12-9-2006

SDS-PAGE and western blotting analyses of Internalin A in Listeria monocytogenes and Listeria spp

Bang-Yuan Chen

SDS-PAGE AND WESTERN BLOTTING ANALYSES OF INTERNALIN A IN *LISTERIA MONOCYTOGENES* AND *LISTERIA* SPP.

By

Bang-Yuan Chen

A Thesis
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in Food Science and Technology
in the Department of Food Science, Nutrition and Health Promotion

Mississippi State, Mississippi

December 2006

SDS-PAGE AND WESTERN BLOTTING ANALYSES OF INTERNALIN A IN *LISTERIA MONOCYTOGENES* AND *LISTERIA* SPP.

By

Bang-Yuan Chen

Approved:

Juan L. Silva Professor of Food Science, Nutrition and Health Promotion (Director of Thesis)	William B. Mikel Head and Graduate Coordinator in the Department of Food Science, Nutrition and Health Promotion
Charles H. White Professor of Food Science, Nutrition and Health Promotion (Committee member)	Mark W. Schilling Assistant Professor of Food Science, Nutrition and Health Promotion (Committee member)

Vance H. Watson
Dean of the College of Agriculture and Life Sciences

Name: Bang-Yuan Chen

Date of Degree: December 08, 2006

Institution: Mississippi State University

Major Field: Food Science, Nutrition and Health Promotion

Major Professor: Dr. Juan L. Silva

Title of Study: SDS-PAGE AND WESTERN BLOTTING ANALYSES OF INTERNALIN A IN *LISTERIA MONOCYTOGENES* AND *LISTERIA* SPP.

Pages in Study: 55

Candidate for Degree of Master of Science

Attachment strength of *Listeria* spp. and their InlA expression was assessed. *Listeria monocytogenes* 19111 exhibited the strongest attachment strength with *L. monocytogenes* 19115, *L. grayi*, *L. innocua*, and *L. monocytogenes* 7644 being the weakest. InlA expression was not detected in silver stained SDS gels but was detected in Western blotting images. Internalin A was only detected in protein extracts of *L. monocytogenes* 19111 and 7644 with band intensities of 50.1 and 2.5 pixels, respectively. Greater InlA expression was correlated with higher attachment strength in *L.*

monocytogenes 19111. *Listeria monocytogenes* 19115 did not express InlA but it had a stronger attachment than *L. monocytogenes* 7644 which demonstrated InlA expression. Intensity of InlA expressed in *L. monocytogenes* 19111 increased when temperature increased from 10 to 40 ^{o}C, and *L. monocytogenes* 7644 only expressed InlA at 40 ^{o}C. It was also determined that InlA was more expressed in nutrient-rich media than in nutrient-poor media.

DEDICATION

I would like to dedicate this research to my parents, Chao-Chi Chen and His- Jen Wu. With their trust and love, I can face up to my difficulties manfully.

ACKNOWLEDGEMENTS

This research would not have been possible without the support of many people. I express sincere appreciation to my adviser, Dr. Juan L. Silva, who brings me into this friendly research group and gives me guidance.

Thanks go to other faculty members, Dr. Taejo Kim for his suggestions and comments throughout the research; Dr. William B. Mikel, Dr. Charles H. White and Dr. Mark W. Schilling for serving as committee members and offering their support. The technical assistance of Dr. Jungyean Jung and writing assistance of Dr. Jong-Yi Fang are gratefully appreciated. And finally, thanks to my family, and friends for their understanding, motivation, and love.

This work was funded in part by the Mississippi Agricultural and Forestry Experiment Station Project No. MIS-371272 and by the USDA-ARS Grant No. 58-0790-0-120.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Listeria monocytogenes, originally named as *Bacterium monocytogenes*, was first described by Murray *et al.* (1926) as a bacterium which can cause large mononuclear leucocytosis in rabbits and guinea-pigs. Pirie (1927) isolated the same organism from sheep and named it *Listerella hepatolytica* in 1927 and *Listeria monocytogenes* in 1940 (Farber and Peterkin, 1991). Since then, more species in the genus *Listeria* have been isolated and recognized. In 1984, eight species under the *Listeria* genus: *L. denitrificans, L. grayi, L. innocua, L. ivanovii, L. murrayi, L. monocytogenes, L. seeligeri,* and *L. welshimeri* were listed in Bergey's Manual of Systematic Bacteriology (Sneath *et al.*, 1984).

Newer methods employed for identification and classification of *Listeria* spp. include sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE), pulsed-field gel electrophoresis (PFGE), plasmid fingerprinting, and genomic fingerprinting. Rocourt *et al.* (1987) based on the 16S ribosomal RNA difference

suggested that *L. denitrificans* be renamed *Jonesia denitrificans*, thus excluding it from the genius *Listeria*. *Listeria grayi* and *L. murrayi* were later combined into *L. grayi* by the same authors in 1992. It is recognized now that only six species belong to the genus *Listeria*: *L. grayi*, *L. innocua*, *L. ivanovii*, *L. monocytogenes*, *L. seeligeri*, and *L. welshimeri*.

Listeria ivanovii, L. monocytogenes and, *L. seeligeri* were reported to cause human and animal infections (Hof and Hefner, 1988; Rocourt *et al.,* 1986; Cummins *et al.*, 1994). However, there is only one case of human infection related to *L. seeligeri* and thus it is now regarded as nonpathogenic. *Listeria innocua*, *L. welshimeri* and *L. grayi*, are also regarded as non-pathogenic (Hof and Hefner, 1988; Rocourt *et al.,* 1983; Rocourt *et al.,* 1986).

Each year, foodborne pathogens cause more than 2,700 deaths in the United States (Paul, 1999). Most of these deaths are caused by *Salmonella, Listeria, Toxoplasma*, *Campylobacter*, and Norwalk-like viruses. Almost 500 deaths are attributed to *L. monocytogenes* alone with a nearly 20% case-fatality rate (Paul, 1999). This reveals that food contaminated with *L. monocytogenes* is a serious food safety problem.

Contaminated food is the primary source of Listeriosis. Heat treatment before

consumption is lethal to *Listeria* spp. After processing, recontamination in ready-to-eat foods is the most common source of *L. monocytogenes* and other *Listeria* spp. (Tompkin, 2002). Thus, there is a "zero tolerance" policy for ready-to-eat foods in the United States (Shank et al., 1996). Ingestion of non-preheated food contaminated with *L. monocytogenes* and *Listeria* spp. will lead to bacterial attachment onto the human gastrointestinal tract. This is the main step that is responsible for human infections.

Listeria monocytogenes can attach and enter into intestinal cells and macrophages by endocytosis, a process known as parasite-directed endocytosis (McGee *et al.*, 1988). This active endocytosis is performed by bacterial surface proteins, also called virulence factors in *L. monocytogenes*. Each strain of *L. monocytogenes* varies in surface protein composition. The variations in protein composition cause different species to have different attachment strengths to intestinal cells (Santiago *et al.* 1999, Dramsi *et al.* 1993). Several surface proteins such as internalin A (InlA), internalin B, *Listeria* adhesion protein, p60 were identified as being responsible for the attachment of *Listeria* cells. Internalin A is the major protein that mediates the adhesion and invasion of *L. monocytogenes* into host epithelium cells (Gaillard *et al.*, 1991; Vázquez-Boland *et al.*, 2001). However, the quantification of the attachment strength of *L. monocytogenes* and

Listeria spp. and the expression of the surface proteins has not been studied.

The objectives of this study were to investigate the attachment strength among different strains of *L. monocytogenes* and *Listeria* spp.; to investigate the potential relationship between attachment strength and expression of the surface protein, internalin A (InlA); and to screen potential InlA expression strains at varying temperatures and in different culture media.

CHAPTER Ⅱ

REVIEW OF LITERATURE

Listeria monocytogenes

Listeria monocytogenes is a gram-positive, nonsporeforming, facultative anaerobic, nonacidfast, diphtheroid-like rod with rounded ends that measure 1.0 to 2.0 μm long by 0.5 μm diameter. This bacteria can grow between -0.4 and 50 ^{o}C (Farber and Peterkin, 1991; Gray and Killinger, 1966). At temperatures from 20 to 25 ^{o}C, flagellins are formed on the surface to produce tumbling motility (Peel *et al.*, 1988). *Listeria monocytogenes* also survives at relatively wide pH range of 4.3 to 9 (Cole *et al.*, 1990).

Listeria monocytogenes can be found in many mammals including human, poultry, fish and crustacean. It can be found in many mammals including human, poultry, fish and crustacean. It is also present in plant, soil, stream water, mud, sewage, slaughter house waste, silage, and sickroom-dust (Gray and Killinger, 1966; Weis and Seeliger, 1975).

Pathogenicity of *Listeria monocytogenes*

Listeria monocytogenes is regarded as a foodborne pathogen that can infect host through food ingestion (Farber and Peterkin, 1991). Host infection usually initiates 20 h after ingestion of food contaminated with *L. monocytogenes*. It initiates from intestinal lumen and later translocates to the central nervous system and fetoplacental units. Common symptoms manifest first as gastroenteritis followed by diarrhea and fever. Hospitalization is required in serious cases known as listeriosis (Dalton *et al.*, 1997; Lecuit *et al.*, 2001). The level of *L. monocytogenes* that causes listeriosis can depend on food matrices, amount of food consumed, and host susceptibility. The infectious level can be as low as 10^2 to 10^4 cells per g of food (Maijala *et al.*, 2001; Vázquez-Boland *et al.*, 2001).

Several methods have been used to determine the possible pathogenicity of different strains of *L. monocytogenes*. Classification by serotyping provides evidence that certain strains of *L. monocytogenes* are more likely to cause illness. Three serotypes of *L. monocytogenes*, 4b, 1/2a, and 1/2b account for more than 90 % of the cases of human listeriosis (Farber and Peterkin, 1991). The evaluation of attachment strength can also contribute to the determination of pathogenicity in different *L. monocytogenes* strains

(Eginton *et al.*, 1995; Allison *et al.*, 2000; and Kim *et al.*, 2005).

Food contaminations by *Listeria*

Listeria monocytogenes can survive and attach onto food processing surfaces (glass, stainless steel, polypropylene, and rubber). The growth of *L. monocytogenes* on these surfaces can further increase the risk of food contamination (Mafu *et al.,* 1990; Blackman and Frank, 1996). It is therefore important to maintain the hygiene of processing environments.

In contaminated environments, cells of *L. monocytogenes* can be found suspended or attached to processing surfaces. Inreversiblely attached *Listeria* cells can develop microcolonies that form biofilms (Costerton *et al.*, 1987). Attached cells and biofilms showed higher resistance to sanitizers and adverse environments than suspended free cells (Frank and Koffi, 1990; Lee and Frank, 1991). For example, it was demonstrated that cells attached for eight days revealed 10 times more resistance than free living cells to 1 ppm chlorine (Lee and Frank, 1990). It was also shown that attached bacterial cells can survive at 70 ^{o}C for five minutes whereas free cells can not.

Listeria cells on food processing surface have shown high resistance to chemical

cleaners and sanitizers. Krysinksi *et al.* (1992) observed that *L. monocytogenes* biofilms can survive through a normal clean up period in a food processing plant. It was also shown that *L. monocytogenes* can acquire higher tolerance to chemicals and intimately survive lethal concentration of chemicals after pretreatment with a sub-lethal concentration of the same chemicals (Pickett and Murano, 1996). Those authors found that pretreatment with a sub-lethal concentration of chemicals did not influence the attachment ability of *Listeria*. This suggests that insufficient cleaning and sanitizing could not completely eradicate *Listeria* cells on food processing surfaces, and food processing industries should make sure that they have adopted adequate cleaning procedures to minimize food contamination by *Listeria*.

Attachment of *Listeria* cells can be affected by several factors such as contact surfaces and time, growth nutrients, growth temperature, cell motility, and cell surface structure (Heald and Zottola, 1989; Mafu *et al.*, 1990; Krysinksi *et al.*, 1992; Gorski *et al.*, 2003; Kim and Frank, 1994). *Listeria* cells can attach to all types of processing surfaces. Mafu *et al.*(1990) found that *L. monocytogenes* could attach to all testing surfaces (stainless steel, glass, polypropylene, and rubber) at both 20 and 4 ^{o}C and contact times as short as 20 min to one hour.

The contact time required for attachment of *Listeria* cells is dependent on the number of cells and the growth phase of the culture. For example, Fletcher (1977) observed that the shortest time necessary for attachment was 2.5 to 3 h in a log phase culture, while a stationary phase culture required 4 h for attachment, and a death phase culture requires 5.5 h. The log phase culture was also shown to have the highest attachment strength followed by the stationary phase and the death phase, respectively.

Nutrients may play an important role in cell metabolism which in turn may affect attachment strength. Kim and Frank (1994) tested *L. monocytogenes* attachment on modified D10 medium (Trivett and Meyer, 1971) with a 10-fold increase of ammonium chloride and one tenth decrease in iron. *Listeria monocytogenes* lost two thirds of its attachment ability when compared to the control culture that was grown on regular D10 medium. Other pathogenic bacteria such as *Pseudomonas*, *Enterobacter*, and *Flexibacter* exhibited a lower attachment ability when grown in nitrogen-sufficient medium when compared to nitrogen-limited medium (Mceldowney and Flecher, 1986). The effect of nitrogen concentration on attachment ability of *Listeria* could be that nitrogen can change the nitrogen/phosphate ratio of the cell surface. An increase in nitrogen/phosphate ratio has been shown to correlate with a decrease in surface charge and hydrophobicity (Mozes

et al., 1988). It was further demonstrated that a low hydrophobicity was correlated with low attachment ability in *Listeria* (Mafu *et al.*, 1991).

Temperature affects the attachment of *L. monocytogenes* through altering the cells metabolism. Bayles *et al.* (1996) reported that cold stress proteins were induced by changing the growth temperature of *L. monocytogenes* from 37 to 5 ^{o}C. These proteins may change the physicochemical properties or the composition of the cell wall. Smoot and Pierson (1998) observed that the attachment strength of *L. monocytogenes* increased when growth temperature was increased from 10 to 45 ^{o}C.

Bacterial attachment ability is also influenced by hydrophobicity (Gilber *et al.*, 1991). For example, these authors reported that a reduction in the attachment ability of *Escherichia coli* and *Staphylococcus epidermidis* was correlated with a decrease in cell surface hydrophobicity. It was also determined that when the cell surface hydrophobicity increased, cells metabolized glucose to lactic acid or acetic acid and this lowered the environmental pH. In *Listeria*, Mafu *et al.* (1991) found that the attachment ability of cells increased when hydrophobicity increased and the environmental pH was brought close to the isoelectric point of the cell surface.

Attachment ability can also be affected by cell surface composition. It was suggested that the presence of flagella in *L. monocytogenes* can affect early stage attachment by functioning as an adhesive structure on the cell surface. However, nonflagellated strains had the same attachment coverage on stainless steel as flagellated strains after three hour of incubation (Vatanyoopaisarn, 2000). Herald and Zottola (1989) compared the attachment ability of *Pseudomonas fragi* following SDS treatment to remove cell surface proteins and polysaccharides and EDTA or mechanical treatment to remove flagella. The result showed that more than 90 % of attached cells pretreated with SDS were effectively removed, whereas less than 10 % of attached cells pretreated mechanically or with EDTA were removed. In addition, Smoot and Pierson (1998) treated *L. monocytogenes* culture with 0.01 % trypsin, a protein enzyme, during cell exposure to the test surface. More than 99 % of the attached cell population was reduced whwn compared to a non-treated control culture. These studies that have been discussed indicate that surface proteins play an important role in the initial attachment process of *L. monocytogenes*.

Infection by *Listeria* contaminated food

Following food ingestion, bacteria enter intestinal cells through phage cells and/or nonphagocytic cells (epithelial cells, fibroblasts, hepatocytes, endothelial cells and nerve cells). Invasion and survival of *L. monocytogenes* in nonphagocytic cells is mediated by several virulence factors which are listeriolysin O (LLO), phosphatidylinositol phospholipase C (PlcA), phosphatidylacholine phospholipase C (PlcB), ActA, internalin B (InlB), and internalin A (InlA) (Geoffroy *et al.*, 1987; Dramsi *et al.*, 1995; Kocks *et al.*, 1992; Gaillard *et al.*, 1991; Vázquez-Boland *et al.*, 2001). These virulence factors are bacterial surface proteins and have been shown to be the cause of listeriosis.

Listeriolysin O was identified using affinity chromatography methods describes by Geoffroy *et al.* (1987). Listeriolysin O is a sulflydryl-activated toxin and has a molecular weight of 60 kDa. It can function at pH 5.5 and lyse the intracellular vacuole membrane. The pore-forming activity of LLO targets the vacuole membrane cholesterol and helps the bacteria escape from the primary and secondary intracellular vacuoles.

Phospholipases, PlcA and PlcB are two enzymes encoded by *plcA* and *plcB* genes in *L. monocytogenes*. The PlcA along with LLO can lyse the membrane of the primary intracellular vacuole, whereas PlcB along with LLO can disrupt the membranes of the

secondary intracellular vacuole (Vázquez-Boland *et al.,* 2001). The ActA is a 610-amino-acid protein encoded by the *actA* gene. The primary function of ActA is to induce actin polymerization which can promote the intracellular movement of *L. monocytogenes* (Kocks *et al.,* 1992).

Internalins are a group of surface proteins that mediate the attachment and penetration of *L. monocytogenes* to nonphagocytic epithelial cells. At present, more than ten internalin derivatives have been found (Vázquez-Boland *et al.,* 2001). The structure of internalins include the N-terminal cap domain, the leucine rich repeat (LRR) domain, the interrepeat region (IR) domain, and the different C-terminal repeats (Gaillard *et al.,* 1991; Schubert *et al.,* 2002). Internalin A and InlB are among the internalin family and are the two most important proteins involved in *L. monocytogenes* invasion in host (Vázquez-Boland *et al.,* 2001).

Internalin B, a 630-amino-acid protein, was shown to be indispensable in the invasion of hepatocytes (HeLa, Vero, CHO, and Hep2 cells) and fibroblasts encoded by the *inlB* gene (Dramsi *et al.,* 1995). Internalin B contains two regions: the N-terminal and the C-terminal. The first region includes seven leucine-rich repeats (LRRs). It is exposed on the bacterial surface and is used to target the host receptor. The second region includes

a 232-amino-acid section which is required for InlB to anchor onto the bacterial surface (Braun *et al.,* 1997). Internalin B uses the C-terminal and lipoteichoic acid as a ligand to anchor onto the bacterial wall and interact with the cytoplasmic membrane (Cossart and Leuit, 1998; Jonquie`res *et al.,* 1999). Based on the affinity chromatography method and enzyme-linked immunsorbent assay, gC1q-R/p32, a C1q-binding protein, was identified as the receptor of InlB on mammalian cells (Braun *et al.,* 2000).

Internalin A, encoded by the *inlA* gene, was first identified in an invasive strain of *L. monocytogenes* EGD-SmR as a 744-amino-acid protein of 80,289 Dalton (Gaillard *et al.,* 1991). Later, InlA was found to be an 800-amino-acids protein with a molecular weight of 86500 Dalton (Dhar *et al.,* 2000). Internalin A contains two regions: the N- and C-terminals. The first region includes 15 leucine-rich repeats (LRRs). It is exposed on the bacterial surface and is used to target the host receptors. The second region includes 75-amino-acids and is followed by a Leu-Pro-X-Thr-Gly (LPXTG) motif. This region connects to the bacterial cell wall through a covalent linkage (Cossart and Leuit, 1998; Cossart *et al.,* 2003). Based on affinity chromatography, E-cadherin, a transmembrane protein, was identified as the receptor of InlA on epithelial cells. Cadherins are transmembrane glycoproteins which regulate homophilic Ca^{2+}-dependent cell adhesion

by the extracellular N-terminal domain. There are 5 cadherins in the cadherin family: N-cadherin was found in the nervous system, skeletal muscle and cardiac muscle; R-cadherin is found in the nervous system and retina; M-cadherin exits in skeletal muscles; P-cadherin is present in the placenta; and E-cadherin functions in epithelial tissues, skin, liver, and the gastrointestinal tract (Geiger and Ayalon, 1992; Taleichi, 1990). Attachment of *L. monocytogenes* to host epithelial cells is mediated by the LRR-domain of InlA in contact with the N-terminal domain of E-cadherin (Lecuit *et al.*, 1999; Schubert *et al.*, 2002).

The intracellular life cycle of *L. monocytogenes* in the host is dependent on all surface proteins described above. The first step of this cycle is the attachment and penetration of *Listeria* cells to the host cell surface. This step involves both InlA and InlB (Gaillard *et al.,* 1991; Dramsi *et al.,* 1995). During penetration, *Listeria* cells are engulfed in a phagocytic vacuole and then enter the host cell (Gaillard *et al.*, 1987). After entering the host cell, *Listeria* cells express two surface proteins, LLO and PlcA, which act alone to disrupt the membrane of vacuole (Geoffroy *et al.,* 1987; Vázquez-Boland *et al.,* 2001). *Listeria* cells are thus released into intracytoplasm for proliferation and dissemination. For dissemination, *L. monocytogenes* produce another surface protein, ActA, which can

stimulate the formation of actin filaments (Kocks *et al.*, 1992). These filaments can induce intracellular movement of *Listeria* cells. When *L. monocytogenes* comes into contact with host cell membranes, it is enveloped by the membrane to form a secondary vacuole. This vacuole which contains bacteria is then engulfed in another host cell and enveloped by this host cell's membrane. This results in a vacuole that contains two membranes. The two membranes of the secondary vacuole are later lysed by two *Listeria* surface proteins, LLO and PlcB (Geoffroy *et al.*, 1987; Vázquez-Boland *et al.*, 2001). Finally, *Listeria* cells are released into the intracytoplasm and this completes its intracellular life cycle. *Listeria monocytogenes* can continually invade and disseminate the entire host body via lymph and blood by the repetition of its intracellular life cycle (Cossart *et al.*, 2003; Vázquez-Boland *et al.*, 2001; Tilney and Portnoy, 1989).

The regulation of the previously described virulence gene is mediated by the PrfA protein. This protein is a transcriptional activator and the only regulator known up to date that is involved in *L. monocytogenes* (Bockmann *et al.*, 1996). PrfA is a 237-amino-acid protein encoded by the *prfA* gene of the virulence gene cluster of *L. monocytogenes* (Mengaud *et al.*, 1991). The function of PrfA relies on its C-terminal which is a DNA-binding helix-turn-helix (HTH) region. This region can bind to PrfA-regulated

promoters and activate the virulence genes of *L. monocytogenes* (Nancy *et al.*, 1993). The regulation of virulence genes can be totally or partially controlled by PrfA. For instance, the expression of *hly, actA, plcA,* and *plcB* genes are totally regulated by PrfA whereas the inlAB operon is partially regulated by PrfA (Vázquez-Boland *et al.*, 2001).

The PrfA-induced virulence gene expression in *L. monocytogenes* can be affected by a number of parameters such as growth temperature and culture medium. Growth temperature has a positive effect on the expression of the virulence gene in *L. monocytogenes*. Santiago *et al.* (1999) reported that higher temperatures (37, and 42 ^{o}C compared to 25 ^{o}C) stimulated a higher level of expression for the *Listeria* adhesion protein (LAP), a 104 kDa protein. Thermoregulated expression has also been observed for the *inlAB* operon, in which higher expression was found at 37 ^{o}C than 25 ^{o}C (Dramsi *et al.,* 1993). This suggests that the LAP and *inlAB* operon expression-dependent PrfA may be affected by growth temperature. For example, Renzoni *et al.* (1997) evaluated the expression of PrfA at 37 and 20 ^{o}C and showed that PrfA was expressed at 37 ^{o}C but not at 20 ^{o}C. The expression of virulence genes can also be affected by culture media that contain different carbon sources. It was observed that fermentable carbohydrates such as cellobiose and glucose can reduce the expression of the *hly* gene, which is totally

controlled by PrfA. However, the expression of PrfA was not affected by these carbon sources (Datta and Kothary, 1993; Milenbachs *et al*., 1997). These carbon sources may affect the activity of PrfA or the binding of PrfA to its target promoters, and thus the repression of *hly* gene expression (Vázquez-Boland *et al*., 2001). Unlike the *hly* gene, the regulation of *inlA* gene expression is only partially controlled by PrfA. The effect of carbon source on *inlA* gene expression has not yet been reported.

CHAPTER Ⅲ

MATERIALS AND METHODS

Materials

Bacterial strains of *L. monocytogenes* (ATCC 19115, serotype 4b), *L. monocytogenes* (ATCC 19111, serotype 1/2a), *L. monocytogenes* (ATCC 7644, serotype 1/2c), *L. grayi* (ATCC 19120), and *L. innocua* (FDA, soft cheese) were used in this study. These bacteria strains were obtained from Mississippi State University food microbiology laboratory.

Bacterial cultures of *L. monocytogenes* and *Listeria* spp. were grown in tryptic soy broth (TSB) (Becton Dickinson, Sparks, MD) at 37 ^{o}C with 110 rpm shaking for 16 h. Grown cultures were then mixed with glycerol at a ratio of 1:1 and maintained as stock cultures at -70 ^{o}C.

Quantification of Bacterial Attachment Strength

Preparation of bacterial culture

Listeria monocytogenes and *Listeria* spp. from stock cultures were incubated in same medium and incubated for another 16 h. The cultured bacteria were then transferred and incubated in TSB at 37 ^{o}C with 110 rpm shaking until the cells' density reached an absorbance of 1.50 AU at 595 nm (~ 9 log cfu/mL).

Preparation of testing surfaces

Borosilicate glass coverslips (22 mm × 22 mm) (Fisher Scientific, Fair Lawn, NJ) were immersed in 70 % (v/v) concentrated nitric acid ((Fisher Scientific) for 1 h. Concentrated nitric acid was rinsed out with distilled water and sterilized with 70 % (v/v) ethanol (Fisher, Fair Lawn, NJ). The coverslips were then dried for 10 min prior to use.

Attachment strength quantification

Attachment strength of *L. monocytogenes* and *Listeria* spp. was quantified using the Blot Succession Method of Eginton *et al.* (1995), Allison *et al.* (2000), and Kim *et al.* (2005). Each coverslip was colonized with 50 µL of bacterial culture and incubated in a

Low Temperature Incubator 815 (Precision Scientific, Chicago, IL) at 37 ^{o}C for 2 h inside a lidded tray (bioMérieux, St. Louis, MO). Coverslips were rinsed five times with 10 mL of sterile phosphate buffered saline (PBS) solution to remove any unattached or loose cells. Excessive PBS solution was dried by incubation at 37 ^{o}C for 30 min. The coverslips were then removed with forceps and placed flat on the surface of Tryptic Soy Agar (TSA) plates. After one minute, each coverslip was transferred to a second plate and the first plate was spread with a sterilized glass spreader. This process was repeated through a succession of 10 TSA plates.

Standard plate counts were conducted after 48 h of incubation at 37 ^{o}C followed by regression analysis using Excel 2003 software (Microsoft, Redmond, WA). For each strain of bacteria, at least four replicate coverslips were used for analysis.

Detection of InlA in *L. monocytogenes* strains and *Literia* spp.

Preparation of bacterial culture

Listeria monocytogenes and *Listeria* spp. from stock cultures were incubated in TSB at 37 ^{o}C with 110 rpm shaking for 16 h. The bacteria were subcultured once on the

same medium and incubated for another 16 h. The cultured bacteria were then transferred and incubated in TSB at 37 ^{o}C with 110 rpm shaking until the cells density reached an absorbance of 1.0 to 1.2 AU at 595 nm (~ 9 log cfu/mL).

Extraction of bacterial surface proteins

Extraction of bacterial surface proteins was performed following the method of Kocks *et al*. (1992) and Olier *et al.* (2003). Cultures (20 mL) of *L. monocytogenes* and *Listeria* spp. were harvested by centrifugation in CU-5000 CENTRIFUGE (Curtin Matheson Scientific, Houston, TX) at 3,000 g for 10 min at room temperature. Cell pellets were suspended in 20 mL of 10 mM Tris-HCl (pH = 8), and recentrifuged. After recentrifugation, cell pellets were immediately resuspended in 1 % (w/v) SDS of 10 mM Tris-HCl (pH = 8) at the ratio of 1,000 μL per U of absorbance (595 nm). Cell suspensions were transferred to 2.0 mL eppendorfs and incubated for 15 min with gentle rotation (30 rotations per min) on a Dynal rotator (Dynal, New Hyde Park, NY). Cell suspensions were then centrifuged at 20,000 g in a Centrifuge 5415C (Brinkmann Instruments Inc. Westbury, NY) for 10 min at room temperature. The supernatants (500 μL) containing bacterial surface proteins were transferred into new eppendorfs and

precipitated with 16 % (v/v) trichloroacetic acid (Sigma, St. Louis, MO) at 4 °C overnight. The precipitated proteins were collected by centrifugation at 20,000 g for 10 min at room temperature and washed twice with 90 % acetone. Finally, the proteins were suspended in 50 μL of 10 mM Tris-HCl (pH = 8) and stored at -70 ^{o}C prior to performing SDS-PAGE and Western blotting analysis.

SDS-PAGE

Five μL of bacterial surface proteins were mixed with 10 μL Laemmli sample buffer (Laemmli, 1970) and heated in a 95 ^{o}C water bath for five minutes prior to SDS-PAGE. SDS-PAGE was run with 10 % polyacrylamide gel (5 % for stacking gel) under 200 volts for 50 min in a Mini-PROTEAN 3 system (Bio-Rad, Hercules, CA). The gels were stained using a silver staining kit (Bio-Rad, Hercules, CA).

Western blotting

The gels from SDS-PAGE were transblotted to Immobilon P membranes (Millipore, Billerica, MA) in a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA). The procedures followed the western blotting Kit instruction manual (Amersham Biosciences, Piscataway, NJ) with some minor changes.

The transblotting procedure is illustrated in Figure 3.1. A fiber pad on the negative side of the cassette was covered on top by a filter paper. The gel was then placed above the filter paper followed by a pre-wetted membrane. The pre-wetted membrane was previously prepared by activation in 100 % methanol (Fisher, Fair Lawn, NJ) for 15 sec and by hydration with a transfer buffer (48 mM Tris, 39 mM glycine, 0.05% (w/v) SDS, and 7.5% (v/v) methanol). Another filter paper and fiber pad were added on top of the membrane. The transblotting was carried out at 30 volts overnight at 4 ^{o}C.

Non-specific sites of transblotted membrane were blocked by immersing in 5% non-fat dried milk with phosphate buffered saline Tween (PBS-T) buffer (0.1% (v/v) Tween 20, 80 mM Disodium Hydrogen Orthophosphate Anhydrous, 20 mM Sodium Dihydrogen Orthophosphate, 100 mM Sodium Choloride). The membrane was then rinsed twice with PBS-T prior to primary antibody incubation. Primary antibody solution was prepared by diluting the InlA monoclonal antibody (Cedarlane, Ontario, Canada) at the ratio of 1:1000 in PBS-T. The membrane was immersed in the primary antibody solution and incubated for one hour at room temperature on an orbital shaker (Bellco, Vineland, NJ). The membrane was rinsed twice with PBS-T. It was then washed with PBS-T for 15 min followed by three washes of five minutes each at room temperature.

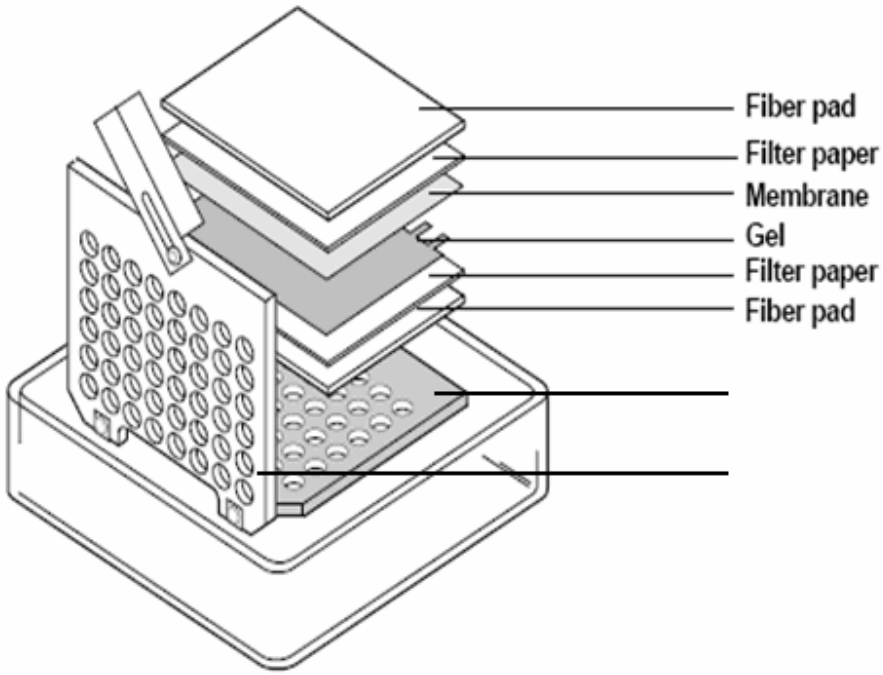

Figure 3.1 Protein transblotting procedure (Bio-Rad, Hercules, CA)

Secondary antibody solution was prepared by diluting the horseradish peroxidase-conjugated secondary antibodies (Dianova, Hamburg, Germany) at the ratio of 1:1000 in PBS-T. The membrane was then immersed in the secondary antibody solution and incubated for one hour at room temperature in an orbital shaker. After incubation, the membrane was rinsed and washed as described after the primary antibody incubation.

The blotted membrane was subject to a Chemiluminescence-based Immunoblot Assay (Amersham Biosciences, Piscataway, NJ). The membrane was first incubated in a mixture of 2.5 mL detection solution 1 and 2.5 mL detection solution 2 for one minute at room temperature. Excess detection solution was drained and the entire membrane surface was wrapped with SaranWrap^{TM} (Sigma, St. Louis, MO). The wrapped membrane was placed in an X-ray film cassette with proteins facing upwards. A Hyperfilm ECL film (Amersham Biosciences, Piscataway, NJ) was placed on top of the membrane and exposed for 15 sec. The film was developed immediately with the Kodak M25A X-OMAT Processor (NorMont NDT Inc., Montana, Canada). A second exposure was necessary if band intensities in the first exposure were too strong or weak for depiction. The second exposure varied from 15 sec to several minutes. The band of InlA (80 kDa)

for different *L. monocytogenes* were quantified using the Un-Scan-It® software program (Silk Scientific, Orem, UT).

Evaluation of InlA expression under different temperature treatments

Preparation of bacterial culture

Listeria monocytogenes ATTCC 19115, *L. monocytogenes* ATTCC 19111, and *L. monocytogenes* ATTCC 7644 from stock cultures were incubated in TSB at 37 ^{o}C with 110 rpm shaking for 16 h. The bacteria were subcultured once on the same medium and incubated for another 16 h. The cultured bacteria were then transferred and incubated in TSB at 40, 25, and 10 ^{o}C with 110 rpm shaking. When the cell density reached absorbance at weave length 595: 1.0 to 1.2 AU, bacteria were used as the working culture (~ 9 log cfu/mL).

Extraction of bacterial surface proteins, SDS-PAGE, Western blotting analysis

The extraction of bacterial surface proteins was conducted following the same procedure as described in page 22. After extraction, bacterial surface proteins were assessed by SDS-PAGE and Western blotting as described in page 23.

Evaluation of InlA expression under different culture media

Preparation of bacterial culture

Listeria monocytogenes ATTCC 19115, *L. monocytogenes* ATTCC 19111, and *L. monocytogenes* ATTCC 7644 from stock cultures were incubated in TSB at 37 ^{o}C with 110 rpm shaking for 16 h. The bacteria were subcultured once on the same medium and incubated for another 16 h. The cultured bacteria were then transferred and incubated in Brain Heart Infusion broth (BHI) and Luria-Bertani Broth (LB) (Becton Dickinson, Sparks, MD) at 37 ^{o}C with 110 rpm shaking. When the cell density reached absorbance of 1.0 to 1.2 and 0.35 to 0.55 at wavelength 595 for BHI and LB, respectively, bacteria were used as working culture (~ 9 log cfu/mL).

Extraction of bacterial surface proteins, SDS-PAGE, Western blotting analysis

The extraction of bacterial surface proteins was conducted following the same procedure as described in page 22. After extraction, bacterial surface proteins were assessed by SDS-PAGE and Western blotting as described in page 23.

CHAPTER Ⅳ

RESULTS AND DISCUSSIONS

Quantification of bacterial attachment strength

The plate blot succession method of Eginton *et al.* (1995), Allison *et al.* (2000), and Kim *et al.* (2005) was used in the present study to assess the number of residual attached bacteria colonies transferred to a succession of plates. In this method, the number of recovered colonies per plate decreased exponentially with plate succession number according to the relationship $CFU = A * 10^{-kN}$ (CFU is the number of colonies transferred, A is the intercept, N is the plate succession number, and k is the removal exponent). The removal exponent, k, reflects the ease of removal of residual attached bacteria from the glass coverslips. Attachment strength of bacteria is assessed based on the *k* value: the smaller is the *k*, the greater is the strength of attachment.

To calculate the extent of attachment strength to glass coverslips of *L. monocytogenes* 19115, 19111, 7644, *L. grayi* and *L. innocua* after incubation for 1h, the mean cfu recovered from each of the 10 TSA plates was determined (Fig. 4.1). The results

demonstrate that under the same growth conditions, the attachment strength were different among *L. monocytogenes* and *Listeria* spp. Among the bacterial strains, *L. monocytogenes* 19111 exhibited the strongest attachment strength followed by *L. monocytogenes* 19115, *L. grayi*, *L. innocua*, and *L. monocytogenes* 7644.

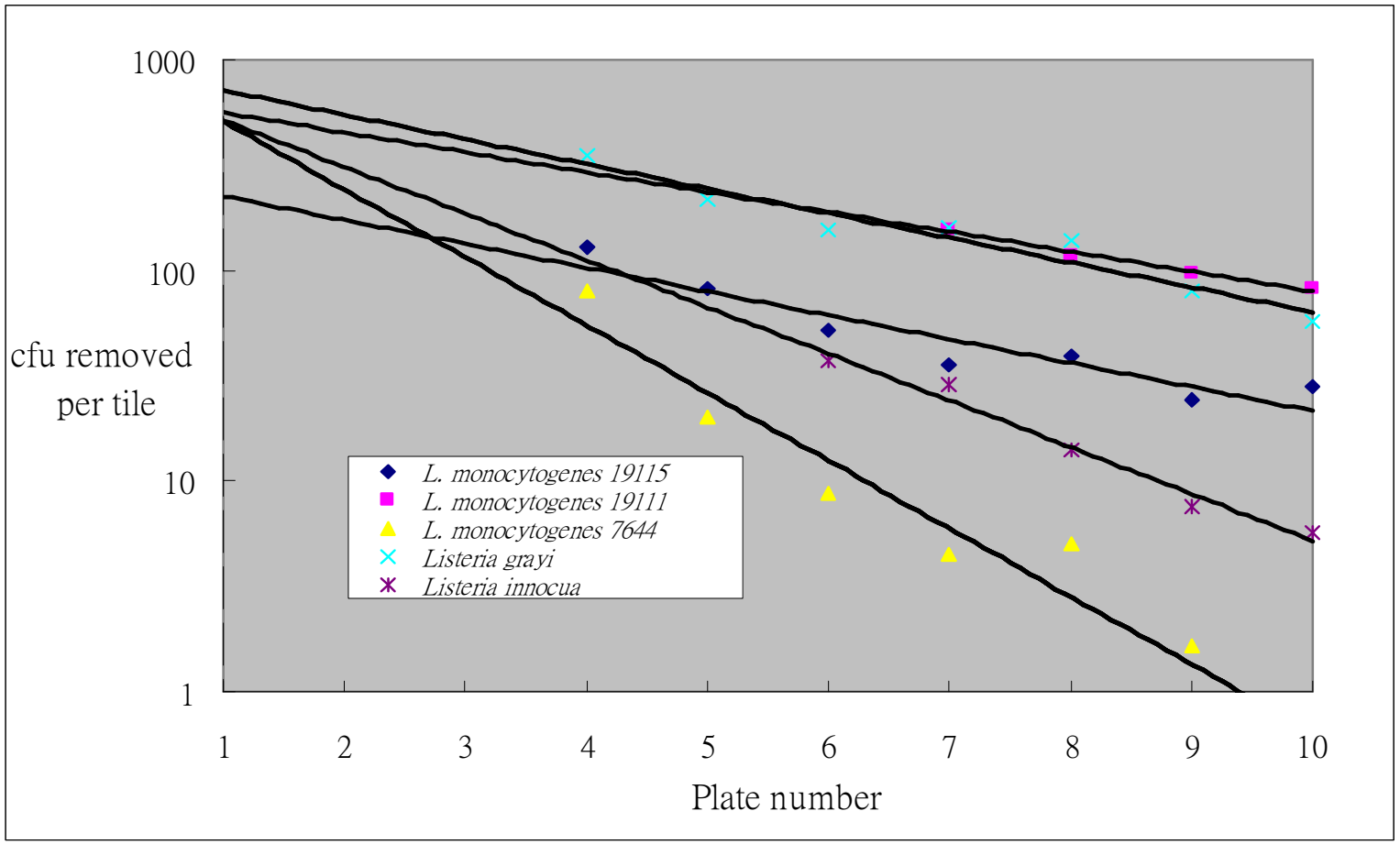

Figure 4.1 Attachment strength of different strains of *L. monocytogenes* and *Listeria* spp. Coverslips were colonized for 2 h by immersion in cultured medium upon successive transferring and blotting onto TSA plates

Table 4.1 Regression analysis of colony forming units removed from test surface upon successive transfer and blotting on tryptic soy agar plates

Bacterial Strain	A (cfu/plate)	k(cfu)	R^2
L. monocytogenes 19115	292	0.260	0.879
L. monocytogenes 19111	696	0.217	0.986
L. monocytogenes 7644	1067	0.741	0.949
L. grayi	938	0.269	0.935
L. innocua	859	0.510	0.976

$CFU = A * 10^{-kN}$, where CFU is the number of colonies transferred, *k* is the removal exponent, A is the intercept and N is the plate succession number. The units for A is cfu/plate and k is cfu also on table.

Detection of Internalin A in *L. monocytogenes* strains and *Listeria* spp.

To determine whether *L. monocytogenes* strains and *Listeria* spp. can express InlA, the bacterial surface extracts were examined by SDS-PAGE and Western blotting, and the results are shown in Figure 4.2 (p. 31). Silver stained SDS gel did not reveal the presence of InlA, which has a molecular weight of 80 kDa, in the five samples tested. However, InlA was detected in the bacterial extracts of *L. monocytogenes* 19111 (lane2) and 7644 (lane3) by Western blotting. The corresponding band intensities were 50.1 and 2.5 pixels, respectively.

According to Smoot and Pierson (1998), proteins play a major role in the initial attachment process of *L. monocytogenes*. Several surface proteins in *L. monocytogenes* such as InlA, InlB, Lap, and p60 have been identified to be involved in bacterial adhesion to human cell lines (Pandiripally *et al.*, 1999; Vázquez-Boland *et al.*, 2001). In *L. monocytogenes*, InlA is the major surface protein that regulates the entry of bacteria into human intestine epithelium cells (Gaillard *et al.*, 1991).

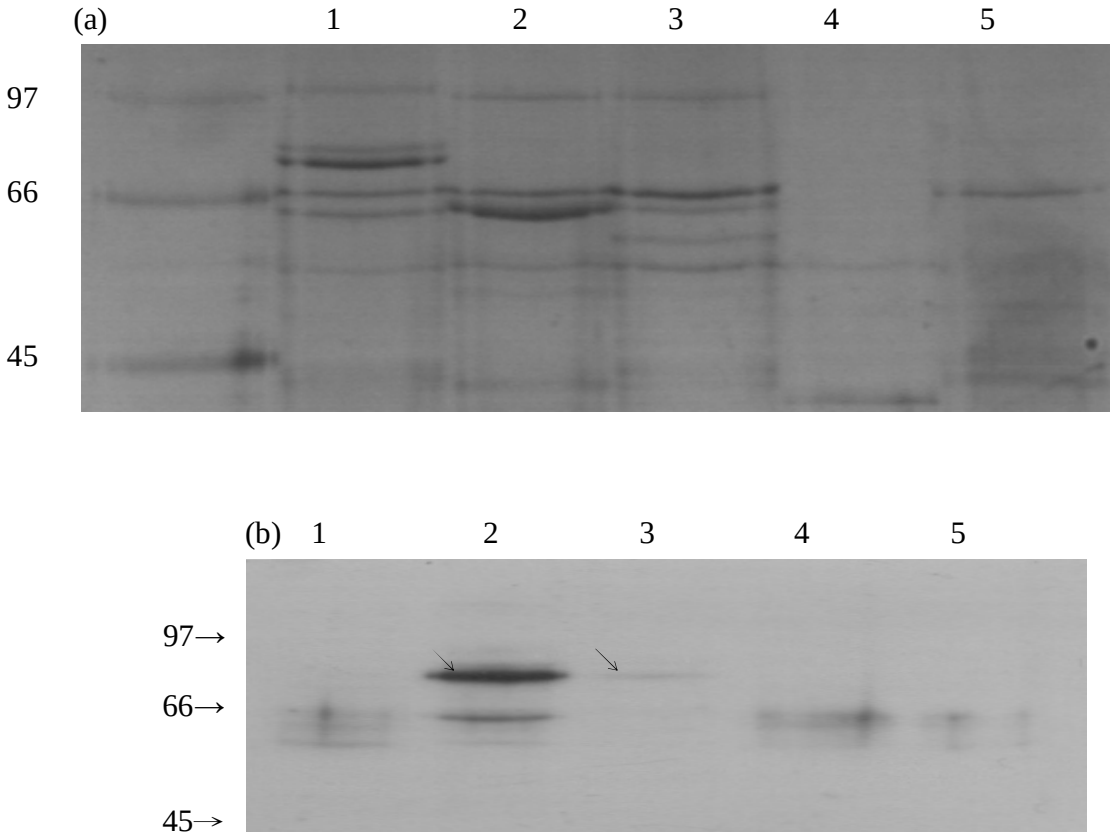

Figure 4.2 Analysis of bacterial surface proteins and InlA expression by silver stained SDS gel (a) and Western blotting (b). *L. monocytogenes*19115 (lane 1), 19111 (lane 2), 7644 (lane 3), *L. grayi* (lane 4), and *L. innocua* (lane 5)

In the present study, it was speculated that a high amount of InlA expression could induce a high attachment strength. Our experiments demonstrated that the presence of InlA was correlated with a high attachment strength in *L. monocytogenes* 19111 (Figures 4.1, 4.2). The potential pathogenicity of *L. monocytogenes* 19111 could possibly be monitored by the expression of InlA. However, *L. monocytogenes* 7644 also expressed InlA, but exhibited an inferior attachment strength when compared to than *L. monocytogenes* 19115 which did not express InlA. A possible explanation for this could be that *L. monocytogenes* 19115 may express surface proteins for adhesion other than InlA and these proteins may have induced stronger attachment than InlA. Or, *L. monocytogenes* 7644 might also express surface proteins other than InlA but in less quantity than in *L. monocytogenes* 19115.

Evaluation of InlA expression under different temperature treatments

The expression of InlA in *L. monocytogenes* strains was studied following growth temperature treatments at 40, 25, and 10 ^{o}C. InlA presence was not detected using the silver staining method (Fig. 4.3a). However, InlA expression could be detected by Western blotting. Internalin A were expressed in *L. monocytogenes* 19111 incubated at 40,

25, and 10 ^{o}C and in *L. monocytogenes* 7644 at 40 ^{o}C. The intensities of InlA in *L. monocytogenes*19111 were 33.6 pixels at 40 ^{o}C (lane 2), 25.6 pixels at 25 ^{o}C (lane 5), and 2.1 pixels at 10 ^{o}C (lane 8). The intensity of InlA in *L. monocytogenes* 7644 was 13.5 pixels at 40 ^{o}C (lane 3). Thus, the higher the growth temperature was, the stronger were the intensities of InlA. However, InlA was not detected in *L. monocytogenes* 7644 at 25 and 10 ^{o}C, and in *L. monocytogenes* 19115 at all the three temperatures (Fig 4.3b).

A 237-amino-acid protein, PrfA, regulates all the virulence genes of *L. monocytogenes*, including the *inlA* gene (Mengaud *et al.*, 1991). Four promoters were found to regulate the *inlA* gene transcription but only one of these was PrfA dependent (Lingnau *et al.*, 1995). According to these authors, the *inlA* gene is regulated by both PrfA-dependent and PrfA-independent mechanisms. The exact regulation of the The PrfA-independent mechanism is still unknown. However, the mechanism of PrfA-dependent *inlA* gene regulation could be influenced by growth temperature. Renzoni *et*

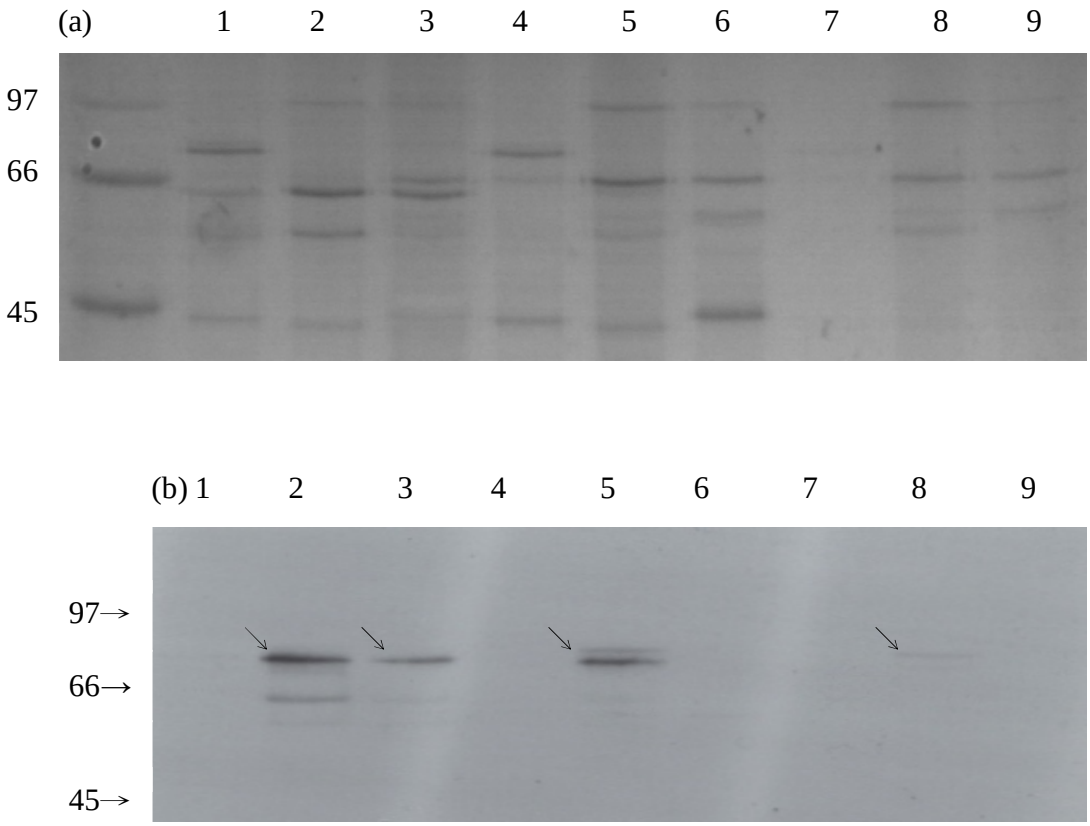

Figure 4.3 Silver stained SDS gel (a) and western blotting (b) analysis of bacterial surface proteins in *L. monocytogenes* strains following different growth temperature treatments. *L. monocytogenes*19115 at 40 ^{o}C (lane 1), 25 ^{o}C (lane 4), and 10 ^{o}C (lane 7), 19111 at 40 ^{o}C (lane 2), 25 ^{o}C (lane 5), and 10^{o}C (lane 8), and 7644 at 40 ^{o}C (lane 3), 25 ^{o}C (lane 6), and 10^{o}C (lane 9)

al. (1997) found that the PrfA protein was undetectable in *L. monocytogenes* when it was incubated at 20 ^{o}C. Similarly, the expression of listeriolysin O, the product of the transcription of another virulence gene *hly*, was lower at 26 ^{o}C than 37 ^{o}C (Datta and Kothary, 1993). In the present study, it was shown that InlA was detected in *L. monocytogenes* 19111 grown at 25 ^{o}C and 10 ^{o}C, temperatures at which PrfA was undetectable (Renzoni *et al.*, 1997). It is therefore speculated that the expression of *inlA* gene in *L. monocytogenes* 19111 might be regulated via a PrfA-independent mechanism. Also, the expression of InlA of *L. monocytogenes* 19111 might be thermo-regulated. In contrast to *L. monocytogenes 19111*, InlA was detected at growth temperatures of 40 ^{o}C and 37 ^{o}C in *L. monocytogenes* 7644 (Figs. 4.2b, 4.3b). This could indicate that *inlA* in *L. monocytogenes* 7644 might be regulated by a PrfA-dependent mechanism. Adoption of either a PrfA-dependent or -independent mechanism could be the means by which each strain of *L. monocytogenes* adapts to environmental changes.

Temperature is one of the environmental parameters that can affect expression of *Listeria* surface proteins. In the present study, *L. monocytogenes* grown at 25 ^{o}C (normal environmental temperature) has a low or no expression of InlA when compared to growth at 37 ^{o}C (warm blood host temperature). At temperatures from 20 to 25 ^{o}C where the

expression of InlA is low, *L. monocytogenes* were reported to express flagellins (Peel *et al.,* 1988). The flagellins could produce tumbling motility that may assist *L. monocytogenes* search for food. At the warm blood host temperature (37 ^{o}C), InlA can be expressed, which can assist *L. monocytogenes* to invade host cell lines (Figure 4.2b).

Evaluation of InlA expression under different culture media

A nutrient-rich broth (BHI), and a nutrient-poor broth (LB), were tested in order to calculate the effect of nutrients on InlA expression in *L. monocytogenes* strains. The silver staining method was not able to detect the expression of InlA (Fig 4.4a). However, InlA was detected by Western blotting in *L. monocytogenes* 19111 cultured on both BHI and LB brothes, with InlA intensities of 11.4 pixels for BHI and 1.6 pixels for LB (Fig. 4.4b).

It was documented that the virulence genes in *L. monocytogenes* are repressed by fermentable carbohydrates such as glucose and β-glucosides cellobiose (Datta and Kothary, 1993; Milenbachs *et al.,* 1997; Brehm *et al.,* 1999), with the exception of glucose-1-phosphate which does not repress the expression of the virulence genes (Ripio

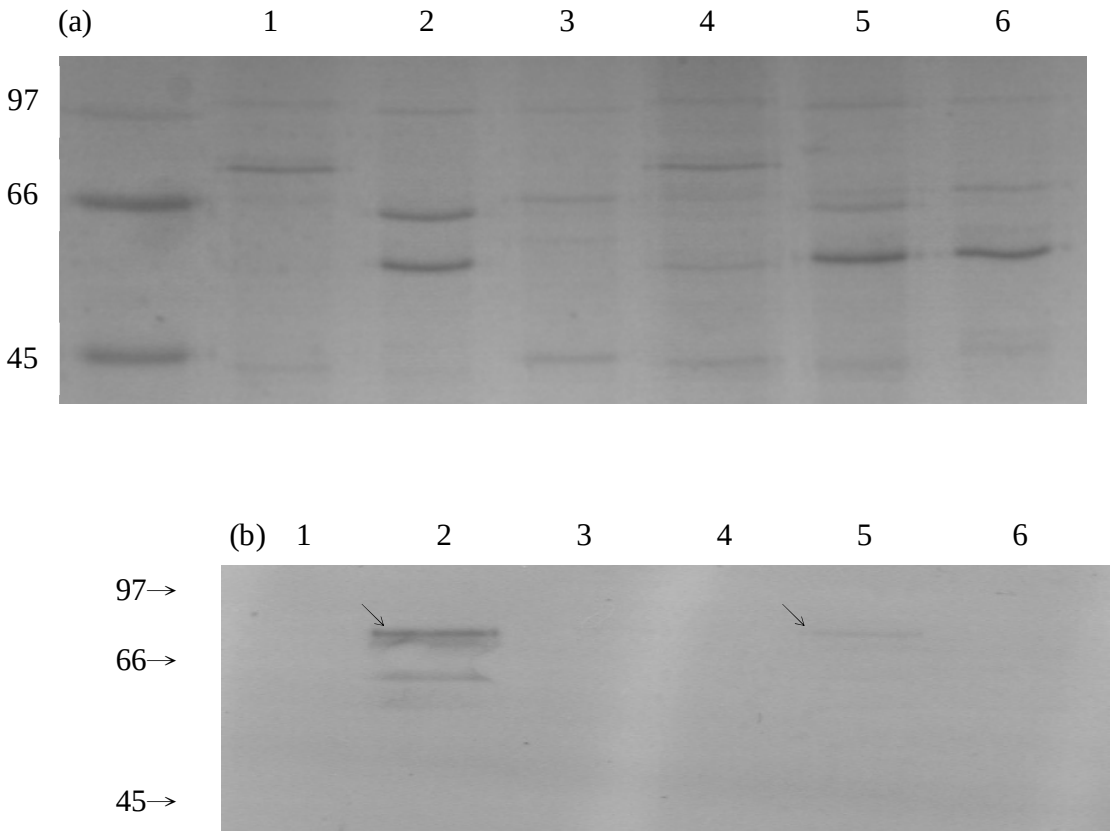

Figure 4.4 Silver stained SDS gel (a) and western blotting (b) analysis of bacterial surface proteins in *L. monocytogenes* strains following different culture media treatments. *L. monocytogenes*19115 in BHI (lane 1) and, LB (lane 4), 19111 in BHI (lane 2), and LB (lane 5), and 7644 in BHI (lane 3), and LB (lane 6)

et al., 1997). Milenbachs *et al.* (1997) found that the transcription of virulence genes *hly* and *plcA* were reduced, when the concentrations of cellobiose reached the repressing level (> 1 mM), although the presence of cellobiose did not affect the amount of PrfA protein produced in *L. monocytogenes*. This suggests that there might be a mechanism other than PrfA-dependent mechanism that is involved in the regulation of virulence genes. This mechanism may reduce the activity of PrfA or have some negative effect on the promoters of virulence genes. It was also reported that the expression of listeriolysin O, a product of the *hly* gene, in *L. monocytogenes* was reduced when the culture medium contained more than 0.3 % of glucose (Datta and Kothary, 1993). However, there is no report on the effects of fermentable sugars on the expression of InlA to date.

In the present study, dextrose was the only carbohydrate in the different culture media tested with 0.014 M in TSB, 0.011 M in BHI, and 0 M in LB Broth (Table 4.2). The expressions of InlA were detected in *L. monocytogenes* 19111 when grown in TSB, BHI, and LB media with band intensities of 50.1 (Fig. 4.2b), 11.4 and 1.6 pixels (Fig. 4.4b), respectively. The concentrations of dextrose used in TSB and BHI may not be high enough to repress virulence genes in *L. monocytogenes*.

Table 4.2 Compositions of culture media used

Tryptic Soy Broth	Pancreatic Digest of Casein	17 g
	Enzymatic Digest of Soybean Meal	3 g
	Dextrose	2.5 g
	Sodium Chloride	5 g
	Dipotassium Phosphate	2.5 g
Brain Heart Infusion Broth	Brain Heart, Infusion from (Solids)	3.5 g
	Peptic Digest of Animal Tissue	15 g
	Pancreatic Digest of Casein	10 g
	Dextrose	2 g
	Sodium Chloride	5 g
	Dipotassium Phosphate	2.5 g
Luria-Bertani Broth	Tryptone	10 g
	Yeast Extract	5 g
	Sodium Chloride	10 g

Listeria monocytogenes 7644, unlike *L. monocytogenes* 19111, expressed InlA only when grown in TSB and not in BHI and LB. It may be that, the concentration of dextrose in TSB was not high enough to repress *inlA* expression. However, InlA expression was not observed in BHI and LB media which contained a lower dextrose concentration than TSB. Therefore, other factors than dextrose might be involved in the expression of InlA in *L. monocytogenes* 7644. For example, the different nutrients in the three culture media other than dextrose, such as enzymatic digest of soybean meal in TSB, brain heart

infusion from (solids), and peptic digest of animal tissue in BHI, and yeast extract in LB, might affect InlA expression and thus should be further investigated. Also, the use of dextrose may go through the same metabolism as glucose-1-phosphate, which is positively coregulated with the activation of PrfA regulon in *L. monocytogenes* (Ripio *et al.,* 1997).

Throughout the experiments, InlA was only detected in Western blotting images but not in silver stained SDS gels. This may be because the InlA does not reduce the silver anions to silver grains to form a band on the SDS gels. As a result, it is recommended to use Western blotting to study InlA expression.

CHAPTER V

SUMMARY AND CONCLUSIONS

The present study aimed to investigate the relationship between the attachment strength of *L. monocytogenes* and *Listeria* spp. and the expression of their surface protein InlA. The effects of temperature and culture media on the expression of InlA were also studied.

The relationship between attachment strength and InlA expression was dependent on the *L. monocytogenes* strain studied. For example, *L. monocytogenes* 19111 exhibited the highest InlA expression and also had the strongest attachment. *Listeria monocytogenes* 19115, however, did not express InlA but it had a stronger attachment than *L. monocytogenes* 7644 which demonstrated InlA expression. This could suggest that the expression of InlA alone cannot explain attachment strength of these *L. monocytogenes* strains. Other surface proteins might play a role in the attachment of *L. monocytogenes* 19115 and 7644.

The expression of InlA in *L. monocytogenes* 19111 was observed when grown at

40, 25 and 10 ^{o}C (33.6, 25.6, and 2.1 pixels respectively) and in *L. monocytogenes* 7644 at 40 ^{o}C. The results demonstrated that the expression of InlA is thermo-regulated between 40 to 10 ^{o}C and that it decreases with decreasing temperature. However, the expression of the *inlA* gene in *L. monocytogenes* 19111 might also involve a PrfA-independent mechanism. This is in agreement with the findings of Renzoni *et al.* (1997) in which PrfA was undetectable when bacteria were grown below 25 ^{o}C.

The expression of InlA was observed in *L. monocytogenes* 19111 when grown in three different culture media (TSB, BHI, and LB) and in *L. monocytogenes* 7644 in TSB. The results demonstrated that the expression of InlA was not affected by dextrose. Other media nutrients need to be studied to understand the mechanism of regulation.

Throughout the experiments, InlA was only detected in Western blotting images but not in silver stained SDS gels. Western blotting analysis is required for further studying of InlA expression.

The present study opens some questions that can be investigated in the future. For example, the attachment strength of *L. monocytogenes* might be directly affected by an array of surface proteins, such as the internalin family, LAP and, p60. It will therefore be useful to identify surface proteins in addition to InlA and evaluate the quantity of these

proteins in relation to *Listeria* attachment strength. This could be carried out by Western blotting. Furthermore, attachment strength of *L. monocytogenes* on human cells via different surface proteins could be investigated.

More work on the regulation factors of surface proteins' expression in *L. monocytogenes* would be of great interest. Understanding how factors such as temperature, nutrient requirement, and incubation condition regulate the expression of the surface proteins would be a useful tool to artificially control the expression of these proteins. For example, down-regulation of bacterial surface proteins could be used to reduce pathogenicity of *L. monocytogenes* and food contamination.

REFERENCES

Allison D.G., Cronin M.A., Hawker J., and Freeman S. 2000. Influence of cranberry juice on attachment of *Escherichia coli* to glass. J. Basic Microbiol. 40:3-6.

Bayles D.O., Annous B.A., and Wilkinson B.J. 1996. Cold stress proteins induced in *Listeria monocytogenes* in response to temperature downshock and growth at low temperatures. Appl. Environ. Microbiol. 62:1116-1119.

Blackman I.C., and Frank J.F. 1996. Growth of *Listeria monocytogenes* as a biofilm on various food-processing surfaces. J. Food Prot. 59:827-831.

Boerlin P., Rocourt J., Grimont P.A.D., Jacqut C., and Piffaretti J.C. 1992. *Listeria ivanovii* subsp nov. Int. J. Syst. Bacteriol., 42: 69-73.

Braun L., Dramsi S., Dehoux P., Bierne H., Lindahl G., and Cossart P. 1997. InlB: an invasion protein of Listeria monocytogenes with a novel type of surface association. Mol. Microbiol. 25:285-294.

Braun L., Ghebrehiwet B., and Cossart P. 2000. gC1q-R/p32, a C1q-binding protein, is a receptor for the InlB invasion protein of *Listeria monocytogenes*. EMBO J. 19:1458-1466.

Brehm K., Ripio M.T., Kreft J., and Vázquez-Boland. 1999. The *bvr* locus of *Listeria monocytogenes* mediates virulence gene repression by β-glucosides. J. Bacteriol. 181:5024-5032.

Cole M., Jones M. and Holyoak C. 1990. The effect of pH, salt concentration and temperature on the survival and growth of *Listeria monocytogenes*. J. Appl. Microbiol. 69:63-72.

Cossart P., Cerda-Piarro J., and Lecuit M. 2003. Invasion of mammalian cells by *Listeria monocytogenes*: functional mimicy to subvert cellular functions. Trends in Cell Biol. 13:23-31.

Cossart P., and Lecuit M. 1998. Interactions of *Listeria monocytogenes* with mammalian cells during entry and actin-based movement: bacterial factors, cellular ligands and signaling. Euro. Mol. Biology Organization J. 17:3797-3804.

Costeron J.W., Cheng K.J., Geesey G.G., Ladd T.I., Nickel J.C., Dasgupta M., and Marrie T.J. 1987. Bacterial biofilms in nature and disease. Ann. Rev. Microbiol 41:435-464.

Cummins A.J., Fielding A.K., and Mclauchlin J. 1994. Listeria ivanovii infection in a patient with AIDS. J. Infect. 28:89-91.

Dalton C.B., Austin C.C., Sobel J., Hayes P.S., Bibb W.F., Graves L.M., Swaminathan B., Proctor M.E., and Griffin P.M. 1997. An Outbreak of Gastroenteritis and Fever Due to *Listeria monocytogenes* in Milk. N. Engl. J. Med. 336:100-105.

Datta A.R., and Kothary M.H. 1993. Effects of glucose, growth temperature, and pH on listeriolysin O production in *Listeria monocytogenes*. Appl. Environ. Microbiol. 59:3495-3497.

Dickneite C., Bökmann R., Spory A., Goebel W. and Sokolovic Z. 1998. Differential interaction of the transcription factor PrfA and the PrfA-activating factor (Paf) of *Listeria monocytogenes* with target sequences. Mol. Microbiol. 27:915–928.

Dhar G., Faull K.F., and Schneewind O. 2000. Anchor structure of cell wall surface proteins in *Listeria monocytogenes*. Biochemistry. 39:3725-3733.

Dramsi S., Kocks C., Forestier C., and Cossart P. 1993. Internalin-mediated invasion of epithelial cells by *Listeria monocytogenes* is regulated by the bacterial growth state, temperature and the pleiotropic activator prfA. Mol. Microbiol. 9:931-941.

Dramsi S., Biswas I., Maguin E., Braun L., Mastroeni P., Cossart P. 1995. Entry of *Listeria monocytogenes* into hepatocytes requires expression of InIB, a surface protein of the internalin multigene family. Mol. Microbiol. 16:251-61.

Eginton P.J., Gibson H., Holah J., Handley P.S., and Gilbert P. 1995. Quantification of the ease of removal of bacteria from surfaces. J. Indust. Microbiol. 15:305-310.

Farber J.M., and Peterkin P.I. 1991. *Listeria monocytogenes*, a food-borne pathogen. Microbiol. Rev. 55:476-511.

Fletcher M. 1977. The effects of culture concentration and age, time, and temperature on bacterial attachment to polystyrene. Can. J. Microbiol. 23:1-6.

Frank J.F., and Koffi R.A. 1990. Surface-adherent growth of *Listeria monocytogenes* is associated with increased resistance to surface sanitizers and heat. J. Food Prot. 53:928-932.

Gaillard J.L., Berche P., Frehel C., Gouin E., Cossart P. 1991. Entry of *L. monocytogenes* into cells is mediated by internalin, a repeat protein reminiscent of surface antigens from gram positive cocci. Cell. 65:1127-41.

Gaillard J.L., Berche P., Mounier J., Richard S., and Sansonetti P. 1987. In vitro model of penetration and intracellular growth of *Listeria monocytogenes* in the human enterocyte-like cell line Caco-2. Infect. Immun. 55:2822-2829.

Gabis D., and Faust R.E. 1988. Controlling microbial growth in food processing environments. Food Technol. 42(4):81-82,89.

Geiger B., and Ayalon O. 1992. Cadherins. Annu.Rev. Cell Biol. 8:307-332.

Geoffroy C., Gaillard J.L., Alouf J.E., and Berche P. 1987. Purification, characterization, and toxicity of the sulfhydryl-activated hemolysin Listeriolysin O from *Listeria monocytogenes*. 55:1641-1646.

Gilbert P., Evans D.J., Evans E., Duguid I.G., and Brown M.R.W. 1991. Surface characteristics and adhesion of Escherichia coli and Staphylococcus epidermidis. J. Appl. Bacteriol. 71:71-77.

Gorski L., Palumbe J.D., and Mandrell R.E. 2003. Attachment of *Listeria monocytogenes* to radish tissue is dependent upon temperature and flagellar motility. Appl. Environ. Microbiol. 69:258-266.

Gouin E., Mengaud,J., and Cossart P. 1994. The virulence gene cluster of *Listeria monocytogenes* is also present in *Listeria ivanovii*, an animal pathogen, and *Listeria seeligeri*, a nonpathogenic species. Infect. Immun. 62:3550–3553.

Gray, M. L., and Killinger A. H. 1966. *Listeria monocytogenes* and listeric infections. Bacteriol. Rev 30:309-382.

Herald P.J. and Zottola E.A. 1989. Effect of various agents upon the attachment of *Pseudomonas fragi* to stainless steel. J. Food Sci. 54:461-464.

Hof H., and Hefner P. 1988. Pathogenicity of *Listeria monocytogenes* in comparison to other Listeria species. Infection 16 (Suppl. 2):141-144.

Jonquie`res R., Bierne., Fiedler., Gounon P. and Cossart P. 1999. Interaction between the protein InlB of *Listeria monocytogenes* and lipoteichoic acid: a novel mechanism of protein association at the surface of gram-positive bacteria. Mol Microbiol. 34:902-914.

Kim K.Y., and Frank J.F.1994. Effect of growth nutrients on attachment of *Listeria monocytogenes* to stainless steel. J. Food Prot. 57:720-726.

Kim T. and Silva J.L. 2005. Quantification of attachment strength of selected foodborne pathogens by the blot succession method. J. Rapid Method. Auto. in Microbiol. 13:127-133.

Kocks C., Gouin E., Tabouret M., Berche P., Ohayon H., and Cossart P. 1992. L. monocytogenes-induced actin assembly requires the actA gene product, a surface protein. Cell. 68:521-531.

Krysinski E.P., Brown L.J., and Marchisello T.J. 1992. Effect of cleaners and sanitizers on *Listeria monocytogenes* attached to product contact surfaces. J. Food Prot. 55: 246-251.

Laemmli U.K. 1970. Cleavage of a structural protein during the assembly of the head of bacteriophage T4. Nature. 227:680–685.

Lecuit M., Ohayon H., Braun L., Mengaud J., and Cossart P. 1997. Internalin of *Listeria monocytogenes* with an intact leucine-rich repeat region is sufficient to promote internalization. Infect. Immun. 65:5309-5319.

Lecuit M., Vandormael- Pournin S., Lefort J., Huerre M., Gounon P., Dupuy C., Babinet C., Cossart P. 2001. A transgenic model for listeriosis: role of internalin in crossing the intestinal barrier. Sci. 292:1722-1725.

Lee S.H., and Frank J.F. 1991. Inactivation of surface-adherent *Listeria monocytogenes* hypochlorite and heat. J. Food Prot. 54:4-6.

Lingnau A., Domann E., Hudel M., Bock M., Nichterlein T., Wehland J., and Chakraborty T. 1995. Expression of the *Listeria monocytogenes* EGD *inl*A and *inl*B genes, whose products mediate bacterial entry into tissue culture cell lines, by PrfA-dependent and –independent mechanisms. Infect. Immun. 63:3896-3903.

Mafu A.A., Roy D., Goulet J., and Magny P. 1990. Attachment of *Listeria monocytogenes* to stainless steel, glass, polypropylene, and rubber surfaces after short contact times. J. Food Prot. 53:742-746.

Mafu A.A., Roy D., Goulet J., and Savone L. 1991. Characterization of physiochemical forces involved in adhesion of *Listeria monocytogenes* to surfaces. Appl. Environ. Microbiol. 57:1969-1973

Maijala R., Lyytikäinen O., Johansson T., Autio T., Aalto T., Haavisto L., Honkanen-Buzalski T. 2001. Exposure of *Listeria monocytogenes* within an epidemic caused by butter in Finland. Int. J. Food Microbiol. 70:97-109.

McEldowney S. and Fletcher M. 1986. Effect of growth conditions and surface characteristics of aquatic bacteria on their attachment to solid surfaces. J. Gen. Microbiol. 132:513-523.

McGee Z. A., G. L. Gorby, P. B. Wyrick, R. Hodinka, and L. H. Hoffman. 1988. Parasite-directed endocytosis. Rev. Infect. Dis. 10 (Suppl. 2):S311-S316.

Mengaud J., Dramsi S., Gouin E., Vázquez-Boland,J. A., Milon G. and Cossart P. 1991. Pleiotropic control of *Listeria monocytogenes* virulence factors by a gene which is autoregulated. Mol. Microbiol. 5:2273–2283.

Mengaud J., Ohayon H., Gounon P., Mege R., and Cossart P. 1996. E-cadherin is the receptor for internalin, a surface protein required for entry of *Listeria monocytogenes* into epithelial cells. Cell. 84:923–932.

Milenbachs A.A., Brown D.P., Moors M., and Youngman P. 1997. Carbon source regulation of virulence gene expression in *Listeria monocytogenes*. Mol. Microbiol. 23:1075–1085.

Mozes N., Leonard A.J., and Rouxhet P.G. 1988. On the relations between the elemental surface composition of yeasts and bacteria and their charge and hydrophobicity. Biochim. Biophys. Acta. 945:324-34.

Murray E. G. D., Webb R. A., and Swann M. B. R. 1926. A disease of rabbits characterized by large mononuclear leukocytosis, caused by a hitherto undescribed bacillus *Bacterium monocytogenes* (n. sp.). J. Pathol. Bacteriol. 29:407-439.

Norwood D.E. and Gilmour A. 2001. The differential adherence capabilities of two *Listeria monocytogenes* strains in monoculture and multispecies biofilms as a function of temperature. Appli. Microbiol. 33:320-324.

Olier M., Pierre F., Rousseaux S., Lemaître J.P., Rousset A., Piveteau P., and Guzzo J. 2003. Expression of truncated internalin A is involved in impaired internalization of some *Listeria monocytogenes* isolates carried asymptomatically by humans. Infect. Immun. 71:1217–1224.

Pandiripally V., Westbrook D.G., Sunki G.R., and Bhunia A.K. 1999. Surface protein p104 is involved in adhesion of *Listeria monocytogenes* to human intestinal cell line, Caco-2. J. Med. Microbiol. 48:117-124.

Paul S. M., Laurence S., Vance D., Linda F. M., Joseph S. B., Craig S. 1999. Food-Related Illness and Death in the United States. Emerging Infect. Dis. 5: 607-625.

Peel M., Donachie W., and Shaw A. 1988. Temperature dependent expression of flagella of *Listeria monocytogenes* studied by electron microscopy, SDS-PAGE, and western blotting. J. Gen. Microbiol. 143:2171-2178.

Pickett E.L., and Murano E.A. 1996. Sensitivity of *Listeria monocytogenes* to sanitizers after exposure to a chemical shock. J. Food Prot. 59:374-378.

Ripio F.X., Brehm K., Lara M., Suarez M., and Vázquez-Boland J.A. 1997. Glucose-1-phosphate utilization by *Listeria monocytogenes* is PrfA dependent and coordinately expressed with virulence factors. J. Bacterial. 179:7174-7180.

Rocourt J., and Grimont P. A. D. 1983. Listeria welshimeri sp. nov. and sp. Listeria seeligeri sp. nov. Int. J. Syst. Bscteriol. 33:866-869.

Rocourt J., Hof H., Schrettenbrunner A., Malinverni R. and Bille J. 1986. Acuter purulent Listeria seeligeri meningitis in an immunocompetent adult. Schweiz. Med. Wochenschr. 116:248-251.

Rocourt J., Wehmeyer U., and Stackebrandt E. 1987. Transfer of *Listeria denitrificans* to a new genus, *Jonesia* gen. nov. as *Jonesia denitrificans* comb. nov. Int. J. Syst. Bacteriol. 37:266-270.

Rocourt J., Boerlin P., Grimont F., Jacquet C., and Piffaretti J.C. 1992. Assignment of *Listeria grayi* and *Listeria murrayi* to a single species, *Listeria grayi*, with a revised description of *Listeria grayi*. Int. J. Syst. Bacteriol. 42:171-174.

Santiago N., Zipf A., and Bhunia A.K. 1999. Influence of temperature and growth phase on expression of a 104-kilodalton Listeria adhesion protein in *Listeria monocytogenes*. Appl. Environ. Microbiol. 65:2765-2769.

Schuber W.D., Urbanke C., Ziehm T., Beier V., Machner M.P., Domann E., Wehland J., Chakraborty T., and Heinz D.W. 2002. Structure of internalin, a major invasion protein of *Listeria monocytogenes*, in complex with its human receptor E-cadherin. Cell 111:825-36.

Shank F.R., Elliot E.L., Wachsmuth I.K., and Losikoff M.E. 1996. US position on *Listeria monocytogenes* in foods. Food Control. 7:229-234.

Smoot L.M. and Pierson M.D. 1998. Influence of environmental stress on the kinetics and strength of attachment of *Listeria monocytogenes* Scott A to Buna-N rubber and stainless steel. J. Food Prot. 61:1286-1292.

Sneath P.H.A., Mair N.S., Sharpe M.E., and Holt J.G. 1984. Bergey's Manual Of Systematic Bacteriology Vol. 2. Williams and Wilkins. 1235-1245.

Stanley P.M. 1983. Factors affecting the irreversible attachment of Pseudomonas aeruginosa to stainless steel. Can. J. Microbiol. 29:1493-1499.

Takeichi M., 1990. Cadherins: a molecular family important in selective cell-cell adhesion. Annu. Rev. Biochem. 59:237-252.

Tilney L.G., and Portnoy D.A. 1989. Actin filaments and the growth, movement, and spread of the intracellular bacterial parasite *Listeria monocytogenes*. J. Cell. Biol. 109:1597-1608.

Tompkin, R. B. 2002. Control of *Listeria monocytogenes* in the food-processing environment. J. Food Prot. 65:709–725.

Vatanyoopaisarn S., Nazli A., Dodd C.E.R., Rees C.E.D., and Waites W.M. 2000. Effect of flagella on initial attachment of *Listeria monocytogenes* to stainless steel. Appl. Environ. Microbiol. 66:860-863.

Vázquez-Boland J.A., Kuhn M. Berche P., Chakraborty T., Dominguez-Bernal G., Goebel W., Gonzalez-zorn B., Wehland J., and Kreft J. 2001. Listeria pathogenesis and molecular virulence determinants. Clinic. Microbiol. 14:584-640.

Weis J., and Seeliger H.P.R. 1975. Incidence of *Listeria monocytogenes* in nature. Appl. Microbiol. 30:29-32.