et al., 1991; Lorber, 1997; Meier and Lopez, 2001). *L. ivanovii* (previously designated *L. monocytogenes* serotype 5) is another species of the *Listeria* genus that is pathogenic exclusively for animals (Cooper and Dennis, 1978). Thus, the only species in the genus that is of great concern for human health is *L. monocytogenes*. Although the first cases of human listeriosis were reported in 1929 in Denmark (Nyfelt, 1929), the link of acquired *Listeria* infections to consumption of *L. monocytogenes*-contaminated food was not established until 1983 (Schlech et al., 1983). Since then, the transmission of this pathogen via contaminated foods (mainly ready-to-eat foods) has been shown to cause both outbreaks of listeriosis and sporadic infections (Chris and Kyriakides, 1998-2004; Meier and Lopez, 2001; Donnelly, 2001; Lorber, 1997; Schlech, 2000). Mother to child transmission is the only documented form of person to person transmission. Factors that may influence the development of an invasive disease include the virulence of infecting strains, the susceptibility of hosts, and the dose of inoculum. Almost all human infections are attributed to serotypes 4b, 1/2a, and 1/2b (Donnelly, 2001; Lorber, 1997). The molecular mechanisms underlying the differential virulence of various *L. monocytogenes* strains are not known. In comparison with healthy individuals, special population groups, including neonates, pregnant women, the elderly, immunosuppressed transplant recipients, and other individuals with impaired cell-mediated immunity such as HIV-infected patients, are at a higher risk of infection and are more likely to develop a serious illness which could lead to death (Meier and Lopez, 2001). Clinical manifestations of listeriosis include septicaemia, meningitis, encephalitis, febrile gastroenteritis, abortion, and stillbirths. Although *L. monocytogenes* is susceptible to a number of antibiotics, penicillin, ampicillin, and amoxicillin are often used for treatment of invasive listeriosis (Schuchat et al., 1991; Meier and Lopez, 2001).

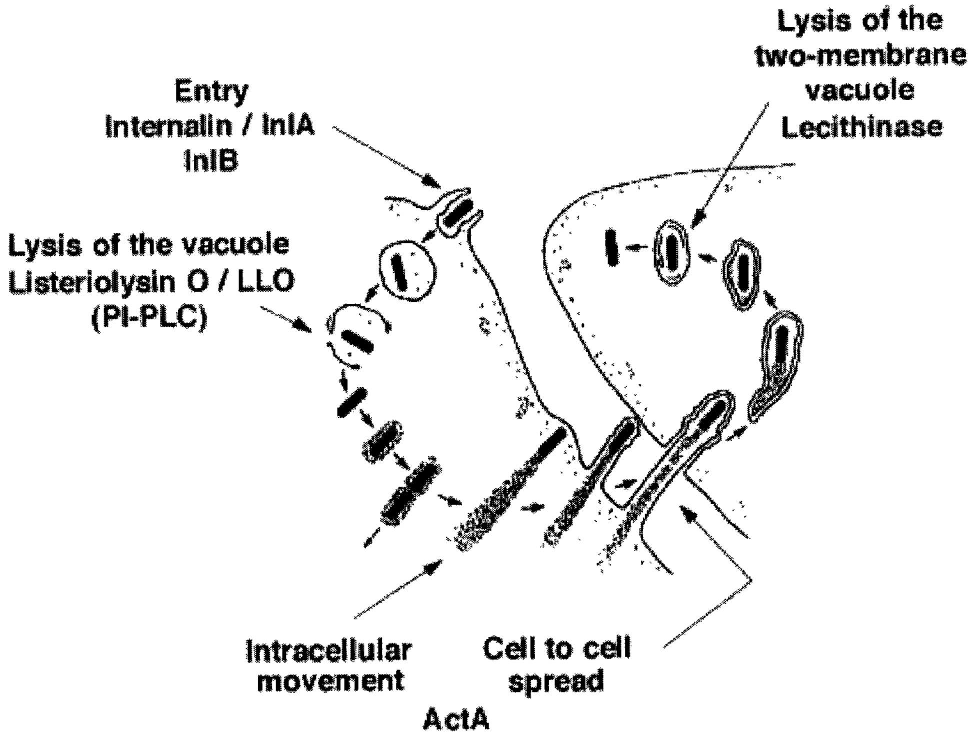
Entry of host tissues. As the major source of listerial infection in both epidemic and sporadic cases can be traced to contaminated food (Farber and Peterkin, 1991; Pinner et al., 1992), the gastrointestinal tract is thought to be the primary site of entry of *L. monocytogenes* into hosts. An early histological study by Racz *et al.* (Racz et al., 1972) with guinea pigs infected intragastrically with *L. monocytogenes* suggested that the bacterium penetrated the hosts by invading the intestinal epithelium. This is further supported by the finding that *L. monocytogenes* is able to penetrate the apical surface of cultured human intestinal epithelial cell line Caco-2 cells

(Karunasagar et al., 1994). Entry into the epithelial cell line is mediated by internalin A (InlA), a surface protein of about 90 kDa, encoded by the gene *inlA* (Lecuit et al., 1997). In contrast, studies with mice inoculated with *L. monocytogenes* demonstrated no invasion of the intestinal villus epithelium; the bacterium instead colonized in the Peyer's patches (MacDonald and Carter, 1980; Marco et al., 1992), suggesting the *L. monocytogenes* gained its entry by invading the M-cell epithelium. Views on the point of entry used by *L. monocytogenes*, based on previous studies with guinea pig, human Caco-2 cell line, and mice models, appear to be controversy but may not be surprising as, recently, Lecuit *et al.* (Lecuit et al., 2001), using the transgenic mouse model and the guinea pig model, showed that *L. monocytogenes* invaded intestinal epithelial cells through binding of InlA to the epithelial transmembrane protein receptor E-cadherin. A single amino acid Pro^{16} in E-cadherin is responsible for the susceptibility of epithelial cells to *L. monocytogenes* infection (Lecuit et al., 2001). A mutation from Pro^{16} to Glu^{16} in mouse E-cadherin rendered mouse epithelium cells nonsusceptible to *L. monocytogenes* invasion. Lack of a functional E-cadherin in mouse epithelium cells provides an explanation for the lack of invasion of the intestinal villus epithelium by the bacterium observed in earlier studies with mouse models (MacDonald and Carter, 1980; Marco et al., 1992).

Intestinal translocation. The study using a mouse model revealed no formation of gross macroscopic or histological lesions in the gut during intestinal translocation of *L. monocytogenes* (Marco et al., 1992). Pron *et al.* (Pron et al., 1998) using a rat ileal loop model of intestinal infection also demonstrated that *Listeria* organisms were rapidly translocated to deep organs within a few minutes. These studies indicate that the bacterium crosses the intestinal barrier without prior intraepithelial replication and suggests that bacterial multiplication in the intestinal mucosa in the early epithelial phase is not required for systemic infection. *L. monocytogenes* mutants defective in virulence factors involved in epithelial cell invasion (InlA and InlB), intracellular survival (LLO) and cell-to-cell spread (ActA), as well as the nonpathogenic species *L. innocua,* were translocated at the same rate as the wild-type *L. monocytogenes* (Pron et al., 1998). It appears that *L. monocytogenes* employs a passive, nonspecific mechanism of intestinal translocation similar to that found for other bacteria (Berg, 1995). After penetration of the intestinal barrier, *L. monocytogenes* disseminates through the lymph or blood into the primary target organs such as liver and spleen (Marco et al., 1992; Pron et al., 1998). Most of the bacteria that reach the primary target organs are killed and cleared from the bloodstream by resident macrophages in the spleen and liver (Conlan and North, 1991; Cousens and Wing, 2000; Lepay et al., 1985; Mackaness, 1962; Ebel et al., 1999). Viable *L. monocytogenes* can infect and replicate within hepatocytes in the liver (Conlan and North, 1992b; Cousens and Wing, 2000; Gaillard and Finlay, 1996; Rosen et al., 1989) and can eventually cause systemic infection and invasion of the secondary target organs such as central nervous system, placenta and fetus (Vazquez-Boland et al., 2001).

Cell-to-cell spread. *L. monocytogenes* not only multiplies within macrophages (Vazquez-Boland et al., 2001) but also invades various types of nonphagocytic cells including epithelial cells (Gaillard et al., 1987; Mengaud et al., 1996a; Mengaud et al., 1996a; Portnoy et al., 1988), fibroblasts (Goebel et al., 1988; Sun et al., 1990), hepatocytes (Dramsi et al., 1995; Wood et al., 1993; Conlan and North, 1992a; Gaillard et al., 1996; Rosen et al., 1989), endothelial cells (Dramsi et al., 1995; Greiffenberg et al., 2000; Schwarzer et al., 1998), dendritic cells (Darji et al., 1997; Kolb-Maurer et al., 2000), and nerve cells such as neurons (Dramsi and Cossart, 1998). Regardless of the types of host cells *L. monocytogenes* is capable of penetrating, whether professional (macrophages) or nonprofessional phagocytes, the bacterium develops a cell-to-cell

Figure 1. Scheme of cell-to-cell spread of *Listeria monocytogenes* in an infected host. Modified from Vázquez-Boland *et al.* (Vazquez-Boland et al., 2001).


Lysis of the
two-membrane
vacuole
Lecithinase
Entry
Internalin / InlA
InlB
Lysis of the vacuole
Listeriolysin O / LLO
(PI-PLC)
Intracellular
movement
Cell to cell
spread
ActA

spread strategy referred to as a zipper-type mechanism with common characteristics (Fig.1; Vazquez-Boland et al., 2001).

Upon interaction of the host cell surface receptors, *L. monocytogenes* is engulfed or internalized in a phagocytic vacuole (phagosome) by induced phatocytosis. A number of different host cell receptors are recognized by *L. monocytogenes*, including the transmembrane glycoprotein E-cadherin (Mengaud et al., 1996a), the C1q complement fraction receptor (Braun et al., 2000), the Met receptor for hepatocyte growth factor (HGF) (Shen et al., 2000), and components of the extracellular matrix such as heparan sulfate proteoglycans (HSPG) (Alvarez-Dominguez et al., 1997a) and fibronectin (Gilot et al., 1999). *L. monocytogenes* surface proteins such as internalins (InlA and InlB), the actin-polymerizing protein ActA, and p60 were reported to be bacterial adhesion ligands (Vazquez-Boland et al., 2001). Several putative adhesins of *L. monocytogenes* including the surface protein Ami (autolysin) (Glaser et al., 2001), the 104-kDa surface protein Lap (Santiago et al., 1999) and 24.6-kDa fibronectin-binding protein (Glaser et al., 2001; Vazquez-Boland et al., 2001) have been identified by transposon mutagenesis or by screening the bacterial genomic expression libraries.

L. monocytogenes resides within the phagosome transiently and then begins to escape from the vacuole to the cytoplasm by disrupting the phagosome membrane mediated by the hemolysin LLO. This membrane disruption step is essential for the intracellular survival and proliferation of the bacterium (Karunasagar et al., 1997). With the mediation of the bacterial surface protein ActA (Goebel et al., 1993; Kocks et al., 1992), bacteria released into the cytoplasm immediately recruit the host cell actin and promote the polymerization of actin molecules, which propels the motility of the bacterial cell intracellualarly. Although this movement is random, bacteria eventually reach and push the cytoplasmic membrane, leading to the formation of protruding pseudopods with a bacterium at the tip (Fig. 1). These pseudopods penetrate the neighboring cells and are in turn internalized by phagocytosis, resulting a double-membrane phagosome with inner membrane originating from the previously infected cell. The bacteria escape from the newly formed vacuole into the cytoplasm by lysing its double membrane mediated by LLO and phospholipases, and the infectious cycle repeats (Vazquez-Boland et al., 2001; Tran and Kathariou, 2002).

1.4 Virulence factors.

Listeriolysin O. The production of a soluble hemolysin by *L. monocytogenes* was demonstrated by Harvey and Faber in early 1940s (Harvey and Faber, 1941). Purification and biochemical characterization of the *Listeria* hemolysin (Jenkins et al., 1964; Wiggins et al., 1974) indicated that it is similar in function and antigenicity to the streptolysin O (SLO) from *Streptococcus pyogenes*. The *Listeria* hemolysin, a 58-kDa secreted protein, is the first important virulence factor identified in *L. monocytogenes* (Vazquez-Boland et al., 2001). The *Listeria* hemolysin has an optimum pH of 5.5 for its activity and is active in a narrow acidic pH range (4.5 to 6.5) (Geoffroy et al., 1987); it is a SLO-related cytolysin belonging to the family of cholesterol-dependent, thiol-activated pore-forming toxins (CDTX) (Geoffroy et al., 1987). These toxins are active only against a cholesterol-containing membrane, reflecting this membrane compound as a key toxin target (Geoffroy et al., 1991). The *L. monocytogenes* hemolysin is referred to as listerolysin O (LLO). LLO-related hymolysin was also produced in the pathogenic species *L. ivanovii* (Vazquez-Boland et al., 1989) and nonpathogenic species *L. seeligeri* (Geoffroy et al., 1989; Leimeister-Wachter et al., 1989).

The gene for LLO, *hly*, was identified and characterized in *L. monocytogenes* by Cossart and others (Mengaud et al., 1987; Mengaud et al., 1988). The isogenic *hly* mutants of *L.*

monocytogenes, generated by transposon mutagenesis (Mengaud et al., 1989; Kathariou et al., 1987; Portnoy et al., 1988), were less virulent in mice, and reintroduction of the *hly* gene into the non hemolytic mutants restored virulence to wild-type levels. Cell culture studies with *hly* mutants showed that hemolysin is required for the survival and proliferation of *L. monocytogenes* within macrophages and nonprofessional phagocytes (Gaillard et al., 1987; Kuhn et al., 1988; Portnoy et al., 1988). These studies established the key role played by LLO in the intracellular growth of *L. monocytogenes*, as a mediator of disruption of the primary phagosomes formed after the uptake of *L. monocytogenes*. LLO is also required for the efficient escape of *L. monocytogenes* from the double-membrane vacuole formed during cell-to-cell spread (Gedde et al., 2000).

As typically found in CDTXs, LLO has a conserved motif ECTGLAWEWWR in the C-terminal region. Single amino acid substitutions in this motif render *L. monocytogenes* mutants with various degrees of attenuation, proportional to the loss of cytolytic activity of the respective LLO variants (Michel et al., 1990). However, membrane binding was not affected in LLO mutants with impaired cytolytic activity due to amino acid substitutions in this motif (Michel et al., 1990). The precise role of the undecapeptide motif is still not understood. Given the high degree of sequence similarity between members of this toxin family, most of the functional features derived from crystallographic studies, functional modeling, and structural analyses with other CDTXs are extrapolable to LLO. CDTXs are composed of four domains of which the first three would be involved in toxin oligomerization and membrane destruction and the fourth primarily in binding to membrane. The mechanism by which CDTXs disrupt membranes involves the transition from monomeric, water-soluble toxin to noncovalently bound oligomeric, insoluble arc- and ring-shaped toxin structures that insert into the membrane, forming large pores about 20 to 30 nm in diameter (Vazquez-Boland et al., 2001). Jacobs *et al.* (Jacobs et al., 1998) demonstrated that binding of cholesterol-complex LLO to target membranes is unaffected but LLO did not oligomerize to form pores. These findings suggest that cholesterol is involved not in the initial membrane binding but at a subsequent stage involved in the interaction with membranes that leads to polymerization and membrane intercalation (Vazquez-Boland et al., 2001).

Internalins. Internalins are a family of proteins associated with the virulence of *L. monocytogenes* and are involved in bacterial internalization by cells not normally phagocytic such as epithelial cells. InlA and InlB, encoded by the *inlAB*, were the first family members that were identified from transposon-induced *L. monocytogenes* mutants for impaired invasiveness in Caco-2 Cell monolayers (Gaillard et al., 1991; Dramsi et al., 1995). A number of studies have shown that *inlAB* mutants are severely impaired in host cell invasion, and both InlA and InlB are required for entry of *L. monocytogenes* into normally susceptible nonphagocytic cells *in vitro* (Krull et al., 1997; Dramsi et al., 1995; Gaillard and Finlay, 1996; Gaillard et al., 1996; Gregory et al., 1996; Greiffenberg et al., 1998; Lingnau et al., 1995). The studies of Dramsi *et al.* (Dramsi et al., 1995) and others (Engelbrecht et al., 1998: Greiffenberg et al., 1997; Parida et al., 1998) indicated that InlA and InlB have different cell specificities. InlA but not InlB mediates the entry into human Caco-2 intestinal cells. InlB, but not InlA, was necessary for invasion of murine TIB73 hepatocytes. In the human hepatocyte cell line HepG-2, both InlA and InlB were required for internalization. The entry of *L. monocytogenes* into HUVECs and brain microvascular endothelial cells was dependent on InlB but not on InlA (Greiffenberg et al., 1998; Greiffenberg et al., 1997; Parida et al., 1998).

InlA is a surface protein of 800 amino acids with a calculated molecular weight of 86.6 kDa. The protein can be divided into two functional regions. The N-terminal half of InlA contains, in addition to a signal peptide (a feature common to all internalins), 15 leucine-rich repeat (LRR) units (A repeats), whereas the C-terminal half contains three longer repeats (B repeats) and a cell wall anchor comprising the sorting motif (LPXTG) followed by a hydrophobic membrane-spanning region of approximately 20 amino acids and a few positively charged amino acids (Vazquez-Boland et al., 2001). The N-terminal region encompassing the LRR domain and the interrepeat region is necessary and sufficient for bacterial entry into permissive cells (Lecuit et al., 1997; Mengaud et al., 1996b). Bacterial entry is meditated via the interaction of InlA with the host receptor E-cadherin, a calcium-dependent intercellular adhesion glycoprotein composed of five extracellualr domains and a cytoplasmic tail (Lecuit et al., 1999). The E-cadherin receptor for InlA was identified in the Caco-2 cell extract by InlA-coupled affinity chromatography and evidenced by transfectin experiments using fibroblasts nonpermissive for invasion by *L. monocytogenes*; expression of the gene of the chicken homolog of E-cadherin, L-CAM, made these fibroblasts fully susceptible to invasion by recombinant *L. innocua* expressing the *inlA* but not to invasion by wild-type *L. innocua* or by an *inlA* deletion mutant of *L. monocytogenes* (Mengaud et al., 1996b). InlA presumably interacts, via its LRR region, with the first extracellular domain of E-cadherin (Lecuit et al., 1999). This interaction leads to bacterial adherence, but entry is mediated by the intracytoplasmic domain of E-cadherin , which presumably leads to actin cytoskeleton rearrangement via a- and ß-catenins (Lecuit et al., 2000). A Pro^{16} in the first extracelluar domain of E-cadherin is critical for this interaction with InlA (Lecuit et al., 1999). A mutation from Pro^{16} to Glu^{16} substitution in mouse E-cadherin rendered mouse epithelium cells insusceptible to *L. monocytogenes* invasion (Lecuit et al., 2001). The data accumulated to date indicate that the role of InlA in invasion is restricted to cells expressing E-cadherin (Vazquez-Boland et al., 2001), which must be functional by maintaining a Pro residue at position 16 (Lecuit et al., 2001).

InlB is a 67 kDa protein of 630 amino acids with seven LRR units in the N-terminal part (Dramsi et al., 1995). In contrast to InlA, InlB does not contain the LPXTG motif and a hydrophobic tail in the C-terminal part. Instead, InlB has a cell surface anchoring domain of 232 amino acids consisting of tandemly arranged repeats about 80 amino acids long, each starting with the sequence GW. This domain is responsible for the attachment of InlB to the bacterial surface (Braun et al., 1997) via interaction with lipoteichoic acid (Jonquieres et al., 1999). The data accumulated to date indicate that InlB mediates entry into a wider range of cell types including certain epithelial and fibroblast cell lines such as Vero, HEp-2, HeLa, CHO, L2 and S180 cells, as well as hepatocytes and endothelial cells (Dramsi et al., 1997; Greiffenberg et al., 1998; Mengaud et al., 1996a; Eugster et al., 1996; Parida et al., 1998). Met, a receptor tyrosine kinase which physiologically serves as ligand for hepatocyte growth factor (HGF), has been identified as a signaling receptor for InlB (Shen et al., 2000). gC1q-R, the cellular ligand of the globular part of the C1q complement fraction was identified as the second receptor for InlB (Braun et al., 2000) by using affinity chromatography. gC1q-R may function as a co-receptor rather than a signaling receptor because it lacks a transmembrane domain (Vazquez-Boland et al., 2001). Like InlA, the LRR region of InlB is sufficient for entry into mammalian cells (Braun et al., 1999; Ireton et al., 1999). The X-ray crystal structure of the InlB LRR domain reveals that it is an elongated structure resembling a bowed tube with an extensive solvent-exposed surface area. Three regions were identified as potential candidates for mediating protein-protein interactions: two were in the concave face of the tube, consisting, respectively, of hydrophobic and negative charged residues; the other was at the tip and contained two calcium ions, which may function as bridging molecules in the interactions with host cell receptors (Marino et al., 1999).

The significant level of residual invasiveness of *inlAB* mutants both *in vitro* and especially *in vivo* may indicate that other members of the internalin family, such as putative surface-associated internalins encoded by *inlC2*, *inlD*, *inlE*, *inlF*, *inlG*, and *inlH* identified in *L. monocytogenes*, play a role similar to that of InlA and InlB (Vazquez-Boland et al., 2001).

ActA. ActA is the bacterial protein involved in actin-based intracellular motility of *L. monocytogenes* after their escape from the vacuole (phagosome) into the cytosol (Domann et al., 1992; Kocks et al., 1992). This protein mediates the recruitment and polymerization of the actin molecules of host cells; these events are responsible for intracellular movement of *L. monocytogenes*. The central role of ActA in listerial intracellular motility and virulence was demonstrated by the unusual phenotype of an isogenic *actA* mutant of *L. monocytogenes*. In infected tissue culture cells (Kocks et al., 1992), the *actA* mutant was capable of escaping into the host cytosol, but was unable to recruit the actin and move intracellularly. The *actA* mutant bacteria were highly attenuated in a mouse infection model ((Domann et al., 1992; Kocks et al., 1992). The essential role of ActA in actin-based motility was also demonstrated by several other lines of other experimental evidence including the expression of the *actA* in epithelial cells by transfection experiments (Pistor et al., 1994) and the heterologous expression of *actA* gene in *L. innocua* (Kocks et al., 1995) and *S. pneumoniae* (Smith et al., 1995b), bacteria that are otherwise incapable of performing actin-based motility.

The ActA protein, encoded by *actA* (Vazquez-Boland et al. 1992), is a secreted 639-amino-acid protein with a 610-residue mature form. The mature protein has three distinct domains: (i) N-terminal cationic residue-rich domain (aa 1 to 234); (ii) central region of prolin-rich repeats (aa 235 to 394); (iii) C-terminal domain (aa 395 to 610), with a highly hydrophobic spanning region (aa 585 to 610) that anchors the protein to the cell surface (Vazquez-Boland et al., 2001). Studies with *actA* deletion constructs expressed in transfected cells (Friederich et al., 1995; Pistor et al., 1994) or in a *Listeria* background and tested in *Xenopus* egg extracts (Lasa et al., 1997; Lasa et al., 1995) have assigned specific functions to these domains. The positively charged residue-rich domain (aa 129 to 153) in the N-terminal region is required for actin assembly and the domain, (aa 117 to 121), is critical for filament elongation. The domain of aa 21 to 97 may play a role in maintenance of the dynamics of actin-based motility. In the central region, a domain of proline-rich repeats (aa 265 to 396) is required for binding to the focal contact proteins vasodilator-stimulated phosphoprotein (VASP) and Mena, which are not essential for the initiation of actin assembly but are important for the efficiency of the process (Lasa et al., 1995; Niebuhr et al., 1997; Smith et al., 1996).

ActA may also be involved in the entry of *L. monocytogenes* into eukaryotic cells, probably by recognition of a HSPG receptor (Alvarez-Dominguez et al., 1997b). Clusters of positively charged amino acids with interdispersed hydrophobic residues in the N-terminal region of ActA are identified as putative heparan sulfate (HS)-binding motifs.

Phospholipases . Phospholipase activity was first described in *L. monocytogenes* in 1962 by Fuzi and Pillis (Fuzi and Pillis, 1962). Two different enzymes with phospholipase C (PLC) activity, PlcA and PlcB, are produced in *L. monocytogenes*. The purified PlcA enzyme is highly specific for phosphatidylinositol (PI), with a pH optimum between 5.5 and 6.5 in Triton X-100-mixed micelles, suggesting that, like LLO, it would be active in acidified phagosome. The gene coding for PI-specific PLC, *plcA*, was identified in *L. monocytogenes* by chromosome walking in the region upstream from *hly* (Camilli et al., 1991; Leimeister-Wachter et al., 1991; Mengaud et al., 1991). PlcA is a 33-kDa protein with 317 amino acids in length, similar to the PI-PLCs from prokaryotic and eukaryotic sources (Glaser et al., 2001; Mengaud et al., 1991). PlcB is a nonspecific listerial PLC characterized as a secreted phosphatidylcholine (PC) cholinephosphohydrolase (PC-PLC; EC3.1.4.3) similar to that responsible for the lecithinase activities of *Bacillus cereus* and *C. perfringens* (Leighton et al., 1975). The purified PlcB is a zinc-dependent enzyme with an apparent molecular weight of 29 kDa, active over a wide pH range (5.5 to 8.0), and with a broad substrate spectrum such as PC, phosphatidylethanolamine (PE), phosphatidylserine (PS), and, to a lesser extent, sphingomyelin (SM). The gene coding for PlcB, *plcB*, was identified by transposon mutagenesis from a lecithinase-deficient, attenuated-virulence mutant (Vazquez-Boland et al., 1992). PclB is secreted into the extracellular medium in an inactive form as a precursor of 264 amino acids, including a N-terminal signal peptide(26 aa residue), which is cleaved by a zinc-metalloprotease Mpl to form an active mature enzyme (Raveneau et al., 1992; Poyart et al., 1993).

The role in virulence for a *L. monocytogenes* phospholipase was demonstrated by a number of studies indicating its involvement in phagosome disruption and in the facilitation of cell-to-cell spread (Vazquez-Boland et al., 2001). A *plcB* mutant resulted from the insertional mutagenesis was found to form smaller plaques in infected J774 macrophage monolayers and accumulated within double-membrane vacuoles (Vazquez-Boland et al., 1992). This indicated that PlcB is required for the efficient lysis of the secondary phagosomes formed during listerial cell-to-cell spread. This is consistent with findings in cells infected by a ActA$^-$ mutant showing that the proteolytic activation of PlcB occurs predominantly in secondary phagosomes (Marquis et al., 1997). PlcB is to be required for intercellular spread from macrophages to different types of mammalian cells, including brain microvascular endothelial cells *in vitro* (Greiffenberg et al., 1998), and for spread in murine brain tissue *in vivo* (Schluter et al., 1998). Insight into the contribution of PlcA to the listerial virulence was obtained by systematic comparison of the behavior of single and double *plcA* and *plcB* deletion mutants in mice and cell culture infection assays (Smith et al., 1995a).

Other virulence factors. The autolytic protein p60, found both in the culture supernatant (Kuhn and Goebel, 1989) and associated with the cell wall (Ruhland et al., 1993), is an invasion-associated protein with a molecular weight of 60 kDa. This protein, encoded by the *iap* gene, consists of 484 amino acids with a central repeat region of Thr-Asn units (Kohler et al., 1990). A defect in the production of p60 in *L. monocytogenes* has resulted in impaired invasiveness, particularly in fibroblasts (Kuhn and Goebel, 1989). Some experimental evidence has been accumulated to support a role of the p60 in intestinal invasion and *in vivo* survival (Hess et al., 1996; Hess et al., 1995). Several other *L. monocytogenes* autolysin including Ami, NamA and Auto (Milohanic et al., 2001; Lenz et al., 2003; Cabanes et al., 2004) have also been demonstrated to be involved in virulence as evidenced in studies with gene mutants. A 64 kDa surface virulence-associated surface protein of 569 amino acids, encoded by *svpA*, was identified recently in *L. monocytogenes* (Borezee et al., 2000; Borezee et al., 2001). This protein can be secreted into culture supernatant and is also surface-exposed. SvpA contains a leader (signal) peptide, a predicted C-terminal transmembrane region and a positively charged tail resembling the ActA protein, suggesting that SvpA might be partially associated with the bacterial surface through its C-terminal membrane anchor. An allelic *L. monocytogenes* mutant disrupting *svpA* exhibited reduced virulence due to a restriction of intracellular growth of mutant bacteria (Borezee et al., 2001). Confocal and electron microscopy studies of bone-marrow-derived macrophages infected with the *svpA* mutant showed that most mutant bacteria remained confined within phagosome, indicating this protein promotes bacterial escape from phagosomes (Borezee et al., 2001). Catalase and superoxide dismutase (SOD) that are produced by bacteria primarily to detoxify the endogenous free oxygen radicals generated during prokaryotic oxidative metabolism appear to have only a minor involvement, if any, in the virulence of *L. monocytogenes*. Loss of catalase activity in an *L. monocytogenes* transposon-induced mutant had no measurable effect on listerial proliferation in mouse organs (Leblond-Francillard et al., 1989). Similarly, loss of SOD activity by deletion of the *sod* gene (Brehm et al., 1992) led to at most a slight decrease in the proliferation capacity of *L. monocytogenes in vitro* in mouse bone marrow-derived macrophages and in the organs of experimentally infected mice. These effects were slightly more profound with a *cat sod* double mutant, indicating that catalase and SOD may together provide some level of resistance to bacterial killing (Vazquez-Boland et al., 2001).

1.5 Humoral immunity

Antibody targets. Antibody responses to *L. monocytogenes* have been known for some years, as demonstrated in earlier studies in rabbits experimentally infected with the organism (Armstrong and Sword, 1966; Belen Lopez et al., 1993) and in other infected hosts such as ewes (Gitter et al., 1986), bovine (Wesley et al., 1990; Bourry and Poutrel, 1996), goats (Miettinen et al., 1990), and mice (Manohar et al., 2001). Only few antigens targeted by antibodies after infection with *L. monocytogenes* have been identified to date. With the purified 58 kDa listeriolysin O (LLO), antibodies to this extracellular protein was first detected in sera from humans infected with *L. monocytogenes* by dot-blots (Berche et al., 1990) and latter by ELISA and Western blots (Traub and Bauer, 1995; Grenningloh et al., 1997). LLO was also shown as the major antigen in inducing the humoral immune response to *L. monocytogenes* in experimentally or naturally infected animals, including goats (Miettinen and Husu, 1991), sheep (Lhopital et al., 1993; Low et al., 1992; Baetz et al., 1996), bovine (Bourry and Poutrel, 1996; Barbuddhe et al., 2000; Boerlin et al., 2003), and lambs (Low and Donachie, 1991). An internalin-related protein IrpA (Lingnau et al., 1996; Domann et al., 1997) was identified as another major protein target of the human humoral response against *L. monocytogenes* (Grenningloh et al., 1997). The IrpA is a PrfA-dependent protein comprising 297 amino acids with strong overall homology to the internalins, InlA and InlB, possessing a calculated molecular weight of 33.104 kDa and an N-terminal 34-amino-acid signal sequence (Domann et al., 1997). Antibodies to internalin B (InlB), ActA and p60 have been demonstrated in human sera of listeriolosis patients (Grenningloh et al., 1997;Gentschev et al., 1992). Recently with the recombinant InlA, antibodies to this protein were demonstrated by ELISA in sera of cattle with bacteriologically proven listeriosis (Boerlin et al., 2003). Several antigens with apparent molecular weights of 93, 106 and 160 kDa, all as yet unidentified and uncharacterized, were recognized by IgG or IgM antibodies in sera of humans and rabbits infected with *L. monocytogenes*.(Delvallez et al., 1979). Other uncharacterized proteins with molecular weights of 96, 60, 40, and 14 kDa were found to react with IgA antibodies from the fragment culture of small intestines of mice orally infected with *act*A$^-$ mutant of *L. monocytogenes* (Manohar et al., 2001).

Antibody-mediated protection. Although antibodies have been demonstrated in listeriosis patients (Armstrong and Sword, 1966; Belen Lopez et al., 1993) and in experimentally infected animals (Gitter et al., 1986; Wesley et al., 1990; Bourry and Poutrel, 1996; Miettinen et al., 1990; Manohar et al., 2001), their contributions to the immune defense against Listeria infection are not known (Bortolussi et al., 1986). Despite this, antibodies are generally considered ineffective in protection against *Listeria* infection. Mackaness and others in the early 1960s showed that serum taken from mice following sublethal infection provided no resistance against *Listeria* when passively transferred to naive mice (Mackaness, 1962; Miki and Mackaness, 1964). The specificity of anti-*Listeria* antibodies in the polyclonal sera was not defined in these investigations. Later the study by Cerny *et al.* (Cerny et al. 1988) also suggested that antibodies played no role in the defense against infection with *L. monocytogenes*. In the model of goats infected with *L. monocytogenes*, Miettinen and Husu (1991) demonstrated that the presence and development of anti-LLO antibodies corresponded with the disappearance of the organism from the gastrointestinal tract. Their findings suggest that an association exists between antibodies to

LLO and acquired resistance to *Listeria* infection. However, the protective role for anti-LLO antibodies can not be determined from the data of Miettinen and Husu (Miettinen and Husu, 1991). Recent studies have revealed a surprising role for natural antibodies (Ochsenbein et al., 1999) and for anti-LLO antibody (Edelson et al., 1999) in the context of *L. monocytogenes* infection.

Ochsenbein *et al* (1999) demonstrated that naive C57bL/6 mice possess low but detectable levels of *Listeria*-specific natural IgM antibodies in sera. In contrast μMT mice, as expected not having this natural anti-*Listeria* IgM antibody, showed approximately 5-10 fold higher bacterial titers in peripheral organs including the liver, kidney and brain but a 79-fold lower bacterial titer in the spleen compared with wild-type mice. This study indicated a role for natural IgM antibodies in the early distribution of *L. monocytogenes* throughout the host. Natural IgM antibodies limit the extent of infection in peripheral organs but increases the localization of *L. monocytogenes* to the spleen. The mechanism whereby IgM alters distribution of the bacterium in organs has not been elucidated (Ochsenbein et al., 1999). Edelson *et al.* (1999) recently showed that the passive administration of a murine anti-LLO mAb, which had been previously shown to be capable of neutralizing LLO-mediated pore formation *in vitro* (Nato et al., 1991), can provide resistance to *L. monocytogenes*. Mice treated with the anti-LLO mAb were protected from a lethal challenge with virulent *L. monocytogenes* and showed a significant reduction in the bacterial titers in the spleen and liver during the first few hours to days post infection. These effects of the mAb were independent of host B or T cells, since treatment with the antibody increased survival in severe combined immunodeficiency (SCID) mice. This study indicates, for the first time, a protective role for a specific anti-*Listeria* antibody in the immune defense against listerial infection.

The study of Edelson *et al.* (1999) suggested the necessity for the re-evaluation of roles of specific antibodies in the defense against the infection with *L. monocytogenes*. Antibodies to other *Listeria* protein components, as yet unidentified, may also influence the resistence to listerial infection. Previous work by Mackaness and others (Mackaness, 1962; Hage-Chahine et al., 1992) showed mice mount very weak anti-*Listeria* antibody responses in total. Recent work of Edelson *et al.* (1999) demonstrated extremely low anti-LLO titers after multiple rounds of infection. This result helps explain the failure of antisera to provide resistance against *L. monocytogenes*.

1.6 Animal models

Mouse. Infection of mice with *L. monocytogenes* has been used extensively as a model for the study of host defense against infections caused by intracellular bacterial pathogens (Kaufmann, 1993). This model has contributed substantially to the concept of T cell-mediated immunity, an important component of host resistance to intracellular pathogens (Mackaness, 1962; Mackaness, 1969; Czuprynski and Brown, 1987; Mielke et al., 1988; North, 1969; North, 1973). This model has also been used to study the virulence and pathogenicity of *L. monocytogenes*. (Rocourt et al., 1986; Kaufmann and Brinkmann, 1984). Oral or intragastric, intraperitoneal, subcutaneous or intravenous routes of inoculation can produce infection (Audurier et al., 1980;Pine et al., 1990; Schlech, 1993). Oral or intragastric rotes often requires the use of high inocula (commonly 10^9 to 10^{10} cells) to cause infection. Repeated oral dosing (5×10^9 over 7 to 10 days) resulted in severe brain lesions (Altimira et al., 1999). The intravenous route required lower doses and is most commonly used (Cossart and Mengaud, 1989). Recent studies have shown that mouse intestinal epithelial cells lack a functional E-cadherin receptor for InlA. The inability of murine E-cadherin to bind InlA was due to a specific residue substitution from Pro^{16} to Glu^{16} substitution (Lecuit et al., 2001). This result explains the requirement of high doses for oral infection. The intravenous route bypasses the intestinal barrier and thus generates infection (liver and spleen) at lower doses. Mice do not appear to be a good model for reproducing listeriosis from inoculation of *L. monocytogenes* by both intragastric and oral routes.

Rabbit and guinea pigs. *L. monocytogenes* was first isolated from infected rabbits by Murray *et al.* (1926). Rabbit listeriosis, resulted from natural infection probably through the oral route, was reported later (Watson and Evans, 1985). The disease has been reproduced in rabbits by both conjunctival and oral routes (Gray et al., 1955). A reproducible model of experimental meningitis due to *L. monocytogenes,* closely paralleled with the human disease, was developed in rabbits through intracisternal incoculation (Scheld et al., 1979). In addition, experimental infection of rabbits was used to assess antibody response to *L. monocytogenes* after oral, intraperitoneal or intragastric inoculation (Armstrong and Sword, 1967; Belen Lopez et al., 1993).

Guinea pigs, which have a functional receptor E-cadherin for InlA required for listerial invasion of human intestinal epithelial cells, has been proposed as a better model (Lecuit et al., 1999; Lecuit et al., 2001) for reproducing listeriolosis. Like human and guinea pigs, rabbits also express the E-cadherin with a conserved Pro^{16} required for binding InlA (Lecuit et al., 2001), suggesting it is a good model of human infection. This is further supported by the earlier studies involved in the reproduction of listeriosis in rabbits (GRAY et al., 1955; Scheld et al., 1979).

Ruminants. Natural listeriosis in domestic animals is most common among ruminants (Blenden et al., 1987; Gray and Killinger, 1966). Experimental infection of ruminants such as goats, sheep and cattle with *L. monocytogenes* has been described in several studies with a focus on characterization of serum antibody responses to the organism (Miettinen et al., 1990; Lhopital et al., 1993; Miettinen and Husu, 1991; Wesley et al., 1990). The increase in specific antibody response was generally observed even in the absence of clinical sign of infection following inoculation by oral route in goats (Miettinen et al., 1990; Miettinen and Husu, 1991) and sheep

(Lhopital et al., 1993) or by intramammary route in cattle (Wesley et al., 1990). Animals such as goats and dairy cattle were found to shed the bacteria consistently in milk. Although more work is needed to establish the role of anti-*L. monocytogenes* antibodies in protective immunity, the findings obtained with experimentally infected goats (Miettinen et al., 1990; Miettinen and Husu, 1991) suggest that an association exists between the humoral immune defense against *Listeria* infections, the clinical course of the infection, and the elimination of the organism from the gastrointestinal tract. Detection of antibodies to *L. monocytogenes* can therefore be used as an indication of previous exposure to the organism (Boerlin et al., 2003).

Monkey. *Macaca fascicularis* (cynomolgus monkey) was used as a nonhuman primate model to established the minimum number of *L. monocytogenes* which must be ingested to cause illness (Farber et al., 1991). This study showed that only animals receiving 10^9 cells of *L. monocytogenes* become noticeably ill, with symptoms of septicemia, irritability, loss of appetite, and occasional diarrhea. It is not clear whether this is also the minimum infectious dose causing human illness. It is to be expected that the number of bacteria causing illness will vary tremendously depending upon a variety of factors, the most important of which are bacterial strain differences and host susceptibility (Farber and Peterkin, 1991).

Chick embryo. The virulence of different *Listeria* isolates was compared using the chick embryo model (Notermans et al., 1991; Avery and Buncic, 1997; Notermans et al., 1998) on the basis of 50 % lethal dose or percentage mortality in infected embryos. An agreement was found with a panel of *L. monocytogenes* strains between the chick embryo model and the mouse model (intravenous route) (Notermans et al., 1991;Notermans et al., 1998).

1.7 Regulated expression of *Listeria* genes

L. monocytogenes experiences the stress or stimulation conferred by numerous external factors such as temperature, nutrients, ions, salt, pH, chemicals (disinfectants and antimicrobial substances) and osmolarity present in the environment including food and its storage conditions (refrigeration, dehydration, freezing and freeze-thawing), food processing facilities, soil, vegetation, and infected warm-blooded hosts. These factors strongly regulate the expression of specific sets of *L. monocytogenes* genes, including the virulence genes and thus influence pathogenicity. Regulated expression of *L. monocytogenes* genes under the influence of external factors are reviewed here.

Regulation by temperature. Temperature is an important factor in the induction of virulence genes in *L. monocytogenes*. Expression of LLO, a major virulence factor of *L. monocytogenes*, which is positively regulated by a transcriptional factor, the *prfA* gene products (Leimeister-Wachter et al., 1990), is thermoregulated. The LLO protein and its hemolytic activity was not detected or reduced in several strains of *L. monocytogenes* when grown at 20, 26 or 0°C (Leimeister-Wachter et al., 1991; Datta and Kothary, 1993). Growth of the bacterium at 37°C resulted in the increased levels of hemolytic activity and enhanced production of the LLO polypeptide in the culture supernatant, which is mediated at the level of transcription. This is consistent with the observation that there are more transcripts corresponding to the *prfA* gene at 37°C than at 20°C (Leimeister-Wachter et al., 1991). Expression of other PrfA-regulated virulence genes, the PI-PLC gene *plcA* and the metalloprotease gene *mpl*, was similarly affected at the transcriptional level by growth temperature.

Using a selective capture of transcribed sequences (SCOTS) procedure, Liu *et al* (2002) have recently identified the transcripts of numerous genes that were expressed at elevated levels in *L. monocytogenes* grown at 10°C compared to 37°C. These included the genes involved in previously described cold-adaptive responses (*flaA* and *flp*), regulatory adaptive responses (*rpoN*, *lhkA*, *yycJ*, *bglG*, *adaB*, and *psr*), general microbial stress responses (*groEL*, *clpP*, *clpB*, *flp*, and *trxB*), amino acid metabolism (*hisJ*, *trpG*, *cysS*, and *aroA*), cell surface alterations (*fbp*, *psr*, and *flaA*), and degradative metabolism (*eutB*, *celD*, and *mleA*), and four additional genes showing no significant similarity to any other previously described genes.

Regulation by acidic pH. Studies of low pH-regulated expression of the *L. monocytogenes* genes are of interest, since the organism is exposed to low pH at several stages during infection, including the acidic environment of the stomach and phagosomal environment, and low pH in acidified foods such as dairy products. In the mouse model, an acid-sensitive *L. monocytogenes* mutant was found to be reduced in virulence, whereas an acid-tolerant mutant had increased virulence (Cotter et al., 1999;Marron et al., 1997; Gahan et al., 1996). Acid-adapted *L. monocytogenes* exhibited enhanced survival in acidified dairy products and other low pH foods (Gahan et al., 1996). All these findings suggest that acid resistance or tolerance of *L. monocytogenes* (Datta and Benjamin, 1997; Davies et al., 1996) is important in pathogenesis and survival in the acidic environment, although no effects on virulence were observed with some *L. monocytogenes* mutants having impaired acid resistance (Wiedmann et al., 1998). The molecular mechanisms underlying the acid resistance or tolerance are not understood. Presumably the expression of certain *L. monocytogenes* genes in response to acid stress will be responsible for the acid tolerance. Using 2D-PAGE and mass spectrometry, Phan-Thanh and Mahouin (Phan-Thanh and Mahouin, 1999) showed acid stress (pH 3.5) and acid adaptation (pH 5.5) resulted in the induction of the expression of 47 and 37 proteins, respectively, including 23 proteins in common. The specific functions of these proteins and their contributions to virulence and survival remain to be determined.

Regulation by sugars. Expression of the PrfA-dependent virulence genes in *L. monocytogenes* is strongly inhibited by fermentable carbohydrates (Brehm et al., 1999; Datta and Kothary, 1993; Milenbachs et al., 1997). Sugar-mediated repression affects not only the divergently transcribed genes, *plcA* and *hly*, which have overlapping promoters, but also *plcB*, belonging to another transcriptional unit with no immediate physical link to *plcA* and *hly* (Vazquez-Boland et al., 2001). The regulation of *L. monocytogenes* virulence genes by sugars appears to be mediated by two separate mechanism, one that is related to catabolite repression (CR) (Milenbachs et al., 1997; Brehm et al., 1999) and the other involving a ß- glucoside-specific sensor encoded by the *bvr* locus (Brehm et al., 1999). The *bvrABC* operon encodes an antiterminator of the BglG family (*bvrA*), a ß- glucoside-specific enzyme II permease component of the phosphoenolpyruvate-sugar phosphotransferase system (*bvrB*), and a putative ADP-ribosylglycohydrolase (*bvrC*). Studies by Brehm *et al.* (1999) showed that transcription of *bvrB* was induced by cellobiose and salicin but not by arbutin. Disruption of the *bvr* operon by replacing part of *bvrAB* with an interposon abolished the repression by cellobiose and salicin but not that by arbutin. These findings indicate that the *bvr* locus encodes a ß- glucoside-specific sensor that mediates virulence gene repression upon detection of cellobiose and salicin. Cellobiose represses expression of the PrfA-dependent virulence genes such as *hly* and *plcA* at the transcriptional level (Milenbachs et al., 1997; Huillet et al., 1999) but has no effect on the levels of PrfA protein (Milenbachs et al., 1997), indicating that this regulation is mediated by a repressor mechanism that either reduces the PrfA activity or blocks PrfA to its target promoters.

Unlike glucose and other fermentable carbohydrates, glucose 1-phosphate (G1P) efficiently stimulates the growth of *L. monocytogenes* without inhibiting the expression of the PrfA-dependant virulence genes (Ripio et al., 1997). This indicates that this sugar follows a catabolic pathway that bypasses the repressing mechanism triggered by other readily metabolized carbon sources. The findings with *L. monocytogenes* mutants generated by Tn917 insertions and *prfA* deletion indicated that G1P utilization by *L. monocytogenes* is under the tight positive control of the central virulence regulator, PrfA, and is coexpressed with PrfA-dependent pathogenicity determinants (e.g., LLO, ActA, and PlcB). PrfA dependence and coexpression with virulence genes suggest that utilization of exogenous G1P may be relevant to *Listeria* pathogenesis. G1P is the precursor metabolite and primary degradation product of glycogen and is therefore available as exogenous carbon sources for bacterial growth within the mammalian cell.

1.8 Research rationales, hypotheses and objectives

It is noted from a thorough review of the relevant literature that antibody responses to listerial infection have been known for some time, which may be traced back to an early study in 1967 with rabbits experimentally infected with *L. monocytogenes* (Armstrong and Sword, 1967). An antibody response occurs during listerial infection even in the absence of clinical signs. In spite of prevalence of anti-*L. monocytogenes* antibodies in sera of infected hosts, their role in defense against infection is not well defined and is generally considered ineffective in protective immunity. This view is supported by the early studies of Mackaness and others (Mackaness, 1962) who showed that serum taken from mice following sublethal infection provided no resistance against *Listeria* when passively transferred to naive mice. The attention was then focused on cell-mediated mechanism of resistance against *Listeria* infection. Antibody-mediated protection was not described in the literature until two recent rapports have demonstrated a surprising role for a specific monoclonal anti-LLO antibody (Edelson et al., 1999; Edelson and Unanue, 2000) and natural antibodies (Ochsenbein et al., 1999). These studies suggest that data obtained with serum transfer studies should not be taken as evidence that a role does not exist for antibody in protection against listeria infection. To fully elucidate the role of antibodies in the context of listerial infection, studies aimed at identification and characterization of antibody targets will be necessary. This has formed one of the rationales for the present study. It is also noted that many of the *L. monocytogenes* proteins such as LLO, InlA, InlB, and p60 targeted by antibodies (Berche et al., 1990; Miettinen and Husu, 1991; Bouwer et al., 1992; Gentschev et al., 1992; Grenningloh et al., 1997; Bhunia, 1997; Boerlin et al., 2003) during infection are also associated with bacterial virulence of the bacterium (Vazquez-Boland et al. 2001). Thus, identification of new antibody targets may allow for the discovery of additional virulence factors which will help further understand the mechanisms by which *L. monocytogenes* uses to evade host defense, cause diseases, and grow intracellularly in the host. As well, these proteins may be instrumental in the development of diagnostic tests, and new antimicrobial agents or vaccines.

Several proteins have been identified in *L. monocytogenes* as antibody targets during infection. These antibody targets includes LLO, InlA, InlB, p60 and IrpA (Berche et al., 1990; Miettinen and Husu, 1991; Bouwer et al., 1992; Gentschev et al., 1992; Grenningloh et al., 1997; Bhunia, 1997; Boerlin et al., 2003). These are a very small number of protein antigens compared to 2853 protein-coding genes identified by analysis of the genome sequence (2944528 bp) of *L. monocytogenes* (Glaser et al., 2001). It is generally recognized that expression of certain bacterial proteins is often influenced by environmental factors such as temperature, nutrients and chemicals. It is most likely that there are more *L. monocytogenes* proteins that are targeted by antibodies during infection but have not identified as yet. Use of traditional immunological techniques to analyze the whole cell proteins of *L. monocytogenes* with antisera from infected hosts has a significant limitation for identification of protein when may not be expressed or the level of their expression remains low in *in vitro* culture. The hypothesis is that *L. monocytogenes* proteins that may not be expressed or may only be expressed at very low levels in *in vitro* conditions could be highly synthesized in infected hosts in response to environmental changes. Some of these induced proteins would trigger the production of antibodies in the hosts, with which the protein targets can be identified from a *L. monocytogenes* protein expression library. To test this hypothesis, two kinds of antisera, one from rabbits infected with live *L. monocytogenes,* the other from rabbits immunized with heat-killed bacterial cells, will be prepared and used for differential screening of a protein expression library derived from *L. monocytogenes* genomic DNA. This approach will lead to the identification of those antibody-reactive bacterial proteins specifically induced during infection. Specific objectives are (i) to produce and evaluate antibody responses to *L. monocytogenes* in rabbit model; (ii) to construct a recombinant protein expression library from *L. monocytogenes* genomic DNA; (iii) to identify the clones expressing the protein products recognized only by antisera from rabbits infected with live *L. monocytogenes* by differential screening of the expression library; (iv) to characterize all the positive clones by DNA sequencing and establish the identity of encoded proteins from the deduced amino acid sequence; and (v) to determine the reactivity of the proteins encoded by the positive clones with antisera from infected hosts of other animal species.

CHAPTER TWO

MATERIALS AND METHODS

2.1 *L. monocytogenes* culture and preparation of inoculum

Listeria monocytogenes (serovar 4b), stored at -70°C in 15% glycerol, was plated on trypticase soy blood agar (TSBA) (Appendix A) and incubated at 37°C overnight. One single colony from the plates was inoculated into 1 ml of Luria–Bertani broth containing MOPS (pH 7.5) (LBMOPS) and cultured for 22 hours; 200 μl of the culture suspension was subcultured in 20 ml of LBMOP broth at 37°C overnight. After determination of OD at 620 nm for the cell culture, the cells were centrifuged at 12,000 × *g* for 1 min and the pellets washed 3 times with physiological saline (0.9%(w/v) NaCl). The cell pellets were resuspended in physiological saline and diluted to obtain $1x10^6$ or $1x10^9$ cells/ml, based on an OD_{620} of 0.61 equivalent to 1×10^9 bacteria/ml (D'Andrea and Ferrera, 1998). *L. monocytogenes* cells were either kept on ice or killed by heat treatment at 75°C for 30 min. The cell number of live *L. monocytogenes* was confirmed (in duplicate) by plating serial dilutions of cells on LBMOPS agar plates. The heat-killed bacteria (100 μl, 1×10^9 cells/ml) were plated on LBMOPS agar at 37°C overnight. No bacteria were grown on the plates. Both cell preparations were used to inoculate animals.

2.2 Exposure of rabbits to *L. monocytogenes*

Two different kinds of antisera, one from rabbits infected with viable *L. monocytogenes*, and the other from rabbits immunized with heat-killed *L. monocytogenes*, were generated for use in the differential screening of a protein expression library derived from *L. monocytogenes* genomic DNA.

Three groups of two New Zealand female rabbits each were used in the experiments.

Preimmune sera were collected from each rabbit at Day 0. The first group of two rabbits were inoculated orally with 1×10^9 viable bacteria in 1 ml saline at day 1. The second group of two rabbits were immunized through the intravenous route with 1×10^6 viable bacteria in 0.25 ml saline. The third group of two rabbits were immunized intravenously with 1×10^9 heat-killed *Listeria monocytogenes* in 0.25 ml saline. At day 30, each group of animals was exposed to the same dose of *L. monocytogenes* via the same route as used at day 1. The animals were bled weekly at days 7, 14, 21, 28, 35, and 44. Analysis of the antisera collected at day 45 by ELISA indicated a high titer of IgG antibody responses in all animals. The rabbits were sacrificed to collect a large volume of blood by cardiac puncture at day 44. The sera were collected and stored at - 20°C until used.

2.3 Exposure of guinea pigs to *L. monocytogenes*

Preimmune sera were collected from four guinea pigs at day 0. The animals which were subsequently infected with 1×10^9 *L. monocytogenes* in 1 ml saline through the oral route at day 1. At day 30, the same dose of the bacteria was orally administered to the guinea pigs. Animals were bled weekly at day 7, 14, 21, 28, 35, and 44. ELISA analysis of the antisera collected at day 44 showed a high titer of IgG antibody responses in all animals. The guinea pigs were sacrificed to collect a large volume of blood by cardiac puncture at day 45. The sera were collected and stored at - 20°C for future use.

2.4 Other sources of antisera

Antisera from two cows experimentally infected with *L. monocytogenes* were a gift from Dr. Irene Wesley, National Animal Disease Center, Ames, Iowa, USA.

2.5 Preparation of *L. monocytogenes* whole cell antigens

A single *L. monocytogenes* colony from a TSBA plate was inoculated into 1-liter of LBMOPS broth and cultured at 37°C for 18 h with constant shaking. The cells were harvested by centrifugation at 5000 rpm for 30 min, washed twice with PBS (pH 7.2), and resuspended in 5 ml of PBS. The cells were lysed using a Vibra-cell sonicator Model GE600 (Johns Scientific Company, USA) with 18 cycles of 30 seconds sonications on ice at full power, followed by a 10 second rest interval. The cell lysate was centrifuged at 15,000 rpm for 30 min and the supernatant (designated whole cell antigens) collected. The whole cell antigens were dialyzed against PBS for 18 h at 4°C and stored at -20°C until used.

2.6 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was performed to analyze the IgG antibody responses in rabbits and guinea pigs exposed to *L. monocytogenes* . Each serum was tested in duplicate. Microtiter plates were sensitized with the whole cell antigen diluted 1:100 in PBS (pH 7.2) by incubation overnight at 4°C. The plates were then washed with PBS containing 0.05% (vol/vol) Tween 20 (PBS-T). The serum samples were serially diluted in PBS containing 1% BSA and added to the wells (100 µl/well), and incubated for 90 min at room temperature. After washing with PBS-T, 100 ul of horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratory Inc.USA) diluted at 1:1000 in PBS were added to the wells and incubated for 60 min at room temperature. After a final washing with PBS-T, 100 µl of a substrate solution containing 2,2' -azinobis (3-ethylbenzthiazolinesulfonic acid) (1mM) and H_2O_2 (0.015 %) in 50 mM sodium citrate buffer (pH 4.5) was added to each well. Following incubation with moderate shaking for 10 min at room tempeature, absorbances were determined at 414 nm on a Labsystems Multiskan Bichromatic plate photometer.

2.7 Extraction of the *Listeria monocytogenes* genomic DNA

The total genomic DNA of *L. monocytogenes* was extracted from 40ml of *L. monocytogenes* culture using a GenomicPrep^{TM} Cells and Tissue DNA Isolation Kit (Amersham Pharmacia Biotech, Quebec, Canada) with some modifications. According to the manufacturer's instructions, the cells (40 ml) were centrifuged in microfuge tubes at 13,000 x *g* for 1 minute, washed 3 times with 1 ml PBS and pelleted at the same speed. The cell pellets were resuspended in 1 ml PBS and mixed with lysozyme (Sigma), added to a final concentration of 2.5 mg/ml. After incubation at 37°C for 1 hour, proteinase K(Sigma) was added to the cell suspension to a final concentration of 2 mg/ml and incubated for another 1 hour. A cell lysis solution (600 μl) was added to 600 μl of the cells suspension, mixed well, and incubated at 80°C for 15 minutes. The cell lysate was cooled to room temperature, mixed with 3 μl of RNase A (100mg/ml) solution and incubated at 37°C for 15 min. The RNase A-treated cell lysate was cooled to room temperature and mixed with 200 μl of protein precipitation solution. The cell lysate was centrifuged at 13000 x *g* for 5 min to remove the precipitated materials. The supernatant containing the genomic DNA was collected in a new 1.5 ml centrifuge tube and mixed with 600 μl of 100% isopropanol by inverting gently 50 times. The DNA was collected by centrifugation at 13000 rpm for 1 min, washed with 600 μl of 70% ethanol, and air dried for 15 min. The DNA pellet was dissolved in 100 μl of DNA hydration solution at room temperature overnight and stored at –20°C.

2.8 Quantification the *Listeris monocytogenes* genomic DNA

The concentration of the genomic DNA extracted from *L. monocytogenes* was determined using a PicoGeen dsDNA Quantitation reagent Kit (Molecular Probe, Inc) with a λDNA standard as per the manufacturer's instructions. The DNA standard samples were prepared by mixing various amount of λDNA (5 - 25 ng) diluted in 1 ml of TE buffer with 1 ml of diluted PicoGreen reagent and incubating for 2 to 5 min at room temperature. Similarly, the genomic DNA samples were diluted in 1 ml of TE buffer and mixed with 1 ml diluted PicoGreen reagent. The fluorescence was measured by using a Luminescence Spectrometer LS50 B (Perkin Elmer) with an excitation wavelength of 480 nm and an emission wavelength of 520 nm. The concentration of *L. monocytogenes* DNA was derived from the standard curve constructed by plotting the fluorescence intensity versus the concentration of the DNA standard.

2.9 Construction of the *L. monocytogenes* expression library

2.9.1 Partial digestion of *L. moncytogenes* genomic DNA

Sau 3A I (New England Biolabs) was used to partially digest the genomic DNA of *L. monocytogenes* to obtain 1.5–2.5 kb DNA fragments. The genomic DNA (24 μg) was incubated with 12 units of *Sau* 3A I (an amount predetermined to give optimal partial digestion) in a 50 μl volume at 37 °C for 30 minutes. The reaction was stopped by adding 1 μl of 0.5 M EDTA and 10 μl of 6 x DNA loading buffer(Appendix A). The digested DNA fragments were separated by gel electrophoresis in 1% low melting agarose. DNA fragments corresponding to the sizes of 1.5 to 2.5 kb were exercised from agarose gels.

2.9.2 Purification of the 1.5–2.5 kb DNA fragments

The 1.5–2.5 kb genomic DNA fragments were purified from agarose gels by using a QIAquick gel extraction kit (QIAGEN Inc.) as per the manufacturer's instruction. DNA fragments contained in approximately 300 mg agarose gel were eluted from the column with 40 μl of H_2O. The concentration of purified DNA product was estimated on an agarose gel by comparison with the intensity of λDNA/*Hin*d III standards.

2.9.3 Partial fill-in of *Sau3A* I digested 1.5–2.5 kb genomic DNA fragment

The *Sau3A* I digested 1.5-2.5 kb fragments were partially filled-in with dGTP and dATP to generate compatible ends for cloning into the vector pScreen 1b$^+$△EcoRV. Briefly, a reaction mixture containing 60µl of the purified *Sau3A* I-digested 1.5-2.5 kb fragments, 7.5 µl of 10 × EcoPol buffer (New England Biolabs), 2.5µl of 1mM dGTP, 2.5µl of 1mM dATP, 0.5µl of BSA (10 mg/ml) and 0.5 µl of Klenow large fragment (5 U/µl) , and 2 µl of H_2O was prepared and incubated at 37°C for 30 min. The reaction mixture was then heated at 75°C for 20 minutes to inactivate the enzyme and stored at -20°C.

2.9.4 Preparation of the expression vector pScreen 1b$^+$△EcoRV

2.9.4.1 Mini-preparation of the recombinant plasmid pScreen-T150

A recombinant plasmid previously constructed by inserting a ~150 bp DNA fragment into the pScreen-T vector (Novagen), designated pScreen-T150 (M. Lin, unpublished data), was propagated in *E. coli* NovaBlue (DE3) in LB broth (Appendix A) containing carbenicillin (50 µg/ml) at 37°C overnight. The plasmid DNA was extracted from 3 ml of overnight culture using a GenElute Plasmid Miniprep kit (Sigma) according to the manufacturer's instructions. The pScreen-T150 plasmid DNA was eluted from the column with 50µl of dH_2O and stored at –20°C.

2.9.4.2 Double digestion of pScreen-T150

The pScreen-T150 plasmid was double digested with *BamH* I and *Nco* I. Briefly, 35 µl of pScreen-T150 (from above), 6 µl of 10 ×*BamH* I buffer , 6 µl of 10×BSA (1mg/ml), 3 µl of

BamHI (20 U/ µl), 3µl of *NcoI*(10 U/ µl) and 7 µl of dH_2O were mixed gently and incubated at 37°C overnight. The *BamHI*-*NcoI* digested pScreen-T150 was separated by gel electrophoresis in 1% low melting agrose and purified from agarose gel by using the QIAquick gel extraction kit as described above.

2.9.4.3 Fill-in and self-ligation of the *BamH* I-*Nco* I digested pScreen-T150 fragment

The *BamH* I-*Nco* I digested pScreen-T150 fragment was made blunt ended by filling-in with dNTP in a reaction mixture containing 40 µl of the DNA fragment, 5 µl of 10 × EcoPol buffer, 2 µl of a dNTP (1mM) mix, 2 µl of Klenow large fragment (5 U/µl) and 1 µl of H_2O. The mixture was incubated at 25°C for 15 min, mixed with 5µl of EDTA (100 mM) and heated at 75°C for 10 minutes to inactivate the enzyme activity. The blunt ended DNA fragment was self ligated with T4 DNA ligase at 16°C overnight in a reaction mixture containing16µl of the DNA fragment, 2 µl of 10 × T4 DNA ligase buffer, and 2µl of T4 DNA ligase(400 U/µl). The recombinant plasmid thus constructed, designated pScreen1b$^+$ △EcoRV was used to transform *E.coli* DH5a using standard procedures (Sambrook and Russel, 2000), and extracted from a selected carbenicillin resistant clone (4 ml of overnight culture) by using the QIAprep Spin Miniprep kit (QIAgen) according to the instructions of the manufacturer. The plasmid pScreen1b$^+$△EcoRV was eluted with 50 µl of 1mM Tris-HCl and was sequenced by an automatic sequencing service provider (Canadian Molecular Research Serviced Inc.) with a T7 promoter primer to verify the accuracy of the ligation point.

2.9.4.4 Linearization of pScreen1b$^+$△EcoRV

pScreen1b$^+$△EcoRV was digested by *Xho* I in a 100 µl reaction mixture containing ~ 4.7 µg of the plasmid (20 µl), 10 µl of 10 × NE Buffer 2 , 10 µl of 10 × BSA (1 mg/ml), 10 µl of *Xho*

I (20 units/µl) and 50 µl of H_2O. The mixture was incubated at 37°C overnight and heated at 75°C for 20 minutes to inactivate the enzyme. The digested pScreen1b^{+}△EcoRV was separated by electrophoresis in a 1% low melting agarose gel and purified with the QIAquick gel extraction kit (see above). The *Xho* I digested pScreen1b^{+}△EcoRV was partially filled-in with dCTP and dTTP to generate compatible ends for cloning the partially filled-in 1.5-2.5 kb genomic DNA fragments. Briefly, a reaction mixture containing 60 µl (4.5 µg) of the purified *Xho* I digested pScreen1b^{+}△EcoRV , 7.5 µl of 10 × EcoPol buffer (New England Biolabs), 2.5 µl of 1mM dCTP, 2.5 µl of 1mM dTTP, 0.5 µl of BSA (10 mg/ml), 0.5 µl of Klenow large fragment (5 U/µl) , and 2 µl of H_2O was prepared and incubated at 37°C for 30 min. The reaction mixture was then heated at 75 °C for 20 min to inactivate the enzyme and stored at -20°C.

2.9.5 Cloning of the 1.5–2.5 kb genomic DNA fragments into pScreen1b^{+}△EcoRV

The partially filled-in 1.5-2.5 kb genomic DNA fragments were ligated with the partially filled-in pScreen1b^{+} △EcoRV vector at a ratio of 1:1 with T4 DNA ligase. This ratio was predetermined to be optimal based on the results of three different insert to vector ratios (1:1, 3:1 and 6:1). The ligation mixture containing 10 µl of 1.5-2.5 kb genomic DNA fragments (130 ng), 2 µl of the above partially filled-in pScreen1b^{+}△EcoRV vector (120 ng), 5 µl of 10 × T_4 DNA ligase buffer, 5µl of 10 × BSA (1 mg/ml), 5 µl of 10 mM ATP, 2 µl of T4 DNA ligase (200 U/ µl) and 22 µl of H_2O was prepared and incubated at 16°C overnight. The ligation mixture, designated the genomic expression library, was stored at -20°C until used.

For the generation of recombinant *E. coli* clones, 1µl of the ligation mixture was used to transform 20 µl of NovaBlue DE3 competent cells (Novagen). Briefly, the competent cells were mixed with 1µl of the ligation mixture and gently stirred with a piptette tip. The cell suspension was placed on ice for 30 min, heated for exactly 40 seconds in a 42°C water bath and placed on ice for 2 min. Eighty microlitres of S.O.C medium (Appendix A) was added to the cells and incubated at 37°C with constant shaking at 225 rpm for 1 hour. The transformed cells were then spread on LB agar plates containing 50µg/ml carbenicillin and incubated overnight at 37°C. Figure 2 summarizes the construction of the *L. monocytogenes* genomic expression library.

2.10 Screening of the *L. monocytogenes* genomic expression library

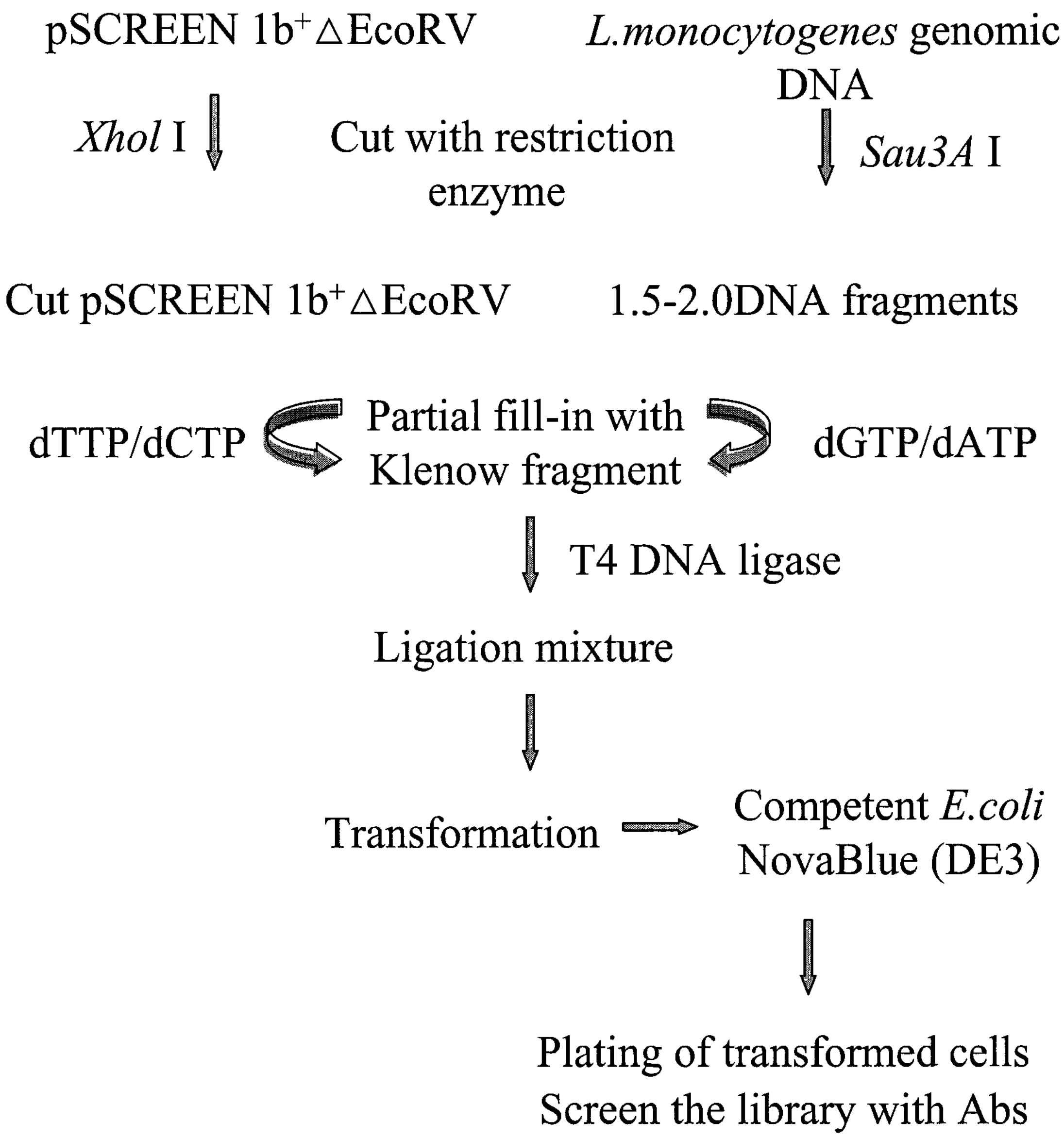
2.10.1 Evaluation of the library by Colony PCR

A number of the library clones (23 colonies) were randomly picked from the LB agar plates containing 50 µg/ml carbenicillin, and each was added to a PCR reaction mixture (50 µl) containing 2 µl dNTP mix (10 mM), 1.5 µl $MgCl_2$ (50mM), 5 µl of 10 × PCR buffer(Invitrogen), 1 µl of Sp6 promoter primer (5'GATTTAGGTGACACTATAG 3'; 25 µM) and 1 µl T7 terminator primer (5'GCTAGTTATTGCTCAGCGG 3'; 25 µM). PCR mixtures were incubated in a thermal cycler for 5 min at 95 °C and 2 min at 94 °C, followed by 40 cycles of 30 seconds at 94°C, 45 seconds at 56°C, and 1.5 min at 72°C, with a final extension at 72°C for 10 min. The PCR products were analyzed by electrophoresis in a 1.5% agarose gel.

2.10.2 Immunoscreening of the protein expression library

The *L. mononcytogenes* expression library was screened with antisera (RaL) from rabbits infected intravenously with viable bacteria and antisera (KaL) from rabbits immunized intravenously with heat-killed bacteria essentially as described in the manual of the ColonyFinder Immunoscreening kit (Invitrogen Inc.). The transformed NovaBlue (DE3) cells were spread on carbenicillin-containing LB plates at the desired density (~1500 colonies per 150 × 15 mm petridish) and incubated at 37°C overnight. The plates were chilled at 4°C for 1.5 hours and

Figure 2. A schematic representation of the construction of a *L. monocytogenes* protein expression library. The library was screened by using a ColonyFinder Immunoscreening kit (Invitrogen) with rabbit anti-*L. monocytogenes* sera.


pSCREEN 1b⁺△EcoRV
L.monocytogenes genomic DNA
XhoI I
Cut with restriction enzyme
Sau3A I
Cut pSCREEN 1b⁺△EcoRV
1.5-2.0DNA fragments
dTTP/dCTP
Partial fill-in with Klenow fragment
dGTP/dATP
T4 DNA ligase
Ligation mixture
Transformation
Competent *E.coli* NovaBlue (DE3)
Plating of transformed cells
Screen the library with Abs

overlaid for 1 min with nitrocellulose filters (132 mm circles). Filters were marked by poking an 18 gauge needle with permanent black India ink in 3 asymmetric places into the filters and plates. After lifting the first filters, a second nitrocellulose filter was overlaid on the same plate for 4 min, marked exactly as the first filter and lifted. The colonies on the filters were lysed by putting the filters into a chloroform vapor chamber for 15 minutes. The filters were then removed from the chamber and placed colony side up on a piece of Whatman 3MM paper saturated with Colony Denaturing Solution for 15 min at room temperature. Filters were washed in petri dishes at room temperature with TBST (20 mM Tris-Cl, 500 mM NaCl, and 0.05% Tween 20) twice for 15 min each with gentle agitation. The filters were probed with RaL diluted at 1: 400 in TBST or probed with KaL diluted at 1: 400 in TBST for 30 min at room temperature. Each serum probe was the mixture of an equal volume of serum from the two rabbits immunized intravenously with live or heat-killed bacteria . The filters were then washed 3 × 10 min with 15–20 ml TBST and incubated with alkaline phosphatase conjugated goat anti-rabbit antibodies, for 30 min. Filters were washed three times with TBST for 10 minutes. The filters were immunostained for 10 minutes with a 10 ml substrates solution containing 4 µl nitro blue tetrazolium (NBT) (83 mg/ml), 4 µl 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (42 mg/ml) and 1 ml of 10 × colour development buffer. The positive clones that reacted only with RaL were selected for further screening. The area containing positive colony was restreaked from the original plate onto fresh plates (90 × 15 mm) and rescreened using the protocol as described above with the exception that 82-mm nitrocellulose filter circles were used. A true positive clone was identified after three rounds of screening.

2.11 Determination of DNA sequences for the positive clones

The RaL reactive clones were cultured in 5 ml LB broth containing 50µg /ml of carbenicillin at 37 °C overnight. The recombinant plasmid DNA was extracted by using the QIAprep Spin Miniprep kit according to the manufacturer's instructions, and sequenced using the automatic sequencing services provided by Canadian Molecular Research Services Inc. or University of Guelph, Department of Molecular Biology and Genetics. Both strands of the inserts were first sequenced with SP6 primer and T7 terminator primer and were completed with additional primers derived from the initially determined sequences. All sequencing primers are listed in Table 3.

2.12 PCR cloning

For those inserts in the immunoreactive clones whose nucleotide sequences contain partial open reading frames (ORFs), PCR was used to amplify the unknown sequence flanking the partial ORFs to obtain the complete ORFs. One of the PCR primer pairs was designed based on the sequence flanking similar ORFs in the published genome sequences of *L. monocytogenes* and *L. innocua* (Glaser et al., 2001). The other primer was synthesized based on the determined sequences of the inserts (Table 4).

PCR was performed by using Expand^{TM} High Fidelity PCR system (Roche) according to the manufacturer's instruction. Briefly, a reaction mixture (100 μl) containing 2 μl dNTP (10 mM), 10μl of 10 × PCR buffer 1, 1 μl of 25μM forward primer, 1 μl of 25μM reverse primer, 1μl of *L. monocytogenes* genomic DNA (0.1 μg/μl), and 1μl of *Taq* and *Pwo* DNA polymerase mixture, and 84 μl of dH_2O was incubated in a thermal cycler at 2 min at 94°C, followed by 35 cycles of amplification at 30 seconds at 94°C, 45 seconds at 55°C, and 2 min at 72°C, with a final extension at 72°C for 10 min. The PCR products were analyzed by electrophoresis in a 1% agarose gel and purified from low melting agarose gel using the QIAquick gel extraction kit as described above.

Table 3. Internal primers for sequencing all positive clones.

Primers	Sequences (5'–3')	Positive Clones
P194 (F)[a]	TTGACTGAACCAGCTAAGC	pSCRN1, 2,
P198 (F)	CGTCAACAAAAGTCGGATAT	pSCRN34, 35
P199 (R)[b]	ATCACCAGTTGTAGGGAGT	pSCRN113, 115
P201 (F)	CGTTGTATGCCCAGTTTAGT	pSCRN88
P202 (R)	ATGGGCGTCACTTGCTCGTG	
P203 (F)	ATGTVACAAGTCTGCACCAA	pSCRN91
P258 (F)	CAACAGGAAATGGCAATG	
P294 (F)	AAATACCACGCTTCCGACA	
P204 (R)	GTCTGTCGCAAAATCCCAT	
P259 (R)	AGTCGCATAATTTCCATAGCCC	
P201 (F)	CGTTGTATGCCCAGTTTAGT	pSCRN93
P205 (R)	AAGATTGGAAAAAGCACATGG	
P270 (R)	ATTTGTTTCGCAGTTGTGGT	
P206 (F)	GGACGGCAAAGAAACAAC	pSCRN95
P207 (R)	CCTACTGCTAACAATCCTATC	
P208 (F)	CATGTGGACGGCAAAGAAAC	pSCRN96
P209 (R)	ACAGGTAGAGCGCATTATCG	
P210 (F)	ATGCTTGGTGCTTGCTCAGG	pSCRN100
P260 (F)	TAGGGCTAATCTGACTATCC	
P211 (R)	CCGCAGAAGACAGTGACGCT	
P261 (R)	GAAACCGAATCAAGTCACTGT	
P212 (F)	GACTTTACAGCCGATACG	pSCRN101
P213 (R)	ATGGGCGTCACTTGCTCGTG	
P212 (F)	GACTTTACAGCCGATACG	pSCRN102
P213 (R)	ATGGGCGTCACTTGCTCGTG	
P214 (F)	AACCAACCCGTGTATTATCA	pSCRN92
P215 (R)	GGTGCTGTTGGTTCTTTAA	
P214 (F)	AACCAACCCGTGTATTATCA	pSCRN105
P215 (R)	GGTGCTGTTGGTTCTTTAA	

P219 (F)	AGAGGGGGCGCAGTATTTCC	pSCRN54
P294 (F)	AAATACCACGCTTCCGACA	
P220 (R)	TGGGTCATTGGCATCTGGGG	
P269 (R)	CCGTTATTTGATTGTTGCTTAG	
P221 (F)	ACGCAGCCACTTMACGCAA	pSCRN65
P222 (R)	GCCAACMMTCCTATCACCAG	pSCRN66
P225 (F)	AAATGGAGAAGAAGTAACGC	pSCRN79
P257 (F)	GGGAACCAATTGCTACGG	
P226 (R)	CTTGAACGCCACCTTGAACA	
P236 (F)	GACTTTACAGCCGATACG	pSCRN106
P273 (F)	ATGTGGTAGGTCAGAAAC	
P237 (R)	TGCAGATGGAACGAGAATTG	
P274 (R)	GCATCATACCATCCGTTAAA	
P264 (F)	TAAGCCCAGTTTCAGGTTTG	pSCRN112
P294 (F)	AAATACCACGCTTCCGACA	pSCRN139
P265 (R)	TCCACCTGTTTTAGCATCAT	pSCRN140
P238 (F)	GGGAATTCATCTAAATGTC	pSCRN114
P249 (F)	GCTATGAATACAGTCC	
P298 (F)	AAGCAACAATCAAATAACGGA	
P240 (F)	GGTACTTGTCCCATTGCC	
P271 (R)	TGGTTGGGTCACTGTTCC	
P280 (F)	ATGATTTAACCCCACTTGC	pSCRN116
P281 (R)	TTGAAAGCGACTGATTGG	
P250 (F)	AATGTGACAGGTAATGCAG	pSCRN119
P299 (F)	TCTCACTCTGTTCTTCATAT	
P242 (F)	ATCTAAATTGCCTGAAGT	
P272 (R)	ACACCCTTACTCTTATATGG	
P243 (F)	AATCCCGTATTAACAACG	pSCRN130
P275 (F)	AAAACGAATGATGGTTCACC	
P244 (R)	TCGTTGGTGCTAGTGGCTC	
P276 (R)	ACCATCATTCGTTTTAGCAT	
P239 (F)	ACGTTTCACAAGCAGAGC	pSCRN143
P277(F)	GCTATGGAAATTATGCGAC	
P240 (R)	GGTACTTGTCCCATTGCC	
P278 (R)	AAGAACCCTGTGCTAATTG	

P251 (F)	CGCAAACAAGTAGAGGAAC	pSCRN153
P252 (R)	TATCTACCCAACCAACTATTT	
P282 (F)	P282 (F) GAGGAAGTACAGGCGGCA	pSCRN159
P283 (R)	P283 (R) CGCTGCTGGTTCTTCTAG	

[a] Forward primers

[b] Reverse primers

Table 4. Primers for PCR cloning of *L. monocytogenes* DNA

Primers	Sequences (5'–3')	Clones
P227 (F)[a]	CATAAAGGGTAGAGGATAACATAAG	pCR227-228
P228 (R)[b]	CTGCATAACTACCACCATCG	
P253 (F)	ATTTGGCTTTCGGGAGTCAC	pCR253-254
P254 (R)	TCACGTTCAATAAAGGTGTAGC	
P293 (F)	ATGAAGAAACGGTGGAATTC	pCR293-263
P263 (R)	CGGGTTGGTTGGTAATTGTTT	
P262 (F)	AACCAGCAGCGCCAACGAAAGC	pCR262-291
P291 (R)	TTCAATTTCTTAGGACTGTGT	
P268 (F)	ATCGCTTCCATTTCATACCT	pCR268-279
P279 (R)	CGCTGTGTGGATGATTCG	
P257 (F)	CAAAGAGCGCAAGACCCA	pCR257-317
P317 (R)	ATCCATTAAGTGGGGCCATT	

[a] Forward primers

[b] Reverse primers

The PCR product was inserted into pCR2.1 vector using a TA Cloning kit (Invitrogen Inc.). The ligation mixture containing 4 µl of purified PCR product, 2µl of pCR2.1 vector (0.025 µg/µl), 1 µl of 10 × T_4DNA ligase buffer, 1 µl of T4 DNA ligase (200 U/µl), and 2 µl of H_2O was prepared and incubated at 14 °C overnight. The ligation mixture was added to 20 µl of *E.coli* INVaF' cells (Invitrogen Inc.) premixed with 2µl of 0.5 M ß-mercaptoethanol (ß-Me), incubated on ice for 30 min, heated for exactly 30 seconds in a 42°C water bath, and put on ice for 2 min. S.O.C medium (250 µl) was added to the cells and incubated at 37°C in a shaker at 225 rpm for 1 hour. The transformed cells (250 µl) were plated on a LB plate containing 50µg/ml kanamycin and X-gal. The white colonies were selected. Colony PCR (as described above) was performed to verify the presence of insert by using the M13 forward primer (5' GTAAAACGACGGCCAGT 3') and M13 reverse primer (5' CAGGAAACAGCTATGAC 3'). One white colony containing the inserted PCR product was cultured in 5 ml LB broth containing 50µg/ml kanamycin. Recombinant plasmid DNA was extracted by using QIAprep Spin Miniprep kit as described above and was sequenced with the M13 forward and reverse primers. The inserted DNA was completely sequenced with additional primers derived from the initially determined sequences.

2.13 SDS-PAGE and Western blots

2.13.1 Preparation of the expressed protein samples

Each positive *E. coli* clone identified from the library screening was cultured on LB agar plates containing 50 µg/ml carbencillin at 37°C overnight. The cells were harvested from the plates by washing with 2 ml LB broth; OD_{590} for the cell suspension was measured. The cell suspension was spun at 14000 × *g* for 1 minute and resuspended in PBS with the volume defined by the formula: $V_{PBS} = (OD_{590} \times V_{\text{cell suspension}})/20$. An equal volume of 2 × SDS-PAGE sample buffer (Appendix A) and 1/5 volume of 10% SDS were added to the samples. The samples were boiled for 10 min and stored at -20°C until used.

2.13.2 SDS-PAGE

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method described by Laemmli (Laemmli, 1970), with 4% stacking gel and 12% resolving gels (Appendix A) and a Bio-Rad minigel apparatus. Protein samples (1 μl) were loaded into the wells and run at 200 V for approximately 45 min. The separated proteins were either stained with Coomassie blue or analyzed by Western blots.

2.13.3 Western blots

After SDS-PAGE, the separated proteins were equilibrated with a transfer buffer (Appendix A) for 1 minute and electrotransferred onto nitrocellulose membranes at 15 V for 30 min by using a Trans-Blot SD semi-dry transfer cell(BioRad) according to manufacturer's instruction. The membrances were blocked with 3% skim milk powder in PBS containing 0.05% Tween 20 (PBS-T) for at least 1 h, then incubated at room temperature for 1 h with rabbit antisera (RaL or RaK) at a dilution of 1:400 in PBS-T containing 3% BSA. Then, the membranes were washed five times for 3 minutes each with 15–20 ml TBST and incubated for 1 h with alkaline phosphatase conjugated goat anti-rabbit IgG at dilution of 1:5000 in PBS-T containing 3% BSA. The membranes were washed 5×3 min with TBST and immunostained with 10 ml substrate solution containing 10 μl NBT (25 mg/ml) and 10 μl BCIP (25 mg/ml) and 1 ml of 10 × AP colour developing buffer (1 M Tris-HCl, 1 M NaCl and 50 mM MgCl) for 10 min.

For Western blots probed with bovine antisera and guinea pig antisera, the procedures were essentially the same as described above. Dilutions at 1:400 for bovine antisera, 1:1200 for guinea pig antisera , and 1:5000 for alkaline phosphatase conjugated goat anti-bovine or anti-guinea pig IgG were used in respective experiments.

2.14 Primer designs and sequence analysis

Oligonucleotide primers were designed using a GeneRunner software package (Hastings Software, Inc., Austin, TX) and synthesized by Sigma Genosys (Oakville, Ontario, Canada). All possible ORFs in the sequenced DNA of *L. monocytogenes* were identified using the software packages available online at ExPAsy Molecular Biology Server (http://ca.expasy.org/). Putative promoters upstream from an ORF were identified using a neural network promoter prediction program (http://www.fruitfly.org/seq_tools/promoter.html). Protein sequence analysis with InterProScan, SignalP and Pfam HMM were carried at the ExPaSy web site (http://ca.expasy.org/tools/). Protein sequence alignment and comparison were performed by using Lasergene (DNASTAR, Inc., Madison, Wis.).

CHAPTER THREE

RESULTS

3.1 Immunogloblin G antibody response of rabbits to infection with *L. monocytogenes*

Sera were collected from two rabbits inoculated intravenously with 1×10^6 viable bacteria and assessed by ELISA for binding to whole cell lysates. Antibodies were first detected at 14 days post infection (dpi) and antibody levels increased linearly up to 28 dpi (Fig. 3). Two rabbits administered intravenously with 1×10^9 heat-killed cells demonstrated a relatively slower antibody response up to 28 dpi. A booster inoculation with viable or killed bacteria on day 28 induced an antibody response in both groups of rabbits to a comparable level. On day 44, rabbits inoculated with the viable bacteria had an antibody titer of 1:3200 to 1:6400, whereas rabbits immunized with heat-killed cells had a titer of 1:1600 to 1:3200. This experiment generated two types of antibodies, one from the animals infected with viable *L. monocytogenes,* the other from animals immunized with heat-killed bacteria. These different antibody preparations will be used in this study to screen a *L. monocytogenes* protein expression library.

3.2 Protein expression library of *Listeria monocytogenes*

The expression library, constructed by ligating *Sau3A*I digested 1.5-2.0 kb *L. monocytogenes* genomic DNA into the *Xhol*I site of pScreen $1b^{+}\triangle$EcoRV vector at a predetermined optimal ratio of insert to vector (1:1), was evaluated by colony PCR using primers for the presence of an insert in the recombinant plasmid after transforming into *E.coli* NovaBlue (DE3). Figure 4 shows the PCR products derived from 23 randomly selected *E. coli* transformants with SP6 and T7 terminator primers. Twenty out of twenty-three clones contained an insert of about 1.5-2.0 Kb. Based on this result, it is estimated that 87 % of the clones in the library have

Figure 3. ELISA analysis of antibody responses of rabbits infected with live *L. monocytogenes* or immunized with heat-killed bacteria. The microtiter plates were sensitized with the whole cell antigen preparation (100μl, 30μg/ml) diluted in PBS, and rabbit sera at a dilution of 1:800 were used. Rabbit 1 (○) and 2 (●) were intravenously injected with viable bacteria (1×10^6); rabbits 3 (▿) and rabbit 4 (▾) were intravenously injected with heat-killed bacteria (1×10^9). Serum samples were collected on various days and analyzed in duplicate.

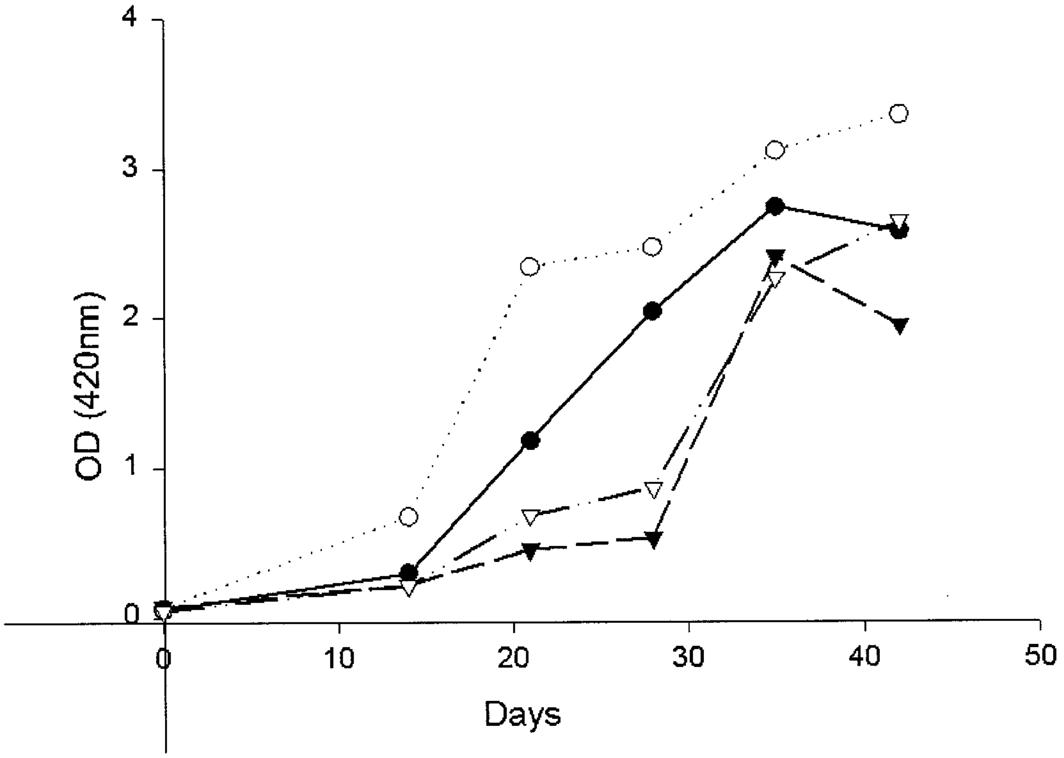

OD (420nm)
0
1
2
3
4
0
10
20
30
40
50
Days

Figure 4. Evaluation of the protein expression library of *L. monocytogenes* by colony PCR. The PCR products derived from 23 randomly selected colonies (Lanes 1-23) were analyzed by 1 % agarose gel electrophoresis. The PCR products representing the *L. monocytogenes* DNA inserts in the vector are indicated by an arrow.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23

L. monocytogenes DNA inserts.

3.3 Identification of positive clones by differentially screening the *L. monocytogenes* protein expression library

Duplicate colony lifts of 3.5×10^4 *L. monocytogenes* library clones blotted onto nitrocellulose membranes were screened using two kinds of antibodies, one from rabbits infected with viable *L. monocytogenes* (RaL), and the other from rabbits immunized by intravenous injection with heat-killed *L. monocytogenes* (RaK). Dilutions of both antisera at 1:400 produced strong immunoreaction signals on Western blots, when the whole cell extract of *L. monocytogenes* was analyzed. This antiserum dilution was used to probe the colony lifts. One hundred and sixty five clones were found to express the proteins that reacted with RaL but not RaK in the initial screening. Each of these positive clones were subjected to a second and third round screening; this process has identified 31 stable clones that react with RaL but not with RaK (Table 5). An example of screening the library clones is presented in Figure 5. Western blot analysis of 31 positive clones harvested from LB agar plates confirmed these clones expressed proteins that reacted only with RaL, but not with RaK.

3.4 Sequence analysis of the immunoreactive clones

All thirty one positive clones that showed reactivity solely with RaL were sequenced. Ten of 31 positive clones, designated pSCRN1, pSCRN2, pSCRN34, pSCRN35, pSCRN65, pSCRN66, pSCRN95, pSCRN96, pSCRN113, and pSCRN115, have a *L. monocytogenes* genomic DNA insert of ~1.5 kb (Fig. 6) with identical nucleotide sequences (Appendix B, Fig 1B, nt 1691 to 3195). The 1.5 kb insert contains an incomplete ORF, which is in frame with the start codon of N-terminal fusion (including a 6-His tag) coding sequence directed by a T7 promoter. This

Table 5. Identification of the clones recognized by antisera from rabbits infected with live *L. monocytogenes* but not by antisera from rabbits receiving the heat-killed bacteria.

Screening (Round)	**Total clones**	**Positive cones**
1^{st}	35000	165
2^{nd}, 3^{rd}		31

Figure 5. Differential screening of the *L. monocytogenes* protein expression library clones with rabbit anti-*L. monocytogenes* sera. The nitrocellouse colony lifts were probed with 1:400 dilution of antiserum from rabbit infected with viable bacteria (RaL) or from the rabbits immunized with heat-killed bacteria (RaK). (**A**) A colony blot was probed with RaL. (**B**) A colony blot, lifted from the same plate as (**A**), was probed with RaK. Two positive clones that reacted with only RaL were picked from (**A**) and subjected to second and third rounds of screening. (**C**) and (**D**), the colony blots of the third round screening of the two positive clones, respectively.

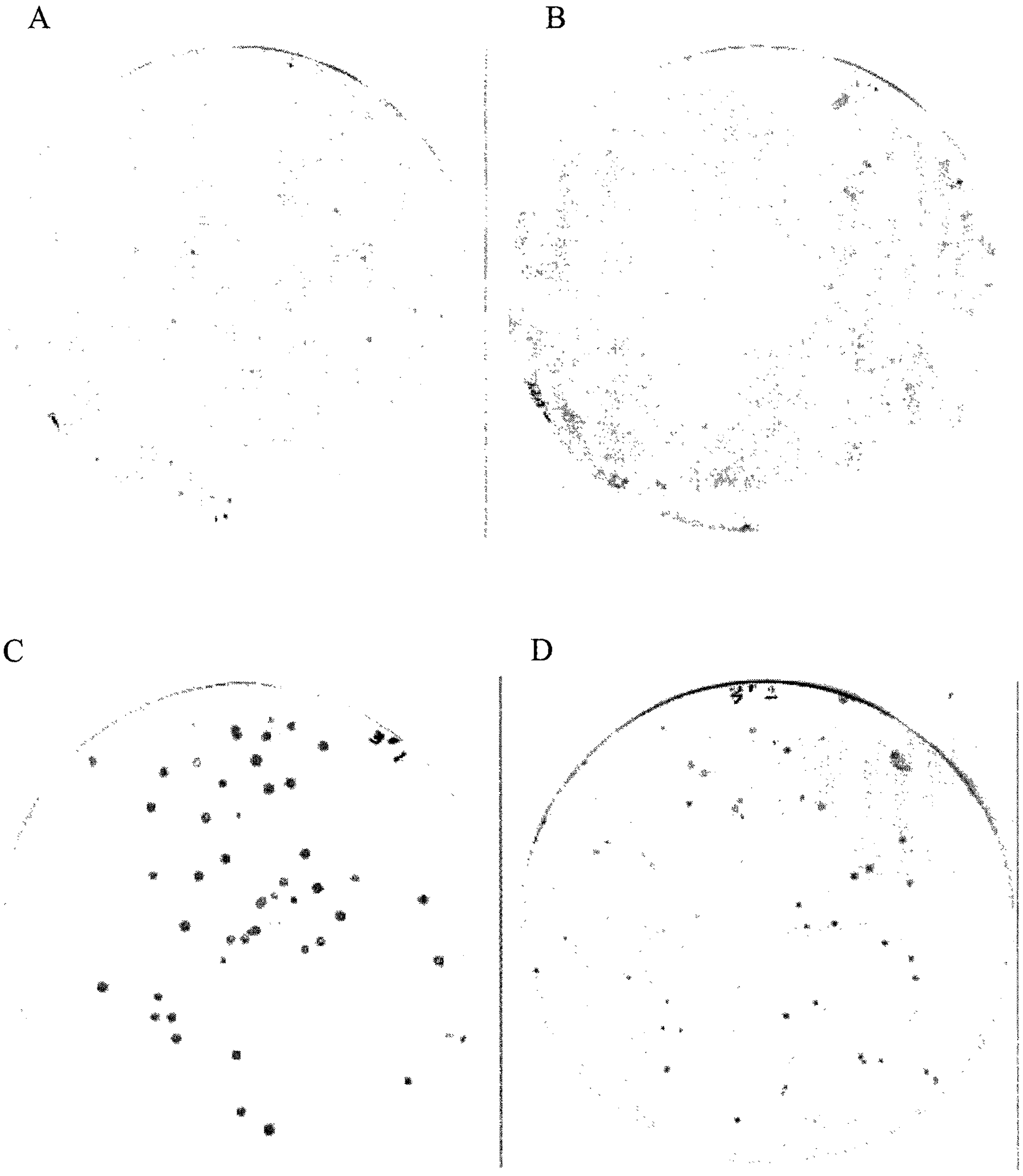
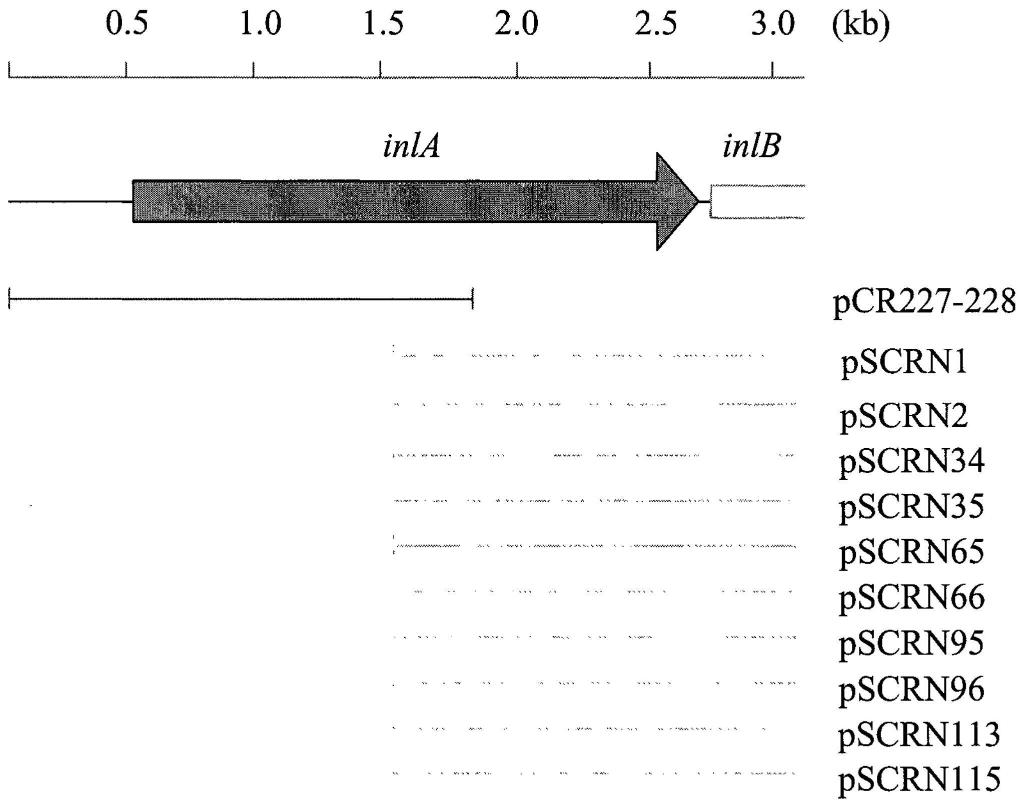

A
B
C
D

Figure 6. Schematic illustration of the protein-coding genes *inlA* and *inlB* in the cloned *L. monocytogenes* (serotype 4b) genomic sequence. The nucleotide sequence is assembled from the inserted DNA of pCR227-228, pSCRN1, pSCRN2, pSCRN34, pSCRN35, pSCRN65, pSCRN66, pSCRN95, pSCRN96, pSCRN113, and pSCRN115.


0.5
1.0
1.5
2.0
2.5
3.0
(kb)
inlA
inlB
pCR227-228
pSCRN1
pSCRN2
pSCRN34
pSCRN35
pSCRN65
pSCRN66
pSCRN95
pSCRN96
pSCRN113
pSCRN115

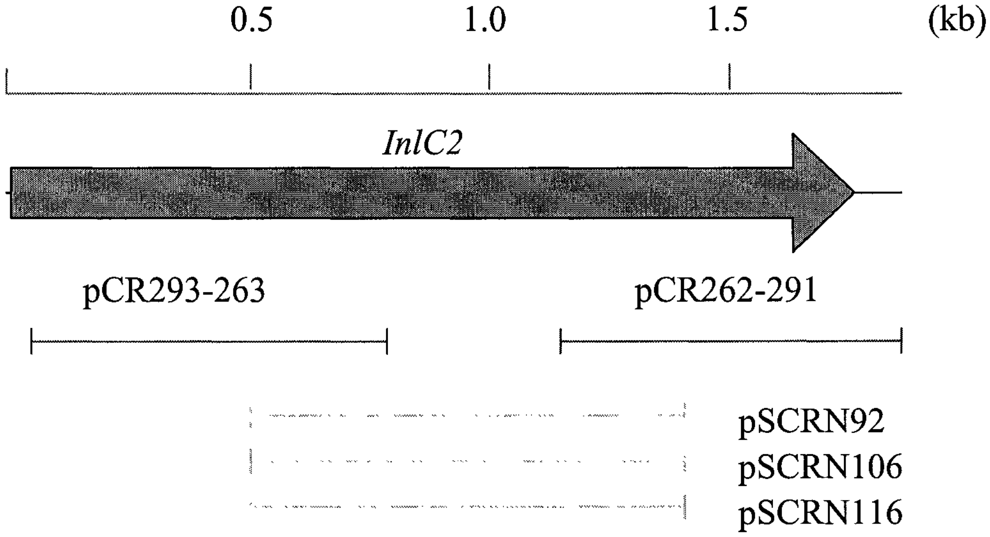
suggests that these clones have expressed a fusion protein recognized by RaL. Both RaL and an anti-His monoclonal antibody (mAb) detected the same protein band on Western blots (data not shown). A similarity search of the deduced amino acid sequence from the partial ORF against the NBCI nr protein database has revealed that it is highly similar to the *L. monocytogenes* InlA protein (Glaser et al., 2001; Cai et al., 2002) (Table 6), indicating the encoded protein is InlA. Based on the genome sequence of *L. monocytogenes* EGD-e (Glaser et al., 2001; Schluter et al., 1998 and the sequence determined here, PCR primers were designed to clone the sequence upstream of the incomplete *inlA* ORF into pCR2.1, and the recombinant plasmid was designated pCR227-228. With additional sequence from this clone, a completed ORF of *inlA* with a 503-bp 5' flanking sequence was obtained. Eighty five bp down stream from the *inlA* there is another incomplete ORF whose gene product shows a high degree of homology to *L. monocytogenes* InlB (Dramsi et al., 1995; Glaser et al., 2001) from the BLAST search.

Three out of the 31 immunoreactive clones, designated pSCRN92, pSCRN106 and pSCRN116, had an insert of ~ 940 bp (Fig. 7) with identical nucleotide sequences (Appendix B, Fig. 2B, nt 532-1472). The insert contains an incomplete ORF, which is in frame with the start codon of N-terminal fusion (including a 6-His tag) coding sequence controlled by T7 promoter. These clones have expressed a fusion protein detected by RaL. The same protein band was also recognized by anti-His mAb on Western blots (data not shown). Similarity search with BLAST revealed that the deduced amino acid sequence from this partial ORF shares a strong homology with the InlC2 protein of *L. monocytogenes* strain EGD (Dramsi et al., 1997) with an E value of e-152. This indicates the encoded protein is InlC2. Two clones, pCR293-263, and pCR262-291, were created by PCR with primers design based on the genomic sequence obtained here and the sequences flanking the *inlC2* gene homolog of *L. monocytogene* strain EGD. A 1832-bp nucleotide sequence was assembled from these clones, which contains a 1647-bp ORF of *inlC2*

Table 6. Similarity search of the deduced amino acid sequences from representative immunoreactive clones against the NCBI nr protein database with BLAST. Three similar proteins found with highest scores are presented.

Positive Clones	Similar Proteins (Accession Number)	Score (bits)	E (Value)
pSCRN34	Internalin A precursor (AAM 95921.1)	602	e-171
	Internalin A precursor (AAM95926.1)	601	e-171
	Internalin A precursor (AAM95919.1)	601	e-171
pSCRN92	Internalin C2 (AAB67969.1)	537	e-152
	Internalin H (NP_463794.1)	527	e-149
	Internalin D (CAC20635.1)	523	e-147
pSCRN114	Internal D (AAB67970.1)	805	0.0
	Internalin H (NP_463794.1)	545	e-153
	Internalin D (CAC20635.1)	539	e-152
pSCRN88	Probable cell surface protein (NP_469717.1)	259	4e-69
	Internalin A precursor (AAM95917.1)	174	2e-43
	Internalin A precursor (AAM95930.1)	174	2e-43
pSCRN79	Hypothetical transmembrane pro (NP_464452.1)	783	0.0
	Hypothetical transmembrane pro (NP_470266.1)	772	0.0
	Sulfatase, Sulfatase(Bacillus) (NP 656814.1)	610	e-173
pSCRN119	Similar to autolysin(amidase) (NP_470401.1)	450	e-125
	Autolysin; amidase(Listeria i) (NP_472032.1)	195	1e-48
	Similar to autolysin (EC 3.5.1) (NP_464601.1)	194	2e-48
pSCRN130	ORF A of *L. seeligeri* (CAA65738.1)	266	5e-70
	Similar to internalin proteins (NP_463863.1)	234	3e-60
	Highly similar to ORFA of *L. s* (NP_470008.1)	222	1e-56
pSCRN100	Putative preptidoglycan bound protein (tr Q8Y8RO)	502	e-141
	Probable cell surface protein (tr Q92DS2)	111	1e-23
	Hypothetical protein Lin1204 (tr Q92 DS2)	110	2e-23

Figure 7. Schematic illustration of the protein-coding gene *inlC2* in the cloned genomic sequence of *L. monocytogenes* (serotype 4b). The nucleotide sequence is assembled from the inserted DNA of pCR293-263, pSCRN92, pSCRN106 and pSCRN116.


0.5
1.0
1.5
(kb)
InlC2
pCR293-263
pCR262-291
pSCRN92
pSCRN106
pSCRN116

and a 184-bp3' flanking sequence.

Eight of 31 immunoreactive expression clones, designated pSCRN54, pSCRN91, pSCRN112, pSCRN139, pSCRN140, and pSCRN143, contain an identical insert of 1790 bp (Fig 8; Appendix B, Fig 3B, nt 500-2288). An incomplete ORF, which is in frame with the start codon of N-terminal fusion (including a 6 His tag) coding sequence under the control of T7 promoter, was identified at the 5' end. The protein band recognized by RaL was also detected by anti-His mAb on Western blot, indicating that a fusion protein was expressed from these clones. The BLAST similarity search of the deduced amino acid sequence from this partial ORF against the NCBI nr protein database showed that the encoded protein shared a high homology with the InlD of *L. monocytogenes* strain EDG (Dramsi et al., 1997). Two of the 31 immunoreactive clones, named pSCRN114 and pSCRN159, contain an identical insert with sequence overlapping with the inserts of pSCRN54, pSCRN91, pSCRN112, pSCRN139, pSCRN140, and pSCRN143. From these overlapping clones, a 2288-bp nucleotide sequence was assembled, which contains a 1707-bp ORF of *inlD*, 388-bp 5'end and 193-bp 3'end flanking sequences. Two hundred and twenty nine bp upstream from the *inlD* gene, there is a partial ORF whose gene product has 98 and 100% identity over 59 residues at the C-terminal to the identified InlD and InlC2, respectively. This indicates that a protein-coding gene similar to the identified *inlD* is present upstream.

One of the 31 immunoreactive expression clones, designated pSCRN93, contains an insert of 1119 bp (Fig 9; Appendix B, Fig 4B, nt 1443-2256). An incomplete ORF, which is in frame with the start codon of N-terminal fusion (including a 6-His tag) coding sequence under the control of T7 promoter, was identified in the 5' end. This suggests that a protein with an N-terminal fusion is expressed from this clone. Western blot analysis showed that the protein band recognized by RaL was also detected by anti-His mAb (data not shown), further demonstrating the expression of a fusion protein. The BLAST similarity search of the deduced amino acid

Figure 8. Schematic illustration of the protein-coding gene *inlD* in the cloned genomic DNA of *L. monocytogenes* (serotype 4b). The nucleotide sequence wass assembled from the inserted DNA of pSCRN54, pSCRN91, pSCRN112, pSCRN139, pSCRN140, pSCRN143, pSCRN114, and pSCRN159.

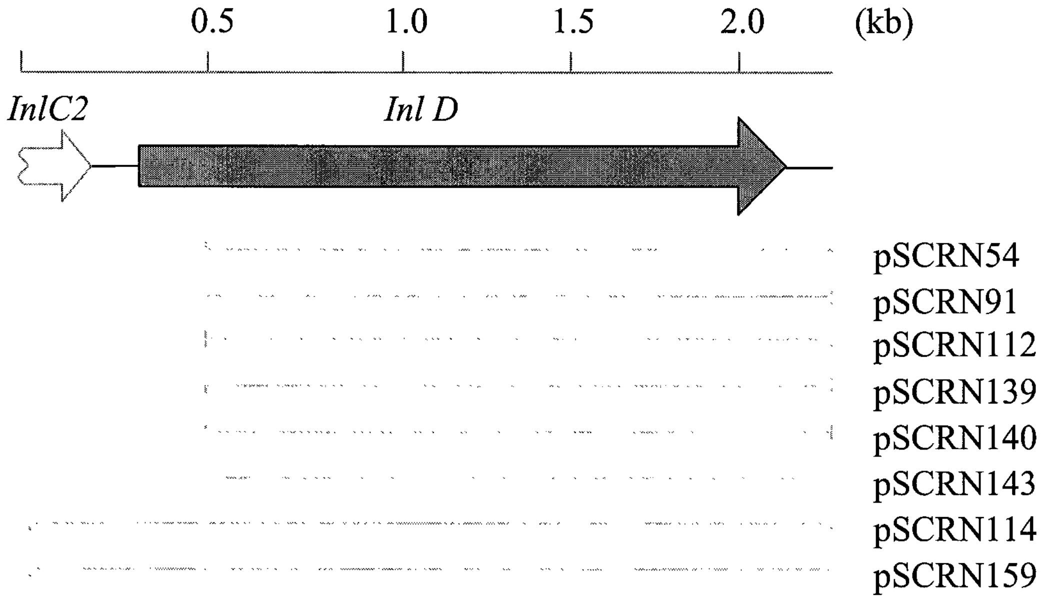
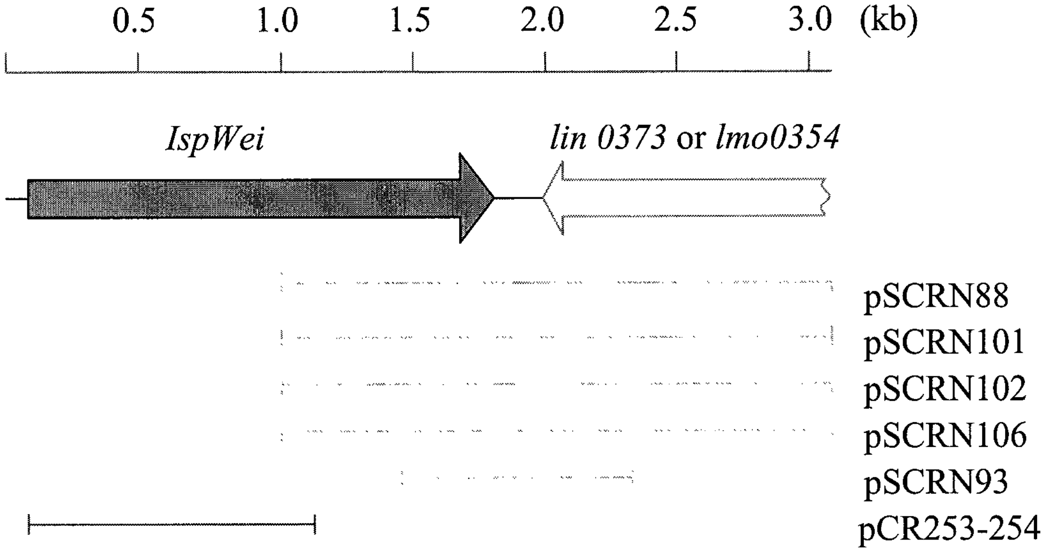

0.5
1.0
1.5
2.0
(kb)
InlC2
Inl D
pSCRN54
pSCRN91
pSCRN112
pSCRN139
pSCRN140
pSCRN143
pSCRN114
pSCRN159

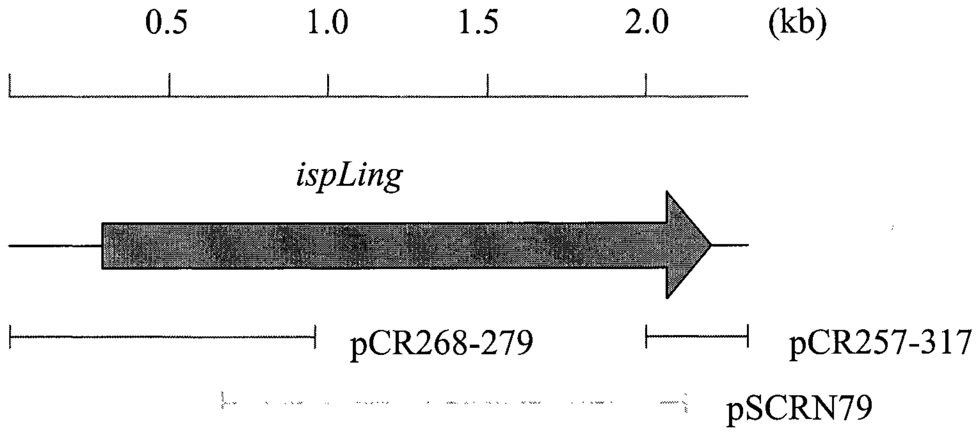
Figure 9. Schematic illustration of the genes coding for the protein IspWei and a putative protein similar to Lin0373 or Lmo0354 in the cloned genomic DNA of *L. monocytogenes* (serotype 4b). The nucleotide sequence is assembled based on the inserted DNA from pCR253-254, pSCRN93, pSCRN88, pSCRN101, pSCRN102 and pSCRN106.


0.5
1.0
1.5
2.0
2.5
3.0
(kb)
IspWei
lin 0373 or lmo0354
pSCRN88
pSCRN101
pSCRN102
pSCRN106
pSCRN93
pCR253-254

sequence from this partial ORF against the NCBI nr protein database reveals that the encoded protein is similar to a probable cell surface protein of *Listeria innocua* strain CLIP11262 (Glaser et al., 2001) (Table 6). Four of the 31 immunoreactive clones, designated pSCRN88, pSCRN101, pSCRN102 and pSCRN106, contain an identical insert of 1936 bp whose sequence is overlapped with the inserted sequence of pSCRN93 (Figure 9; Appendix B, Fig. 4B, nt 1140-3074). The inserts from these four clones contain an incomplete ORF coding for a protein having similarity to a probable cell surface protein of *L. innocua* (Table 6). The identified protein was named IspWei. One hundred and thirty three bp downstream there is another 1020 bp incomplete ORF (in the reverse orientation) whose translated product is similar to *L. innocua* Lin0373 or *L. monocytogenes* Lmo0354 (Glaser et al., 2001). To obtain the complete ORF of *IspWei*, a clone, designated pCR253-254, was created by PCR with primers designed on the basis of the nucleotide sequence determined here and the sequence 5' flanking the ORF for a probable cell surface protein (Lin0372) in *L. innocua* strain CLIP11262 (Glaser et al., 2001). A 3074-bp nucleotide sequence was assembled from all these clones, which contains a 1770-bp ORF coding for IspWei and 156 bp 5' and 128 bp 3' flanking sequences.

One of the 31 immunoreactive expression clones, designated pSCRN79, contained an insert of 1361 bp (Fig. 10; Appendix B, Fig. 5B, nt 819-2179). An incomplete ORF, which is in frame with the start codon of N-terminal fusion (including a 6 His tag) coding sequence under control of a T7 promoter, was identified. This suggests that a fusion protein was expressed from pSCRN79. Expression of a fusion protein was demonstrated by Western blot showing that the same protein band was recognized by both RaL and anti-His mAb. The BLAST similarity search of the deduced amino acid sequence from this partial ORF against the NCBI nr protein database revealed that the encoded protein is similar to a hypothetical transmembrane protein Lmo0927 of *L. monocytogenes* strain EDG-e and a hypothetical transmembrane protein Lin0927 of *L.*

Figure 10. Schematic illustration of the protein-coding gene *ispLing* in the cloned genomic DNA of *L. monocytogenes*. The nucleotide sequence is assembled from the inserted DNA of pCR268-279, pSCRN79, and pCR257-317.


0.5
1.0
1.5
2.0
(kb)
ispLing
pCR268-279
pCR257-317
pSCRN79

innocua strain Clip11262 (Glaser et al., 2001) (Table 6). The newly identified protein was named IspLing. To obtain the complete ORF of *ispLing*, two clones, pCR268-279, and pCR257-317, were generated by PCR cloning with primers designed on the basis of the genomic sequence obtained here and the sequence flanking the *lmo0927* gene of *L. monocytogenes* strain EDG-e. A 2371-bp nucleotide sequence is assembled based on the inserted DNA from these clones, which are composed of a 1958-bp ORF coding for IspLing and 270 bp 5' and 143 bp 3' flanking sequences.

Two of the 31 immunoreactive expression clones, designated pSCRN119 and pSCRN153, have an identical insert of 1325 bp (Fig 11; Appendix B, Fig 6B, nt 1-1325). The insert contained an incomplete ORF (978 bp) at the 5' end, which is in frame with the start codon of N-terminal fusion (including a 6 His tag) coding sequence under the control of a T7 promoter. This suggests that a fusion protein is expressed from these two clones. This further was confirmed by Western blot showing that both RaL and anti-His mAb detected the same protein band (data not shown). The BLAST similarity search of the deduced amino acid sequence from this partial ORF against the NCBI nr protein database reveals that the encoded protein is similar to putative amidase-like proteins (Lin1064 and Lmo1076) and the amidases Ami (autolysin) from *L. monocytogenes* and *L. innocua* (Glaser et al., 2001).The newly identified immunoreactive protein was named IspYu. Sixty five (65) bp downstream from the *ispYu* ORF, there is an incomplete ORF (nt 1043-1325) in the reverse orientation coding for a protein similar to *L. innocua* protein Lin1065 of unknown function.

One of the 31 immunoreactive expression clones, designated pSCRN130, contained an insert of 1812 bp (Fig 12; Appendix B, Fig 7B, nt 1-1812*)*. An incomplete ORF, which is in frame with the start codon of N-terminal fusion (including a 6-His tag) coding sequence under the control of a T7 promoter, was identified in the 5' region. This suggests that a fusion protein is

Figure 11. Schematic illustration of the genes coding for ispYu and a protein similar to Lin1065 in the cloned genomic DNA of *L. monocytogenes* (serotype 4b). The nucleotide sequence is assembled from the inserted DNA of pSCRN119 and pSCRN153.

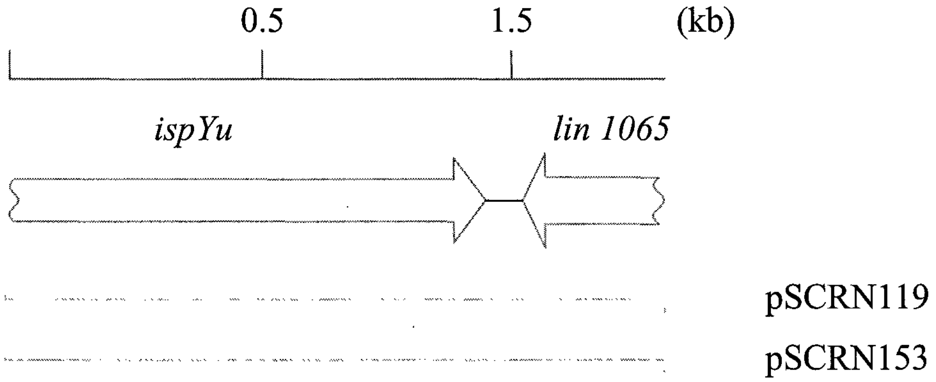
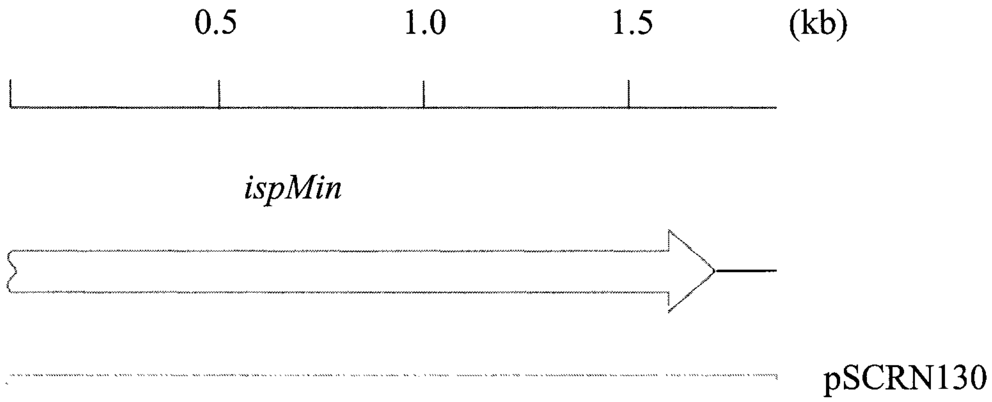

0.5
1.5
(kb)
ispYu
lin 1065
pSCRN119
pSCRN153

Figure 12. Schematic illustration of the protein-coding gene *ispMin* in the cloned genomic DNA of *L. monocytogenes* (serotype 4b). The nucleotide sequence is derived from the inserted DNA of pSCRN130.


0.5
1.0
1.5
(kb)
ispMin
pSCRN130

expressed from pSCRN130. The fusion protein was confirmed by Western blot analysis showing that RaL and anti-His mAb recognized the same protein band (data not shown). The BLAST similarity search of the deduced amino acid sequence from this partial ORF against the NCBI nr protein database reveals that the encoded protein is similar to a putative protein ORFA of *L. seeligeri* (Genbank Accession No. CAA65738) and a putative peptidoglycan bound protein Lmo0333 of *L. monocytogenes* DEG-e (Glaser et al., 2001). This newly identified immunoreactive protein was named IspMin.

One of the 31 immunoreactive expression clones, designated pSCRN100, contained an insert of 1478 bp (Fig 13; Appendix B, Fig 8B, nt 1-1478). No partial ORF is found in frame with the start codon of N-terminal fusion (including a 6-His tag) coding sequence under the control of a T7 promoter. This is consistent with Western blot analysis showing that anti-His mAb failed to detect a protein product (data not shown). No ORF potentially coding for a protein was found in the 5' to 3' direction. However, two partial ORFs were identified in the 3' to 5' orientation. One partial ORF spanning from nt 1 to 925 encodes a protein similar to a putative protein Lmo0835 of *L. monocytogenes* (Glaser et al., 2001). Another partial ORF spanning from nt 876 to 1478 encodes a putative protein similar to the *L. monocytogenes* protein Lmo0834 (Glaser et al., 2001). The two ORFs are overlapped by 50 bp. It is unlikely that the protein similar to Lmo0834 is expressed from pSCRN100 because no promoter is in place to direct this expression. Most likely the protein similar to Lmo0835 is expressed and detected by RaL. Consistent with this result was the observation that putative promoters were identified in the 552- bp sequence upstream of the ATG codon of the ORF coding for a putative protein similar to Lmo0835, using a neural network promoter prediction program (http://www.fruitfly.org/cgi-bin/seq_tools/promoter.html). This newly identified protein was named IspLin.

All proteins, expressed from the 31 clones and detected by RaL but not by RaK, are listed

Figure 13. Schematic illustration of the protein-coding genes *ispLin* and *lmo0834* in the cloned genomic DNA of *L. monocytogenes* (serotype 4b). The nucleotide sequence is derived from the inserted DNA of pSCRN100.

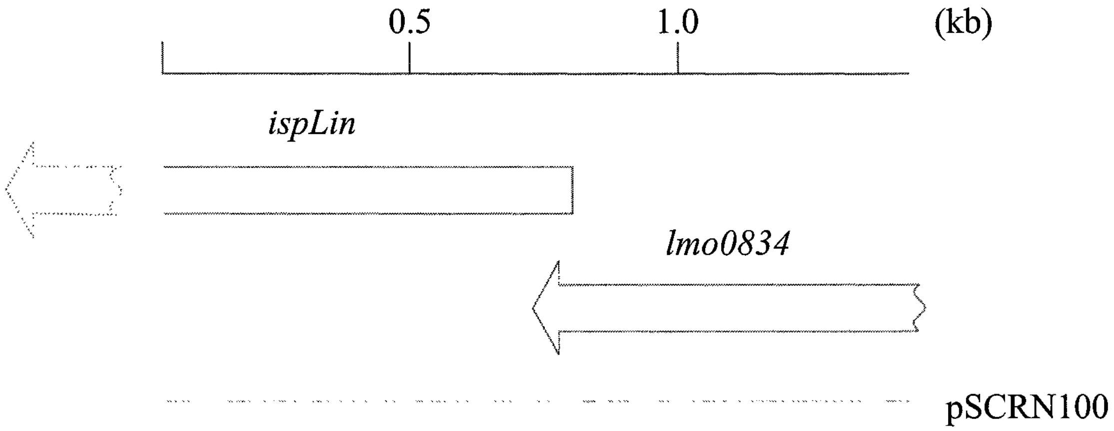

0.5
1.0
(kb)
ispLin
lmo0834
pSCRN100

Table 7. Summery of the protein product encoded by positive clones reacting with antiserum RaL from rabbits infected with live *L. monocytogenes* but not by antiserum RaK from rabbits receiving the heat-killed bacteria.

Immunoreactive Clones	Encoded Product	# of Clones identified	Gene Name	Function in *L. monocytogenes*
pSCRN1, 2, 34, 35, 65, 66, 95, 96, 113, 115	internalin A	10	*inlA*	mediation of bacterial internalization
pSCRN92, 105, 116	internalin C2	3	*inlC2*	unknown
pSCRN54, 91, 112, 139, 140, 143, 114, 159	internalin D	8	*inlD*	unknown
pSCRN93, 88, 101, 102, 106	IspWei	5	*ispWei*	unknown, similar to a probable cell surface protein
pSCRN79	IspLing	1	*ispLing*	Unknown, similar to hypothetical transmembrane protein
pSCRN119, 153	IspYu	2	*ispYu*	unknown, similar to putative amidase-like proteins of *L. monocytogenes* and *L. innocua*
pSCRN130	IspMin	1	*ispMin*	unknown, similar to the putative protein ORFA of *L. seeligeri*
pSCRN100	IspLin	1	*ispLin*	unknown, similar to other LPXTG proteins

in Table 7.

3.5 Immunological characterization of the recombinant proteins expressed from positive clones

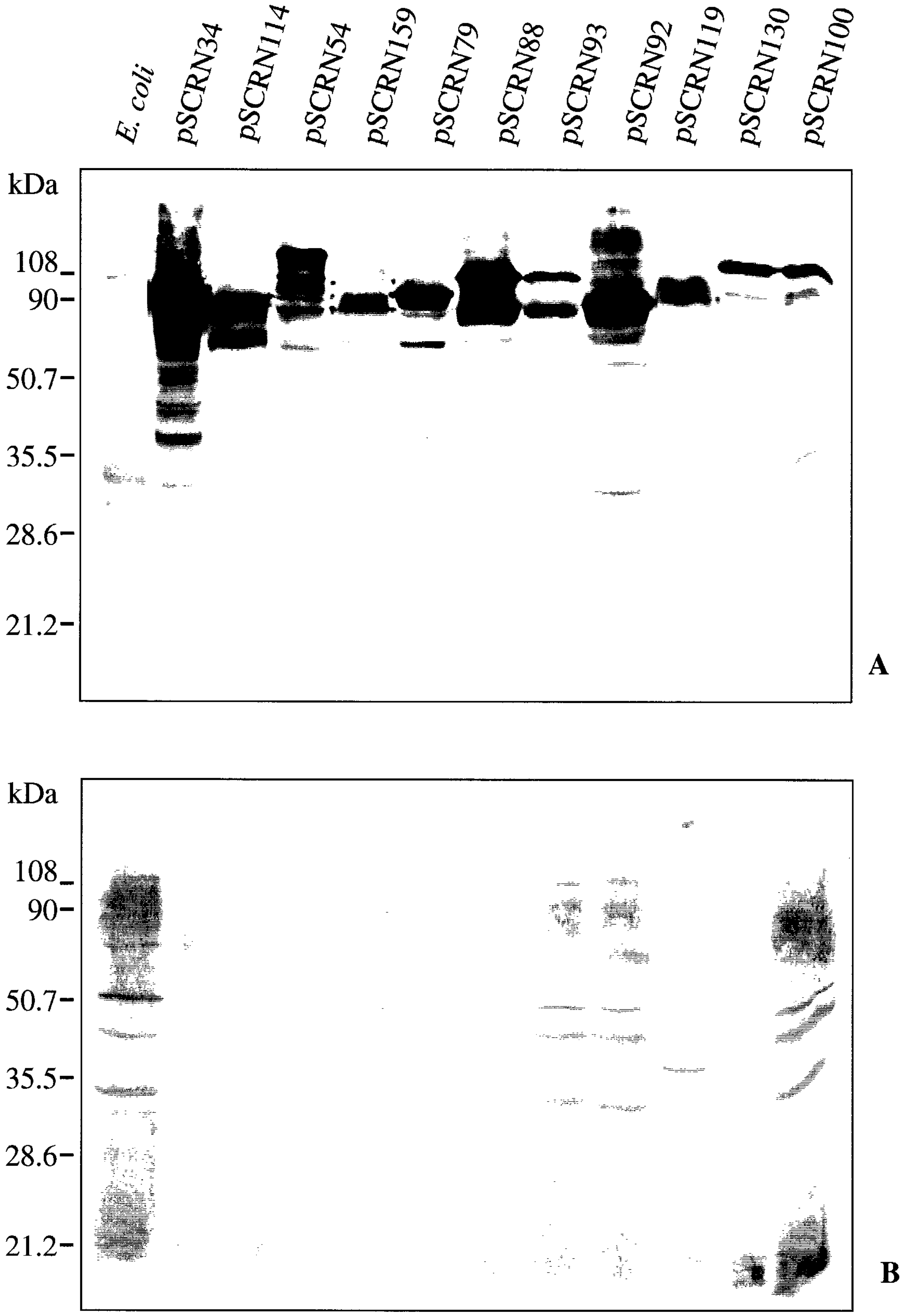
InlA. The whole cell lysates of representative positive clones were analyzed by Western blots to further characterize their immunological reactivity with RaL and RaK (Fig. 14). In pSCRN34, RaL strongly recognized smeared protein bands of sizes ranging from 62 to 85 kDa, which contain a major protein band of approximately 78 kDa, close to the predicated size of 71.98 kDa of the InlA fusion protein encoded by the clone.

InlC2. RaL recognized a protein of approximately 73 kDa in pSCRN92, which is similar to the predicated size of 70.6 kDa for the InlC2 fusion protein.

InlD. A protein band of ~75kDa was detected by RaL in two identical clones pSCRN114 and pSCRN159. The apparent molecular mass is almost 2-fold larger than the predicated size of 41.9 kDa for the InlD fusion protein encoded by these two clones. The reason for this discrepancy is not clear. It is possible that the fusion protein has been aggregated or associated with other *E. coli* components to form a stable complex, which is not completely denatured in the experiment condition. In pSCRN114 and pSCRN159, a smaller immunoreactive protein of ~61 kDa may represent a degraded form of the stable complex. RaL detected a protein of 91kDa in pSCRN54, which is in good agreement with the predicated molecular mass of 93.3kDa for the InlD fusion protein expressed from the clone. Multiple protein bands, smaller than the 91 kDa protein, are likely its degraded products. A strong immunoreactive protein band of 105 kDa may represent a stable protein complex containing the 91kDa protein.

IspWei. Two proteins with apparent molecular masses of 74 kDa and 108 kDa were detected by RaL in pSCRN88; the size of the smaller band is close to the predicted molecular

Figure 14. Western blot analysis of the recombinant proteins expressed from selected immunoreactive clones with rabbit antisera. Proteins transferred onto nitrocellulose membranes were probed with RaL (A) or RaK (B) at a dilution of 1:400. Each lane contains total proteins from cells equivalent to 1 ml of culture with an OD_{590} of 0.02. All clones but pSCRN100 express a truncated *L. monocytogenes* protein with a N-terminal fusion. *E.coli* harboring the cloning vector pScreen1$b^+\Delta$EcoRV was used as control.


E. coli
pSCRN34
pSCRN114
pSCRN54
pSCRN159
pSCRN79
pSCRN88
pSCRN93
pSCRN92
pSCRN119
pSCRN130
pSCRN100
kDa
108
90
50.7
35.5
28.6
21.2
A
kDa
108
90
50.7
35.5
28.6
21.2
B

mass of 64.2 kDa for the IspWei fusion protein encoded by pSCRN88. The larger protein may represent a stable protein complex containing the 74 kDa polypeptide.

IspLing. pSCRN79 expressed a protein of 79 kDa detected by RaL, which is slightly smaller than the size of 87 kDa predicted for the IspLing fusion protein encoded by the clone.

IspYu. pSCRN119 expressed a protein doublet of about 73 to 75 kDa recognized by RaL, which is in good agreement with the predicated size of 71.8 kDa for the IspYu fusion protein encoded by the clone.

IspMin. A protein band of about 90 kDa was detected by RaL in pSCRN130, which is close to the predicated size of 97.3 kDa for the IspMin fusion protein encoded by the clone.

IspLin. pSCRN100 expressed a protein of about 90 kDa detected by RaL but not by anti-His mAb (data not shown here). This indicated that the protein encoded by pSCRN100 did not contain a N-terminal 6-His tag fusion and that expression of the immunoreative product was directed by a *L. monocytogenes* promoter. The predicted size of the IspLin fusion protein encoded by pSCRN100 is 39.0 kDa with a C-terminal fusion, which is much smaller than the protein band detected by RaL. As discussed previously, this may be due to a stable protein aggregate or complex, which was not completely denatured in the present experimental condition.

No protein bands, corresponding to those recognized by RaL, were detected in the selected clones by RaK (Fig. 14B). Lack of specific antibodies to the identified gene products (Table 7) in the antiserum from rabbits immunized with heat-killed bacteria further confirmed the results of differential immunoscreening of the *L. monocytogenes* expression library.

3.6 Immunological analysis of the identified gene products with antisera from other animal species infected with *L. monocytogenes*

The immunoreactive clones, identified during differential screening of the *L. monocytogenes* library, were further analyzed to determine whether the same gene products are expressed during infection of other animal species. The fusion proteins expressed from all identified clones but pSCRN100 are recognized by a bovine anti-*L monocytogenes* serum on Western blots (Fig. 15). Strong reactions were found with InlA, InlC2, IspWei, and IspMin. Other proteins such as InlD, IspLing and IspYu appeared to react weakly with the bovine anti-*L. monocytogenes* serum. No specific immunoreaction was observed with a normal bovine serum (data not shown). These results indicate induced expression of InlA, InlC2, InlD, IspWei, IspLing, IspYu, and IspMin during bovine infection. Contrasting results were obtained with Western blotting analysis of the positive clones with antisera from guinea pigs infected with *L. monocytogenes* (Fig. 16). Only the InlA fusion protein encoded by pSCRN34, the InlC2 fusion protein encoded by pSCRN92 and the IspYu fusion protein encoded by pSCRN119 were detected by guinea pig antisera. No immunoreaction was found with pre-immune sera from guinea pigs (data not shown).

3.7. Sequence characteristics of the immunoreactive protein products encoded by positive clones

The protein product encoded by pSCRN1, pSCRN2, pSCRN34, pSCRN35, pSCRN65, pSCRN66, pSCRN95,pSCRN96, pSCRN113, and pSCRN115 was identified as truncated InlA based on a similarity search with BLAST. InlA functions as an invasin, mediating bacterial internalization by host cells (Gaillard et al., 1991). The InlA protein of *L. monocytogences* serotype 4b derived from its complete ORF (Apendix B) has 800 amino acid residues with a calculated molecular weight of 86.598 kDa and predicted pI of 4.673. It contains 58 (7.3 %) basic amino acids (K, R), 74 (9.2 %) acidic amino acids (D, E), 266 (33.2 %) hydrophobic amino acids

Figure 15. Western Blot analysis of the recombinant proteins expressed from selected immunoreactive clones with bovine antiserum. Proteins transferred onto nitrocellulose membranes were probed with antiserum from a bovine experimentally infected with *L. monocytogenes* at a dilution of 1:400. Each lane contains total proteins from cells equivalent to 1 ml of culture with an OD_{590} of 0.02. All clones but pSCRN100 express a truncated *L. monocytogenes* protein with a N-terminal fusion. *E.coli* harboring the cloning vector pScreen1b^+ △EcoRV was used as control.

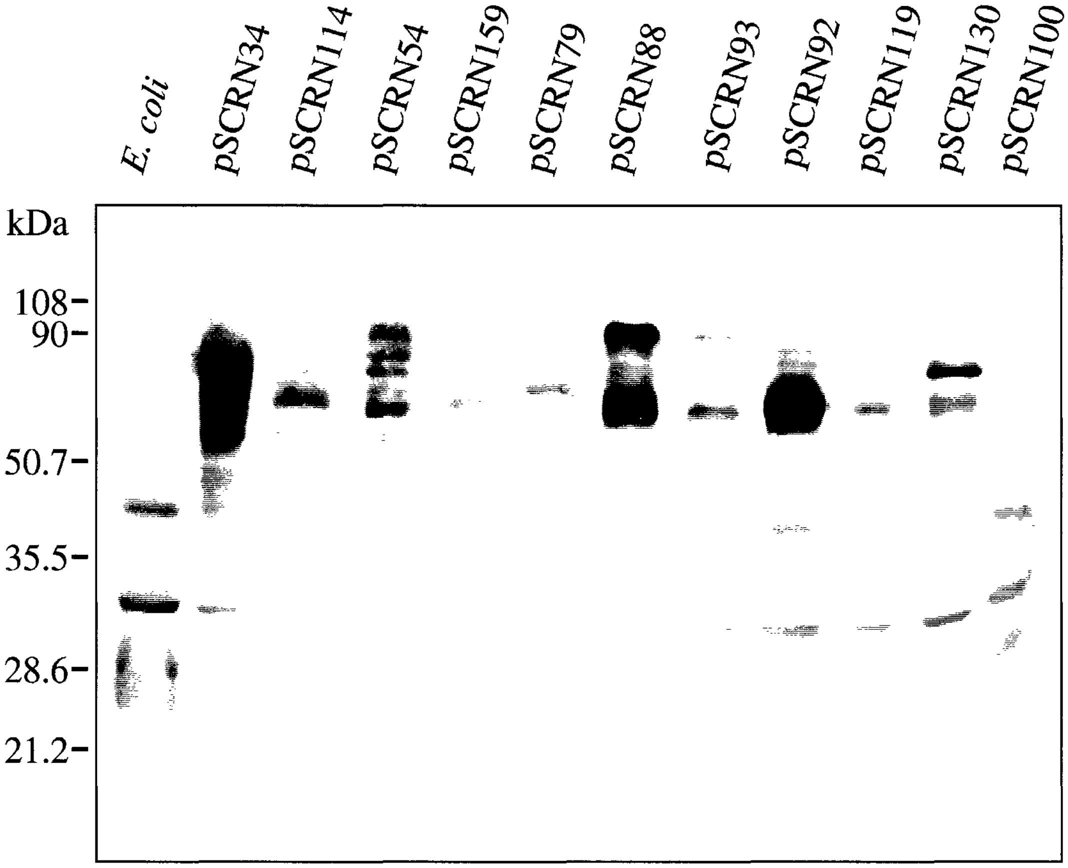
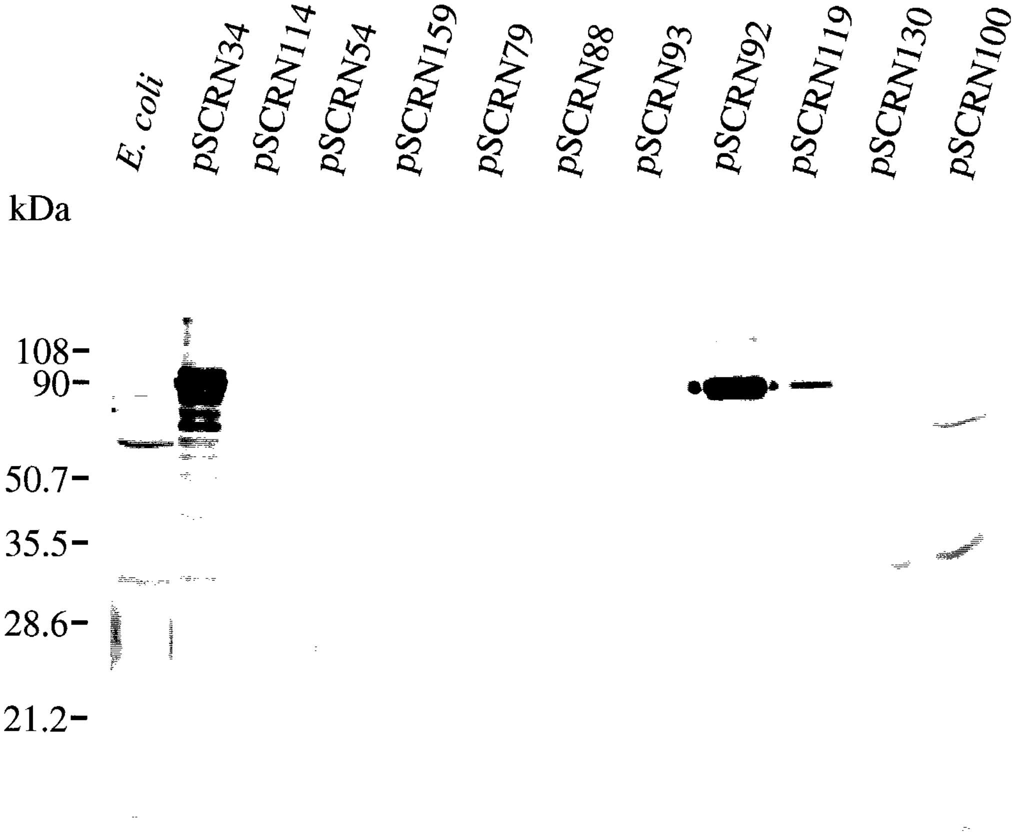

E. coli
*p*SCRN34
*p*SCRN114
*p*SCRN54
*p*SCRN159
*p*SCRN79
*p*SCRN88
*p*SCRN93
*p*SCRN92
*p*SCRN119
*p*SCRN130
*p*SCRN100
kDa
108
90
50.7
35.5
28.6
21.2

Figure 16. Western Blot analysis of the recombinant proteins expressed from selected immunoreactive clones with guinea pig antisera. Proteins transferred onto nitrocellulose membranes were probed with antiserum from a guinea pig experimentally infected with *L. monocytogenes* at a dilution of 1:1200. Each lane contains total proteins from cells equivalent to 1 ml of culture with an OD_{590} of 0.02. All clones but pSCRN100 express a truncated *L. monocytogenes* proteins with a N-terminal fusion. *E.coli* harboring the cloning vector pSCREEN1b$^+$ △EcoRV was used as control.


E. coli
pSCRN34
pSCRN114
pSCRN54
pSCRN159
pSCRN79
pSCRN88
pSCRN93
pSCRN92
pSCRN119
pSCRN130
pSCRN100
kDa
108
90
50.7
35.5
28.6
21.2

(A, I, L, F, W, V) and 306 polar amino acids (38.3 %). Alignment of the 4b InlA with the homologs of other *L. monocytogences* strains (Appendix C, Fig. 1C) reveals that its sequence contains 97.6 % homology (identity) to those of the strains EGD-e and EGD-SmR. The 4b protein sequence has general characteristics similar to those of the InlA proteins from other *L. monocytogences* strains (Vazquez-Boland et al., 2001, including the N-terminal domain containing 14 leucine-rich repeats (LRR) and a C-terminal sorting signal consisting of a conserved LPXTG motif (aa 767-771) followed by a hydrophobic domain (~ 20 aa) and a tail of positively charged amino acids. The sorting signal retains the polypeptide in the cell membrane compartment. After cleavage between the threonine and glycine residues of the LPXTG motif, the carboxyl group of threonine residue is covalently linked to the *meso*-diaminopimelic acid in cell wall, catalyzed by a membrane protein sortase (Cossart and Jonquieres, 2000; Navarre and Schneewind, 1994; Navarre and Schneewind, 1999).

The protein product encoded by pSCRN92, pSCRN106 and pSCRN116 was identified as truncated InlC2 based on a similarity search with BLAST. The InlC2 protein of *L. monocytogences* serotype 4b derived from its complete ORF (Apendix B, Fig. 2B) has 548 amino acids with a calculated molecular weight of 58.684 kDa and predicted pI of 4.665. It contains 36 (6.7 %) basic amino acids (K, R), 46 (8.4 %) acidic amino acids (D, E), 193 (35.2 %) hydrophobic amino acids (A, I, L, F, W, V) and 196 (35.8 %) polar amino acids. Alignment of the 4b InlC2 with the homologs of other *L. monocytogences* strains (Appendix C, Fig. 2C) reveals that its sequence has 94.9 %, 94.0 % and 94.2 % homology (identity) to those of the EGD InlC2 (Dramsi et al., 1997), EGD-e InlH (Glaser et al., 2001) and EGD (serotype 1/2a) InlD (Genbank Accession No. CAC20635)Peters et al., 2003; Dramsi et al., 1997, respectively.

The protein product encoded by pSCRN114, pSCRN159, pSCRN54, pSCRN91, pSCRN112, pSCRN139, and pSCRN143 was identified as truncated InlD based on a similarity search with BLAST. The InlD protein of *L. monocytogences* serotype 4b derived from its complete ORF (Appendix B, Fig. 3B) has 568 amino acids with a calculated molecular weight of 61.441 kDa and predicted pI of 4.391. It contains 38 (6.7 %) basic amino acids (K, R), 61 (10.7 %) acidic amino acids (D, E), 199 (35.0 %) hydrophobic amino acids (A, I, L, F, W, V) and 199 (35.0 %) polar amino acids. Alignment of the 4b InlD with the *L. monocytogences* strain EGD InlD (Dramsi et al., 1997) reveals that its sequence has strong homology (99.5 % identity) to that of EGD strain (Appendix C, Fig. 3C). The 4b InlD also has a 61.4 % and 64.1 % sequence identity to *L. monocytogences* EGD-e InlH (Glaser et al., 2001) and EGD (serotype 1/2a) InlD (Genbank Accession No. CAC20635), respectively.

The protein IspWei encoded by pSCRN88, and pSCRN93, pSCRN101, pSCRN102, and pSCRN106 represents a novel protein of unknown function based on a similarity search with BLAST. The homolog of this protein is not found in *L. monocytogences* EGD-e (Glaser et al., 2001) and also has not been described in other *L. monocytogences* strains. The IspWei protein derived from its complete ORF (Apendix B, Fig. 4B) has 589 amino acids with a calculated molecular weight of 63.578 kDa and predicted pI of 4.092. It contains 29 (4.9 %) basic amino acids (K, R), 62 (10.5%) acidic amino acids (D, E), 208 (35.3%) hydrophobic amino acids (A, I, L, F, W, V) and 212 (36.0 %) polar amino acids. Alignment of IspWei with a probable cell protein of *L. inocau* (Appendix C, Fig. 4C) reveals that its sequence has 93.5 % identity to that of *L. innocua* Clip11262 (Glaser et al., 2001). Ispwei has sequence characteristics similar to an internalin molecule, including a signal peptide (aa 1-31) (predicted by SignalP), nine LRR repeats with 26 aa per repeat (aa 94-291), a region of two inter-repeat (B repeat) (aa 384-502), and a LPXTG sorting motif (aa 559-563) at the C-terminus. In addition, IspWei also has 47.1 % and 47.8 % identity to InlD and InlH of *L. monocytogences* strains EGD and EGD-e (Dramsi et al., 1997; Glaser et al., 2001), respectively. Based on these analysis, IspWei is an internalin-like

LPXTG protein (Cabanes et al., 2002).

A BLAST search of protein databases reveals the protein IspLing encoded by pSCRN79 resembles a hypothetical transmembrane protein of unknown function described in other *Listeria* strains (Glaser et al., 2001). The IspLing protein derived from its complete ORF (Apendix B, Fig. 5B) has 653 amino acids with a calculated molecular weight of 74.693 kDa and predicted pI of 6.224. It contains 65 (10.0 %) basic amino acids (K, R), 71 (10.9%) acidic amino acids (D, E), 227 (34.8 %) hydrophobic amino acids (A, I, L, F, W, V) and 199 (30.5 %) polar amino acids. Alignment of IspLing with the homologs of other *L. monocytogences* strains (Apendix C, Fig. 5C) reveals that its sequence has 99.8 % and 97.7 % (identity) to hypothetical transmembrane proteins of *L. monocytogences* EGD-e and *L. innocua* Clip11262 (Glaser et al., 2001), respectively. IspLing exhibits significant homology (62% and 23% identity) to the sulfatases of *Bacillus anthracis* A20121 and *E. coli* K1 (Read et al., 2003; Hoffman et al., 2000), respectively. This protein contains a signal peptide (aa 1-33) and a C-terminal domain (aa 253-574) similar to a sulfatase domain (predicted by SignalP and InterProScan respectively).

The immunoreactive product encoded by pSCRN119 and pSCRN153 was the C-terminal region (325 amino acids) of a protein of unknown function (designated IspYu) resembling the amidases (autolysins) of *L. innocua* and *L. monocytogenes* (Glaser et al., 2001;Braun et al., 1997; McLaughlan and Foster, 1998; Milohanic et al., 2001; Lecuit et al., 1997). The C-terminal sequence (325 amino acids) of IspYu has 32.3 % and 32.6 % homology (identity) to the corresponding regions of *L. monocytogenes* and *L. innocua* amidases (Apendix C, Fig. 6C), respectively. Putative amidase-like proteins have been derived from the genome sequences of *L. innocua* and *L. monocytogenes* (Glaser et al., 2001). The C-terminal region (325 amino acids) of IspYu has 68.9 % and 35.4 % homology (identity) to the corresponding regions of the amidase-like proteins of *L. innocua* and *L. monocytogenes* (Glaser et al., 2001), respectively.

The immunoreactive product encoded by pSCRN130 represents the C-terminal region (570 amino acids) of a novel protein (designated IspMin) whose function is not known in *L. monocytogenes* based on the BLAST similarity search. The C-terminal sequence (570 residues) of IspMin has 32.9 %, 33.9 %, and 30.0 % homology (identity) to the corresponding regions of a putative protein ORFA of *L. seeligeri,* a putative peptidoglycan protein of *L. monocytogenes* EGD-e, and a putative *L. innocau* protein highly similar to the *L. seeligeri* ORFA (Appendix C, Fig.7C). The available 570-amino acid sequence (i. e., the C-terminal region) of IspMin contains two LLR units (aa 16-70) with 14 residues per repeat and four polycystic kidney disease (PKD) repeats (Cabanes et al., 2002), and no LPXTG motif at the C-terminus.

The immunoreactive polypeptide encoded by pSCRN100 is a truncated form of a protein of unknown function (designated IspLin) having high homology (92.2% identity over 294 amino acids at the N-terminus) to a putative peptidoglycan bound protein of *L. monocytogenes* EGD-e (Glaser et al., 2001. This protein contain a signal peptide (aa 1-47) (predicted by SignalP) and two repeats (aa 58-107 and 1aa 85-237, respectively) of unknown function in the available amino acid sequence. These repeats may be a domain anchoring the protein to the cell wall.

CHAPTER FOUR

DISCUSSION

4.1 General discussion

A significant effort was made in the present study to discover the *L. monocytogenes* proteins induced specifically during host infection by immunoscreening of a *L. monocytogenes* genomic expression library with two kinds of rabbit antisera, one (RaL) from the rabbits experimentally infected with live *L. monocytogenes*, and other (RaK) from the animals immunized with the heat-killed organism. A similar immunoscreening strategy has led to the successful identification of the bacterial antigens induced only during infection with *Borrelia burgdorferi* (Suk et al., 1995) and *Mycobacterium avium* subsp. *paratuberculosis* (Bannantine and Stabel, 2001. This approach has allowed for the identification of numerous positive clones coding for the proteins recognized by RaL but not by RaK, including InlA, InlC2, InlD, IspWei, IspLing, IspYu, IspMin and IspLin. Lack of antibodies to these proteins in the RaK antiserum indicated that (i) these proteins are not expressed or expressed at extremely low levels in the *in vitro* culture conditions employed in the study; (ii) they are induced in infected hosts due to the influence from the external factors (physical, chemical, and biological) encountered in the new environment. Of eight proteins identified, three proteins (InlA, InlC2 and InlD; 37.5 %) belong to the internalin family in which InlA and InlB are well known bacterial virulence factors (Vazquez-Boland et al., 2001; Bergmann et al., 2002; Dramsi et al., 1993; Dramsi et al., 1995; Lingnau et al., 1995). The protein IspYu shows some degree of homology (32.3% identity) in the C-terminal region (over 325 amino acids) to the *L. monocytogenes* amidase Ami (Braun et al., 1997; McLaughlan and Foster, 1998), a known virulence factor (Milohanic et al., 2001). The other four proteins IspWei, IspLing, IspMin and IspLin (50%) of unknown function are predicted cell surface-exposed proteins based on their amino acid sequences and are first demonstrated being expressed in *L. monocytogenes*. The cell wall of Gram-positive bacteria serves as a docking site for proteins that interact with the environment and that are of primary importance in bacterial adherence, invasion and interaction with the host (Cabanes et al., 2002). Demonstration of the five novel proteins, IspWei, IspWling, IspYu, IspMin and IspLin, co-expressed with other known virulence factors during infection, may suggest that these novel proteins play a role in adhesion and/or signaling in bacterial pathogenesis and virulence.

The experimental data indicated that the identified internalins are not expressed or only expressed at extremely low levels in the culture conditions used in the present study and induced specifically in infected hosts. This is consistent with the previous findings that the internalin genes are not constitutively expressed in *L. monocytogenes* and regulated in *in vitro* cultures by the influence of external factors such as nutrition, temperature, and pH (Vazquez-Boland et al., 2001). The level of transcription of the *inlAB* operon in both epithelial cells and macrophages is low, and is lower than those under extracellular conditions in BHI rich media (Bubert et al., 1999). The *inlAB* operon is maximally expressed during the exponential phase of growth in BHI medium at 37 °C. In contrast, at the same conditions inlC2, inlD and inlE are expressed early and transiently (Bubert et al., 1999). These cell-wall associated internalin proteins are preferentially expressed extracelluarly and with the exception of *inlAB* operon, are independent of PrfA (Bubert et al., 1999; Engelbrecht et al., 1996). The expression of *inlC2*, *inlD* and *inlE* are regulated by sigmaB, a stress responsive alternative sigma factor (Kazmierczak et al., 2003), whereas that of *inlAB* operon are PrfA-dependent (Bohne et al., 1996).

The presence of antibody responses to InlA, InC2 and InlD in infected hosts is consistent with the fact that they are cell surface molecules, which contain a signal peptide and various numbers of N-terminal LRR repeats and an LPXTG motif at their C-terminal ends (Vazquez-

Boland et al., 2001; Cabanes et al., 2002). The LPXTG motif is the site where the mature internalins are covalently bound to the bacterial cell wall after the cleavage between threonine and glycine residues (Glaser et al., 2001). With the exception of antibodies to InlA, which has been recently demonstrated in the sera of dairy cows suffering from sub-clinical listeria-related mastitis and abortion (Boerlin et al., 2003), antibodies to InlC2 and InlD are first demonstrated in *L. monocytogenes*-infected hosts. Boerlin *et al.* (2003) described the use of recombinant InlA (aa 2-710) and LLO (aa 26-529) as antigens in ELISA tests for the specific detection of anti-*L. monocytogenes* antibodies in cattle and showed that that the InlA ELISA outperformed LLO ELISA in terms of specificity and sensitivity. Unlike the *inlA* and *inlB* genes which were identified in all *L. monocytogenes* strains tested and are species-specific (Doumith et al., 2004), the *hly* gene also exists in other *Listeria* species including *L. ivanovii*, and *L. seeligeri.* Antibody cross-reaction with LLO between *Listeria* species may thus limit the specificity of the LLO-based immunoassay (Low and Donachie, 1997). In this study, InlA and InlC2 were consistently found to be the major antibody targets in rabbit, bovine and guinea pig experimentally infected with *L. monocytogenes*, indicating the two proteins have the great potential for use in serological tests.

The immunoreactive polypeptide encoded by pSCN119 and pSCN153 represents a truncated form (the C-terminal region) of a protein of unknown function (designated IspYu), which shows significant homology at the C-terminus to a putative amidase-like protein and the autolysin Ami of *L. innocua* or *L. monocytogenes* (Glaser et al., 2001). Attempts to clone the 5' part of the *ispYu* ORF by PCR were unsuccessful in the present study. IspYu was first demonstrated being expressed by *L. monocytogenes* during infection and most likely represents a different form of the *Listeria* peptidoglycan hydrolases based on the sequence similarity. In *L. monocytogenes*, the autolytic activity has been demonstrated for at least five proteins Ami, P60, NamA, P45 and Auto (Wuenscher et al., 1993; Braun et al., 1997; McLaughlan and Foster, 1998;

Schubert et al., 2000; Lenz et al., 2003; Cabanes et al., 2004). Only antibodies to p60 were described in human sera of listeriosis patients (Gentschev et al., 1992). The deduced amino acid sequence of *L. monocytogenes* Ami contains three characteristic domains (Braun et al., 1997; McLaughlan and Foster, 1998;Milohanic et al., 2001): (i) a 30-amino-acid putative signal sequence, (ii) a 179-amino-acid N-terminal domain similar to the N-acetylmuramoyl-L-alanine amidase domain of the *Staphylococcus aureus* autolysin Atl (Sugai et al., 1995) and a C-terminal region (aa 262 to 917) made up of four tandem repeats of approximately 160 amino acids; each repeat can be divided into two modules containing dipeptide GW ("GW modules"), module 1 consisting 82-83 amino acids and module 2 of 78 amino acids. This C-terminal region of Ami has homology with the 232-amino-acid cell wall-anchoring region of InlB, which is made up of three GW modules (Braun et al., 1997). A similar domain organization was also found in the *L. monocytogenes* Auto protein (572 amino acids) with a signal sequence, a N-terminal autolysin domain and a C-terminal cell wall-anchoring domain made up of four GW modules (Cabanes et al., 2004). Like the Ami, Auto and InlB of *L. monocytogenes*, IspYu contains repeated GW modules at the C-terminal region; which likely function as a cell wall anchoring domain. The immunogenicity of the C-terminal region of IspYu suggests that the putative cell wall anchoring domain is cell surfaced-exposed. This is consistent with the finding that the cell wall anchor of repeated GW modules in Ami contributes to the adhesion of *L. monocytogenes* to eukaryotic cells (Milohanic et al., 2001).

The autolysins Ami, P60, NamA and Auto are of importance in *L. monocytogenes* virulence (Milohanic et al., 2001; Lenz et al., 2003; Cabanes et al., 2004). The *Ami* mutant was defective in adhesion of *L. monocytogenes* to eukaryotic cells and attenuated in the livers of mice inoculated intravenously. In contrast, studies with an *Auto* mutant indicated that Auto was not essential for cell adherence and but was required for entry into eucaryotic cells (Cabanes et al., 2004). A reduced virulence was observed following intravenous inoculation of mice and oral infection of guinea pigs with the *Auto* mutant. The *iap*-encoded autolytic p60 (Wuenscher et al., 1993) was an invasion-associated protein involved in intestinal invasion and *in vivo* survival (Hess et al., 1996; Hess et al., 1995). Deficiencies of p60 or NamA in gene deletion mutants caused intermediate reductions in bacterial virulence *in vivo* , yet showed no defect for infection of cultured cells. Restoration of virulence in p60 mutant required full-length p60 with an intact catalytic domain, suggesting that peptidoglycan hydrolysis by p60 is crucial for *L. monocytogenes* (Lenz et al., 2003). Collectively, data accumulated from previous studies with *L. monocytogenes* autolysins may imply that IspYu is likely another form of petidoglycan hydrolase, although its autolytic activity remains as yet unknown and hence may have a role in *L. monocytogenes* virulence.

The nucleotide sequence derived from the immunoreactive clone pSCRN88 and the PCR clone pCR253-254 contains a 1770-bp ORF coding for a 590-amino acid protein of unknown function (designated IspWei) having similarities to a probable cell surface protein of *L. monocytogenes* or *L. innocau* (Appendix C, Fig. 4C). The sequence characteristics of IspWei indicate that it is an internalin-like LPXTG protein and appears to be a new member of the *Listeria* internalin family. Specific induction of IspWei in *L. monocytogenes* and the presence of antibodies to this protein were first demonstrated during rabbit infection. These results, together with the sequence characteristics of IspYu, suggests that the protein has a role for *L. monocytogenes* virulence involved in host colonization *in vivo*.

The immunoreactive polypeptide encoded by pSCRN130 is a truncated form of a protein of unknown function (designated IspMin) having a significant homology (32.9 % identity over 570 amino acids) to a putative protein ORFA of *L. seeligeri* (GenBank Accession No. CAA65738Lampidis,). The present study has provided the first experimental evidence indicating the regulated expression of IspMin in *L. monocytogenes* and the presence of antibodies to this protein during infection. Like several other internalin-like IPXTG proteins (Cabanes et al., 2002), IspMin contains both LRR and PKD repeats, which may imply some similar functions for the protein. The internalin N-terminal region, compassing the LRR domain and the inter-repeat (IR) region, is necessary and sufficient for bacterial entry into permissive cells (Lecuit et al., 1997; Mengaud et al., 1996b). The PKD domain was first identified in the polycystic kidney disease protein PKD1, and contains an Ig-like fold. Although PKD1 is involved in adhesive protein-protein and protein-carbohydrate interactions, it is not clear if the PKD domains mediate any of these interactions (Cabanes et al., 2002). Most of these domains are present in the extracellular parts of proteins involved in interactions with other proteins. PKD domains have been found in the extracellular regions of proteins from higher organisms, bacteria, and archaea (Cabanes et al., 2002). Consistent with this is the observed imunogenicity associated with the C-terminal region of IspMin. Since domains with Ig-like folds have been shown to form ligand-binding sites in cell surface proteins (Cabanes et al., 2002). The IspMin protein containing PKD domains, which is expressed specifically during infection, may have a similar role in adhesion and or signaling.

The DNA sequence derived from the immunoreactive clone pSCRN79 and the PCR clones (pCR268-279 and pCR257-317) contains a 1962-bp ORF encoding a 653-amino acid protein of unknown function (designated IspLing) having strong homology to a hypothetical transmembrane protein of *L. monocytogenes* or *L. innocau* (Appendix C, Fig. 5C). This study first demonstrated induced expression of IspLing in *L. monocytogenes* which is targeted by antibodies during infection. IspLing contains a signal peptide (aa 1-33) and a C-terminal domain (aa 253-574) similar to a sulfatase domain. Based on the sequence characteristics of IspLing, it is likely that this protein may also serve as a sulfatase (EC:3.1.6) that canalizes the hydrolysis of various sulphate esters (von Figura et al., 1998), and may thus has a role in sulphate metabolism. Studies have shown that eukaryotic and also some prokaryotic enzymes homologous to sulfotransferase and sulfatase are involved in modulation of extracellular processes such as cell adhesion and receptor/ligand interactions by the addition and removal of sulfate ester (Mougous et al., 2002). Despite the sulfatase activity demonstrated in bacterial crude extracts for some time, the biological functions of most bacterial sulfatases remain unclear (Mougous et al., 2002). In a few cases, bacterial sulfatases have been implicated in facilitating host-pathogen interactions. For example, deletion of the sulfatase gene *aslA* from a cerebrospinal isolate of *E. coli* K1 (the casual agent of neonatal *E. coli* meningitis) has reduced the ability of this pathogen to invade the brain microvasculature (Ball et al., 2000). *L. monocytogenes* is known to invade the blood-brain barrier and cause meningitis. This implies that the novel immünogenic protein IspLing, although its sulfatase activity is as yet unknown, may have a similar role in the invasion of the brain microvasculature.

The immunoreactive polypeptide encoded by pSCRN100 is a truncated form of a protein of unknown function (designated IspLin) having high homology (92.2% identify over 294 amino acids) to a putative peptidoglycan bound protein of *L. monocytogenes* EGD-e (Glaser et al., 2001). A signal peptide (aa 1-47) and two repeats (aa 58-107 and 185-237, respectively) of unknown function are found in the available amino acid sequence of IspLin. These repeats may function as a cell wall anchoring domain. This protein was first demonstrated being induced in *L. monocytogenes* and also targeted by antibodies during infection, suggesting it is surfaced-exposed. Cloning of the full-length protein encoding gene and generation of a gene knock-out bacterial mutant will be necessary for the study of its functional roles in *L. monocytogenes* virulence and pathogenesis.

The data obtained from the present study indicate that differential immunoscreening of a protein expression library appears to be a powerful tool for identification of *L. monocytogenes* proteins specifically induced during infection. The genomic library constructed in the study appears to cover well the bacterial genome based on the fact that eight different gene products are identified with multiple clones (identical or/and overlapping) encoding each of the six truncated proteins. Like any other techniques used for identification of *in vivo*-induced bacterial genes or gene products, the current system has limitations. This approach is not capable of identifying every *L. monocytogenes* gene product induced during infection, only those proteins that elicit antibodies during the infectious process. The current system identified a set of the *in vivo*-induced gene products, which are immunogenic and very different from those found induced *in vivo* upon entry of *L. monocytogenes* into the host cells by using other strategies including *in vivo* expression technology (IVET) (Dubail et al., 2000; Gahan and Hill, 2000) and Fluorescence-Activated Cell Sorting (FACS) (Wilson et al., 2001). By using an IVET tool based on the *L. monocytogenes hly* gene as a reporter for monitoring protein expression, Dubail *et al.* (2000) identified nine *in vivo*-induced loci of *L. monocytogenes*, including genes encoding (i) the previously known *in vivo*-inducible phosphatidylinositol phospholipase C (PI-PLC), a major virulence factor; (ii) a putative N-acetylglucosamine epimerase (possibly involved in teichoic acid biosynthesis) homologous to YvyH of *B. subtilis* (62%), Cps19ka of *Streptococcus pneumoniae* (58% identity), and Cap5P of *S. aureus* (62% identity); (iii) a short protein of unknown function (designated Orf104) having similarities to a putative protein of the archaebacterium *Aeropyrus pernix*, which possesses a putative membrane lipoprotein lipid attachment site. Ganhan and Hill (2000) have used a similar *hly*-based IVET system to identify nine *in vivo*- induced loci including genes encoding DNA topoisomerase III, a cellobiose transporter and a fumarase. Using a GFP-based FACS technique similar to that developed by Valdivia and Falkow (Valdivia and Falkow, 1997), Wilson *et al.* (2001) have identified twelve *L. monocytogenes* genes that appear to be induced when the bacteria are in the intracellular environment of a macrophage. Of twelve genes identified by Wilson *et al.* (2001), only one gene *actA* encodes a virulence factor necessary for the nucleation of host cell actin and motility of *Listeria* within the host cell. The other eleven loci encoded proteins either involved in basic metabolism (e.g., sulfate transporter, UDP galactose epimerase and xylose repressor and PTS mannose-specific IIAB system) or in information pathway (e.g., BvrA antiterminator and E-Tu elongation factor Tu). It appears that the current system has some advantages over the IVET and FACS systems in the discovery of *in-vivo* induced virulence factors of *L. monocytogenes*. One possible explanation is that the gene products identified by the current system are immunogenic during infection with *L. monocytogenes* and are thus most likely surface-exposed proteins. The surface-exposed characteristics imply that these induced proteins are accessible to interaction with host cells and thus are of importance in bacterial adherence and invasion of host cells.

Discovery of the immunogenic proteins induced during infection is particularly interesting in the context of *L. monocytogenes*. The roles of cell-mediated immunity in protection against listerial infection has been clearly established and the role of antibodies is generally believed to be ineffective in protective immunity, although the roles of natural antibodies (Ochsenbein et al., 1999) and anti-LLO antibody (Edelson et al., 1999) have been suggested in the recent studies. Thus, the *in vivo*-expressed novel immunogenic proteins of *L. monocytogenes* will become new candidates that may lead to a deeper understanding of the role of humoral immune response in protection against listerial infection. Identification of these novel immunogens may provide new insights for developing improved reagents for vaccination, diagnosis and treatment of listeriolosis. Also importantly, identification of the *in vivo*-expressed novel proteins of *L. monocytogenes* may lead to a better understanding of the molecular pathogenesis of listeriosis and provide further insights into the survival strategy of the organism in the hosts.

In conclusion, this study has developed a rabbit model for identification of the *L.*

monocytogenes gene products specifically induced during infection through differential immunoscreening of a *L. monocytogenes* genomic expression library. Of the eight gene products identified, five are novel proteins (IspWei, IspLing, IspYu, IspMin, and IspLin) that are demonstrated for the first time being expressed in *L. monocytogenes*, and the presence of antibodies to seven of the proteins was first shown in an infected host. Some of the *in vivo*-induced gene products identified from the rabbit model do not appear to be induced during guinea pig infection. Sequence characteristics and immunogenicity of the five novel proteins and their co-expression with several internalin family members (known virulence factors) appear to suggest that these novel proteins are involved in *L. monocytogenes* virulence and pathogenesis.

4.2 Future work

Future studies will be focused on (i) generation of the full length recombinant forms of the identified *in vivo*-induced *L. monocytogenes* proteins which is to be used to (a) develop monospecific polyclonal antibodies for protein localization and for detection of expression of target proteins in *L. monocytogenes* culture and in infected tissues and (b) to detect the presence of antibodies to the proteins in listeriosis hosts; (ii) detection of the gene transcripts for the identified proteins by RT-PCR in in *L. monocytogenes* culture and in infected tissues; (iii) study of the protein/gene function by generation of *Listeria monocytogenes* mutants through gene knockout and destruction; (iv) biochemical and structural characterization of the novel *in vivo*-induced *L. monocytogenes* proteins; (v) study of immunological roles of the identified proteins; and (vi) use of the identified immunogens to develop improved diagnostics tools.

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APPENDIX A

Preparation of Culture Medium, Buffers and Solutions

Trypticase soy blood agar (TSBA) plates

Per liter:

Trypticase Soy Agar	40 g
Defibrinated Sheep Blood	50 ml

LB Medium (Luria-Bertani Medium)

Per liter:

Trytptone	10 g
Yeast Extract	5 g
Sodium Chloride	10 g

LBMOPS Medium

Per liter:

Trytptone	10 g
Yeast Extract	5 g
Sodium Chloride	10 g
MOP	0.5 g

Adjust pH to 7.4

LB Agar containing Kanamycin

Per liter:

Tryptone Peptone	10 g
Yeast Extract	5 g
Sodium Chloride	10 g
Bacto Agar	12 g

Add kanamycin to 50 μg/ml (final concentration)

SOB Medium

Tryptone	20 g
Yeast Extract	5.0 g
NaCl	0.5 g

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

SOC Medium

Add 1 ml of filter sterilized 2 M glucose to 100 ml S. O. B medium

Prepare just prior to use.

6 × DNA Loading Buffer

0.25% (w/v) bromophenol blue

0.25% (w/v) xylene cyanol FF

15% (w/v) Ficoll (Type 400) in water

6 mM EDTA (pH 8.0) (dilution from 0.5 M stock)

2 × SDS-PAGE Sample Buffer
100 mM Tris.Cl (pH 8.0)
200 mM Dithiotreitol (DTT)
4% (w/v) Sodium dodecyl sulfate (SDS; electrophoresis grade)
0.2% (w/v) Bromophenol Blue
20% (v/v) Glycerol

12% Separating SDS-Polyacrylamide Gel
Per 10 ml:
4.0 ml of acrylamide /bis (30%T, 2.67% C)
2.5 ml of 1.5 M Tris-HCL(pH8.8)
3.35 ml of dH_2O
100 µl of 10% (w/v) SDS
50 ul of 10% (w/v) ammonium persulfate
5 µlof TEMED.

4% Stacking Gel:
Per 5 ml:
0.65 ml Acrylamide /bis (30%T (w/v), 2.67% C)
1.25 ml of 0.5 M Tris-HCL(pH6.8)
3.05 ml of dH_2O
50µl of 10% (w/v) SDS
25µl of 10% (w/v) APS
5µl of TEMED,

Protein Transfer Buffer
Per liter:
5.82 g Tris
2.93 g glycine
Add dH_2O to 1L

APPENDIX B

Nucleotide Sequences and Deduced Amino Acid Sequences of the Positive Clones Identified in the Study

Figure 1B. Nucleotide sequences of the *L. monocytogenes* (serotype 4b) genes *inlA* and *inlB* (partial) and deduced amino acid sequences of the corresponding proteins. Nucleotides 1691 to 3195 were derived from inserts in pSCRN1, pSCRN2, pSCRN34, pSCRN35, pSCRN65, pSCRN66, pSCRN95,pSCRN96, pSCRN113, and pSCRN115. Nucleotides 1 to 1866 was obtained from pCR227-228. Nucleotides 504 to 2906 represent the ORF of *inlA*. The partial ORFof *inlB* is indicated by nt 2991 to 3195.

Figure 1B

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1                                         CATAAAGGGTAGAGGATAACATA 23
24   AGATGGTGGTAGTTATGCAGCATAAAGGGTAGAGGATAACATAAGTTAATTCTTTTTTGG 83
84   AGAAAAATAGCTATTATTATTTAATGGGCTTTGAAAGGGGTGATTATATAGAAGAAGTGA 143
14   AAAAAAGAGAGTTTTGGCGGGAAGAGTGCGGGGCTTGAGTTCGTCAATAATAGACCATTT 203
204  TAAAAGGTGGAATGACACGTGTGGTATCAACATAACATGATTTGCATGGTTGAATTCCTA 263
264  TTAAGCTTCGATAGTGAAATACATAAAAATATTATATATAGGGAAAATGTGCTGGAACGA 323
324  ACGAGATGAAAGATATCACTAAGCAGCTCCGTAGACAGATTAACTACTGAATACCATAGG 383
384  AACAATTCATGGGGCGCATAGTTTAATAAAGTAAGCAGACATGATTTTTCGGATGCAGGA 443
444  GAAAATCCTATACAACGAAACCTGATATTGTTTCTAACTATTGAAAAAGGAGTGTATATA 503
     INLA
504  GTGAGAAGAAAACGATATGTATGGTTGAAAAGTATACTAGTAGCAATATTAGTATTTGGC 563
1     V  R  R  K  R  Y  V  W  L  K  S  I  L  V  A  I  L  V  F  G  20
564  AGTGGAGTATGGATTAACACGAGTAACGGGACAAATGCTCAGGCAGCTACAATTACACAA 623
21    S  G  V  W  I  N  T  S  N  G  T  N  A  Q  A  A  T  I  T  Q  40
624  GATACTCCTATTAATCAGATTTTTACAGATACAGCTCTAGCGGAAAAAATGAAGACGGTC 683
42    D  T  P  I  N  Q  I  F  T  D  T  A  L  A  E  K  M  K  T  V  60
684  TTAGGAAAAACGAATGTAACAGACACGGTCTCGCAAACAGATCTAGACCAAGTTACGACG 743
63    L  G  K  T  N  V  T  D  T  V  S  Q  T  D  L  D  Q  V  T  T  80
744  CTTCAGGCGGATAGATTAGGGATAAAATCTATCGATGGATTGGAATACTTGAACAATTTA 803
84    L  Q  A  D  R  L  G  I  K  S  I  D  G  L  E  Y  L  N  N  L  100
804  ACACAAATAAATTTCAGCAATAATCAACTTACGGACATAACTCCACTTAAAGATTTAACT 863
105   T  Q  I  N  F  S  N  N  Q  L  T  D  I  T  P  L  K  D  L  T  120
864  AAGTTAGTTGATATTTTGATGAATAATAATCAAATAGCAGATATAACTCCGCTAGCTAAT 923
126   K  L  V  D  I  L  M  N  N  N  Q  I  A  D  I  T  P  L  A  N  140
924  TTGTCGAATCTAACTGGTTTGACTTTGTTCAACAATCAGATAACGGATATAGACCCGCTT 983
147   L  S  N  L  T  G  L  T  L  F  N  N  Q  I  T  D  I  D  P  L  160
984  AAAAATCTAACAAATTTAAATCGGCTAGAACTATCTAGTAACACGATTAGTGATATTAGT 1043
168   K  N  L  T  N  L  N  R  L  E  L  S  S  N  T  I  S  D  I  S  180
1044 GCGCTTTCAGGTTTAACTAGTCTACAGCAATTATCTTTTGGTAATCAAGTGACAGATTTA 1103
189   A  L  S  G  L  T  S  L  Q  Q  L  S  F  G  N  Q  V  T  D  L  200
1104 AAACCATTAGCTAATTTAACAACACTAGAACGACTAGATATTTCAAGTAATAAGGTGTCG 1163
210   K  P  L  A  N  L  T  T  L  E  R  L  D  I  S  S  N  K  V  S  220
1164 GATATTAGTGTTCTGGCTAAATTAACCAATTTAGAAAGTCTTATCGCTACTAACAACCAA 1223
231   D  I  S  V  L  A  K  L  T  N  L  E  S  L  I  A  T  N  N  Q  240
1224 ATAAGTGATATAACTCCACTTGGGATTTTAACAAATTTGGACGAATTATCCTTAAATGGT 1283
252   I  S  D  I  T  P  L  G  I  L  T  N  L  D  E  L  S  L  N  G  260
1284 AACCAGTTAAAAGATATAGGCACATTGGCGAGTTTAACAAACCTTACAGATTTAGATTTA 1343
273   N  Q  L  K  D  I  G  T  L  A  S  L  T  N  L  T  D  L  D  L  280
1344 GCAAATAACCAAATTAGTAATCTAGCACCACTGTCGGGTCTAACAAAACTAACTGAGTTA 1403
274   A  N  N  Q  I  S  N  L  A  P  L  S  G  L  T  K  L  T  E  L  300
1404 AAACTGGGAGCTAACCAAATAAGTAACATCAGTCCCCTAGCAGGTCTAACCGCACTCACT 1463
295   K  L  G  A  N  Q  I  S  N  I  S  P  L  A  G  L  T  A  L  T  320
1464 AACTTAGAGCTAAATGAAAATCAGTTAGAAGATATTAGCCCAATTTCTAACCTGAAAAAT 1523
316   N  L  E  L  N  E  N  Q  L  E  D  I  S  P  I  S  N  L  K  N  340
1524 CTCACATATTTAACGTTGTACTTTAATAATATAAGTGATATAAGCCCAGTTTCTAGTTTA 1583
337   L  T  Y  L  T  L  Y  F  N  N  I  S  D  I  S  P  V  S  S  L  360
1584 ACAAAGCTTCAAAGATTATTTTTCTATAATAACAAGGTAAGTGACGTAAGCTCACTTGCG 1643
358   T  K  L  Q  R  L  F  F  Y  N  N  K  V  S  D  V  S  S  L  A  380
1644 AATTTAACCAATATTAATTGGCTTTCGGCTGGGCATAACCAAATTAGCGATCTTACACCA 1703
379   N  L  T  N  I  N  W  L  S  A  G  H  N  Q  I  S  D  L  T  P  400
1704 TTGGCTAATTTAACAAGAATTACCCCATTAGGATTGAATGACCAAGAATGGACAAATCCA 1763
400   L  A  N  L  T  R  I  T  P  L  G  L  N  D  Q  E  W  T  N  P  420
1764 CCAGTGAACTACAAAGTAAATGTATCCATTCCAAACACGGTGAAAAATGTGACGGGCGCT 1823
421   P  V  N  Y  K  V  N  V  S  I  P  N  T  V  K  N  V  T  G  A  440
1824 TTGATTGCACCAGCTACTATTAGCGATGGTGGTAGTTATGCAGAACCTGATATAACATGG 1883
442   L  I  A  P  A  T  I  S  D  G  G  S  Y  A  E  P  D  I  T  W  460
1884 AATTTACCTAGTTATACCAATGAAGTAAGTTATACCTTTAACCAATCTGTCACCATTGGA 1943
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463   N  L  P  S  Y  T  N  E  V  S  Y  T  F  N  Q  S  V  T  I  G  480
1944 AAAGGAACGACAACATTTAGTGGAACTGTGACGCAGCCACTTAAGGCAATTTTTAATGCT 2003
484   K  G  T  T  T  F  S  G  T  V  T  Q  P  L  K  A  I  F  N  A  500
2004 AAGTTTCATGTGGACGGCAAAGAAACAACCAAAGAAGTGGAAGCTGGGAATTTATTGACT 2063
505   K  F  H  V  D  G  K  E  T  T  K  E  V  E  A  G  N  L  L  T  520
2064 GAACCAGCTAAGCCTGTAAAAGAAGGTTATACATTTGTTGGGTGGTTTGATGCCCAAACC 2123
526   E  P  A  K  P  V  K  E  G  Y  T  F  V  G  W  F  D  A  Q  T  540
2124 GGCGGAACTAAATGGAATTTCAGTACGGATAAAATGCCGACAAATGACATCGATTTATAT 2183
547   G  G  T  K  W  N  F  S  T  D  K  M  P  T  N  D  I  D  L  Y  560
2184 GCGCAATTTAGTATTAACAGCTACACAGCAACGTTTGATAATGACGGTGTAACAACATCT 2243
568   A  Q  F  S  I  N  S  Y  T  A  T  F  D  N  D  G  V  T  T  S  580
2244 CAAACAGTAGATTATCAAGGCTTGCTACAAGAACCTACGGCACCAACAAAAGAAGGTTAT 2303
589   Q  T  V  D  Y  Q  G  L  L  Q  E  P  T  A  P  T  K  E  G  Y  600
2304 ACTTTCAAAGGCTGGTATGACGCAAAAACTGGTGGTGACAAGTGGGATTTTGCAACTAGT 2363
610   T  F  K  G  W  Y  D  A  K  T  G  G  D  K  W  D  F  A  T  S  620
2364 AAGATGCCTGCTAAAAACATCACCTTATATGCTCAATATAGCGCCAATAGCTATACAGCA 2423
631   K  M  P  A  K  N  I  T  L  Y  A  Q  Y  S  A  N  S  Y  T  A  640
2424 ACCTTTGATGTTGATGGAAAAACAACGACTCAAGCAGTAGACTATCAAGGACTTCTAAAA 2483
652   T  F  D  V  D  G  K  T  T  T  Q  A  V  D  Y  Q  G  L  L  K  660
2484 GAACCAAAAACGCCAACAAAAGCCGGATATACTTTCAAAGGTTGGTATGACGAAAAAACA 2543
673   E  P  K  T  P  T  K  A  G  Y  T  F  K  G  W  Y  D  E  K  T  680
2544 GATGGTAAAAAATGGGATTTTGCGACAGATAAAATGCCAGCAAATGATATTACGCTGTAC 2603
694   D  G  K  K  W  D  F  A  T  D  K  M  P  A  N  D  I  T  L  Y  700
2604 GCTCAATTCACGAAAAATCCTGTGGCACCACCAACAACTGGAGGGAACACTCCGCCGACT 2663
714   A  Q  F  T  K  N  P  V  A  P  P  T  T  G  G  N  T  P  P  T  720
2664 ACAAATAACGGAGGGAACACTACACCACCTTCCGCAAATATACCTGGAAGCAACACATCT 2723
735   T  N  N  G  G  N  T  T  P  P  S  A  N  I  P  G  S  N  T  S  740
2724 AACACATCAACTGGGAATTCAGCTAGCACAACAAGTACAATGAACGCTTATGACCCTTAT 2783
756   N  T  S  T  G  N  S  A  S  T  T  S  T  M  N  A  Y  D  P  Y  760
2784 AATTCAAAAGAAGCTTCACTCCCTACAACTGGTGATAGCGATAATGCGCTCTACCTGTTG 2843
777   N  S  K  E  A  S  L  P  T  T  G  D  S  D  N  A  L  Y  L  L  780
2844 ATAGGATTGTTAGCAGTAGGAACTGCAGTGGCTCTTACTAAAAAAGCACGTGCTAGTAAA 2903
798   I  G  L  L  A  V  G  T  A  V  A  L  T  K  K  A  R  A  S  K  800
2904 TAGAAGTAGTGTAAAGAGCTAGATGTGGTTTTCGGACTATATCTAGCTTTTTTATTTTTT 2963
2964                                  AATAACTAGAATCAAGGAGAGGATAGT 2990
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INLB

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2991 GTGAAAGAAAAGCACAACCCAAGAAGGAAATATTGTTTAATCTCAGGTTTAGCTATTATT 3050
      V  K  E  K  H  N  P  R  R  K  Y  C  L  I  S  G  L  A  I  I  20
3051 TTTAGTTTATGGATAATTATTGGAAACGGGGCGAAAGTACAAGCGGAGACTATCACCGTG 3110
21    F  S  L  W  I  I  I  G  N  G  A  K  V  Q  A  E  T  I  T  V  40
3111 TCAACGCCAATCAAGCAAATTTTTCCAGATGATGCTTTTGCAGAAACAATCAAAGACAAT 3170
42    S  T  P  I  K  Q  I  F  P  D  D  A  F  A  E  T  I  K  D  N  60
3171 TTAAAGAAAAAAAGTGTGACGGATC                                    3195
63    L  K  K  K  S  V  T  D                                      71
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Figure 2B. Nucleotide sequence of the *L. monocytogenes* (serotype 4b) gene *inlC2* and deduced amino acid sequence of the corresponding protein. Nucleotides 532 to 1472 were derived from inserts in pSCRN92, pSCRN105, and pSCRN106. Nucleotide 1 to 806 and nt 1025 to 1832 were obtained from inserted DNA from pCR227-228 and pCR262-291. Nucleotides 2 to 1648 represents the ORF of *inlC2*.

Figure 2B

```
     InlC2
1    TATGAAGAAACGGTGGAATTCTGTATTTAAACTAGTTTTAATGGTAGCTGCTATTCTCGGT 61
1     M  K  K  R  W  N  S  V  F  K  L  V  L  M  V  A  A  I  L  G   20
62   ATTAGTCTATATGTAACGACAAGTCAAGGTGCGGAGGTTCGCGCGGAAAGCATTGCGCAG  121
21    I  S  L  Y  V  T  T  S  Q  G  A  E  V  R  A  E  S  I  A  Q   40
122  CCAACCCCAATTAATGTTATTTTCCCTGATCCGGCTCTTGCGAATGCAGTTAAAACAGCG  181
41    P  T  P  I  N  V  I  F  P  D  P  A  L  A  N  A  V  K  T  A   60
182  ACTGGAAAATCTAATGTAACAGACACTGTTACGCAAGCAGATTTAGATGGAATAGCTACT  241
61    T  G  K  S  N  V  T  D  T  V  T  Q  A  D  L  D  G  I  A  T   80
242  TTATCAGCATTTAATACTGGAGTAACAACGATAGAAGGAATACAATACTTAAATAATTTG  301
81    L  S  A  F  N  T  G  V  T  T  I  E  G  I  Q  Y  L  N  N  L   100
302  ATAGGGTTAGAACTTAAAGATAACCAAATAACTGATTTAACTCCTCTTAAAAATTTAACG  361
101   I  G  L  E  L  K  D  N  Q  I  T  D  L  T  P  L  K  N  L  T   120
362  AAAATAACAGAGCTTGAATTATCTGGAAATCCGTTAAAAAATGTGAGCGCAATTGCTGGG  421
121   K  I  T  E  L  E  L  S  G  N  P  L  K  N  V  S  A  I  A  G   140
422  TTACAAAGCATTAAAACGCTAGATTTAACTTCTACGCAAATTACAGATGTGGCTCCACTT  481
141   L  Q  S  I  K  T  L  D  L  T  S  T  Q  I  T  D  V  A  P  L   160
482  GCAGGTCTTTCCAATTTGCAGGTATTATATTTGGACCTCAATCAAATAACCGATCTAAGT  541
161   A  G  L  S  N  L  Q  V  L  Y  L  D  L  N  Q  I  T  D  L  S   180
542  CCGCTCGCAGGACTAACTAATTTACAATACTTATCAATCGGAAATAACCAAGTAAATGAT  601
181   P  L  A  G  L  T  N  L  Q  Y  L  S  I  G  N  N  Q  V  N  D   200
602  TTAACCCCACTTGCTAATTTATCTAAACTAACGACTTTAAGAGCTGATGATAATAAAATA  661
201   L  T  P  L  A  N  L  S  K  L  T  T  L  R  A  D  D  N  K  I   220
662  AGTGATATTTCGCCACTTGCGAGTTTACCTAACCTTATAGAAGTTCATTTGAAAGATAAT  721
221   S  D  I  S  P  L  A  S  L  P  N  L  I  E  V  H  L  K  D  N   240
722  CAAATTAGTGATGTCAGCCCACTTGCTAATTTATCGAACTTATTTATAGTCACTTTAACA  781
241   Q  I  S  D  V  S  P  L  A  N  L  S  N  L  F  I  V  T  L  T   260
782  AATCAAACAATTACCAACCAACCCGTGTATTATCAAAATAATCTTGTCGTTCCTAATGTA  841
261   N  Q  T  I  T  N  Q  P  V  Y  Y  Q  N  N  L  V  V  P  N  V   280
842  GTAAAAGGTCCTTCTGGCGCGCCTATTGCACCTGCTACTATTAGCGACAATGGAACATAC  901
281   V  K  G  P  S  G  A  P  I  A  P  A  T  I  S  D  N  G  T  Y   300
902  GCTAGTCCAAATTTAACATGGAATTTAACTAGTTTTATTAATAATGTTAGCTACACGTTT  961
301   A  S  P  N  L  T  W  N  L  T  S  F  I  N  N  V  S  Y  T  F   320
962  AACCAATCAGTCGCTTTCAAAAATACAACGGTTCCTTTCAGTGGAACAGTTACCCAACCA  1021
321   N  Q  S  V  A  F  K  N  T  T  V  P  F  S  G  T  V  T  Q  P   340
1022 TTAACAGAAGCTTACACTGCGGTTTTTGATGTGGATGGAAAACAAACAAGTGTGACAGTC  1081
341   L  T  E  A  Y  T  A  V  F  D  V  D  G  K  Q  T  S  V  T  V   360
1082 GGCGCGAATGAATTAATTAAAGAACCAACAGCACCAACGAAAGAAGGTTACACATTCACA  1141
361   G  A  N  E  L  I  K  E  P  T  A  P  T  K  E  G  Y  T  F  T   380
1142 GGCTGGTATGATGCCAAAACTGGCGGGAATAAATGGGATTTCAGCACGGACAAAATGCCA  1201
381   G  W  Y  D  A  K  T  G  G  N  K  W  D  F  S  T  D  K  M  P   400
1202 GCAGAAAATATCACATTGTATGCGCAGTTCACGATTAATAGCTATACTGCCACATTTGAC  1261
401   A  E  N  I  T  L  Y  A  Q  F  T  I  N  S  Y  T  A  T  F  D   420
1262 AATGATGGAAAAATAACGACGCAAAAAGTCACTTATCAAAGTCTGTTAGAAGAACCAGCA  1321
421   N  D  G  K  I  T  T  Q  K  V  T  Y  Q  S  L  L  E  E  P  A   440
1322 GCGCCAACGAAAGCAGGTTATACGTTCAAAGGTTGGTACGATGCTAAAACGGGTGGAACT  1381
441   A  P  T  K  A  G  Y  T  F  K  G  W  Y  D  A  K  T  G  G  T   460
1382 AAATGGGATTTTGCTACTGGTAAAATGCCGGCGGGAAATATTACACTATATGCCCAATTC  1441
461   K  W  D  F  A  T  G  K  M  P  A  G  N  I  T  L  Y  A  Q  F   480
1442 ACTAAAAATGATAGCCCAAATCCAAATGATCCAACTCCTAATACCCCAACAGGAAATGGT  1501
481   T  K  N  D  S  P  N  P  N  D  P  T  P  N  T  P  T  G  N  G   500
1502 GATGGTACAAGTAATCCAAGTGATTCAGGCGGTAATACCACACTTCCAACAGCTGGGGAC  1561
501   D  G  T  S  N  P  S  D  S  G  G  N  T  T  L  P  T  A  G  D   520
1562 GAAAATACTATGCTTCCAATTTTTATCGGAGTTTTCTTGTTAGGGACAGCAACGCTAATT  1621
521   E  N  T  M  L  P  I  F  I  G  V  F  L  L  G  T  A  T  L  I   540
1622                                  CTCCGCAAAACAATCAAAGTGAAATAA  1648
541                                   L  R  K  T  I  K  V  K  *   548
```

1649 CAACAAAAACAGCTGCGGTTTATAGTTTCTTTATAGACTTCAGCTGTTTTTTGTTAATAG 1708
1709 GGGAATTCATCTAAATGTCACAACTAATCAATAATGAGTCTAATTCTAGTGATTAAACGA 1768
1769 AATTTGATTAAGTTATAATGAAATAATTCAGCTATGAATACATACACAGTCCTAAGAAAT 1828
1829 TGAA 1832

Figure 3B. Nucleotide sequence of the *L. monocytogenes* (serotype 4b) gene *inlD* and the deduced amino acid sequence of the corresponding protein. Nucleotides 500 to 2288 were derived from pSCRN54, pSCRN91, pSCRN112, pSCRN139, and pSCRN143. Nucleotides 1 to 2288 were derived from inserts in pSCRN114, and pSCRN159. Nucleotides 389 to 2095 represent the ORF of *inlD*. A partial ORF coding for a protein similar to InlD is located 178 bp upstream of *inlD*. A predicted promoter (-10 and -35 elements) and a putative ribosome binding site (AAGAGA) upstream of *inlD* are underlined. A potential stem-loop structure (16-bp inverted repeats) immediately followed by a string of Ts, similar to a typical prokaryotic *rho*-independent transcription terminator, is indicated by two facing arrow.

Figure 3B

```
     Inl
1    GATCCAACTCCTAATACCCCAACAGGAAATGGTGATGGTACAAGTAGTCCAAGTGATTCA  60
1     D  P  T  P  N  T  P  T  G  N  G  D  G  T  S  S  P  S  D  S   20
61   GGCGGTAATACCACACTTCCAACAGCTGGGGACGAAAATACTATGCTTCCAATTTTTATC  120
21    G  G  N  T  T  L  P  T  A  G  D  E  N  T  M  L  P  I  F  I   40
121  GGAGTTTTCTTGTTAGGGACAGCAACGCTAATTCTCCGCAAAACAATCAAAGTGAAATAA  180
41    G  V  F  L  L  G  T  A  T  L  I  L  R  K  T  I  K  V  K  *   59
181  CAACAAAAACAGCTGCGGTTTATAGTTTCTTTATAGACTTCAGCTGTTTTTGTTAATAG  240
                                                          -35
241  GGGAATTCATCTAAATGTCACAACTAATCAATAATGAGTCTAATTCTAGTGATTAAACGA  300
                  -10
301  AATTTGATTAAGTTATAATGACATAATTCAGCTATGAATACATACACAGTCCTAAGAAAT  360
                  RBS           InlD
361  AGAATAATATTATAAAAGAGATAAGTTAATGAAAAGAAAAAAAACAGCATTACGTATCTTG 421
1                                 M  K  R  K  K  T  A  L  R  I  L  11
422  GTAACTCTGGCTGTAGTAATGGCAATTAGCTTTTGGGTAGGGACTAGCTCAAAAGAGGAA  481
12    V  T  L  A  V  V  M  A  I  S  F  W  V  G  T  S  S  K  E  E   31
482  GTACAGGCGGCAGTAATTGATCAACCGACGCCAATTAATGAAATTTTTACAGATGAGAAT  541
32    V  Q  A  A  V  I  D  Q  P  T  P  I  N  E  I  F  T  D  E  N   51
542  TTAGCAAACGCAATAAAAGCAACTTTAAATAAACCTAGTACTACATCTGACGTTTCACAA  601
52    L  A  N  A  I  K  A  T  L  N  K  P  S  T  T  S  D  V  S  Q   71
602  GCAGAGCTAGATTCTATAAGTGAAGTAACGGCAGAAAGTAGCAATATAGCTTCTTTAGAG  661
72    A  E  L  D  S  I  S  E  V  T  A  E  S  S  N  I  A  S  L  E   91
662  GGGGCGCAGTATTTAAATAATATAGATACATTAATTTTAAATAATAATAAAATAACGGAT  721
92    G  A  Q  Y  L  N  N  I  D  T  L  I  L  N  N  N  K  I  T  D   111
722  TTAAATTCACTTGCAGGACTATCTAAATTAAGGATATTAGAAGCAAATGACAATCAATTA  781
112   L  N  S  L  A  G  L  S  K  L  R  I  L  E  A  N  D  N  Q  L   131
782  AGCGATATAAGTGCACTAGCAAATGTGACAAGTCTGCACCAATTAAGATTAGCTGGAAAT  841
132   S  D  I  S  A  L  A  N  V  T  S  L  H  Q  L  R  L  A  G  N   151
842  CAAATAAAACAGTTAAATGGAGTTAGTAATTTAATCAATCTAGAAACCATAGAACTAAGC  901
152   Q  I  K  Q  L  N  G  V  S  N  L  I  N  L  E  T  I  E  L  S   171
902  AACAATCAAATAACGGATATAAGCCCAGTTTCAGGTTTGAAAAACTTAATTGGTCTTGGA  961
172   N  N  Q  I  T  D  I  S  P  V  S  G  L  K  N  L  I  G  L  G   191
962  ATAGATTATAATGATATCAGTGATTTAAGCCCAATTGCTGGCTTAGCAAAGTTAAGTCAT  1021
192   I  D  Y  N  D  I  S  D  L  S  P  I  A  G  L  A  K  L  S  H   211
1022 TTAACTGCAGACAATAACCAAATAAGTGATTTGAGTCCTATTTCAAGCTTAGGGGCTATG  1081
212   L  T  A  D  N  N  Q  I  S  D  L  S  P  I  S  S  L  G  A  M   231
1082 GAAATTATGCGACTAGATAAAAATCAAATCAGTGATGTAACCCCAATTGCGAATTTGGCT  1141
232   E  I  M  R  L  D  K  N  Q  I  S  D  V  T  P  I  A  N  L  A   251
1142 AATTTAAGCTATGTATTTTTAGCTGAAAATCAAATCAGTGATATTAGTTCTTTACAACCA  1201
252   N  L  S  Y  V  F  L  A  E  N  Q  I  S  D  I  S  S  L  Q  P   271
1202 CTTTTTAATTCACCTAATTTTTTTGGTATTACTTTAGACAATCAAAAAATAACGAGTGAA  1261
272   L  F  N  S  P  N  F  F  G  I  T  L  D  N  Q  K  I  T  S  E   291
1262 CCAGTATTGTACCAACAAGAGCTTGTGGTCCCCAATAACATTAAGGATGAAATGGGAGCG  1321
292   P  V  L  Y  Q  Q  E  L  V  V  P  N  N  I  K  D  E  M  G  A   311
1322 TTAATCTCACCAGATACAATTAGCGATAATGGTGTTTATGCAAGCCCGAATATTAAGTGG  1381
312   L  I  S  P  D  T  I  S  D  N  G  V  Y  A  S  P  N  I  K  W   331
1382 AACTTGGCTAATTATACTAACCAAGTAAGTTACACATTTAATAAACAATTAGCACAGGGT  1441
332   N  L  A  N  Y  T  N  Q  V  S  Y  T  F  N  K  Q  L  A  Q  G   351
1442 TCTTTTAGCGGAACAGTGACCCAACCACTTCACAATGCTTATACAGCGACATTTGACGTA  1501
352   S  F  S  G  T  V  T  Q  P  L  H  N  A  Y  T  A  T  F  D  V   371
1502 GATGGAGTGAAAACAAATGAAGTAGTGGAAGAAACGAAATTGCTTCAAGAACCAACAGCT  1561
372   D  G  V  K  T  N  E  V  V  E  E  T  K  L  L  Q  E  P  T  A   391
1562 CCAACGAAAGAAGGATACACTTTCACAGGCTGGTATGATGCGAAAACTGGCGGGAATAAA  1621
392   P  T  K  E  G  Y  T  F  T  G  W  Y  D  A  K  T  G  G  N  K   411
1622 TGGGATTTTGCGACAGACAAAATGCCAGCAGAAGACATCACATTATACGCGCAGTTCACG  1681
412   W  D  F  A  T  D  K  M  P  A  E  D  I  T  L  Y  A  Q  F  T   431
```

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1682 ATTAATAGCTATACAGCCACATTCGATATCGATGGGAAATTAACGACGCAAAAGTCACT  1741
432   I  N  S  Y  T  A  T  F  D  I  D  G  K  L  T  T  Q  K  V  T   451
1742 TATCAAAGCTTGCTAGAAGAACCAGCAGCGCCAACGAAGGATGGCTATACGTTCATAGGT  1801
452   Y  Q  S  L  L  E  E  P  A  A  P  T  K  D  G  Y  T  F  I  G   471
1802 TGGTATGATGCTAAAACAGGTGGAACTAAATGGGATTTTGCGACAGACAAAATGCCAGCT  1861
472   W  Y  D  A  K  T  G  G  T  K  W  D  F  A  T  D  K  M  P  A   491
1862 GGAAATATAACGCTATACGCCCAGTTTACTAAAAATGCCACCCCAGATTCCAATGACCCA  1921
492   G  N  I  T  L  Y  A  Q  F  T  K  N  A  T  P  D  S  N  D  P   511
1922 ACAACGGTTACCCCAACAGGAAATGGCAATGGGACAAGTACCCCAAGCAATTCAGGTGGA  1981
512   T  T  V  T  P  T  G  N  G  N  G  T  S  T  P  S  N  S  G  G   531
1982 AATACCACGCTTCCGACAGCTGGGGACGAAAATACCATGCTTCCAATTTTTGTAGGAGTT  2041
532   N  T  T  L  P  T  A  G  D  E  N  T  M  L  P  I  F  V  G  V   551
2042 TTCTTGTTAGGAACAGCAACGCTTATTCTCCGCAAAACAATCAAAGTAAAATAACAACAA  2101
552   F  L  L  G  T  A  T  L  I  L  R  K  T  I  K  V  K *          569
2102 AAAAACAGCTGAGGTCTATAGTTTTTCTATAGATTTCGGCTGTTTTTTTATTAGCAAAGA  2161
          ────────────────►     ◄────────────────

2162 AATTCATCTAAACGTCACAACCTAAAACGGTAACAAGTCTAATTTTAGTGATTGAATGAA  2221
2222 AACCTACTACGCTATAATATAGTAACTCAACTACGAGAACATACAAATTTCTAAAAGCAG  2281
2282 AACGATC                                                       2288
```

Figure 4B. Nucleotide sequences of the *L. monocytogenes* (serotype 4b) gene *ispWei* and a gene (in the reverse orientation) coding for a protein similar to Lin0373 or Lmo0354 and the deduced amino acid sequences of the corresponding proteins. Nucleotides 1439 to2258 were derived from the insert in pSCRN93. Nucleotides 1140 to 3075 were derived from inserts in pSCRN88, pSCRN101, pSCRN102, and pSCRN106. Nucleotides 1 to 1150 were obtained from pCR253-254. Nucleotides 156 to 1926 represent the ORF of *ispWei*. The partial ORF of a gene coding for a protein similar to Lin0373 or Lmo0354 is indicated by nt 2054 to 3075

Figure 4B

```
1                                 ATTTGGCTTTCGGGAGTCAGCACCAGCTTCAATCG  34
35   GAATGATTTACCAAATGAAATGAAAACAGCTCGGGGACGCCCTCAAGCTGTTTTTTTATG  95
96   TTTCTGGTGATAAGAAAATGGGAACAAGATACTATTAAACTTTTTGTCAGGGGGAGCAAG  156
     IspWei
157  ATGAAATCCAAAGGGAGACTGTTTTTGTACGTTGTTTTGGCGTTGTCGATTGTTATTGGG  217
1     M  K  S  K  G  R  L  F  L  Y  V  V  L  A  L  S  I  V  I  G   20
218  ACGAATGTATTTATAAAAATAGACGCACATGCAGCCGCTGCACCACCAGCTGCAATCAAC  277
21    T  N  V  F  I  K  I  D  A  H  A  A  A  A  P  P  A  A  I  N   40
278  CAGATTTTTCCCGATGATGCTTTAGCGACAGAAATTCAAACTACACTCGGAAAGTCTAGT  337
41    Q  I  F  P  D  D  A  L  A  T  E  I  Q  T  T  L  G  K  S  S   60
338  ACAGCAGAGGTTGTCACTCAAACAGATTTAGATACTATTAATTCATTAACCCTTACTTCT  397
61    T  A  E  V  V  T  Q  T  D  L  D  T  I  N  S  L  T  L  T  S   80
398  AAAGGTATCAGCTCCCTAGAAGGTATGAACTACTTGACCAATTTAGGAACTTTAATTTTA  457
81    K  G  I  S  S  L  E  G  M  N  Y  L  T  N  L  G  T  L  I  L   100
458  ACTGGTAATCAAGTAAGCGATATTTCGCCACTTAAAGGCTTAACTAACTTGACCATGCTT  517
101   T  G  N  Q  V  S  D  I  S  P  L  K  G  L  T  N  L  T  M  L   120
518  CAGCTGAGTGGAAATCCGATTAGCGACATTAGCGCACTTAGTAACTTGAAAAACTTACAA  577
121   Q  L  S  G  N  P  I  S  D  I  S  A  L  S  N  L  K  N  L  Q   140
578  GCTCTAGATATTAACGACGCGCAAGTTACTGACATCACGCCACTGAGCGGTTTAACAAAT  637
141   A  L  D  I  N  D  A  Q  V  T  D  I  T  P  L  S  G  L  T  N   160
638  CTAAAGGGTTTAGGCTTATATAATAATCAATTGGAGAACCTTAGTGGAGTAAATAGTTTA  697
161   L  K  G  L  G  L  Y  N  N  Q  L  E  N  L  S  G  V  N  S  L   180
698  CATCAACTCCGCTCCTTAAATGTTAGTAATAATAAGCTTACGAACCTAGATGAGCTACAA  757
181   H  Q  L  R  S  L  N  V  S  N  N  K  L  T  N  L  D  E  L  Q   200
758  GCGCTAAGCAATTTAAGCGTTCTATATGCAAATGAAAACCAAATTAACAATCTTCAGGGG  817
201   A  L  S  N  L  S  V  L  Y  A  N  E  N  Q  I  N  N  L  Q  G   220
818  CTAAGTAATTTAAATAACCTTTTCTTGCTTGATTTATCAGCGAACCAAATTGTGGATACT  877
221   L  S  N  L  N  N  L  F  L  L  D  L  S  A  N  Q  I  V  D  T   240
878  ACTCCACTCGCTGGCTTAACTAAGGTACAAACATTATACGTATCAAACAATCAAATTAGT  930
241   T  P  L  A  G  L  T  K  V  Q  T  L  Y  V  S  N  N  Q  I  S   260
931  GATGTAACTGGTCTTTCGAGTTTAATAAATCTAGATTGGCTCGATATAAGCCAAAATAAA  990
261   D  V  T  G  L  S  S  L  I  N  L  D  W  L  D  I  S  Q  N  K   280
991  ATTAGTAACATTAGACCTTTAAACAGTTTAACGAAGCTAACAATTATTCAAATGACTAAT  1050
281   I  S  N  I  R  P  L  N  S  L  T  K  L  T  I  I  Q  M  T  N   300
1051 CAATTAATCGTAAACGAACCGATAAGTTTCGAAAGCACTGTAACGATTCCTAATTTAATA  1110
301   Q  L  I  V  N  E  P  I  S  F  E  S  T  V  T  I  P  N  L  I   320
1111 AAAAACATTGCGGAACAAACGATTGATCCCGATACAATTAGCGATAACGGTGTGTATGCG  1170
321   K  N  I  A  E  Q  T  I  D  P  D  T  I  S  D  N  G  V  Y  A   340
1171 AATGAAGCTGTTACATGGAATTTACCTAGTTATATTCCTAAAGTCAGCTACACCTTTATT  1230
341   N  E  A  V  T  W  N  L  P  S  Y  I  P  K  V  S  Y  T  F  I   360
1231 GAACGTGATACGATTGGTAATGCAACTGGAAATTTCAGTGGGACTGTAGAACAACCATTA  1290
361   E  R  D  T  I  G  N  A  T  G  N  F  S  G  T  V  E  Q  P  L   380
1291 GTACAATATTTTAAAGCAACTTTCAATATTGACGGGCAAGAAACAACGGAAAATGTCGAA  1350
381   V  Q  Y  F  K  A  T  F  N  I  D  G  Q  E  T  T  E  N  V  E   400
1351 ACAGGGACACTTTTACAAGAACCACCAACCCCAGTCAAAGAGGGTTATACATTTAACGGA  1410
401   T  G  T  L  L  Q  E  P  P  T  P  V  K  E  G  Y  T  F  N  G   420
1411 TGGTATGATGCTGAAACAGGTGGAACAAAATGGGACTTTACAGCCGATACGATGCCAGCA  1470
421   W  Y  D  A  E  T  G  G  T  K  W  D  F  T  A  D  T  M  P  A   440
1471 AATGATATAACGTTGTATGCCCAGTTTAGTATTAATAGTTATACCGCAACTTTTGATGTA  1530
441   N  D  I  T  L  Y  A  Q  F  S  I  N  S  Y  T  A  T  F  D  V   460
1531 GATGGCGTCATTTCTACTCAAGCGGTAGAGTATCAAGGTTTACTGGAAGAGCCACCAGCG  1590
461   D  G  V  I  S  T  Q  A  V  E  Y  Q  G  L  L  E  E  P  P  A   480
1591 CCAACTAAGGATGGTTATACATTTAAAGGTTGGTATGATGCCAAAACTGGTGGAACAAAA  1650
481   P  T  K  D  G  Y  T  F  K  G  W  Y  D  A  K  T  G  G  T  K   500
1651 TGGGACTTCACAAACAATCAAATGCCAGCAAATGATATAACCTTGTATGCCCAGTTTAGT  1710
501   W  D  F  T  N  N  Q  M  P  A  N  D  I  T  L  Y  A  Q  F  S   520
1711 AAAGATGCATCTTCTGGTGGGGACGGTGGAGGAACCGATGAAGGTGGAGGTAACTCTGAA  1770
521   K  D  A  S  S  G  G  D  G  G  G  T  D  E  G  G  G  N  S  E   540
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1771 AATAGTACAGAGGGAGCTCCTAATACGAGTGATATAGATACTATAAACCACATCGTACTA  1830
541   N  S  T  E  G  A  P  N  T  S  D  I  D  T  I  N  H  I  V  L   560
1831 CCAGCAACAGGAGATGACCATGTGCTTTTTCCAATCTTTATTGGAACATTTCTTACGAGT  1890
561   P  A  T  G  D  D  H  V  L  F  P  I  F  I  G  T  F  L  T  S   580
1891                              TTAGCATTACTTACACTTAGAAGAAAATAA   1927
581                                L  A  L  L  T  L  R  R  K  *    589
1928 AGGAAAAAGCAGCTGCATAACGCAGCTGCTTTTTTGAAACTTATTTTTTCAATTCTCGTT  1987
1988 ATCTGCAAACGTTGTACTTTCATTGTTGCAGTACGAGGGATTTCATCATATTTCATGACA  2045
2046 ATTGGCTCATTCATATGTGGTAGGTCAGAACTGCGTCCCACCATGCATCCCAATTCATTT  2105
               *  I  H  Y  T  L  V  A  D  W  W  A  D  W  N  M  E
2106 CTTTATCATCATGAACTGCGATAATTGGTTGCGGGCTGCCATTTTCACCACGGATAATGA  2165
      K  D  D  H  V  A  I  I  P  Q  P  S  G  N  E  G  R  I  I  V
2166 CAACTTCATCTAAGAAAGTCAGCGTATCAAGCAGTTTATCTTCAATTGCGAGTGTACTAT  2225
      V  E  D  L  F  T  L  T  D  L  L  K  D  E  I  A  L  T  S  D
2226 CAATTGTTTCTACTAAATCGACTTGACGATCTTGCAAGAATAACCGGCCTTGTTCATCTT  2285
      I  T  E  V  L  D  V  Q  R  D  Q  L  F  L  R  G  Q  E  D  K
2286 TCATACCATAATCGCCGCTATCCCACCAAGGTCCGTACACATTCTCTTCAAAACGAGCTT  2345
      M  G  Y  D  G  S  D  W  W  P  G  Y  V  N  E  E  F  R  A  E
2346 CTTCTTTGTAATAAGTAAGCGCGCGACCTTTGGAAAGCATTTGAATATTACCACTAACCC  2405
      E  K  Y  T  T  L  A  R  G  K  S  L  M  Q  I  N  G  S  V  G
2406 CAGCTGGAACAGGATTACCATCTTGGTCAACAATCCGAACTTCTGTTAAACCAGGAACAC  2465
      A  P  V  P  N  G  D  Q  D  V  I  R  V  E  T  L  G  P  V  G
2466 CAATTCCCATATCGCGCGCATTTAGCGTTTCAATGGATTGAAGCGTATGACCACGTAAAA  2525
      I  G  M  D  R  A  N  L  T  E  I  S  Q  L  T  H  G  R  L  I
2526 TCATCGGACCACATTCACTTTGTCCATAAATTTGTAAGAAAATTGGTTTTTTATATTCGG  2585
      M  P  G  C  E  S  Q  G  Y  I  Q  L  F  I  P  K  K  Y  E  S
2586 AAGTACGAAGGAAAACCGCCATTGTTTCTTTATTAATCGCATCAAAAGTAGAATGATAAA  2645
      T  R  L  F  V  A  M  T  E  K  N  I  A  D  F  T  S  H  Y  F
2546 ATTTGATACTTTGGAAAACGTCTGGTTTTTCACGAGCAAGTGACGCCCATTGAACAAAAT  2705
      K  I  S  Q  F  V  D  P  K  E  P  A  L  S  A  W  Q  V  F  H
2706 GATTTGGATGCGTTTCGAGTACGTACGGTTTGTATTCGCGTAGCACTTTTTCGACGTTCG  2765
      N  P  H  T  E  L  V  Y  P  K  Y  E  R  L  V  K  E  V  N  A
2766 CTTTAGTCGGATTGGCAATTGGCAGAAGCGGGAAACCGAGCGACATCAAGGATGAAACAC  2825
      K  T  P  N  A  I  P  L  L  P  F  G  L  S  M  L  S  S  V  G
2826 CAATATTAAAACGAGAATGTACTGGTGAGATATGGAAAGCTACAAGCCCTCTTGGTTTGA  2885
      I  N  G  R  S  H  V  P  S  I  H  G  A  V  L  G  R  P  K  I
2886 TGAAATTAAGAATACGTCGTTGGTATTTCGTTCTCCAACCCATCGAATTCGCAGAGTGAG  2945
      F  N  L  I  R  R  Q  Y  K  T  R  W  G  M  S  N  A  S  H  A
2946 CGATTAATTTTGGCACACCAGTTGTTCCAGAAGTGTGAGTCATATAAGCAATCATATCTT  3004
      I  L  K  P  V  G  T  T  G  S  T  H  T  M  Y  A  I  M  D  K
                                     Similar to Lin0373 or Lmo0354
3005 TTTGGTAATTCTTCTTGTTCGCATGTGTAGCCATCTAGTGGCGCTTTAAACAATTGTTCAG 3065
      P L  T  E  Q  E  C  E  Y  G   D   L  P  A  K  F  L  Q  W  Q

3066  ATTGATC                                                      3074
       N  I
```

Figure 5B. Nucleotide sequence of the *L. monocytogenes* (serotype 4b) gene *ispLing* and deduced amino acid sequence of the corresponding protein. Nucleotides 819 to 2179 were derived from inserts in pSCRN79. Nucleotides 1 to 920 and nucleotides1971 to 2371 were obtained from pCR268-279 and pCR257-317. Nucleotides 270 to 2232 represent the ORF of *ispLing*.

Figure 5B

```
1    TATCGCTTCCATTTCATACCTCAATGTTTTCTTTTTATTCTAGCAGAATTCTATTCCAAA  60
61   TGGTTCTTTTTTGACTAGTTATATCAGCCTATTTCCCCTAAAATCCTACTTCAGACGTAT  120
121  AGGTTATAACTTGCCTTTTATTAATCTTGTGTTAGAATTATGTTAAAGTGGGTGCTGTAT  180
181  GAAAATGTTTCTTGTTACATAATACAGCTTCTTTTTTTCATTTCAAAATAACAATTAAAA  240
                                    IspLing
241  CGAGTAAATAGTGTGTAGGAGAGGTGTTTCATGAAGGATTGGAAAATAAAAATCCAAACG  300
1                                    M  K  D  W  K  I  K  I  Q  T   20
301  TTTTTAAGCAAGAATTATGGATTTTTTGTTCTAGCCGTCATTCTTTATTGGCTAAAAACG  360
21    F  L  S  K  N  Y  G  F  F  V  L  A  V  I  L  Y  W  L  K  T   40
361  TATATTGCATATCAACTTGAATTTAAACTTGGCATAGAAAATCTGATGCAGCAAATTTTG  420
41    Y  I  A  Y  Q  L  E  F  K  L  G  I  E  N  L  M  Q  Q  I  L   60
421  CTGTTCATTAATCCATTAAGTGGGGCCATTTTCTTTATGGGTCTTGCGCTCTTTGCTAAG  480
61    L  F  I  N  P  L  S  G  A  I  F  F  M  G  L  A  L  F  A  K   80
481  GGACGTCGTTCATTCATTTGGATTATCGTCATTGACTTTTTAATGAGTTTTATTCTTTAC  540
81    G  R  R  S  F  I  W  I  I  V  I  D  F  L  M  S  F  I  L  Y   100
541  GCAAATATTGTATATTATCGATTCTTTAGTGATTTCATTACACTTCCGAATCTAAACGCA  600
101   A  N  I  V  Y  Y  R  F  F  S  D  F  I  T  L  P  N  L  N  A   120
601  AAACAAATGCAAAACATGGGCGACATGGGAAGTAGTATCACAGCGTTACTCAGCTGGCAC  660
121   K  Q  M  Q  N  M  G  D  M  G  S  S  I  T  A  L  L  S  W  H   140
661  GATATTATTTACTTCGCGGATATTATCATTTTAATTGCACTACTTGCTTTCCGCTTTGTA  720
141   D  I  I  Y  F  A  D  I  I  I  L  I  A  L  L  A  F  R  F  V   160
721  AAACCAAACAAAACAGCCCGTATTCGTGCAAGAAAAGTAGTTGGGGTTCTGACGCTTGGT  780
161   K  P  N  K  T  A  R  I  R  A  R  K  V  V  G  V  L  T  L  G   180
781  ATCGCAATGTTCTTTGGAAACTTGGGACTTGCAGAAATTGATCGTCCACAACTTCTAACA  840
181   I  A  M  F  F  G  N  L  G  L  A  E  I  D  R  P  Q  L  L  T   200
841  AGAACGTTTGATAGAAATTATATTGTTAAATATCTTGGTATGACTAACTATCAAATCTAT  900
201   R  T  F  D  R  N  Y  I  V  K  Y  L  G  M  T  N  Y  Q  I  Y   220
901  GACGCAGTGAAAAGTACCGAATCATCCACACAGCGTGCACTTGCTGATAGTAGTGATGTT  960
221   D  A  V  K  S  T  E  S  S  T  Q  R  A  L  A  D  S  S  D  V   240
961  ACAGAAGTCTTAAACTATACCAAATCCAAATATGCCGCGCCAAATCCGGAATATTTTGGT  1020
241   T  E  V  L  N  Y  T  K  S  K  Y  A  A  P  N  P  E  Y  F  G   260
1021 AAAGCAAAAGGCAAAAACGTCATTTATATCCATTTAGAGAGTTTCCAACAATTCCTAGTA  1080
261   K  A  K  G  K  N  V  I  Y  I  H  L  E  S  F  Q  Q  F  L  V   280
1081 AATTACAAATTAAATGGAGAAGAAGTAACGCCGTTTATTAACTCTTTCTTTAAAGACCAA  1140
281   N  Y  K  L  N  G  E  E  V  T  P  F  I  N  S  F  F  K  D  Q   300
1141 AATACACTAAGCTTTACAAACTTCTTCCACCAAACAGGTCAAGGTAAAACAGCTGACTCC  1200
301   N  T  L  S  F  T  N  F  F  H  Q  T  G  Q  G  K  T  A  D  S   320
1201 GACATGTTACTTGAGAACTCGCTTTACGGCTTGCCTCAAGGTTCTGCCTTTACAACTAAA  1260
321   D  M  L  L  E  N  S  L  Y  G  L  P  Q  G  S  A  F  T  T  K   340
1261 GGGCAAAACACTTATGAATCTGCATCTGCTATTTTAGGCCAACAAGGCTATACAAGTGCT  1320
341   G  Q  N  T  Y  E  S  A  S  A  I  L  G  Q  Q  G  Y  T  S  A   360
1321 GTATTCCACGGTAACTATAAGAGCTTCTGGAATCGTGATGAAATTTATAAACAATTTGGT  1380
361   V  F  H  G  N  Y  K  S  F  W  N  R  D  E  I  Y  K  Q  F  G   380
1381 TACGATAATTTCTTTGATGCTAGTTACTACGATATGAACGAAGCAGATGTTTCTAACTAC  1440
381   Y  D  N  F  F  D  A  S  Y  Y  D  M  N  E  A  D  V  S  N  Y   400
1441 GGACTTAAAGATAAACCATTCTTTAAGGAATCCGAAGAATATCTATCGTCATTACAACAA  1500
401   G  L  K  D  K  P  F  F  K  E  S  E  E  Y  L  S  S  L  Q  Q   420
1501 CCGTTCTACACGAAATTTATTACGCTTACGAACCACTTCCCTTATCCAATTGATGAGAAA  1560
421   P  F  Y  T  K  F  I  T  L  T  N  H  F  P  Y  P  I  D  E  K   440
1561 GATGCTTCGATTGCTCCGGCAACAACAGGCGATTCATCTGTAGATACGTATTTCCAAACA  1620
441   D  A  S  I  A  P  A  T  T  G  D  S  S  V  D  T  Y  F  Q  T   460
1621 GCTCGTTATTTAGACGAATCTGTGAAGAGCTTTGTTGATTACTTGAAGAAATCTGGTCTT  1680
461   A  R  Y  L  D  E  S  V  K  S  F  V  D  Y  L  K  K  S  G  L   480
1681 TACGATAACTCTGTCATTATCATGTACGGTGACCATTATGGTATTTCCGACAACCATGAA  1740
481   Y  D  N  S  V  I  I  M  Y  G  D  H  Y  G  I  S  D  N  H  E   500
1741 GAAGCAATGACAAAAATTCTTGGTAAAGATTACAACACTTTTGAAAATGCCCAAGCGCAA  1800
501   E  A  M  T  K  I  L  G  K  D  Y  N  T  F  E  N  A  Q  A  Q   520
1801 CGTGTTCCTTTAATGATTCACGTTCCTGGTGTTCAAGGTGGCGTTCAAGAACAATATGGT  1860
521   R  V  P  L  M  I  H  V  P  G  V  Q  G  G  V  Q  E  Q  Y  G   540
```

```
1861 GGCCAAGTTGACTTACTTCCGACATTACTTCACTTACTTGGTGTTGATAATAAAGAGTAC  1920
541   G  Q  V  D  L  L  P  T  L  L  H  L  L  G  V  D  N  K  E  Y  560
1921 TTGCAATTTGGTACAGATTTACTTTCTAAAGACCATAAACAACTTGTTCCATTCCGAAAT  1980
561  L  Q  F  G  T  D  L  L  S  K  D  H  K  Q  L  V  P  F  R  N   580
1981 GGCGATTATATAACTCCAACCTATTCAATGATTGGTGGTAACATGTATAATCAACAAACT  2040
581   G  D  Y  I  T  P  T  Y  S  M  I  G  G  N  M  Y  N  Q  Q  T  600
2041 GGGGAACCAATTGCTACGGAAACAAAAGAAATGAAAGAAACAAAAGAAAAAGTAGCAAAA  2100
601   G  E  P  I  A  T  E  T  K  E  M  K  E  T  K  E  K  V  A  K  620
2101 GAATTAGAGCTTTCAGATTCTGTACTACAAGGTGACTTGTTACGTTTCTATGCTCCTGAT  2160
621   E  L  E  L  S  D  S  V  L  Q  G  D  L  L  R  F  Y  A  P  D  640
2161 GGTTTCAAAAAAGTGGATCCAAGTAAATACAATTACAATAAGAAAAAATCAACTGATTCA  2220
641   G  F  K  K  V  D  P  S  K  Y  N  Y  N  K  K  K  S  T  D  S  660
2221 TCCGATAAATGAGAAAAAGAGTCCAGAATATCTGGACTCTTTTTTACTTACCTAAACCTC  2280
661   S  D  K  *                                                  663
2281 TCATAATATACGCCACCGTATCATCAATTTCTTTGGCATCATCCCACTCCACGTCGGGCA  2340
2341 TCACGACAAATCTTGTTAGCATAAATCCGAC                               2371
```

Figure 6B. Nucleotide sequence of the *L. monocytogenes* (serotype 4b) gene *ispYu* (partial) and deduced amino acid sequence of the corresponding protein. Nucleotides 1 to 1325 were derived from inserts in pSCRN119 and pSCRN153. Nucleotides 1 to 978 represent the partial ORF of *ispYu*. The partial ORF of a gene (in the reverse orientation) coding for protein similar to Lin1065 is indicated by nucleotides 1043 to 1325.

Figure 6B

```
     IspYu
1    GATCAAGAGGAATACAATAAAACAGTTGCTATTGACGCAGTAGTAAAAAATGTGAAGGGT  60
1     D  Q  E  E  Y  N  K  T  V  A  I  D  A  V  V  K  N  V  K  G   20
61   AATGCTGTATGGACAGAACCTTACCGTACAGTTGGTACAAAATTAATCGGACCAGCGGAA  120
21    N  A  V  W  T  E  P  Y  R  T  V  G  T  K  L  I  G  P  A  E   40
121  ACTTACTTGAATAAAGAAGTGGAAGTCGTCCGTGAAGCAAAAACGCCAAAAGGAACTTAC  180
41    T  Y  L  N  K  E  V  E  V  V  R  E  A  K  T  P  K  G  T  Y   60
181  TACCAATTTAAATCTGGTGGCAAAGTAATCGGCTGGTTAGATAAAAAAGCTTTCGATGTA  240
61    Y  Q  F  K  S  G  G  K  V  I  G  W  L  D  K  K  A  F  D  V   80
241  TATGACAATATTAATTACAACAAAGCGGTTAATTTAGATGCTGTAGTGGAAAATGTGACA  300
81    Y  D  N  I  N  Y  N  K  A  V  N  L  D  A  V  V  E  N  V  T   100
301  GGTAATGCAGTTTGGACGGCTCCTTATAAGAGTAAAGGTGTTAAACTTGTTACTTCAGCA  360
101   G  N  A  V  W  T  A  P  Y  K  S  K  G  V  K  L  V  T  S  A   120
361  GCAACCTATAAAGGCAAGGCAACAAAAATAACTCGTGAAGCGCAAACAAGTAGAGGAACA  420
121   A  T  Y  K  G  K  A  T  K  I  T  R  E  A  Q  T  S  R  G  T   140
421  TATTACGAGTTTAGTGTTGATGGTAAAGTCATTGGCTGGTTAGATAAAAAAGCTTTCGAT  480
141   Y  Y  E  F  S  V  D  G  K  V  I  G  W  L  D  K  K  A  F  D   160
481  GTATATGACAATATTAATTACAACAAAGCGGTTAACTTAGATGCTGTAGTGGAAAATGTG  540
161   V  Y  D  N  I  N  Y  N  K  A  V  N  L  D  A  V  V  E  N  V   180
541  ACAGGCAACGCAGTTTGGACTGCTCCATATAAGAGTAAGGGTGTTAAATTAGTTACTTCA  600
181   T  G  N  A  V  W  T  A  P  Y  K  S  K  G  V  K  L  V  T  S   200
601  GCAGCCACATATAAAGATAAAGCAACTAAGATAACTCGAGAAGCGCAAACAAGTAGAGGA  660
201   A  A  T  Y  K  D  K  A  T  K  I  T  R  E  A  Q  T  S  R  G   220
661  ACTTACTACGAATTTAGCGTAAACGGCAAAGTAATCGGTTGGTTAGATAAAAAAGCTTTT  720
221   T  Y  Y  E  F  S  V  N  G  K  V  I  G  W  L  D  K  K  A  F   240
721  GATGTATATGATTCTATTGAGTACAATAAAGCGATTAATATGACTGGATTACTTAGCAAC  780
241   D  V  Y  D  S  I  E  Y  N  K  A  I  N  M  T  G  L  L  S  N   260
781  GCGCCAGGTAATGGCATTTGGACAGAGCCGTATAGAGTTATTGGCACAAAAAATGTAGGA  840
261   A  P  G  N  G  I  W  T  E  P  Y  R  V  I  G  T  K  N  V  G   280
841  CAAGCAACTGCTTATGCTAACAAGACAGTACAGTTGATACGCGAGGCTAAGACTACACGT  900
281   Q  A  T  A  Y  A  N  K  T  V  Q  L  I  R  E  A  K  T  T  R   300
901  GCAACTTACTATCAAATGAGTGTAAATGGTAAAATAGTTGGTTGGGTAGATAAACGAGCT  960
301   A  T  Y  Y  Q  M  S  V  N  G  K  I  V  G  W  V  D  K  R  A   320
961  TTTACAAACGTTAAATAGACTAAAAATAATCATATAAAAACAGAGTGAGATTTCTCACTC  1020
321   F  T  N  V  K  *                                             325
1021 TGTTTTTATATGATTATAGATTAAGATTTAGTGATGTAAATAAGTTCCTTCAAGAAGTCT  1080
1081 TTATAATAAGGGGATTCGAAGTGAGAAAAGGAATGCCCAAATGGATAGTTGAAATCACCT  1140
1141 ATATAGCCTTTTTTCGAAAAGTCGATTACTTTTGCTTCAGGCAATTTAGATAAGAAATAA  1200
1201 TTATTTAAACGTTCCCAGAAGCCCTTATTCTTTTCGATGGCCATTTTGTTTTTGTAAGTT  1260
1261 GCGACTTCTCCATCTTCATCATAATAAGACGTTGTAAATCCACCTAGGTTTAAAATAACC  1320
1321 CGATC                                                         1325
```

Figure 7B. Nucleotide sequence of the *L. monocytogenes* (serotype 4b) gene *ispMin* (partial) and deduced amino acid sequence of the corresponding proteins. Nucleotides 1 to 1812 were derived from inserted DNA of pSCRN130. Nucleotides 1 to 1713 represent the partial ORF of *ispMin*.

Figure 7B

```
     IspMin
1    GATCGTTGCGCGATAGAAGAGTTAGTAATTAATAACCTTCCAAAAAAATCAATGGTAAAT  60
1     D  R  C  A  I  E  E  L  V  I  N  N  L  P  K  K  S  M  V  N   20
61   ATAAGTAACAACAAAATTACTACACTAGAAGGACTTGAAAATTTATCTGCAGTAAACACT  120
21   I  S  N  N  K  I  T  T  L  E  G  L  E  N  L  S  A  V  N  T    40
121  TTATACGTATCTGAGAATTTAGTGACTGAAATAGAGAACCTACATGCGTTCCCTAAATTA  180
41    L  Y  V  S  E  N  L  V  T  E  I  E  N  L  H  A  F  P  K  L   60
181  CAGACACTCACCGTAGACAATAACCATATCAGTGTATTGCCAACAAGTTTGAAAACGGAA  240
61    Q  T  L  T  V  D  N  N  H  I  S  V  L  P  T  S  L  K  T  E   80
241  AATCCCGTATTAACAACGCTAAGTGCGATGAATCAAACAATCACTCTAAAGCAAAAAGTT  300
81    N  P  V  L  T  T  L  S  A  M  N  Q  T  I  T  L  K  Q  K  V   100
301  ATCGTGTCAGACCTAGTTCTTGATAATGAAGTGAAGAATTTCGGTCAAATAACCACTGCC  360
101   I  V  S  D  L  V  L  D  N  E  V  K  N  F  G  Q  I  T  T  A   120
361  AAATCCATCTCTAATAAGGGAACCTATCAAAATAACCAAATCAAGTGGCTTTTTGAAGAT  420
121   K  S  I  S  N  K  G  T  Y  Q  N  N  Q  I  K  W  L  F  E  D   140
421  ATAAAAAGCGTGAATGCCGTTGATTATCAATTTAGTGAACCTGTTCAAGAGGCAACTATT  480
141   I  K  S  V  N  A  V  D  Y  Q  F  S  E  P  V  Q  E  A  T  I   160
481  CAAGGAACTTTTTCGGGGAAAGTGACACAACCAATCAAAGCATCTAAAGTACCAGTTATT  540
161   Q  G  T  F  S  G  K  V  T  Q  P  I  K  A  S  K  V  P  V  I   180
541  AGCGCAGATGCAGAGATGAATTACCCGAAAAACGAAACGGTATCAGAAGCTGCCTTTTTC  600
181   S  A  D  A  E  M  N  Y  P  K  N  E  T  V  S  E  A  A  F  F   200
601  AAAGATATTTCTGCAAGCGTAACGGATGATGCAACACTAACTTCTGATTTTGAAAGTGTT  660
201   K  D  I  S  A  S  V  T  D  D  A  T  L  T  S  D  F  E  S  V   220
661  GTGGACTTTGCAAAAGCGGGAACGTATGAAGTGACATTAAATGCAGTGAATGAGGATGGA  720
221   V  D  F  A  K  A  G  T  Y  E  V  T  L  N  A  V  N  E  D  G   240
721  GTAAAAGCGACTTCGGTGACTGTATTAGTGCATATCGCTAAGTCGCCAGCGCCAGTAATT  780
241   V  K  A  T  S  V  T  V  L  V  H  I  A  K  S  P  A  P  V  I   260
781  ACCGCAGATAAAGAAATCACATACACTAAAAACGCGGAAGTCAGCATCACGGAATATCTT  840
261   T  A  D  K  E  I  T  Y  T  K  N  A  E  V  S  I  T  E  Y  L   280
841  GCAGCGATTCATGCTAAAACGAATGATGGTTCACCAATTGAAAGTGATTTCGCTACGGCT  900
281   A  A  I  H  A  K  T  N  D  G  S  P  I  E  S  D  F  A  T  A   300
901  GTAAATTGGGGCACTGCAGGAGATTATACCGTAACGCTAAGGTCTACAAATGAAGATGGA  960
301   V  N  W  G  T  A  G  D  Y  T  V  T  L  R  S  T  N  E  D  G   320
961  GTTGAAGCAATCCCTGTAGAAGTAACTGTGCACATCGCCAAGTCGCCAGCGCCAGTAATT  1020
321   V  E  A  I  P  V  E  V  T  V  H  I  A  K  S  P  A  P  V  I   340
1021 ACCGCAGATAAAGAAATTACGTACGCTAAAAACGCGGAAGTCAGCATCACGGAATATCTT  1080
341   T  A  D  K  E  I  T  Y  A  K  N  A  E  V  S  I  T  E  Y  L   360
1081 GCAGCGATTCATGCTAAAACGAGTGATGGTTCATCAATTGAAGCTGATTTAGATACGGCT  1140
361   A  A  I  H  A  K  T  S  D  G  S  S  I  E  A  D  L  D  T  A   380
1141 GTAACATGGGGCACTGTAGGAGGTTATACCGTAACGCTAAGGTCTACAAATGAAGACGGA  1200
381   V  T  W  G  T  V  G  G  Y  T  V  T  L  R  S  T  N  E  D  G   400
1201 GTAGAAGCAATCCCTGTAGAAGTAACTGTGCACATCGCTAAGTCACCAGCACCAGTAATT  1260
401   V  E  A  I  P  V  E  V  T  V  H  I  A  K  S  P  A  P  V  I   420
1261 ACCGCAGATAAAGAAATCACGTACGCTAAAAACGCGGAAGTCAGCATGACGGAATTTCTT  1320
421   T  A  D  K  E  I  T  Y  A  K  N  A  E  V  S  M  T  E  F  L   440
1321 GCAGCGATTCATGCTAAAACGAGTGATGGTTCACCAATTGAAAGTGATTTCGCTACGGCT  1380
441   A  A  I  H  A  K  T  S  D  G  S  P  I  E  S  D  F  A  T  A   460
1381 GTAATATGGAGCACTGCAGGAGATTATACCGTAACGTTAAAATCTACAAATGAAGATGGA  1400
461   V  I  W  S  T  A  G  D  Y  T  V  T  L  K  S  T  N  E  D  G   480
1441 GTAGAAGCAATCCCTGTAGAAGTAAAGGTGCATATCGTAGAGCCACTAGCACCAACGATT  1460
481   V  E  A  I  P  V  E  V  K  V  H  I  V  E  P  L  A  P  T  I   500
1501 TCGAATGTGACATTTGATGTGGATGATGTACAAACGACAGAATCTCTTGAAGCTGGAGAG  1520
501   S  N  V  T  F  D  V  D  D  V  Q  T  T  E  S  L  E  A  G  E   520
1561 CTAATTTCTGAACCATTGAGCCCAACAAAAGAAGGCTATACTTTTATTGGTTGGTATGAC  1580
521   L  I  S  E  P  L  S  P  T  K  E  G  Y  T  F  I  G  W  Y  D   540
1621 TCGAAAACTGGTGGTAATAAATGGGATTTTACAACAGATAAAATGCCAGCATATAATATT  1640
541   S  K  T  G  G  N  K  W  D  F  T  T  D  K  M  P  A  Y  N  I   560
```

```
1681 ATTCTTTATGCTCAGTTTAGTAAAGATACGTAATAAAGCAGAAGCGGCCGGTGGAGATAA  1700
561   I  L  Y  A  Q  F  S  K  D  T  *
1741 GCCCTCAACACCCTCTTCTATAAAAGTAAGTCCAACAGGTCAGTCCGAGAGTGGAAACTT  1760
1801 GGAAAATAGATC                                                  1812
```

Figure 8B. Nucleotide sequence of the *L. monocytogenes* gene *ispLin* (partial, in the reverse orientation) and deduced amino acid sequence of the corresponding protein. Nucleotides 1 to 1478 were derived from inserted DNA in pSCRN100. Nucleotides 1 to 876 represent the partial ORF of *ispLin*. The partial ORF of a gene coding for a putative protein similar to Lmo0834 is indicated by nt 827 to 1478. The two genes are overlapped by 49 bp. Two predicted promoters (-35 and -10 elements) and a putative ribosome binding site upstream of *ispLin* are underlined.

Figure 8B

```
1    GATCTTTCGTCTCTTGTTTTTTTTGTTCAACTTTGGTTAATTTCAATGCTTTTGGTTCGAT  61
294   D  K  T  E  Q  K  K  Q  E  V  K  T  L  K  L  A  K  P  E  I   275
62   TTTTGCTTTTGATATAGTTCGCTTTTCTGTGGTTTCTATTTTTGATTTCTTTATTTTTGC  121
274   K  A  K  S  I  T  R  K  E  T  T  E  I  K  S  K  K  I  K  A   255
122  TTTTTCTACTGGCGGATTTTCTGGAACAGCTGTATCAGGTAATGGTTCCATGCTTGGTGC  181
254   K  E  V  P  P  N  E  P  V  A  T  D  P  L  P  E  M  S  P  A   235
182  TTGCTCAGGCGCTTTTTTTTGATAAATGATGTTGATTGTTTGTGGTGATTCGGTAAAAAC  241
234   Q  E  P  A  K  K  Q  Y  I  I  N  I  T  Q  P  S  E  T  F  V   215
242  GCCAAGAATTTCCGAATCGTATTTTAATAAAGTATAACCTTTAATTTTTTTCATCGGAAC  301
214   G  L  I  E  S  D  Y  K  L  L  T  Y  G  K  I  K  K  M  P  V   195
302  ATTGTATGCTTCGTTGAAAAGTCCGCTCAAATATAATGACGGGGCAATTGAAGTATTGTT  361
194   N  Y  A  E  N  F  L  G  S  L  Y  L  S  P  A  I  S  T  N  N   175
362  TTCATCCAGATAATTTACAGTGACTTGATTCGGTTTCCCGAACTCTGGAATGGTAGAAAT  421
174   E  D  L  Y  N  V  T  V  Q  N  P  K  G  F  E  P  I  T  S  I   155
422  ATTCTTTTTATAGCGAAAAATGATATGCAAGCCATCTATGCTAATGTCTTTTCTTACAAA  481
154   N  K  K  Y  R  F  I  I  H  L  G  D  I  S  I  D  K  R  V  F   135
482  TGTATAGTCAGGTATTGTCTCTAAAATAGCTGCTTCGCTATCGATGATTTCTACTACTGG  541
134   T  Y  D  P  I  T  E  L  I  A  A  E  S  D  I  I  E  V  V  P   115
542  TAATAAGTCTTTGCCAGTTTCATCTACGTAGCTTACATCGATTATTTTATCATATATGTA  601
114   L  L  D  K  G  T  E  D  V  Y  S  V  D  I  I  K  D  Y  I  Y   95
602  ACGCACGATAATCCCAGTATCTGTGATATTGCCTGTCGCATTATGAGGTATTTCTCTTAA  661
94    R  V  I  I  G  T  D  T  I  N  G  T  A  N  H  P  I  E  R  L   75
662  TTTATAGCCAGGAATATCTTTTGGTGTAGATACATAATGATAATCAGTTAATGTATCTGA  721
74    K  Y  G  P  I  D  K  P  T  S  V  Y  H  Y  D  T  L  T  D  S   55
722  AGGAGCAATCTCGGCTCCGTCTGCATCAACATATTCCACTTTAACCGTGAAATTATTTGT  781
54    P  A  I  E  A  G  D  A  D  V  Y  E  V  K  V  T  F  N  N  T   35
782  GGCTGCACTTGCTTTTATAGGTGAGATAAACCCACTCGCCAATCCTACTGTCATTATTAA  841
34    A  A  S  A  K  I  P  S  I  F  G  S  A  L  G  V  T  M  I  L   15
842  TGCTAAGATTGTTATCACTATTTTTTTTACATTTTTACTCACTGACCATTCCTCCAAAGT  901
14    A  L  I  T  I  V  I  K  K  V  N  K  S  M           RBS          1
                                         IspLin       -10
902  TCTCATACATTTTGCAACGTACATTTAATTCATCTGGTAAAAGTACATACGATACAAATT  961
                -35
962  CGTTTTTTTCATCTACTAAAACTAAAAAATCAACAAATCGAAGTTCGGCCCAGTTTTTCC  1021
1022 AAAATGATAGAAAAAACTTTAATTCTGTAATATTGTTTAAGAGCTTCGACTTTTGCGTTA  1081
1082 AACTATATTTCAATTCATCAATATAGGGCGTAAAAATCAGCGTTTGTTCGATGGCACCAC  1141
1142 TTTCAATATGCTTTGAAATAAAATAATGCCACTTTCTCAACATTTTCAACTTCCCTTTCA  1201
1202 AAAAAGCGTCACTGTCTTCTGCGGAAAAATGTTCCTTTGAATTAGGGCTAATCTGACTAT  1261
1262 CCCCAACTAGTTCAATCGTTATTCGCCGGCTATCTTTCTGCAAATTCTCAAGCAACAGCA  1321
                -10                     -35
1322 TTGTTTCCCGAAAATATAATTGTTGAAAGGAAATTTTAACAATAAAGTGCTGATTGGGAT  1381
1382 AAACTTGTAGAACTTTTTTAACTTCCATTTCATACCAATTGATAAATTTTCCATACTCTG  1441
1442 TTTGTAACACATAACAAATGCGTAGTTAGATC                              1478
```

APPENDIX C

Alignment results of deduced proteins of all positive clones with other similar proteins found with BLABT search.

Figure 1C. Alignment of the *L. monocytogenes* serotype 4b InlA sequence deduced from pSCRN1, pSCRN2, pSCRN34, pSCRN35, pSCRN65, pSCRN66, pSCRN95,pSCRN96, pSCRN113, and pSCRN115 with selected similar proteins found with BLAST search. Lm 4b, the deduced amino acid sequence of the *L. monocytogenes* serotype 4b InlA (the present study); EGD-e, the InlA sequence of *L. monocytogenes* strain EGD-e (GenBank Accession No.NP_ 463962) (Glaser et al., 2001); EGD-SmR, the InlA sequence of *L. monocytogenes* strain EGD-SmR (GenBank Accession No. S37387) (Gaillard et al., 1991). Amino acid residues that match exactly with those of the 4b InlA (Lm4b) are represented by dots. Gaps are indicated by dashes.

```
Lm 4b     MRRKRYVWLKSILVAILVFGSGVWINTSNGTNAQAATITQDTPINQIFTDTALAEKMKTV   60
EGD-e     ..K.........................................................   60
EGD-SmR   ..K...............................................A.........   60

Lm 4b     LGKTNVTDTVSQTDLDQVTTLQADRLGIKSIDGLEYLNNLTQINFSNNQLTDITPLKDLT  120
EGD-e     .................................V.......................N..  120
EGD-SmR   ............................................................  120

Lm 4b     KLVDILMNNNQIADITPLANLSNLTGLTLFNNQITDIDPLKNLTNLNRLELSSNTISDIS  180
EGD-e     .....................T......................................  180
EGD-SmR   .....................T......................................  180

Lm 4b     ALSGLTSLQQLSFGNQVTDLKPLANLTTLERLDISSNKVSDISVLAKLTNLESLIATNNQ  240
EGD-e     ............................................................  240
EGD-SmR   ......N.....................................................  240

Lm 4b     ISDITPLGILTNLDELSLNGNQLKDIGTLASLTNLTDLDLANNQISNLAPLSGLTKLTEL  300
EGD-e     ............................................................  300
EGD-SmR   ............................................................  300

Lm 4b     KLGANQISNISPLAGLTALTNLELNENQLEDISPISNLKNLTYLTLYFNNISDISPVSSL  360
EGD-e     ............................................................  360
EGD-SmR   ............................................................  360

Lm 4b     TKLQRLFFYNNKVSDVSSLANLTNINWLSAGHNQISDLTPLANLTRITPLGLNDQEWTNP  420
EGD-e     ................................................Q......A...A  420
EGD-SmR   ................................................Q......A...A  420

Lm 4b     PVNYKVNVSIPNTVKNVTGALIAPATISDGGSYAEPDITWNLPSYTNEVSYTFNQSVTIG  480
EGD-e     .....A...........................T...................S.P....  480
EGD-SmR   .....A...............................................S.P....  480

Lm 4b     KGTTTFSGTVTQPLKAIFNAKFHVDGKETTKEVEAGNLLTEPAKPVKEGYTFVGWFDAQT  540
EGD-e     ...................V.............................H..........  540
EGD-SmR   ...................V.............................H..........  540

Lm 4b     GGTKWNFSTDKMPTNDIDLYAQFSINSYTATFDNDGVTTSQTVDYQGLLQEPTAPTKEGY  600
EGD-e     .................N..........................................  600
EGD-SmR   .................N..............E....................P......  600

Lm 4b     TFKGWYDAKTGGDKWDFATSKMPAKNITLYAQYSANSYTATFDVDGKTTTQAVDYQGLLK  660
EGD-e     ...............................................S............  660
EGD-SmR   ...............................................S............  660

Lm 4b     EPKTPTKAGYTFKGWYDEKTDGKKWDFATDKMPANDITLYAQFTKNPVAPPTTGGNTPPT  720
EGD-e     ...A........................................................  720
EGD-SmR   ...A........................................................  720

Lm 4b     TNNGGNTTPPSANIPGSNTSNTSTGNSASTTSTMNAYDPYNSKEASLPTTGDSDNALYLL  780
EGD-e     .................D..........................................  780
EGD-SmR   .................D..........................................  780

Lm 4b     IGLLAVGTAVALTKKARASK                                          800
EGD-e     L........M..........                                          800
EGD-SmR   L........M..........                                          800
```

Figure 2C. Alignment of the *L. monocytogenes* (serotype 4b) InlC2 sequence deduced from pSCRN92, pSCRN105, and pSCRN106 with selected similar proteins found with BLAST search. Lm 4b, deduced amino acid sequence of the *L. monocytogenes* (serotype 4b) InlC2 (this study); EGD, InlC2 sequence of *L. monocytogenes* strain EGD (GenBank Accession No. AAB67969; Dramsi et al., 1997); EGD-e, InlH sequence of *L. monocytogenes* strain EGD-e (GenBank Accession No. NP_463794; Glaser et al., 2001); EGD 1/2a, InlD sequence of *L. monocytogenes* strain EGD (serotype1/2a)(GenBank Accession No. CAC20635). Amino acid residues that match exactly with those of the 4b InlC2 are represented by dots. Gaps are indicated by dashes.

```
Lm 4b    MKKRWNSVFKLVLMVAAILGISLYVTTSQGAEVRAESIAQPTPINVIFPDPALANAVKTA   60
EGD      ...............T....L.........V.......T...A.............I.I.   60
EGD-e    ...............T....L.........V.......T...A.............I.I.   60
EGD1/2a  ..............................V.......T...A.............I.I.   60

Lm 4b    TGKSNVTDTVTQADLDGIATLSAFNTGVTTIEGIQYLNNLIGLELKDNQITDLTPLKNLT  120
EGD      A.................T.....G........V..........................  120
EGD-e    A.................T.....G........V..........................  120
EGD1/2a  A.................T.....G........V..........................  120

Lm 4b    KITELELSGNPLKNVSAIAGLQSIKTLDLTSTQITDVAPLAGLSNLQVLYLDLNQITDLS  180
EGD      .....................................T...................NI.  180
EGD-e    .....................................T...................NI.  180
EGD1/2a  .....................................T...................NI.  180

Lm 4b    PLAGLTNLQYLSIGNNQVNDLTPLANLSKLTTLRADDNKISDISPLASLPNLIEVHLKDN  240
EGD      ...............A..S..............K........................N.  240
EGD-e    ...............A..S..............K........................N.  240
EGD1/2a  ...............A..S..............K........................N.  240

Lm 4b    QISDVSPLANLSNLFIVTLTNQTITNQPVYYQNNLVVPNVVKGPSGAPIAPATISDNGTY  300
EGD      ..........T..................F.........I....................  300
EGD-e    ..........T..................F..............................  300
EGD1/2a  ..........T..................F..............................  300

Lm 4b    ASPNLTWNLTSFINNVSYTFNQSVAFKNTTVPFSGTVTQPLTEAYTAVFDVDGKQTSVTV  360
EGD      ........................T...................................  360
EGD-e    ........................T...................................  360
EGD1/2a  ........................T...................................
360

Lm 4b    GANELIKEPTAPTKEGYTFTGWYDAKTGGNKWDFSTDKMPAENITLYAQFTINSYTATFD  420
EGD      ..................................GV.....................S..  420
EGD-e    .............................T....A.......D.................  420
EGD1/2a  .............................T....A.......D.................  420

Lm 4b    NDGKITTQKVTYQSLLEEPAAPTKAGYTFKGWYDAKTGGTKWDFATGKMPAGNITLYAQF  480
EGD      ....L...................T...................................  480
EGD-e    I...L..............V....D....T..............................  480
EGD1/2a  I...L..............V....D....T...........S..................  480

Lm 4b    TKNDSPNPNDPTPNTPTGNGDGTSNPSDSGGNTTLPTAGDENTMLPIFIGVFLLGTATLI  540
EGD      ...........................N................................  540
EGD-e    ....N...D...T..............N................................  540
EGD1/2a  ....N...D...T..............N................................  540

Lm 4b    LRKTIKVK                                                      548
EGD      ........                                                      548
EGD-e    ........                                                      548
EGD1/2a  ........                                                      548
```

Figure 3C. Alignment of the *L. monocytogenes* (serotype 4b) InlD sequence deduced from PSCRN114, pSCRN54, pSCRN91, pSCRN112, pSCRN139, and pSCRN143 with selected similar proteins found with BLAST search. Lm 4b, deduced amino acid sequence of the *L. monocytogenes* (serotype 4b) InlD (this study); EGD, InlD sequence of *L. monocytogenes* strain EGD (GenBank Accession No. AAB67960; Dramsi et al., 1997). Amino acid residues that match exactly with those of the 4b InlD are represented by dots. Gaps are indicated by dashes.

```
Lm 4b MKRKKTALRILVTLAVVMAISFWVGTSSKEEVQAAVIDQPTPINEIFTDENLANAIKATL      60
EGD   ...N..........................-....E.G.........................T..      59

Lm 4b NKPSTTSDVSQAELDSISEVTAESSNIASLEGAQYLNNIDTLILNNNKITDLNSLAGLSK     120
EGD   S....A.A...V....VRD.............V.....L...V..........P....T.     119

Lm 4b LRILEANDNQLSDISALANVTSLHQLRLAGNQIKQLNGVSNLINLETIELSNNQITDISP     180
EGD   .S....SN.........S...N......D............................A...     179

Lm 4b VSGLKNLIGLGIDYNDISDLSPIAGLAKLSHLTADNNQISDLSPISSLGAMEIMRLDKNQ     240
EGD   .......V.....N.K.......S..S..N.....S......R.L.N.A...V....G..     239

Lm 4b ISDVTPIANLANLSYVFLAENQISDISSLQPLFNSPNFFGITLDNQKITSEPVLYQQELV     300
EGD   .............N..............................................     299

Lm 4b VPNNIKDEMGALISPDTISDNGVYASPNIKWNLANYTNQVSYTFNKQLAQGSFSGTVTQP     360
EGD   .............A.A.............N...P...............Y..........     359

Lm 4b LHNAYTATFDVDGVKTNEVVEETKLLQEPTAPTKEGYTFTGWYDAKTGGNKWDFATDKMP     420
EGD   ..................A..........I..............................     419

Lm 4b AEDITLYAQFTINSYTATFDIDGKLTTQKVTYQSLLEEPAAPTKDGYTFIGWYDAKTGGT     480
EGD   .......................................V.........T..........     479

Lm 4b KWDFATDKMPAGNITLYAQFTKNATPDSNDPTTVTPTGNGNGTSTPSNSGGNTTLPTAGD     540
EGD   ......G................DN.NPD....N......D...N...............     539

Lm 4b ENTMLPIFVGVFLLGTATLILRKTIKVK.                                   569
EGD   ........I...................                                    567
```

Figure 4C. Alignment of the *L. monocytogenes* (serotype 4b) IspWei sequence deduced from pSCRN93, pSCRN88, pSCRN101, pSCRN102, and pSCRN106 with selected similar proteins found with BLAST search. 4b IspWei, deduced amino acid sequence of the *L. monocytogenes* (serotype 4b) IspWei protein (this study); Li PCSP, a probable cell surface protein sequence of *L. monocytogenes* strain EGD-e (GenBank Accession No. NP_469717; Glaser et al., 2001). Amino acid residues that match exactly with those of the 4b IspWei protein are represented by dots. Gaps are indicated by dashes.

```
4b IspWei MKSKGRLFLYVVLALSIVIGTNVFIKIDAHAAAAPPAAINQIFPDDALATEIQTTLGKSS    60
Li PCSP   .............................Y...................K....A.....    60

4b IspWei TAEVVTQTDLDTINSLTLTSKGISSLEGMNYLTNLGTLILTGNQVSDISPLKGLTNLTML   120
Li PCSP   .........................I..............SS..................   120

4b IspWei QLSGNPISDISALSNLKNLQALDINDAQVTDITPLSGLTNLKGLGLYNNQLENLSGVNSL   180
Li PCSP   ............................I.............................N.   180

4b IspWei HQLRSLNVSNNKLTNLDELQALSNLSVLYANENQINNLQGLSNLNNLFLLDLSANQIVDT   240
Li PCSP   Q........................G.....G..........T.K........T......   240

4b IspWei TPLAGLTKVQTLYVSNNQISDVTGLSSLINLDWLDISQNKISNIRPLNSLTKLTIIQMTN   300
Li PCSP   .......N.....L..............................................   300

4b IspWei QLIVNEPISFESTVTIPNLIKNIAEQTIDPDTISDNGVYANEAVTWNLPSYIPKVSYTFI   360
Li PCSP   .............I.....................S.....GS......T..........   360

4b IspWei ERDTIGNATGNFSGTVEQPLVQYFKATFNIDGQETTENVETGTLLQEPPTPVKEGYTFNG   420
Li PCSP   ....V.........................E.............................   420

4b IspWei WYDAETGGTKWDFTADTMPANDITLYAQFSINSYTATFDVDGVISTQAVEYQGLLEEPPA   480
Li PCSP   ...........................................T...T.D..........   480

4b IspWei PTKDGYTFKGWYDAKTGGTKWDFTN-----------------------------------   505
Li PCSP   .........................NQMPAKDITLYAQFSINSYTATFDVDGVISTQTVD   540

4b IspWei -----------------------------------NQMPANDITLYAQFSKDASSGGDGG   530
Li PCSP   YQGLLEEPPAPTKDGYTFKGWYDAKSGGTKWDFAN.....K..I.........A......   600

4b IspWei GTDEGGGNSENSTEGAPNTSDIDTINHIVLPATGDDHVLFPIFIGTFLTSLALLTLRRK    589
Li PCSP   .............D.TT.---..............HR............G...F.....    656
```

Figure 5C. Alignment of the *L. monocytogenes* (serotype 4b) IspLing protein sequence deduced from pSCRN79 with selected similar proteins found with BLAST search. Lm 4b, deduced amino acid sequence of the *L. monocytogenes* (serotype 4b) IspLing protein (this study); Li, a hypothetical transmembrane protein sequence of *L. innocua* (GenBank Accession No. NP_464452; Glaser et al., 2001); EGD-e HTP, a hypothetical transmembrane protein sequence of *L. monocytogenes* strain EGD-e (GenBank Accession No.NP_ 470266; Glaser et al., 2001). Amino acid residues that match exactly with those of the 4b IspLing protein are represented by dots. Gaps are indicated by dashes.

```
IspLing    MKDWKIKIQTFLSKNYGFFVLAVILYWLKTYIAYQLEFKLGIENLMQQILLFINPLSGAI    60
Li         ...........................................................V    60
EGD-e HTP  ............................................................    60

IspLing    FFMGLALFAKGRRSFIWIIVIDFLMSFILYANIVYYRFFSDFITLPNLNAKQMQNMGDMG   120
Li         ..L...............V.........................................   120
EGD-e HTP  ............................................................   120

IspLing    SSITALLSWHDIIYFADIIILIALLAFRFVKPNKTARIRARKVVGVLTLGIAMFFGNLGL   180
Li         ................................D......................A....   180
EGD-e HTP  ............................................................   180

IspLing    AEIDRPQLLTRTFDRNYIVKYLGMTNYQIYDAVKSTESSTQRALADSSDVTEVLNYTKSK   240
Li         ............................................................   240
EGD-e HTP  ............................................................   240

IspLing    YAAPNPEYFGKAKGKNVIYIHLESFQQFLVNYKLNGEEVTPFINSFFKDQNTLSFTNFFH   300
Li         .........................................Y.......K..........   300
EGD-e HTP  ............................................................   300

IspLing    QTGQGKTADSDMLLENSLYGLPQGSAFTTKGQNTYESASAILGQQGYTSAVFHGNYKSFW   360
Li         ..........E.................................................   360
EGD-e HTP  ..........E.................................................   360

IspLing    NRDEIYKQFGYDNFFDASYYDMNEADVSNYGLKDKPFFKESEEYLSSLQQPFYTKFITLT   420
Li         .......................D..............Q...K.....K...........   420
EGD-e HTP  ............................................................   420

IspLing    NHFPYPIDEKDASIAPATTGDSSVDTYFQTARYLDESVKSFVDYLKKSGLYDNSVIIMYG   480
Li         ...............................................T............   480
EGD-e HTP  ............................................................   480

IspLing    DHYGISDNHEEAMTKILGKDYNTFENAQAQRVPLMIHVPGVQGGVQEQYGGQVDLLPTLL   540
Li         .........................................E..................   540
EGD-e HTP  ............................................................   540

IspLing    HLLGVDNKEYLQFGTDLLSKDHKQLVPFRNGDYITPTYSMIGGNMYNQQTGEPIATETKE   600
Li         ............................................................   600
EGD-e HTP  ............................................................   600

IspLing    MKETKEKVAKELELSDSVLQGDLLRFYAPDGFKKVDPSKYNYNKKKSTDSSDK          653
Li         ........S..........................................T.          653
EGD-e HTP  .....................................................          653
```

Figure 6C. Alignment of the *L. monocytogenes* (serotype 4b) IspYu sequence deduced from pSCRN119 and pSCRN153 with selected similar proteins found with BLAST search. Lm 4b, deduced amino acid sequence of the *L. monocytogenes* (serotype 4b) IspYu protein (this study); Lm Ami, *L. monocytogenes* amidase (autolysin) (GenBank Accession No. CAC20640; Braun et al., 1997; McLaughlan and Foster, 1998); Li Ami, *L. innocua* amidase (autolysin) (GenBank Accession No. NP_ 472032; Glaser et al., 2001). Amino acid residues that match exactly with those of the 4b IspYu protein are represented by dots. Gaps are indicated by dashes.

```
IspYu  DQEEYNKTVAIDAVVKNVKGNAVWTEPYRTVGTKLIGPAETYLNKEVEVVREAKTPKGTY   60
Lm Ami .KI..D.G.TAY.R..TAP.....K....E.S..VNQLSV.QG.NMRIL.....VIT.W   60
Li Ami .KI..D.G.TAY.R..TAP.....K....E.S..VNQLSV.QG.NMRIL......IT.W   60

IspYu  YQFKSGGKVIGWLDKKAFDVYDNINYNKAVNLD-AVVENVTGNAVWTAPYKSKGVKLVTS  119
Lm Ami ...SID......V.TR.L.TFYKQSME.DT..TRY.IA.KVNE.YYKV.VVDAD.RWG.-  119
Li Ami ...SID......V.TR.L.TFYKQSMEQST..TRY.TA.K..E.YYKV.VADSD..WG.-  119

IspYu  AATYKGKATKITREAQTSRGTYYEFSVDGKVIGWLDKKAFDV---YDNINYNKAVNLDAV  176
Lm Ami L.A..DQKLTVDKQ.TVEGQLW.RVRTSTTF...TKASNLTTTTP..K.E.D.GATAY.R  179
Li Ami L....NEKLTVDSQ.TVEGQLW.RVRTSTTF...TKAENLTTTPPF.A.E.D.G.TAY.R  179

IspYu  VENVTGNAVWTAPYKSKGVKLVTSAATYKDKATKITREAQTSRGTYYEFSVNGKVIGWLD  236
Lm Ami .KTAP.....K..RTE.S...NQLSV.QG.NIANL...K.VIT.W.Q..ID......V.  239
Li Ami .KTAP.....K..RTE.S...NQLSV.QG.NMR.L...K.VIT.W.Q..ID......V.  239

IspYu  KKAFDVYDSIEYNKAINMTGLLSNAPGNGIWTEPYRVIGTKNVGQATAYANKTVQLIREA  296
Lm Ami TR.L.TFYKQSME.DT.L.RYVIANKV.EAYYKVPV.DADVRW.TLA..KGQKLTVDKQ.  299
Li Ami TR.L.TFYKQSME.DT.L.RYVIANKV.EAYYKVPV.DADVRW.TLA..KGQKLTVDKQ.  299

IspYu  KTTRATYYQMSVNGKIVGWVDKRAFTNVK                                325
Lm Ami TVEGQLW.RIRTSSTFI..TKASNLSAT.                                328
Li Ami TVEGQLW.RIRTSSTFI..TKASNLSAT.                                328
```

Figure 7C. Alignment of the *L. monocytogenes* (serotype 4b) IspMin sequence deduced from pSCRN130 with selected similar proteins found with BLAST search. IspMin, deduced amino acid sequence of the *L. monocytogenes* (serotype 4b) IspMin protein (this study); Ls, a putative protein OFRA of *L. seeligeri* (GenBank Accession No. CAA65738); EGD-e, a putative peptidoglycan proein of *L. monocytogenes* strain EGD-e (GenBank Accession No. NP_463863; Glaser et al., 2001). Li, a putative *L. innocua* protein highly similar to the *L. seeligeri* ORFA (GenBank Accession No. NP_470008; Glaser et al., 2001). Amino acid residues that match exactly with those of the 4b IspMin protein are represented by dots. Gaps are indicated by dashes.

```
IspMin DRCAIEEL-VINNLPKKSMVNISNNKITTLEGLENLSAVN--------------TLYVSE      45
Ls     ------------------------------------------------------------       0
EGD-e  .SNK..DISALS..TNLQELTLE....ENISA.SD.ENL.--------------K.V..K      46
Li     CTIPFKGISTKTRVSFDIDT.VDGKPG..DPY.VS..S.SSATKSLEFYPVNNADTKMTA      60

IspMin NLVTEIENLHAFPKLQTLTVDNNHISVLPTSLKTENPVLTTLSAMNQTITLKQKVIVSDL     105
Ls     ----------EDENGAAI.F.DYSEAAFAGE.NEDG-----------EYEIDG.FLYNST      39
EGD-e  .KIID.SPVANMVNRGAIVTAS.QTYT...V.SYQS--------S---F.IDNP..WY.-      94
Li     AFFGKVTTRFEDELGNPV.FNDYSTVTT.GKVNQDG-----------KFEIAEPFLHNSV     109

IspMin VLDNEVKNFGQITTAKSISNKGTYQNNQIKWLFEDIKSVNAVDYQFSEPVQEATIQGTFS     165
Ls     QS-------VDKHAFDEMLEDEK.KLVDVSSENQLTETE----DNLNIQIKLG-----YE      83
EGD-e  ---------.TLLAPS..G.S.N.KDGK.T.TNMTAT.S-STLFN.NR-LKDG--L-...     140
Li     QN-------VDKHAYD.LL.TNK.KLLEVTSPNKLSETA----DNL.IQIKQG-----YQ     153

IspMin GKVTQPIKASKVPVISADAEMNYPKNETVSEAAFFKDISASVTDDATLTSDFESVVDFAK     225
Ls     ND.LYK..KLQK...Q.LP.IE.S.TVVKTKEE.LG.VE.TTDIPSVIDC.LKD-.EWGV     142
EGD-e  .T....Y.S--AAKVT....QT.TIGD.I..EQ.L..VN.KSS.G.PV....AT...LNT     198
Li     ND.LYK...LQK.....LP.IE.S.TVNRTMEE.LE.VE.KTDIP.DIKC.LTN-.KWGV     212

IspMin AGTYEVTLNAVNEDGVKATSVTVLVHIAKSPAPVITADKEITYTKNAEVSITEYLAAIHA     285
Ls     P.D.P.VIT..K..NQE.DP...M...L.N.....SV.P....K.TVTKTDD.L.TDVN.     202
EGD-e  F.E.....TSE-K..IQGD.CK.I.KVLHG-....S..QT.S.D.H.TITEKQF.ED...     256
Li     P.D.P.LIT.....NQA.DP.P.TIK.S.N......V.P..I.D.TVTKDESTL.NEVN.     272

IspMin KTNDGSPIESDFATAVNWGTAGDYTVTLRSTNEDGVEAIPVEVTVHIAKSPAPVITADKE     345
Ls     R.....V.T..I.DK.K..VP...E...NAM.....A.ESKTF....L.....I.AV.P.     262
EGD-e  S.DLDTA.TTN.S....LNKG.....A.N.E.....K.ET.Y...TVN.D...I.S.KT.     316
Li     R.....T.T.NINDK.K..VP...E...NAV.....A.EAKTFI.R.L.....I.AV.P.     332

IspMin ITYAKNAEVSITEYLAAIHAKTSDGSSIEADLDTAVTWGTVGGYTVTLRSTNEDGVEAIP     405
Ls     ...L.STTKTEP.L..DV..Q.N...P.VS.ML.E.K..VP.E.P...N.M.....A.ES     322
EGD-e  ...D.FSKKTEAAF.DD.D.D.N...IVTSNFA...NLDKA.D.....N.I.S...AGT.     376
Li     ...P.TVTKTEA.L.Q.VN.Q.N...PLVS.MNDK.K..VP.D.E...NAV.....A.EA     392

IspMin VEVTVHIAKSPAPVITADKEITYAKNAEVSMTEFLAAIHAKTSDG---SPIESDFATAVI     462
Ls     KTFI.K.L.D...I..V.P....DSKTKKEVA.L.TEV..Q.N..---.A.T..AE.Q.N     379
EGD-e  TAII..VE.EKIAT.STNTAQQ.E.Y.KINE.Q..KDV..SINASPTTAVL....E.V.K     436
Li     KTFI.R.L.....I..V.P.VS.DSAIIKNE..L.KEVR...N.N---.A.T..APDK.K     449

IspMin WSTAGDYTVTLKSTNEDGVEAIPVEVKVHIVEPLAPTISN---VTFD----------VDD     509
Ls     .GMP.E.P...NAM.....A.E.KTFI.K.LKNP..V.TVDPEI.Y.SKTKKEVAELLAE     439
EGD-e  LDVP.T....ITA.....GVSA.K..S.IVRKIP..E.TADKEI.YPKFDEVSEAEFLN.     496
Li     .Q.P.S.....NAV....IP.D..TFI.....AKKAP.VIE-ENPA.TPTK---------     499

IspMin VQTTES-------------------------------------------------LEAGE     520
Ls     .HAQTNDGSAISSDAETQVVWGAPGDYLVTLNAVNEHEIAAEPVSFTVHIVEAKEKPVPK     499
EGD-e  IHA.I.D----------------------------------------------KNVAITS     510
Li     -----------------------P----------------------------------SK     502

IspMin LISEPLSPTKEGYTFIGWYDSKTGGNK----------WDFTTDKMPAYNIILYAQFSKDT     570
Ls     DKPKNKLTV..K-KLPQTG.RNSE.VLGF-----------SALCLGLWLFFRNGRLK        544
EGD-e  NF.TDVNLN.A.DYTVTLNATNED.V.ATPVEVIVHVQQGERPVIT.DAT.S.DK.ANI.     570
Li     QKPKEKIVI.KTTKLPRTG.TQSKAILG------------GVLCLG.WFLVRKK           544
```

Figure 8C. Alignment of the *L. monocytogenes* (serotype 4b) IspLin sequence deduced from pSCRN100 with selected similar proteins found with BLAST search. IspLin, deduced amino acid sequence of the *L. monocytogenes* (serotype 4b) IspLin protein (this study); EGD-e PBP, a putative peptidoglycan bound protein of *L. monocytogenes* strain EGD-e (GenBank Accession No. NP_464362;Glaser et al., 2001). Amino acid residues that match exactly with those of the 4b IspLin protein are represented by dots. Gaps are indicated by dashes.

```
IspLin    MSKNVKKIVITILALIMTVGLASGFISPIKASAATNNFTVKVEYVDADGAEIAPSDTLTD    60
EGD-e PPB ..........LF..............A......D......................I...    60

IspLin    YHYVSTPKDIPGYKLREIPHNATGNITDTGIIVRYIYDKIIDVSYVDETGKDLLPVVEII   120
EGD-e PBP .......................................T...R................   120

IspLin    DSEAAILETIPDYTFVRKDISIDGLHIIFRYKKNISTIPEFGKPNQVTVNYLDENNTSIA   180
EGD-e PBP N....V....S.....K......................D...............K.P..   180

IspLin    PSLYLSGLFNEAYNVPMKKIKGYTLLKYDSEILGVFTESPQTINIIYQKKAPEQAPSMEP   240
EGD-e PBP ............................................................   240

IspLin    LPDTAVPENPPVEKAKIKKSKIETTEKRTISKAKIEPKALKLTKVEQKKQETKD         294
EGD-e PBP ...PT...S.Q...S..............T..V..A..................         294
```

HIGHLIGHTS OF QUALIFICATIONS:

1. Excellent laboratory and organization skills
2. Knowledge of research and techniques in Immunology, Microbiology, molecular biology and biochemistry
3. Competent in guiding students to complete their research
4. Honest, reliable, precise and cooperative person

EMPLOYMENT EXPERIENCE:

BRTD of Animal Diseases Research Institute, CFIA	2003-now
Employee	
BRTD of Animal Diseases Research Institute, CFIA	2001-2003
Graduate student	
Study English in St. lucy school	1999-2001

Department of Immunology
1992~1999
Harbin Medical University, Harbin ~P.R.China
Assistant professor

RESEARCH INTEREST

1. Developing rapid diagnosis about AIV influnanza virus and *E.coli*
2. The gene products of *Listeria monocytogenes* induced specifically during rabbit infection, such as molecular cloning, generating genes constructs for protein expression, immunological assays and protocols
3. Researching of the effects of Astragalus on the old mice and the old people, for example: the effects on CIC, Cks, Complement and CMSC of the old subjects, on the immunological function of the mice with the low immunological function
4. Studying the function of cytokines in allograft rejection, such as the rental and heart transplantation, monitoring the survival time of the organs

EDUCATION:

University of Ottawa, Ottawa-Canada	September 2001-2004
Master of Science, Faculty of Medicine, Department of Biochemistry, Microbiology and Immunology	
Harbin Medical University, Harbin-China	September 1989-July 1992
Master of Immunology, Department of Immunology	
Bachelor of Medicine	September 1984-July 1989
Harbin Medical University	