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PROTEOMIC ANALYSIS OF *LISTERIA MONOCYTOGENES*

By

Sana Mujahid

A Thesis
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in Veterinary Medical Science
in the College of Veterinary Medicine

Mississippi State, Mississippi

December 2007

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PROTEOMIC ANALYSIS OF *LISTERIA MONOCYTOGENES*

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Listeria monocytogenes is a deadly, Gram-positive foodborne pathogen that is ubiquitous in the environment. The bacterium expresses a number of virulence and stress adaptation proteins that support its pathogenic capabilities. Two-dimensional gel electrophoresis (2-DE) was used to map *L. monocytogenes* surface proteins, which play a central role in virulence, and to examine protein expression by *L. monocytogenes* grown on ready-to-eat meat, an important source of *Listeria* infections. A novel method for solubilization of surface proteins from *L. monocytogenes* for 2-DE was developed. Additionally, the unique proteome expressed by *L. monocytogenes* grown on a meat matrix was uncovered. The developed solubilization method will facilitate efforts to identify and routinely compare surface proteins of *Listeria* by 2-DE. Furthermore, the 2-DE database of proteins expressed by *L. monocytogenes* grown on a meat matrix will allow further understanding of the interactions of *Listeria* with its food environment that influence its ability to cause disease.

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ACKNOWLEDGEMENTS

I sincerely thank Dr. Chinling Wang, my major professor, for guiding me throughout my research. I also thank the other members of my thesis committee, Dr. John Boyle and Dr. Peter Ma, for their invaluable aid and direction. In addition, special thanks go to my lab mates, fellow graduate students, and members of the faculty and staff of the Department of Basic Sciences as well as the staff of the Life Sciences and Biotechnology Institute for their cooperation and help.

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CHAPTER I

INTRODUCTION

Listeria monocytogenes is a pathogenic, Gram-positive foodborne bacterium that causes listeriosis, a severe invasive illness, with symptoms including encephalitis, septicemia, meningitis, and spontaneous abortion (Vazquez-Boland et al., 2001; Wing and Gregory, 2002). A series of ongoing epidemic outbreaks in humans in North America and Europe have established listeriosis as an important foodborne infection of public health concern (Kathariou, 2002; Vazquez-Boland et al., 2001). Most cases of listeriosis occur among the elderly, pregnant women, and immunocompromised individuals. The fatality rate from listeriosis among these susceptible populations can be as high as 30% (ILSI RSI, 2005; Ramaswamy et al., 2007; Roberts and Wiedmann, 2003; Vazquez-Boland et al., 2001; Wing and Gregory, 2002).

The primary means of acquiring listeriosis is through consumption of contaminated food. *L. monocytogenes* is widespread in nature and, consequently, contaminates the raw materials used in the preparation of industrially processed foods and the production plants as well (CDC, 2005; Vazquez-Boland et al., 2001). The pathogen is able to survive various food processing and storage environments due to its ability to tolerate high concentrations of salt, grow over a wide range of pHs, and multiply at refrigeration temperatures (Kathariou, 2002; Roberts and Wiedmann, 2003; Vazquez-Boland et al., 2001). All of these characteristics make *L. monocytogenes* of

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particular concern in ready-to-eat (RTE) foods, especially processed meat products, which have long refrigerated shelf lives and can be consumed without being reheated or cooked after purchase (FAO/WHO, 2000; ILSI RSI, 2005; Johnson et al., 1990; Kathariou, 2002). Consequently, RTE foods are considered “high risk” for listeriosis (ILSI RSI, 2005), particularly RTE turkey deli meat, which supports the growth of *L. monocytogenes* exceptionally well and has been linked to a number of listeriosis outbreaks (Frye et al., 2002; Glass et al., 1989; Gottlieb et al., 2006; Kathariou et al., 2006; Lianou et al., 2007; Lin et al., 2006; Mead et al., 2006; Olsen et al., 2005).

The extensive virulence capabilities of *L. monocytogenes* are principally determined by proteins attached to the surface (cell wall and membrane) of the bacterium. These proteins interact with the environment and/or infected hosts and are of primary importance in bacterial adherence, invasion, and interaction with the host immune system (Bierne and Cossart, 2007; Cabanes et al., 2002; Glaser et al., 2001; Navarre and Schneewind, 1999; Popowska and Markiewicz, 2004). Moreover, there is evidence for a link between stress response and virulence in *L. monocytogenes*. The changes in protein expression that *L. monocytogenes* undergoes in order to survive environmental stresses may contribute to the bacterium’s ability to mount a successful infection (FAO/WHO, 2000; Roberts and Wiedmann, 2003; Vazquez-Boland et al., 2001). For example, *L. monocytogenes* adapted to stress may express certain stress response proteins that make it better quipped to infect a host, and/or adaptation to environmental stresses may directly activate certain virulence factors of the bacterium.

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Two-dimensional gel electrophoresis (2-DE) remains the highest resolution technique for protein separation that can be used for mapping several hundred to a few thousand protein molecules on the basis of differences in their p*I* and molecular mass values (Herbert, 1999; Righetti et al., 2003). It is therefore essential to be able to monitor *L. monocytogenes* protein expression using a high resolution technique such as 2-DE. However, only a small percentage of the characterized surface proteome of *L. monocytogenes* has been mapped by 2-DE (Schaumburg et al., 2004). The primary reasons for the low abundance of surface proteins in 2-DE analyses are their tight linkage to the thick, multilayered peptidoglycan sheet structure of the Gram-positive cell wall (Navarre and Schneewind, 1999) and their intrinsically hydrophobic nature due to transmembrane spanning regions (Santoni et al., 2000). These properties lead to difficulties in solubilizing surface proteins for 2-DE, where protein solubility must be maintained during cell lysis and initial solubilization for protein isolation and during the first dimension isoelectric focusing (IEF) stage, when hydrophobic proteins tend to precipitate at their application positions (Rabilloud, 1999; Santoni et al., 2000). Consequently, an effective method for *L. monocytogenes* surface protein solubilization for 2-DE has not been established, and efforts to gain further insight into *L. monocytogenes* virulence by identifying and routinely comparing the bacterium's surface proteins by 2-DE proteomic maps have been limited.

Along with being able to identify and compare surface proteins by 2-DE, it is essential to analyze total protein expression by *L. monocytogenes* under different growth environments to determine the bacterium's expression of known and novel stress and virulence proteins that can play a role in pathogenesis (Kathariou, 2002; Roberts and

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Wiedmann, 2003; Vazquez-Boland et al., 2001). Of particular importance is monitoring protein expression induced in *L. monocytogenes* when it grows on high-risk foods. This data can be valuable in understanding food matrix effects that influence the pathogen's potential to cause disease. Such analyses are largely just beginning to be undertaken, and so far no information is available on protein expression induced in *L. monocytogenes* when it grows on RTE meat, one of the primary foods associated with listeriosis cases.

The aim of this project was to develop a method for improved solubilization of surface proteins from *L. monocytogenes* for 2-DE in order to expand the currently available *L. monocytogenes* surface proteome reference map and allow for more comprehensive comparative 2-DE proteomic analyses of changes in surface protein expression. Additionally, protein expression by *L. monocytogenes* grown on a RTE-meat matrix was analyzed by 2-DE in order to gain insight into meat matrix effects that influence *L. monocytogenes* survival on the matrix and its potential to cause disease.

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CHAPTER II

LITERATURE REVIEW

***Listeria monocytogenes* and Listeriosis**

Listeria monocytogenes is a pathogenic Gram-positive bacterium transmitted to humans by the foodborne route. The bacterium is a facultative anaerobic rod of 0.4 by 1 to 1.5 µm that does not form spores, has no capsule, and is motile at 10 to 25°C (Vazquez-Boland et al., 2001). The bacterium is ubiquitous in nature and can be found in soil, water, effluents, a large variety of foods, and the feces of humans and animals (Vazquez-Boland et al., 2001). The infectious disease caused by *L. monocytogenes* is known as listeriosis. Listeriosis is a severe invasive disease that can lead to encephalitis, septicemia, or meningitis in newborns and immunocompromised individuals or miscarriages and stillbirths in pregnant women (Roberts and Wiedmann, 2003; Vazquez-Boland et al., 2001; Wing and Gregory, 2002). Immunocompromised individuals affected by listeriosis include transplant patients, infants and elderly patients receiving chemotherapy, individuals with diabetes or liver disease, and patients with AIDS. In healthy individuals, *L. monocytogenes* has been reported to cause gastroenteritis (ILSI RSI, 2005; Wing and Gregory, 2002). Generally, listeriosis is a very acute disease with a mean mortality rate among at-risk people of 20 to 30% or higher even with early antibiotic treatment (Ramaswamy et al., 2007; Vazquez-Boland et al., 2001). Since the late 1970s, a series of epidemic outbreaks in humans in North America and Europe have

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established listeriosis as an important foodborne disease of public health concern (Kathariou, 2002; Vazquez-Boland et al., 2001).

L. monocytogenes can be found in a variety of raw foods, in food production plants, and in processed foods (CDC, 2005; Vazquez-Boland et al., 2001). The pathogen possesses unique characteristics that allow it to survive various food processing and storage environments. For example, *L. monocytogenes* has the ability to tolerate high concentrations of salt (up to 10%), grow over a wide range of pHs (4.3-9.6), and multiply at refrigeration temperatures (as low as 1°C) (Kathariou, 2002; Roberts and Wiedmann, 2003; Vazquez-Boland et al., 2001). All of these characteristics make *L. monocytogenes* a significant threat to food safety and thus places the bacterium among microorganisms that most concern the food industry (Vazquez-Boland et al., 2001). *L. monocytogenes* is of particular concern in ready-to-eat (RTE) foods, especially processed meat products, which have long refrigerated shelf lives and can be consumed without being reheated or cooked after purchase (FAO/WHO, 2000; FDA/FSIS, 2003; ILSI RSI, 2005; Johnson et al., 1990; Kathariou, 2002). Consequently, RTE foods are considered “high risk” for listeriosis (ILSI RSI, 2005). Several studies have found that RTE poultry products, particularly turkey deli meat, provide an exceptionally favorable environment for growth of *L. monocytogenes*, even during refrigerated storage (Glass and Doyle, 1989; Lianou et al., 2007; Lin et al., 2006). Indeed, RTE turkey deli meat has been linked to a number of recent listeriosis outbreaks. In 1998-99, a multistate outbreak of listeriosis in the United States was attributed to contaminated RTE meats (meat frankfurters and chicken and turkey deli meat). A total of 108 cases were identified with 14 associated fatalities and 4

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miscarriages or stillbirths (Kathariou et al., 2006; Mead et al., 2006). Another multistate outbreak of listeriosis occurred in the year 2000 and was linked to the ingestion of contaminated RTE turkey deli meat. The outbreak involved 30 reported cases with 4 associated fatalities and 3 miscarriages or stillbirths (Olsen et al., 2005). In 2001, an outbreak of acute febrile gastroenteritis among 16 healthy attendants of a catered party in Los Angeles, CA was again associated with contaminated RTE turkey deli meat (Frye et al., 2002). Moreover, RTE turkey deli meat was implicated in a 2002 multistate outbreak of *L. monocytogenes* infection. Fifty-four case patients were identified, among which 8 died and 3 pregnant women had fetal deaths (Gottlieb et al., 2006; Kathariou et al., 2006). *L. monocytogenes* has 13 known serovars, and among these, only 3 serovars, 1/2a, 1/2b, and 4b, account for more than 90% of all human and animal cases of listeriosis (Popowska and Markiewicz, 2004; Vazquez-Boland et al., 2001). However, serotype 4b strains alone cause over 50% of all human listeriosis cases worldwide (FDA/FSIS, 2003; Ramaswamy et al., 2007; Vazquez-Boland et al., 2001).

Listeria monocytogenes **Pathogenesis**

Information about the pathophysiology of *Listeria* infection in humans and animals is mainly derived from interpretation of epidemiological, clinical, and histopathological findings as well as observations from experimental infections in animals (Vazquez-Boland et al., 2001). The primary site of entry by *L. monocytogenes* into the host is thought to be the gastrointestinal tract, given that the majority of epidemic and sporadic cases of infection are caused by the consumption of contaminated food

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(Farber and Peterkin, 1991; Pinner et al., 1992; Vazquez-Boland et al., 2001). It usually takes about 20h after the ingestion of heavily contaminated food for the clinical course of infection to begin in cases of gastroenteritis (Dalton et al., 1997). However, for the invasive illness, the incubation period can be around 20 to 30 days (Linnan et al., 1988; Riedo et al., 1994). The dose of *L. monocytogenes* required to cause clinical infection in humans is still not exactly known. However, based upon murine experiments and data from listeriosis cases in humans, the infectious dose can vary from around 10^2 to 10^9 bacteria depending on the pathogenicity and virulence of the *L. monocytogenes* strain and the host risk factors (Vazquez-Boland et al., 2001).

L. monocytogenes is able to cross the intestinal barrier, multiply in the liver, colonize the gravid uterus and fetus, and invade the brain of hosts (Vazquez-Boland et al., 2001). After ingestion, *L. monocytogenes* first has to withstand the acidic environment of the stomach. Gastric acidity is thought to destroy a significant number of *L. monocytogenes* organisms ingested with contaminated food (Vazquez-Boland et al., 2001). *L. monocytogenes* organisms able to survive the harsh conditions of the stomach reach the intestine. The point of entry and the mechanism of intestinal translocation used by *L. monocytogenes* are disputed among researchers. Some studies have suggested that *L. monocytogenes* penetrates the host by invading the intestinal villous epithelium (Karunasagar et al., 1994; Racz et al., 1972). While other studies suggest that *L. monocytogenes* penetrates the host through the M cells overlying the Peyer's patches of the intestinal epithelium (Jensen et al., 1998; MacDonald and Carter, 1980; Marco et al., 1992; Marco et al., 1997). Translocation from the intestine to deep organs by *L.*

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monocytogenes has been shown to be a very rapid process of a few minutes that occurs without bacterial multiplication in the intestinal mucosa (Pron et al., 1998). However, intestinal translocation studies have not explained how *L. monocytogenes* causes enteritis. As *L. monocytogenes* is able to pass directly from cell to cell using host cell actin polymerization, it is theorized that regardless of the mechanism of entry used, the bacteria that penetrate the intestinal wall may then invade neighboring enterocytes by basolateral spread, leading to enteritis (Vazquez-Boland et al., 2001).

L. monocytogenes bacteria that cross the intestinal barrier are transported to the mesenteric lymph nodes, the spleen, and the liver by the lymph or blood (Marco et al., 1992; Pron et al., 1998). Studies have demonstrated that resident macrophages in the spleen and liver are able to quickly clear *L. monocytogenes* organisms from the bloodstream (Conlan and North, 1991; Cousens and Wing, 2000; Mackaness, 1962). Ninety percent of the bacterial load collects in the liver, where resident macrophages kill the majority of the ingested bacteria. This causes a decrease in the size of the viable bacterial population in the liver during the first 6 h after infection (Ebe et al., 1999). The macrophages also initiate the development of antilisterial immunity by inducing the antigen-dependent proliferation of T lymphocytes and the secretion of cytokines (Gregory and Wing, 1990). *L. monocytogenes* cells not destroyed by the macrophages start to grow, multiplying for 2 to 5 days (Cheers et al., 1978; Conlan and North, 1991; De Chastellier and Berche, 1994; Fleming and Campbell, 1997; Lepay et al., 1985; Mandel and Cheers, 1980; Mitsuyama et al., 1978), principally in the hepatocytes of the liver (Conlan and North, 1992; Cousens and Wing, 2000; Gaillard et al., 1996; Gregory et

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al., 1992; Gregory and Liu, 2000; Rosen et al., 1989). *L. monocytogenes* goes through the complete intracellular infectious cycle in the hepatocytes, including actin-based intracellular spread (Gaillard et al., 1996; Siddique et al., 1978; Vazquez-Boland et al., 2001). Under normal conditions, *L. monocytogenes* bacteria start to disappear from organs between 5 to 7 days postinfection, until infected cells are completely destroyed through gamma interferon (IFN-γ) mediated macrophage activation and the induction of an acquired immune response primarily mediated by CD8+ lymphocytes (Vazquez-Boland et al., 2001). This is thought to be the most common outcome of *L. monocytogenes* infections in healthy individuals, given that there is a high frequency of exposure to the bacterium via contaminated food and clinical disease rarely occurs. It has in fact been reported that *L. monocytogenes* reactive T lymphocytes are commonly found in healthy individuals (Munk and Kaufmann, 1988), most likely due to the chronic stimulation of the immune system by *L. monocytogenes* antigens that are probably continuously supplied to the immune system by the ingestion of contaminated food (Vazquez-Boland et al., 2001).

If *L. monocytogenes* infection is not restricted by an adequate immune response in the liver, as may occur in pregnant women or immunocompromised individuals, the bacteria may be released into the circulation. Basically *L. monocytogenes* is a multisystemic pathogen that can infect a wide range of host tissues, however, clinical forms of listeriosis show that *L. monocytogenes* has a pathogenic tropism towards the gravid uterus and the central nervous system (CNS) (Vazquez-Boland et al., 2001). As mentioned in the previous section, *L. monocytogenes* can cause abortion and stillbirth in

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pregnant women. The pathogen gains access to the fetus by hematogenous penetration of the placental barrier. Once *L. monocytogenes* reaches the fetal bloodstream, it causes generalized infection and subsequent death of the fetus in utero or it leads to premature birth of a severely infected neonate (Vazquez-Boland et al., 2001). The decline in cell-mediated immunity during pregnancy is thought to play an important part in the development of listeriosis (Vazquez-Boland et al., 2001). Studies in laboratory rodents indicate that there is a disruption in immune resistance to *L. monocytogenes* infection during pregnancy (Bortolussi et al., 1984; Luft and Remington, 1982; Nakane et al., 1985; Redline and Lu, 1987; Redline and Lu, 1988), and the course of primary infection in the liver is significantly prolonged (Abram and Doric, 1997). The reason why mothers suffering *Listeria*-induced miscarriage never themselves develop CNS infection or observable septicemia is still a mystery (Vazquez-Boland et al., 2001).

L. monocytogenes infection of the CNS in humans primarily presents in the form of meningitis. The mechanisms of this type of infection by *L. monocytogenes* are still largely unknown. Numerous studies have examined listerial meningoencephalitis in susceptible animal hosts and laboratory rodents, yet with variable success. One proposed mechanism by which *L. monocytogenes* may cross the blood-brain barrier is through direct uptake by endothelial cells of bacteria circulating free in the blood (Vazquez-Boland et al., 2001). *L. monocytogenes* has been shown to be able to invade cultured human brain microvascular endothelial cells (BMEC) in vitro (Greiffenberg et al., 2000; Greiffenberg et al., 1998). The pathogen replicates efficiently for long periods of time within brain microvascular cells, not causing any apparent damage, but creating heavily infected centers from which bacteria spread to neighboring cells by actin-based motility.

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This allows the bacteria to reach and easily spread into the protected spaces of the CNS (Vazquez-Boland et al., 2001).

Inside all the cells that *L. monocytogenes* is able to penetrate, it develops an intracellular life cycle with common characteristics (Vazquez-Boland et al., 2001). The cells include macrophages and non-phagocytic cells such as epithelial cells, fibroblasts, hepatocytes, endothelial cells, and various types of nerve cells including neurons. The cycle initiates with bacterial adhesion to the surface of the host cell and subsequent penetration of the bacterium into the cell (Vazquez-Boland et al., 2001). During invasion, *L. monocytogenes* becomes engulfed in a phagocytic vacuole (Gaillard et al., 1987). The bacteria begin to disrupt the phagosome membrane around 30 minutes after entry (Gaillard et al., 1987), and within 2 h, approximately 50 % of the intracellular bacteria is free in the cytoplasm (Tilney and Portnoy, 1989) where they multiply with a doubling time of about 1 h (Gaillard et al., 1987; Portnoy et al., 1988). The intracytoplasmic bacteria form actin tails, which allow them to reach the cell periphery, come into contact with the cell membrane, and push it to form finger-like protrusions with a bacterium at the tip. These pseudopods penetrate uninfected neighboring cells and the cycle continues (Vazquez-Boland et al., 2001).

Listeria monocytogenes **Proteins Involved in Virulence**

The extensive virulence capabilities of *L. monocytogenes* are principally determined by proteins attached to the surface (cell wall and membrane) of the bacterium. The primary function of a cell wall is to provide a tough exoskeleton for protection

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against mechanical and osmotic cell lysis. However, in gram-positive bacteria such as *L. monocytogenes*, the cell wall also serves as an attachment site for proteins that interact with the environment and are critical to the viability and virulence of the cell (Navarre and Schneewind, 1999).

The *L. monocytogenes* cell wall is mainly composed of murein, a peptidoglycan that contains muramic acid. Specifically, this peptidoglycan is a polymer of alternating units of the disaccharide *N*-acetylmuramic acid (MurNAc)-(β-1,4)-*N*-acetylglucosamine (GlcNAc), cross-linked by peptidic bridges in a strong molecular network. The muropeptides, L-alanyl-γ-D-glutamyl-*meso*-diaminopimelyl-D-alanine-D-alanine, are bound to the MurNAc residue and are connected by a direct link between the D-Ala residue of one lateral peptide and the *meso*-diaminopimelyl residue of the other stem peptide (Bierne and Cossart, 2007). The cross-linkage of murein in gram-negative bacteria is usually 20-30%, whereas in gram-positive bacteria this value is significantly higher and can approach 100% (Popowska and Markiewicz, 2004). The secondary polymers of the *L. monocytogenes* cell wall are teichoic acids (TAs) and lipoteichoic acids (LTAs). The TAs are covalently bound to the peptidoglycan, while the LTAs are embedded into the plasma membrane. These polymers have important roles in anchoring of surface proteins and transport of ions, nutrients, and proteins. The polymers are also the main determinants of surface immunogenicity, providing most of the basis of the serotype diversity known in *L. monocytogenes* (Bierne and Cossart, 2007).

The membrane of *L. monocytogenes* is approximately 90 Å thick and made up of 55 to 60% protein, including known virulence factors, 30 to 35% lipid, and 1.3 to 2.3%

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carbohydrate. The carbohydrates include glucose, galactose, ribose and arabinose, and the lipids include mainly phospholipids, such as phosphatidylglycerol, diphosphatidylglycerol, and phosphoglycolipid. Greater than 90% of the total fatty acid content of the membrane is branched-chain fatty acids. The fatty acid composition is known to modulate upon temperature variation in order to maintain optimal membrane fluidity. However, the change in membrane composition during *Listeria* cellular infection and how the change affects anchoring of membrane proteins are not known (Bierne and Cossart, 2007).

Proteins attached to the cell wall and membrane of *L. monocytogenes* are involved in important processes such as bacterial growth, sensing of and protection from environmental stresses, adhesion to and invasion of host cells, signaling, cell-to-cell spread, and interaction with the host immune system. Classes and functions of *L. monocytogenes* surface proteins are described in reports by Bierne and Cossart (2007), Cabanes et al. (2002), Glaser et al. (2001), and Popowska and Markiewicz (2004). The general information that follows is based on these reports.

According to genome sequence analysis, *L. monocytogenes* has more surface proteins than any other bacteria with a known genome sequence. The surface proteins are classified according to their anchoring mechanisms. Basically, the proteins can be divided into three groups: proteins covalently linked to the peptidoglycan, proteins with noncovalent association to the cell wall, and membrane-bound proteins.

Under proteins covalently linked to the peptidoglycan, there are two main subgroups. These subgroups are proteins with a LPXTG sorting signal and proteins with

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a NXXTX sorting signal. *L. monocytogenes* has 41 proteins with a LPXTG sorting signal. This signal consists of a conserved LPXTG sequence motif (where X=any amino acid), followed by a hydrophobic domain of about 20 amino acids and a tail of positively charged amino acids. The sorting signal retains the polypeptide in the cell membrane compartment, after which sortase, a membrane-bound transpeptidase, cleaves the LPXTG motif between the threonine and glycine residues and catalyzes the formation of an amide link between the carboxyl group of the threonine and cell wall precursors. There are several proteins with a LPXTG sorting signal known or suggested to be involved in *L. monocytogenes* virulence. For instance, Internalin A (InlA) has been shown to play a significant role in crossing of the intestinal barrier by *L. monocytogenes* (Lecuit et al., 2001). Four other internalins with a LPXTG motif, InlE, InlF, InlG, and InlH, are important for colonization of host tissues in vivo (Raffelsbauer et al., 1998; Schubert et al., 2001), and the LPXTG protein Vip is required for *L. monocytogenes* entry into some mammalian cells (Cabanes et al., 2005). Furthermore, the LPXTG protein lmo2026 has been identified as a new factor involved in virulence in a murine model of *Listeria* infection, and a role for lmo2026 in bacterial invasion and multiplication in the brain has been suggested but remains to be demonstrated (Autret et al., 2001). Also, it has been suggested that the LPXTG proteins lmo0331 and lmo0333 may play a role in adhesion and/or signaling, lmo1666 may mediate adhesion to host cells, and lmo0842 may be involved in virulence and/or biofilm formation (Cabanes et al., 2002; Popowska and Markiewicz, 2004).

Proteins with a NXXTX sorting signal, the second subgroup under proteins covalently linked to the peptidoglycan, are cleaved by a different sortase type, sortase B

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(SrtB). There are only two proteins with a NXXTX sorting signal, lmo2185 (SvpA) and lmo2186 (SvpB), encoded in the *L. monocytogenes* genome. Of these, SvpA has been shown to be required for intracellular survival of *L. monocytogenes* (Borezee et al., 2001).

Under proteins with noncovalent association to the cell wall, there are also two main subgroups. These subgroups are proteins with GW modules and proteins belonging to the P60 family. *L. monocytogenes* has nine proteins with GW modules. The first characterized protein of this type in *L. monocytogenes* was InlB. This internalin family protein is required for *L. monocytogenes* entry into many eukaryotic cell types (Bierne and Cossart, 2002; Dramsi et al., 1995; Hamon et al., 2006). The InlB carboxy-terminal region contains three highly conserved modules of around 80 amino acids, which are termed GW modules as they begin with the dipeptide Gly-Trp. GW modules are necessary and sufficient to anchor InlB to the bacterial surface by binding LTAs. Among the eight other *L. monocytogenes* proteins with GW modules, seven are putative autolysins, while one is of unknown function. The autolysins include two known virulence factors, Ami and Auto. Ami is involved in the adhesion of *L. monocytogenes* to eukaryotic cells (Milohanic et al., 2001), while Auto has been reported to be required for *L. monocytogenes* entry into eukaryotic cells and virulence (Cabanes et al. 2004).

L. monocytogenes has four proteins belonging to the P60 family, the second subgroup under proteins with noncovalent association to the cell wall. The P60 protein, also known as Iap, has murein hydrolase activity that is implicated in cell division. In addition, P60 has been shown to play a role in the invasion of host cells by *L. monocytogenes* (Kuhn and Goebel, 1989; Park et al., 2000). The P60 protein contains two

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LysM domains, a bacterial Src homology 3 (SH3) domain, and a carboxy-terminal NLPC/P60 domain. The function of the NLPC/60 domain is unknown, although it is found in several other prokaryotic surface proteins or lipoproteins. The exact functions of the other two domains are also not known, however, the LysM domain is suggested to have a general peptidoglycan binding function, and the SH3 domain is thought to be involved in cell wall binding and interaction with host signaling molecules. The three other proteins with P60-like sequences are the P45 protein, lmo0394, and lmo1104. P45 has been characterized as a surface-exposed protein exhibiting peptidoglycan lytic activity (Schubert et al., 2000), while the functions of lmo0394 and lmo1104 are not known.

Under the membrane bound proteins group, there are two main subgroups as well. These subgroups are lipoproteins and proteins with hydrophobic tails. The lipoproteins are anchored to the membrane by covalent N terminal lipidation. This process is directed by a specific signal peptide sequence characterized by a lipobox with a conserved cysteine residue. *L. monocytogenes* has a total of 68 lipoproteins, of which 28 are putative substrate binding proteins of ABC transporter systems, 19 are predicted to be involved in different enzymatic activities or other function, and 21 are of unknown function. Among the lipoproteins, TcsA has been shown to be presented by major histocompatibility complex (MHC) class II molecules and mediate $CD4^+$ T cell activation (Sanderson et al., 1995). Another lipoprotein, OppA, mediates the transport of oligopeptides and is required for bacterial growth at low temperatures. OppA has been shown to be involved in the intracellular survival of *L. monocytogenes* (Borezee et al., 2000).

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The second subgroup of membrane bound proteins, proteins with hydrophobic tails, consists of 11 proteins. These proteins are attached to the bacterial cell surface by a carboxyl terminus consisting of a hydrophobic domain followed by positively charged residues thought to serve as a stop-transfer signal. One of the hydrophobic tail proteins, ActA, is known to promote *L. monocytogenes* intracellular motility (Kocks et al., 1992). Transfection experiments in mammalian cells have shown that the carboxy-terminal membrane anchor of ActA directs this polypeptide to mitochondria and can be used to direct heterologous proteins to this location (Pistor et al., 1994).

Although the extensive virulence capabilities of *L. monocytogenes* are principally mediated by surface proteins, there is also evidence for a link between stress response and virulence in *L. monocytogenes*. That is to say, the changes in protein expression and resulting physiological changes that *L. monocytogenes* undergoes in order to survive environmental stresses may contribute to the bacterium's ability to mount a successful infection (FAO/WHO, 2000; Roberts and Wiedmann, 2003; Vazquez-Boland et al., 2001). For example, *L. monocytogenes* adapted to stress may express certain stress response proteins that make it better quipped to infect a host, and/or adaptation to environmental stresses may directly activate certain virulence factors of the bacterium.

Survival under stress involves an adaptive response mediated by a set of conserved proteins that are upregulated in vitro upon exposure to specific stresses such as heat shock, low pH, oxidative agents, toxic chemical compounds, starvation, or any other situation in which bacterial growth is arrested. These proteins can be chaperones that assist in the proper refolding or assembly of stress-damaged proteins or proteases that degrade damaged proteins, ensuring that essential physiological pathways function

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correctly in stressed cells (Vazquez-Boland et al., 2001). Certain stress response proteins of *L. monocytogenes* are associated with virulence. One of these proteins is ClpC, an ATPase of the Clp (caseinolytic protein) family important for stationary phase survival and resistance to low pH, oxidation, osmotic stress, high temperature, and iron deprivation (Roberts and Wiedmann, 2003; Vazquez-Boland et al., 2001). It has been shown that the ClpC protein is required for adhesion and invasion by *L. monocytogenes*, possibly by modulating the expression of surface proteins InlA, InlB, and ActA (Nair et al., 2000).

Another member of the Clp family, ClpE, is required for prolonged survival at 42°C and involved in cell division and virulence of *L. monocytogenes* (Nair et al., 1999). In a murine model, a double *clpC clpE* mutant was found to be totally avirulent. In addition, *clpE* expression was found to be upregulated in a *clpC* mutant, indicating that ClpC and ClpE have redundant roles in stress tolerance and that the absence of one is compensated for by upregulation of the other (Nair et al., 1999). The stress protease ClpP has also been shown to be required for growth under stress conditions and for survival by *L. monocytogenes* in macrophages in vitro and in mouse tissues in vivo (Gaillot et al., 2000). Adaptation to environmental stresses can also directly activate certain virulence factors of *L. monocytogenes*. For example, it has been shown that heat stress results in the synthesis of proteins regulated by the virulence gene regulator PrfA and known virulence proteins ActA, listeriolysin O, PlcA, and PlcB (Sokolovic et al., 1993).

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Two-Dimensional Gel Electrophoresis

It is essential to use high resolution protein analysis techniques to identify new proteins that contribute to *L. monocytogenes* virulence and to study proteins already known to be involved in virulence. To date, two-dimensional gel electrophoresis (2-DE) remains the most successful method of resolving proteins. This high resolution technique can be used to reliably and reproducibly map several hundred to a few thousand protein molecules based on differences in their isoelectric points (p*I*) and molecular mass values (Herbert, 1999; Righetti et al., 2003; Westermeier and Naven, 2002). Usually, multiple comparative samples are produced for an experiment and separated in a number of 2-D gels. Proteins are separated by their p*I* in the first dimension isoelectric focusing (IEF) stage and by their molecular weight in the second dimension SDS polyacrylamide gel electrophoresis stage. Sample treatment and the separation of proteins are usually performed rapidly to avoid protein modification. Once the proteins are isolated in the gel matrix, the single proteins are much more stable and can be further identified and characterized by mass spectrometry (Westermeier and Naven, 2002).

Over the past decade, the technology of 2-DE has been considerably improved, with advances in the resolution, reliability, and reproducibility of the method. Additionally, protein labeling techniques using fluorescent tags and stable isotopes have been made available for differential analyses of related samples. This allows quantitative changes in protein expression levels to be uncovered. Moreover, novel ionization techniques and detectors for mass spectrometry have been invented, which allow the analysis of proteins and peptides with high sensitivity, accuracy, and throughput (Westermeier and Naven, 2002).

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Producing quality 2-D gels requires skill and a number of challenges have to be considered before using the technique. For example, some very hydrophobic proteins, such as membrane proteins, do not go in solution, while others are lost during sample preparation and the first dimension IEF stage. Moreover, proteins that are expressed in very low copy numbers cannot be detected easily on 2-D gels. To overcome this, a highly sensitive method such as radiolabelling has to be applied or more protein must be loaded. Great care must also be taken to ensure that no proteins get lost during sample preparation and the analysis process, as proteomics is a method of quantitative comparisons, and the detection method must give reliable quantitative information. Lastly, it is of highest importance that the results are reproducible. Otherwise it is impossible to find variations of proteins between different samples (Westermeier and Naven, 2002). Overcoming these challenges can require method optimization for each sample type. To aid with this, a large amount of literature is available on 2-DE analyses of various types of proteins from different organisms. Moreover, a number of commercial kits are available to facilitate 2-DE analysis.

Mass spectrometry, a technique for analyzing the molecular weight of molecules based upon the motion of a charged particle in an electric or magnetic field, is routinely used for protein identification after 2-DE. A mass spectrometer measures the mass-to-charge ratio *(m/z)* of ionized atoms or molecules. The instrument is comprised of three parts: an ion source, a mass analyzer, and an ion detector. There are two modes of operation of a mass spectrometer, MS and MS/MS. In MS mode, the actual mass of the analyte is measured and this mass is indicative of the composition of the analyte. In the MS/MS mode, the analyte ion of interest is mass measured, specifically selected, and

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fragmented within the mass spectrometer to yield structural information. This method is routinely used for determining small molecule structure and peptide sequence (Westermeier and Naven, 2002).

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) is one of the most useful and commonly used mass spectrometry techniques. Relative to other mass spectrometry techniques, MALDI provides high sensitivity and excellent tolerance of interfering buffer components (Henzel and Stults, 1996). MALDI ions are created by mixing the analyte with a small, organic molecule called the matrix, which absorbs light at the wavelength of the laser. The analyte becomes incorporated into the crystal lattice of the matrix and is then irradiated with a laser. The laser causes the desorption and ionization of the matrix and analyte, either by protonation or cationation (positively charged ions) or by deprotonation (negatively charged ions). The ions are then accelerated into the MS analyzer (Westermeier and Naven, 2002). Most often the MALDI ion source is coupled with a time-of-flight (ToF) mass analyzer from which the high mass range, resolution, and mass accuracy characteristics are derived (Henzel and Stults, 1996). Ions produced at the ion source are accelerated by high voltage into the ToF analyzer, acquiring an initial velocity that is dependent on their mass. Mass measurement is recorded by the time of flight of an ion in the ToF flight tube (Westermeier and Naven, 2002). Recently, a TOF/TOF analyzer has been coupled with a MALDI ion source to generate peptide mass fingerprint (PMF) data and peptide sequence derived by high energy collision induced dissociation. In this combination, two ToF analyzers are separated by a collision cell, with the first ToF analyzer used for precursor ion selection. High energy collisions occur within the collision cell, and the second ToF

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analyzer resolves the ions. With this combination, high sensitivity and high resolution are obtained in both MS and MS/MS mode (Westermeier and Naven, 2002). Peptide mass data obtained by mass spectrometry is compared with databases of known protein sequences to determine the identity of proteins.

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CHAPTER III

IMPROVED SOLUBILIZATION OF SURFACE PROTEINS FROM *LISTERIA MONOCYTOGENES* FOR TWO-DIMENSIONAL GEL ELECTROPHORESIS

Abstract

Solubilization of bacterial surface (cell wall and membrane associated) proteins for two-dimensional gel electrophoresis (2-DE) is challenging, particularly in the case of Gram-positive bacteria. This is primarily due to strong protein association with the cell wall peptidoglycan and protein hydrophobicity. We solubilized surface proteins for 2-DE from the Gram-positive pathogen *L. monocytogenes* using mutanolysin, which digests cell wall peptidoglycan, and one of three different mixtures of zwitterionic detergent and chaotropes: (1) CHAPS/Urea (2) ASB-14/Urea/Thiourea (3) SB3-10/Urea/Thiourea. Cell lysis with mutanolysin followed by solubilization with ASB-14/Urea/Thiourea gave the highest overall protein yield with the best 2-DE resolution. Protein spot identification by MALDI-TOF/TOF-MS analysis revealed 29 characterized surface proteins of *L. monocytogenes*, 17 of which have not previously been reported on the surface proteome map. This is the first report describing the successful solubilization and 2-D electrophoresis of *L. monocytogenes* proteins bound to the cell surface via an LPXTG motif or by a hydrophobic tail. The increase in surface proteome coverage obtained by mutanolysin and ASB-14/Urea/Thiourea solubilization suggests the utility of this method

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for future analytical and comparative studies of surface proteins from *Listeria,* and possibly other Gram-positive bacteria, using 2-DE proteomic analysis. An updated 2-DE reference map of *L. monocytogenes* surface proteins is presented.

Introduction

The Gram-positive bacterium *Listeria monocytogenes* is a facultative intracellular pathogen that has the ability to cross the intestinal tract and blood-brain and fetoplacental barriers in humans and other animals. It can cause listeriosis, a severe food-borne illness, which leads to infections such as encephalitis, septicemia, and meningitis in newborns and immunocompromised individuals, or miscarriages and stillbirths in pregnant women (Jacquet et al., 2004; Vazques-Boland et al., 2001). The extensive invasive capabilities of *L. monocytogenes* are principally determined by proteins attached to the surface (cell wall and membrane) of the bacterium by LPXTG motifs, GW modules, hydrophobic tails, lipo-boxes or P60-like domains (Cabanes et al., 2002; Cabanes et al., 2004; Cabanes et al., 2005; Cossart et al., 2003; Cossart and Sansonetti, 2004; Dramsi et al., 2004; Glaser et al., 2001; Khelef et al., 2006; Lecuit et al., 2001; Lecuit et al., 1997; Muller et al., 1998; Navarre and Schneewind, 1999; Popowska and Markiewicz, 2004; Sabet et al., 2005). This notable role of surface proteins in *L. monocytogenes* virulence and their potential as pharmacological targets (Santoni et al., 2000) make it essential to investigate the proteins in-depth; however, only a small percentage of the characterized surface proteome of *L. monocytogenes* has been mapped by two-dimensional gel electrophoresis (2-DE) and does not include any proteins attached to the cell surface by LPXTG motifs, GW modules, or hydrophobic tails (Schaumburg et al., 2004). This is consistent with

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proteomic analyses of other Gram-positive bacteria, where examination of 2-D gel maps reveals that cell wall and membrane associated proteins are notably under-represented (Cordwell et al., 2002; Len et al., 2003; Santoni et al., 2000). The primary reasons for the low abundance of surface proteins in such analyses are their tight linkage to the thick, multilayered peptidoglycan sheet structure of the Gram-positive cell wall (Navarre and Schneewind, 1999) and their intrinsically hydrophobic nature due to transmembrane spanning regions (Santoni et al., 2000). These properties lead to difficulties in solubilizing surface proteins for 2-DE analysis, where protein solubility must be maintained during cell lysis and initial solubilization for protein isolation and during the first dimension isoelectric focusing (IEF) stage, when hydrophobic proteins tend to precipitate at their application positions (Rabilloud, 1999; Santoni et al., 2000). Compared to other Gram-positive bacteria, the *Listeria* cell wall is particularly resistant to lysis (Fliss et al., 1991). However, the enzyme mutanolysin, an endo-N-acetyl muramidase, has been shown to be effective in lysing the *Listeria* cell wall peptidoglycan (Fliss et al., 1991) and fractionating other Gram-positive cells to isolate cell wall components without cytoplasmic contamination (Cockayne et al., 1998; Hughes et al., 2002; Mazmanian et al., 2003). Additionally, a number of solubilizing agents including chaotropes and novel detergents have been reported to increase hydrophobic and membrane protein solubility during IEF for various cell types (Chevallet et al., 1998; Herbert, 1999; Molloy, 2000; Molloy et al., 1999; Rabilloud, 1996; Rabilloud, 1998; Rabilloud, 1999; Rabilloud et al., 1997; Santoni et al., 1999; Santoni et al., 2000; Vuillard et al., 1995).

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In this study, we improved the solubilization of surface proteins from *L. monocytogenes* for 2-DE analysis by using a mutanolysin based digestion buffer for cell wall lysis and surface protein isolation and testing various mixtures of detergents and chaotropes in the solubilization buffer for IEF. Our results indicated that isolation of surface proteins with mutanolysin followed by solubilization with ASB-14 (amidosulfobetaine-14)/Urea/Thiourea gave the highest number of protein spots with the best 2-DE resolution. A total of 82 proteins were identified by matrix assisted laser desorption/ionization-time of flight/time of flight-mass spectrometry (MALDI-TOF/TOF-MS) analysis of protein spots. The identified proteins include 29 characterized surface proteins, of which 17 proteins have not been reported before on the published surface proteome map of *L. monocytogenes* (Schaumburg et al., 2004). Among the remaining identified proteins, 3 have unknown localization and function, 23 are known cytoplasmic proteins with potential "moonlighting" functions at the cell surface (Schaumburg et al., 2004; Hughes et al., 2002; Wilkins et al., 2003; Ling et al., 2004; Liou et al., 2001; Zuobi-Hasona et al., 2005; Lenz et al., 2003; Braunstein et al., 2003), while 27 proteins are only known to have cytoplasmic functions and are likely contaminants. With the presented method, we have largely expanded the currently available *L. monocytogenes* surface proteome reference map and allowed for more comprehensive comparative 2-DE proteomic analyses of changes in surface protein expression.

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Materials and Methods

Bacterial Strain and Surface Protein Preparation

L. monocytogenes serotype 1/2a strain EGD-e was grown in 500 mL brain-heart-infusion broth at 37°C and 180 rpm in a Series 25 incubator shaker (New Brunswick Scientific Co., Edison, New Jersey) until mid-log growth phase ($A_{600} \approx 0.9$). The cells were harvested by centrifugation at 2,600 × g for 15 min and washed twice with Tris-buffered sucrose (pH 7.0, 10mM Tris, 250 mM sucrose). To prepare cell surface proteins, cells were resuspended in 60 mL of osmotic digestion buffer containing 20% sucrose in 20mM Tris-HCl, pH 7.0, 10 mM $MgCl_2$, protease inhibitor cocktail (1 mM phenylmethylsulfonyl fluoride, 10 μM iodoacetic acid, 1 μM pepstatin A, and 10 mM 1,10-phenanthroline), and 5,000 U of mutanolysin. Enzymatic digestion was allowed to proceed for 2 h at 37º C. The majority of intact protoplasts were removed by centrifugation at 2,900 × g for 1 h. The supernatant was then subjected to microcentrifugation at 16,000 × g (Eppendorf 5415C) for 30 min to remove cell debris and any remaining protoplasts. The supernatant containing solubilized cell surface proteins was treated with the ReadyPrep 2-D cleanup kit (Catalog # 163-2130, Bio-Rad, Hercules, CA) to remove substances from the digestion process that interfere with IEF. The resulting pellets were resuspended in IEF solubilization buffer containing 50 mM DTT, 0.2 % 100× Bio-lyte 3/10 ampholyte and one of the following solubilizing cocktails: (1) CHAPS (3-[(3 cholamidopropyl) dimethylamino]-1-propane sulfonate): 9.5 M urea, 4 % CHAPS; (2) ASB-14 (amidosulfobetaine-14): 7 M urea, 2 M thiourea, 1 % ASB-14; or (3) SB3-10 (N-decyl-N,N'-dimethyl-3-ammonio-1-propanesulfonate): 5 M

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urea, 2 M thiourea, 1% SB3-10. The samples were clarified by centrifugation at 16,000 × g (Eppendorf 5415C) for 5 min, and protein concentrations were determined using a Bradford Protein Assay (Bio-Rad, Hercules, CA).

Two-Dimensional Electrophoresis

In the first dimension, IEF was performed using a PROTEAN™ IEF cell (Bio-Rad, Hercules, CA). Approximately 300 µg of protein suspended in solubilization buffer were loaded onto the IEF focusing tray with ReadyStrip™ IPG strips (7cm, pH 4-7 or pH 3-10 NL) and rehydrated overnight (12 h) at 23° C and 50 V. IEF was carried out at as follows: 250 V for 15 min, followed by voltage ramping, linear mode, to 4000 V for 2 h, and final focusing at 4000 V for 20,000 volt-hours. The current was limited to 50 µA per IPG strip, and the temperature was kept at 23° C for all focusing steps. Following IEF, the IPG strips were stored at -80ºC. Prior to electrophoresis in the second dimension, IPG strips were thawed and incubated for 30 min in 5 mL of equilibration solution containing 6 M urea, 2 % SDS, 20 % glycerol, 25 % 1.5 M Tris pH 6.8, 5 % 2-mercaptoethanol, and 0.002 % bromophenol blue. Lastly, the IPG strips were positioned on top of 12% polyacrylamide gels and sealed in place with 1% agarose. The second dimension was performed in a Mini-PROTEAN 3 Electrophoresis cell (Bio-Rad, Hercules, CA). Gels were run at 50 mA until the marker dye reached the bottom of the gel. Following electrophoresis, the gels were stained using Coomassie Blue R-250. Gel images were acquired using a ChemiImager™ 5500 imaging system (Alpha Innotech, San Leandro, CA). Three independent replicates were performed for each solubilizing condition with each replicate started from an independent bacterial cultivation.

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Protein Identification

Gel images were compared and protein spots were counted using PDQuest 2-D Analysis Software (Bio-Rad, Hercules, CA). To confirm the consistency and reproducibility among gels, all the spots were counted and compared by the software and further confirmed by visual inspection. For protein identification by mass spectrometry, the spots of interest were robotically excised from the 2-D gels by a Proteome Works Spot Cutter (Bio-Rad, Hercules, CA). In-gel trypsin digestion was performed using the ProPrep robotic digester/spotter (Genomic Solutions, Ann Arbor, MI). The method used included disulfide bond reduction and alkylation with DTT (dithiotreitol) and iodoacetamide, respectively. The resulting peptide mix was desalted with C18 ZipTips (Millipore) and spotted on a MALDI plate in a solution of 70% acetonitrile, 0.1% trifluoroacetic acid, and 5mg/ml matrix (alfa-cyano-4-hydroxycinnamic acid).

The samples were analyzed using an ABI 4700 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Foster City, CA), and protein identification (ID) was performed using the Result Dependent Analysis (RDA) of ABI GPS Explorer software, version 3.5 (Applied Biosystems, Foster City, CA). The parameters were set as follows: MS (precursor-ion) peak filtering: 800 - 4000 m/z interval, monoisotopic, minimum S/N=10, mass tolerance = 75 ppm; MSMS (fragment-ion) peak filtering: monoisotopic, $M+H^+$, minimum S/N=3, MSMS fragment tolerance = 0.2 Da; database used: *Listeria* taxonomic sub-database of “nr” (non redundant) database of National Center for Biotechnology Information (NCBI). During the initial MS scan, the data were analyzed as peptide mass fingerprinting (PMF) and preliminary protein ID was done by searching against the database using the MASCOT (Matrix Science, Boston,

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MA) algorithm. Proteins with high confidence ID (Cross Confidence Interval C.I.% > 95%) were automatically selected for "*in silico*" digestion, and their three most prevalent corresponding peptides-precursor ions present in the MS spectra were selected for MSMS analysis – RDA_1 (top protein confirmation). The sample spots not yielding high confidence ID after preliminary PMF ID and/or after RDA_1 ID were subjected to RDA_2 by selecting the first 20 most intensive precursor ions in the MS spectra for MSMS analysis. The spectral data from the PMF (initial MS scan), RDA_1 and RDA_2 MSMS were together subjected to a combined MASCOT search. Only proteins with total Protein Score C.I.% > 95% were considered as a positive ID. The functional categories of the identified proteins were determined according to the ListiList *Listeria* genome database (http://genolist.pasteur.fr/ListiList/).

Results and Discussion

The surface proteins of *L. monocytogenes* have been characterized by genome sequence analysis (Glaser et al., 2001) and can be divided into groups depending on the unique signal peptide or other motif that anchors them to the bacterial surface e.g. LPXTG motif, GW module, hydrophobic tail, lipo-box, and P60-like domain (Cabanes et al., 2002; Glaser et al., 2001; Popowska and Markiewicz, 2004). As many of the surface proteins are known to be involved in virulence and interaction with a variety of eukaryotic host cells (Cabanes et al., 2002; Cabanes et al., 2004; Cabanes et al., 2005; Cossart et al., 2003; Cossart and Sansonetti, 2004; Dramsi et al., 2004; Glaser et al., 2001; Khelef et al., 2006; Lecuit et al., 2001; Lecuit et al., 1997; Muller et al., 1998; Navarre and Schneewind, 1999; Popowska and Markiewicz, 2004; Sabet et al., 2005),

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comprehensive comparative proteomic analyses of changes in surface protein expression are needed. However, the strong attachment of surface proteins to the bacterial cell wall and their hydrophobic nature due to transmembrane spanning regions make it difficult to solubilize these proteins, and an effective method for *L. monocytogenes* surface protein solubilization for 2-DE has not been established. Consequently, efforts to identify and routinely compare *L. monocytogenes* surface proteins by 2-DE proteomic maps have been limited. In this study, we improved the solubilization of surface proteins from *L. monocytogenes* for 2-DE analysis.

Surface Protein Solubilization

Protein solubility has to be considered at two stages of the 2-DE process: (1) during cell lysis and initial solubilization for protein isolation and (2) during the first dimension IEF stage when hydrophobic proteins tend to precipitate at their application positions (Rabilloud, 1999; Santoni et al., 2000). We used a mutanolysin based digestion buffer to lyse the thick, multilayered peptidoglycan sheet structure of the *Listeria* cell wall and solubilize surface proteins. Mutanolysin, an endo-N-acetyl muramidase from *Streptomyces globisporus*, is a muralytic enzyme that provides gentle cell lysis by cleaving the β (1-4) N-acetylmuramyl-N-acetylglucosamine linkage of the bacterial cell wall polymer peptidoglycan-polysaccharide (Lichtman et al., 1992). Mutanolysin treatment is effective in lysing the *Listeria* cell wall peptidoglycan (Fliss et al., 1991) and has been used in a number of studies for the isolation of cell wall components without cytoplasmic contamination including surface proteins of streptococci and staphylococci (Cockayne et al., 1998; Hughes et al., 2002; Mazmanian et al., 2003). This suggested the

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suitability of mutanolysin treatment for the initial cell lysis and solubilization of surface proteins from *L. monocytogenes* in the present study.

After isolation of surface proteins using mutanolysin, we solubilized them for the first dimension in an IEF solubilization buffer containing CHAPS/Urea, ASB-14/Urea/Thiourea, or SB3-10/Urea/Thiourea and compared the effect of each buffer on protein solubility and resolution on 2-DE gels. Each solubilization buffer was distinct with regard to the solubilizing power of the included detergent and the overall chaotrope concentration of the solution. High chaotropicity of the medium used for IEF, generally associated with enhanced solubilizing power, can be achieved by using urea alone or a mixture of urea and thiourea, the latter being the recommended approach (Rabilloud, 1999; Rabilloud et al., 1997; Rabilloud, 1998). Rabilloud et al. (1997) introduced the use of thiourea in combination with urea to increase the solubility of proteins during IEF and showed that thiourea had a major positive effect on the number of proteins visualized in the second dimension.

Formerly, CHAPS was the detergent of choice for IEF and was used in high concentrations of urea. However, it was found that the solubilization efficiency of CHAPS reduces in the presence of thiourea, thus limiting the chaotropic power of CHAPS buffers (Herbert, 1999; Rabilloud et al., 1997). Sulfobetaines with long linear alkyl tails such as SB3-10 are more efficient than CHAPS although they suffer from poor solubility in concentrations of urea greater than 5M, hence the overall chaotrope concentration of SB3-10 buffers is relatively low. In contrast, the linear alkyl tail amidosulfobetaine ASB-14 can be used with thiourea and high concentrations of urea and has been shown to be comparatively more efficient than CHAPS and SB3-10 (Chevallet

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et al., 1998; Herbert, 1999; Molloy et al., 1999). Considering these published reports, we optimized our solubilization cocktail concentrations to make the most effective use of the solubilizing power of the included detergent and its limits of chaotrope compatibility. Interestingly, we found that there was little variation in the overall spot patterns among gels from samples treated with different solubilization buffers (Fig. 3.1). However, the number of protein spots resolved varied slightly depending on the solubilization buffer used, with the buffers showing increasing solubility in the pH 4-7 range in the following order SB3-10 (~113 spots) < CHAPS (~126-128 spots) < ASB-14 (~147-149 spots). The spot numbers were consistent over three biological replicate gels for each solubilizing condition. The ASB-14 gels showed ~19 unique spots in addition to all protein spots visualized in the other detergent gels. Similarly, the resolution of proteins as determined by streaking and sharpness of spots also differed depending on the buffer. The use of the ASB-14 solubilization buffer resulted in large, sharp protein spots, almost no streaking, and overall exceptional protein resolution (Fig. 3.1A), whereas the CHAPS buffer showed moderate protein solubility and resolution over the pH range with some streaking (Fig. 3.1B). The SB3-10 buffer exhibited the lowest protein separation and resolution with a general shift of the protein profile towards the acidic end of the gel and horizontal streaking in that region resulting in none or low solubilization of important surface proteins with acidic p*I* such as LPXTG proteins and membrane associated lipoproteins that could be seen on the ASB-14 and CHAPS gels (Fig. 3.1C). Our results support the observations of Chevallet et al. (1998) and Molloy et al. (1999) that ASB-14 solutions give greater protein solubility, compared to solutions of SB3-10 and CHAPS, probably due to the increased efficiency of ASB-14 and the fact that thiourea and high

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concentrations of urea can be used with ASB-14, whereas thiourea cannot be used with CHAPS and only up to 5M urea can be used with SB3-10.

Protein Identification by MALDI-TOF/TOF-MS

Replicable protein spots solubilized by mutanolysin and ASB-14/Urea/Thiourea treatment were excised and subjected to MALDI-TOF/TOF-MS analysis. A total of 81 protein spots corresponding to 82 proteins (Fig. 3.2 & Table 3.1) were identified following a search of the MASCOT database, and their functional categories were determined. Three spots (8, 19, & 59) matched two distinct proteins with C.I.% of 100, indicating that these were most likely cases of comigration of proteins with similar electrophoretic properties. Additionally, lmo2186– spot 26 and lmo1068– spot 79 were represented by two spots each. These multiple spots may be analytical artifacts, but possibly represent post-translationally modified protein forms.

The identified proteins include 29 characterized surface proteins, of which 17 proteins have not been reported before on the published surface proteome map of *L. monocytogenes* (Schaumburg et al., 2004). Specifically, the identified surface proteins include three proteins with a LPXTG sorting motif (lmo0130-spot 22, inlA-spot 23, lmo1666-spot 24), two proteins with a hydrophobic C-terminal anchoring domain (lmo2185-spot 25, lmo2186-spot 26), two P60-like proteins (p60/iap-spot 1, p45/spl-spot 3), fourteen lipoproteins (lmo0135-spot 5, lmo0152-spot 6, lmo0181-spot 7, lmo0285-spot 8, lmo1041-spot 11, lmo1388-spot 12 , lmo1738-spot 13, lmo1847-spot 14, lmo2196-spot 15, lmo0355-spot 17, lmo2637-spot 27, lmo0047-spot 73, lmo2636- spot 77, lmo1068-spot 79), and eight other characterized cell wall or membrane associated

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proteins (lmo1082-spot 2, lmo2754-spot 4, lmo2478-spot 8, lmo1002-spot 9, lmo1003-spot 10, lmo2415-spot 16, lmo2471-spot 18, lmo2531-spot 19). The initial proteomic map of *L. monocytogenes* surface proteins was generated in a recent study by Schaumburg et al. (2004) using serial extraction of proteins by different salts. In that study, however, certain surface proteins could not be solubilized and were absent from the map such as those linked to the cell surface by a LPXTG motif or by a hydrophobic carboxy-termimal domain. In comparison, we found that using a mutanolysin and ASB-14/Urea/Thiourea solubilization method increases the number of characterized surface proteins solubilized and separated by 2-DE.

Proteins covalently linked to the cell wall by LPXTG motifs or anchored to the cell membrane by hydrophobic carboxy-terminal domains are difficult to solubilize and separate by 2-DE (Navarre and Schneewind, 1999; Osaki et al., 2002; Schaumburg et al., 2004). Essentially, the identification of *L. monocytogenes* cell surface bound LPXTG proteins and hydrophobic tail proteins has been limited to non-gel-based approaches using quadruple time-of-flight mass spectrometry (Q-TOF-MS) or two-dimensional nanoliquid chromatography coupled to mass spectrometry (2DnLC-MS/MS) (Bierne et al., 2002; Calvo et al., 2005). This is the first report describing the successful solubilization and identification of *L. monocytogenes* proteins bound to the cell surface via an LPXTG motif or by a hydrophobic tail using a gel-based approach. With the mutanolysin and ASB-14/Urea/Thiourea method, the LPXTG proteins lmo0130-spot 22, inlA-spot 23, and lmo1666-spot 24 were solubilized. InlA is a known virulence factor that mediates *L. monocytogenes* crossing of the intestinal barrier and entry into human intestinal epithelial cells by binding to an epithelial transmembrane host receptor called

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E-cadherin (Lecuit et al., 2001). InlA and lmo1666 have no ortholog in the apathogenic strain *L. innocua* and their genes have a binding site for the virulence gene regulator PrfA (Glaser et al., 2001). PrfA, which is absent from *L. innocua*, regulates most known virulence genes of *L. monocytogenes*. In comparison to the whole *Listeria* genome, a significant percentage of LPXTG protein genes contain the PrfA box sequence in their promoter region, indicating the involvement of LPXTG proteins in virulence (Cabanes et al., 2002). Along with inlA and lmo1666, the genes of five other solubilized proteins have a putative binding site for PrfA: lmo1082-spot 2, lmo1847-spot 14, lmo1305-spot 38, lmo0223-spot 45, and lmo1856-spot 51 (Glaser et al., 2001). Also, lmo1082 and the lipoprotein lmo1068-spot 79 have no ortholog in *L. innocua* (Glaser et al., 2001), and thus are unique for *L. monocytogenes* like inlA and lmo1666. Two proteins that are attached to the cell surface via the hydrophobic carboxy-terminal anchoring domain NXZTN were solubilized in this study: lmo2185-spot 25 (svpA) and lmo2186-spot 26 (svpB). A recent report suggested that the NXZTN sorting motif is recognized by a new class of *L. monocytogenes* sortase, sortase-B (srtB), to covalently anchor proteins to the cell wall peptidoglycan. The virulence factor svpA has been specifically identified as one of the srtB-anchored proteins (Bierne et al., 2004).

We were also able to solubilize *L. monocytogenes* surface proteins belonging to the P60 family: P60/iap-spot 1 and P45/spl-spot 3. Both P60/iap and P45/spl display peptidoglycan lytic activity and may promote bacterial pathogenesis (Kuhn and Goebel, 1989; Park et al., 2000; Schubert et al., 2000). Recently, it has been reported that P60 secretion is dependent on a virulence related auxiliary protein secretion system called SecA2 (Lenz et al., 2003). Two other groups of *L. monocytogenes* surface proteins are

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known to play an important role in host-pathogen interaction and bacterial virulence. These groups are lipoproteins that are linked to the *L. monocytogenes* cell membrane via an N-acyldiglyceride moiety and proteins with a GW repeat anchoring motif for surface association (Cabanes et al., 2002). Among the 14 lipoproteins solubilized in our study, lmo1847-spot 14 (LpeA) and lmo2196-spot 15 (OppA), have been reported to be involved in invasion and intracellular survival, respectively (Borezee et al., 2000; Reglier-Poupet et al., 2003). Five of the solubilized lipoproteins are of unknown function, while the majority of the remainder are binding proteins. None of the GW-proteins were solubilized in our study or reported on the initial published 2D-E surface proteome map of *L. monocytogenes* (Schaumburg et al., 2004). The possibility that GW-proteins along with other surface proteins were insoluble in the detergents used, were lost during the 2-DE procedure, or were undetectable or underrepresented on the gels may explain why some of these proteins were missing.

Despite the care taken to minimize contamination by cytoplasmic proteins, a number of proteins with known functions in the cytoplasm were present in the surface protein fraction. It is not uncommon for abundant bacterial proteins to be found in enriched fractions despite the unlikely occurrence of these proteins on the cell surface (Cordwell et al., 2001). However, at least 23 of the identified cytoplasmic proteins have potential "moonlighting" functions at the cell surface**.** The majority of these proteins have been previously identified in the defined surface proteome of *L. monocytogenes* (Spot Nos. 21, 33, 37, 41, 43, 44, 45, 56, 61, 62, 63, 64, 65, 71, 78) (Schaumburg et al., 2004). For instance, enolase-spot 41 was shown to be present in the cell wall of *L. monocytogenes* by immunoelectron microscopy even though it lacks a known cell wall

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retention signal or a secretion signal (Schaumburg et al., 2004). Enolase has also been shown by others to be cell surface associated (Hughes et al., 2002; Ling et al., 2004; Wilkins et al., 2003) and reported to be membrane associated in *E. coli* and *S. mutans* (Liou et al., 2001; Zuobi-Hasona et al., 2005). Furthermore, *L. monocytogenes* enolase, gap-spot 44, EF-Tu-spot 61, and DnaK-spot 64 were found to be able to bind human plasminogen in overlay blots and surface plasmon resonance (SPR) experiments. It has been suggested that these proteins may be involved in *L. monocytogenes* crossing of the intestinal barrier (Schaumburg et al, 2004). The specific mechanism by which these proteins are translocated has not been identified as yet. However, enolase, DnaK, and EF-Tu, along with GroEL-spot 65 are among proteins reported to be dependent on the virulence related SecA2 auxiliary protein secretion system (Lenz et al., 2003). Additionally, superoxide dismutase (Sod)-spot 71, a protein present in surface extracts yet without a classical export signal sequence, has been identified as a protein dependent on SecA2 for secretion in *Mycobacterium tuberculosis,* and a role for SodA in helping *M. tuberculosis* survive the oxidative attack of macrophages has been proposed (Braunstein et al., 2003). Furthermore, several of the identified proteins have been reported to be associated with the cell surface of *S. oralis* (Wilkins et al., 2003), *S. agalactiae* (Hughes et al., 2002), and *S. pneumoniae* (Ling et al., 2004). These proteins include ldh-spot 28, 6-phosphogluconate dehydrogenase-spot 30, fbaA-spot 33, pgi-spot 40, tpi-spot 42, pgk-spot 43, gap-spot 44, NADP-specific glutamate dehydrogenase-spot 46, tsf-spot 56, gatA-spot 60, EF-Tu-spot 61, fus-spot 62, frr-spot 63, dnaK-spot 64, and sod-spot 71. Of these proteins, pgk, gap, and pgi have also been identified in membrane preparations of *S. mutans* (Zuobi-Hasona et al., 2005). Also, the genes of three identified proteins not

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characterized to be surface proteins, lmo1305-spot 38 (tkt), lmo0223-spot 20 (cysK) and lmo1856- spot 51 (deoD), have a putative binding site for the virulence gene regulator PrfA (Glaser et al., 2001), suggesting a potential function of the proteins at the cell surface. Of the remaining proteins identified in this study, 3 have unknown localization and function (lmo0558-spot 74, lmo1726-spot 75, and lmo2256-spot 76), while 27 proteins are only known to have cytoplasmic functions and are likely contaminants.

Concluding Remarks

Surface proteins of Gram-positive pathogens promote bacterial adhesion to specific tissues, resistance to phagocyte killing, or invasion of host cells. Many successful drugs act by modulating the activity of these surface proteins. It is therefore essential to be able to monitor surface protein expression using high resolution techniques such as 2-D electrophoresis. However, a major limiting factor in surface proteome analysis is the complexity of protein solubilization and separation due to their strong attachment to the cell wall and their hydrophobic nature caused by transmembrane spanning regions. In this study, we show that a protein solubilization protocol using mutanolysin and ASB-14/Urea/Thiourea is effective in solubilizing surface proteins from the Gram-positive pathogen *L. monocytogenes* for 2-DE analysis. This solubilization method helped to solubilize certain surface proteins, in particular LPXTG proteins and hydrophobic tail proteins, which had not previously been reported on the surface proteome map of *L. monocytogenes*. To date, our 2-DE reference map of *L. monocytogenes* surface proteins is the most comprehensive, revealing proteins over a wide range of molecular weights and p*I*. The increase in surface proteome coverage obtained by mutanolysin and ASB-

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14/Urea/Thiourea solubilization suggests the utility of this method for future analytical and comparative studies of surface proteins from *Listeria,* and possibly other Gram-positive bacteria, using 2-DE proteomic analysis. Proteins predicted by genome sequence analysis may or may not truly exist in the organism. The proteomic approach can prove the existence of the genomic-predicted or –unpredicted proteins and allow further characterization of the identified proteins. Improvements in surface protein solubilization techniques for 2-DE are still required for the establishment of efficient methods to isolate defined proteomes without cytoplasmic contamination and with comprehensive coverage of characterized proteins.

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Table 3.1 Proteins of *L. monocytogenes* identified by MALDI-TOF/TOF-MS following solubilization with mutanolysin and ASB-14/Urea/Thiourea

Spot	Gene	Description	Functional Category[a]	Protein Score C.I.%	M_r	p*I*	Notes
1	lmo0582	iap, P60 extracellular protein, invasion associated protein Iap	1.1	100	46 911	9.34	surface
2	lmo1082	similar to dTDP-sugar epimerase	1.1	100	21 091	5.63	surface, new
3	lmo2505	spl, peptidoglycan lytic protein P45	1.1	100	42 685	8.56	surface
4	lmo2754	similar to D-alanyl-D-alanine carboxypeptidase (penicillin-binding protein 5)	1.1	100	48 056	6.93	surface, new
5	lmo0135	similar to oligopeptide ABC transport system substrate-binding proteins, lipoprotein	1.2	100	58 250	5.04	surface, new
6	lmo0152	similar to oligopeptide ABC transporter-binding protein, lipoprotein	1.2	100	62 032	5.69	surface, new
7	lmo0181	similar to sugar ABC transporter, sugar-binding protein, lipoprotein	1.2	100	46 604	4.53	surface, new
8	lmo0285	Unknown, putative lipoprotein	1.2	100	29 792	5.04	surface
8	lmo2478	trxB, thioredoxin reductase	1.4	100	34 152	4.79	surface, new
9	lmo1002	ptsH, PTS phosphocarrier protein Hpr (histidine containing protein)	1.2	100	9 403	4.46	surface, new
10	lmo1003	phosphotransferase system enzyme I	1.2	100	63 172	4.73	surface, new
11	lmo1041	similar to molybdate ABC transporter binding protein, lipoprotein	1.2	100	27 849	5.1	surface
12	lmo1388	tcsA, CD4+ T cell-stimulating antigen, lipoprotein	1.2	100	38 391	5.02	surface
13	lmo1738	similar to amino acid ABC transporter (binding protein), lipoprotein	1.2	100	30 846	5.06	surface

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Table 3.1 (continued)

Spot	Gene	Description	Functional Category[a]	Protein Score C.I.%	M_r	p*I*	Notes
14	lmo1847	LpeA, similar to adhesion binding proteins and lipoproteins with multiple specificity for metal cations (ABC transporter)	1.2	100	34 514	5.16	surface
15	lmo2196	similar to pheromone ABC transporter (binding protein) Peptide binding protein OppA, lipoprotein	1.2	100	62 548	5.3	surface, new
16	lmo2415	similar to ABC transporter, ATP-binding protein	1.2	100	29 135	4.56	surface
17	lmo0355	similar to Flavocytochrome C Fumarate Reductase chain A, lipoprotein	1.4	100	54 428	5.7	surface, new
18	lmo2471	similar to NADH oxidase	1.4	100	36 982	6.38	surface, new
19	lmo2531	atpA, highly similar to H+-transporting ATP synthase chain alpha	1.4	100	55 052	5.33	surface, new
19	lmo2747	SerS, seryl-trna synthetase	3.7.2	100	49 047	5.26	cytoplasmic
20	lmo0196	similar to B. subtilis SpoVG protein	1.7	100	11 249	4.46	cytoplasmic
21	lmo0197	similar to B. subtilis SpoVG protein	1.7	100	11 403	4.23	cytoplasmic, moonlighting
22	lmo0130	similar to 5'-nucleotidase, putative peptidoglycan bound protein (LPXTG motif)	1.8	100	82 459	4.96	surface, new
23	lmo0433	inlA, nternalin A	1.8	100	74 257	4.69	surface, new
24	lmo1666	Unknown, peptidoglycan linked protein (LPxTG)	1.8	100	184 414	4.48	surface, new
25	lmo2185	svpA	1.8	100	63 341	6.44	surface, new
26	lmo2186	svpB	1.8	100	22 253	8.96	surface, new
27	lmo2637	conserved lipoprotein	1.8	100	32 721	6.25	surface
28	lmo0210	ldh, similar to L-lactate dehydrogenase	2.1.1	100	34 191	5.05	cytoplasmic, moonlighting
29	lmo0982	similar to glucanase and peptidase	2.1.1	100	38 649	5.67	cytoplasmic
30	lmo1376	similar to 6-phosphogluconate dehydrogenase	2.1.1	100	52 382	5.11	cytoplasmic, moonlighting
31	lmo1570	pykA, highly similar to pyruvate kinases	2.1.1	100	62 544	5.45	cytoplasmic

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Table 3.1 (continued)

Spot	Gene	Description	Functional Category[a]	Protein Score C.I.%	M_r	p*I*	Notes
32	lmo1571	pfk, highly similar to 6-phosphofructokinase	2.1.1	100	34 398	5.46	cytoplasmic
33	lmo2556	fbaA, similar to fructose-1,6-bisphosphate aldolase	2.1.1	100	30 048	5.2	cytoplasmic, moonlighting
34	lmo2700	similar to aldo/keto reductase	2.1.1	100	32 592	5.77	cytoplasmic
35	lmo2743	tall, similar to transaldolse	2.1.1	100	23 081	4.97	cytoplasmic
36	lmo1052	pdhA, highly similar to pyruvate dehydrogenase (E1 alpha subunit)	2.1.2	99.988	41 241	6.05	cytoplasmic
37	lmo1055	PdhD, highly similar to dihydrolipoamide dehydrogenase, E3 subunit of pyruvate dehydrogenase complex	2.1.2	100	49 456	5.24	cytoplasmic, moonlighting
38	lmo1305	tkt, highly similar to transketolase	2.1.2	100	71 781	5.15	cytoplasmic, moonlighting
39	lmo2205	2,3-biphosphoglycerate-dependent phosphoglycerate mutase (Phosphoglycero-mutase) (PGAM) (BPG-dependent PGAM) (dPGM)	2.1.2	100	26 400	5.6	cytoplasmic
40	lmo2367	pgi, glucose-6-phosphate isomerase	2.1.2	100	49 865	5.27	cytoplasmic, moonlighting
41	lmo2455	eno, highly similar to enolase	2.1.2	100	46 443	4.7	cytoplasmic, moonlighting
42	lmo2457	tpi, highly similar to triose phosphate isomerase	2.1.2	100	26 861	4.73	cytoplasmic, moonlighting
43	lmo2458	pgk, highly similar to phosphoglycerate kinase	2.1.2	100	42 078	4.97	cytoplasmic, moonlighting
44	lmo2459	gap, highly similar to glyceraldehyde 3-phosphate dehydrogenase	2.1.2	100	36 263	5.2	cytoplasmic, moonlighting
45	lmo0223	cysK, highly similar to cysteine synthase	2.2	100	32 197	5.32	cytoplasmic, moonlighting
46	lmo0560	similar to NADP-specific glutamate dehydrogenase	2.2	100	49 235	5.54	cytoplasmic, moonlighting
47	lmo1299	glnA, highly similar to glutamine synthetases	2.2	100	50 429	4.98	cytoplasmic
48	lmo1611	similar to aminopeptidase	2.2	100	38 795	5.57	cytoplasmic

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Table 3.1 (continued)

Spot	Gene	Description	Functional Category[a]	Protein Score C.I.%	M_r	p*I*	Notes
49	lmo2188	similar to oligoendopeptidase	2.2	100	68 531	4.83	cytoplasmic
50	lmo2414	similar to aminotransferase	2.2	100	48 071	5.61	cytoplasmic
51	lmo1856	deoD, purine nucleoside phosphorylase	2.3	99.998	25 341	4.87	cytoplasmic, moonlighting
52	lmo2538	Uracil phosphoribosyltransferase (UMP pyrophosphorylase) (UPRTase)	2.3	100	22 930	5.7	cytoplasmic
53	lmo0970	similar to enoyl- acyl-carrier protein reductase	2.4	100	28 276	5.46	cytoplasmic
54	lmo1673	menB, similar to dihydroxynapthoic acid synthetase	2.5	100	30 005	5.15	cytoplasmic
55	lmo2101	similar to a protein required for pyridoxine synthesis	2.5	100	31 700	5.35	cytoplasmic
56	lmo1657	tsf, translation elongation factor, EF-TS	3.5.3	100	32 617	5.2	cytoplasmic, moonlighting
57	lmo0249	rplA, ribosomal protein L1	3.7.1	100	24 517	9.32	cytoplasmic
58	lmo0250	rplJ, ribosomal protein L10	3.7.1	100	17 682	5.36	cytoplasmic
59	lmo2617	rplF, ribosomal protein L6	3.7.1	100	19 388	9.75	cytoplasmic
59	lmo2620	rplE, ribosomal protein L5	3.7.1	100	19 982	9.02	cytoplasmic
60	lmo1755	gatA, glutamyl-tRNA(Gln) amidotransferase (subunit A)	3.7.2	100	52 319	4.92	cytoplasmic, moonlighting
61	lmo2653	tufA, highly similar to translation elongation factor EF-Tu	3.7.4	100	43 314	4.81	cytoplasmic, moonlighting
62	lmo2654	fus, highly similar to translation elongation factor G, EF- G	3.7.4	100	76 801	4.85	cytoplasmic, moonlighting
63	lmo1314	frr, highly similar to ribosome recycling factors	3.7.5	100	20 743	5.25	cytoplasmic, moonlighting
64	lmo1473	dnaK, class I heat-shock protein (molecular chaperone) DnaK	3.9	100	65 972	4.57	cytoplasmic, moonlighting
65	lmo2068	groEL, class I heat-shock protein (chaperonin) GroEL	3.9	100	57 331	4.72	cytoplasmic, moonlighting

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Table 3.1 (continued)

Spot	Gene	Description	Functional Category[a]	Protein Score C.I.%	M_r	p*I*	Notes
66	lmo0211	ctc, similar to B. subtilis general stress protein	4.1	100	22 640	4.44	cytoplasmic
67	lmo0906	similar to glutathione Reductase	4.1	100	48 964	5.66	cytoplasmic
68	lmo0943	fri, non-heme iron-binding ferritin (ferritin-like protein)	4.1	100	18 035	4.86	cytoplasmic
69	lmo1138	similar to ATP-dependent Clp protease proteolytic component	4.1	100	21 334	4.98	cytoplasmic
70	lmo2468	clpP, ATP-dependent Clp protease proteolytic subunit	4.1	100	21 605	4.94	cytoplasmic
71	lmo1439	sod, superoxide dismutase	4.2	100	22 617	5.23	cytoplasmic, moonlighting
72	lmo2785	kat, catalase	4.2	100	55 853	5.38	cytoplasmic
73	lmo0047	unknown, putative lipoprotein	5.2	100	22 693	4.45	surface
74	lmo0558	unknown, conserved hypothetical protein	5.2	100	37 361	5.34	unknown
75	lmo1726	unknown, similar to hypothetical proteins	5.2	100	36 718	4.98	unknown
76	lmo2256	similar to unknown proteins	5.2	100	19 060	5.22	unknown
77	lmo2636	unknown, conserved hypothetical lipoprotein	5.2	100	39 777	5.3	surface
78	lmo1059	unknown	6	100	19 915	4.83	cytoplasmic, moonlighting
79	lmo1068	unknown, lipoprotein	6	100	30 768	5.85	surface

[a] Functional class codes according to ListiList Listeria genome database (http://genolist.pasteur.fr/ListiList/): 1.1, cell wall; 1.2, transport/binding proteins and lipoproteins; 1.4, membrane bioenergetics; 1.7, cell division; 1.8, cell surface proteins; 2.1.1, metabolism of carbohydrate specific pathways; 2.1.2, main glycolytic pathways; 2.2, metabolism of amino acids and related molecules; 2.3, metabolism of nucleotides and nucleic acids; 2.4, metabolism of lipids; 2.5, metabolism of coenzymes and prosthetic groups; 3.5.3, RNA synthesis – elongation; 3.7.1, protein synthesis - ribosomal proteins; 3.7.2, protein synthesis - aminoacyl-tRNA synthetases; 3.7.4, protein synthesis – elongation; 3.7.5, protein synthesis – termination; 3.9, protein folding; 4.1, adaptation to atypical conditions; 4.2, detoxification; 5.2, similar to unknown proteins from other organisms; 6, no similarity

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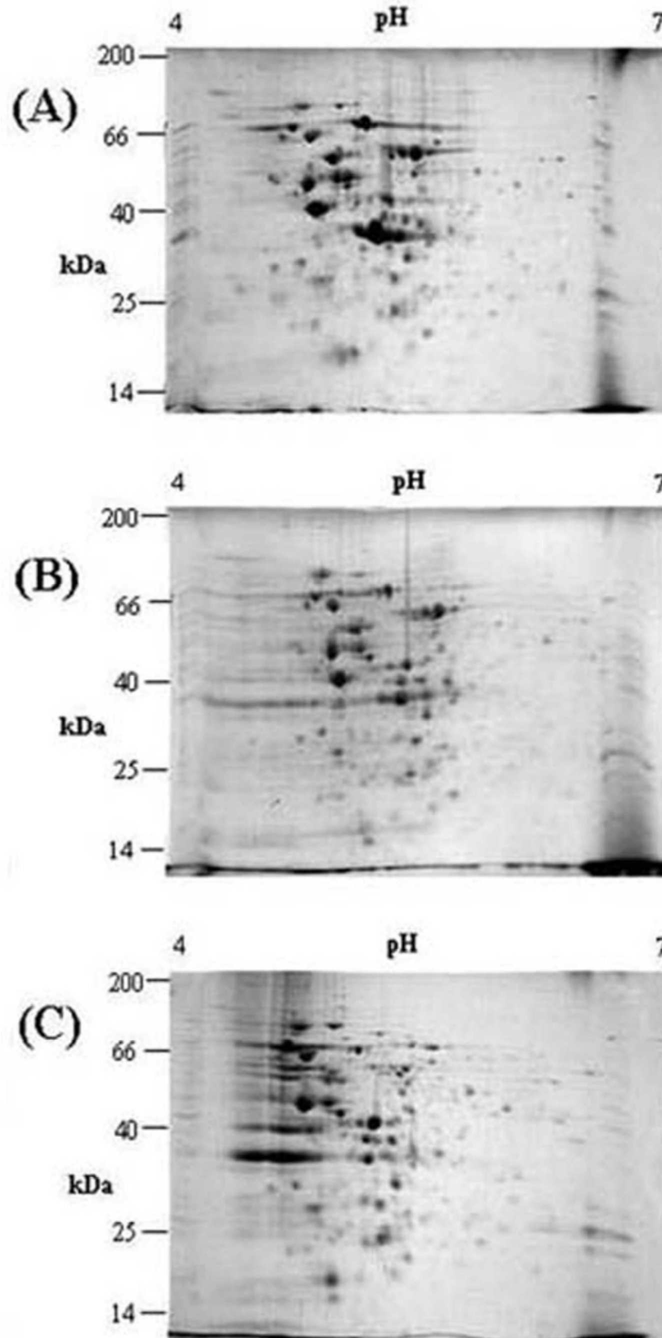

Figure 3.1 The type of zwitterionic detergent and chaotrope concentration present during IEF affects protein solubility and resolution on a 2-D gel.

Surface protein-enriched fractions of *L. monocytogenes* solubilized with mutanolysin and isoelectrically focused using (A) ASB-14/Urea/Thiourea (B) CHAPS/Urea (C) SB3-10/Urea/Thiourea.

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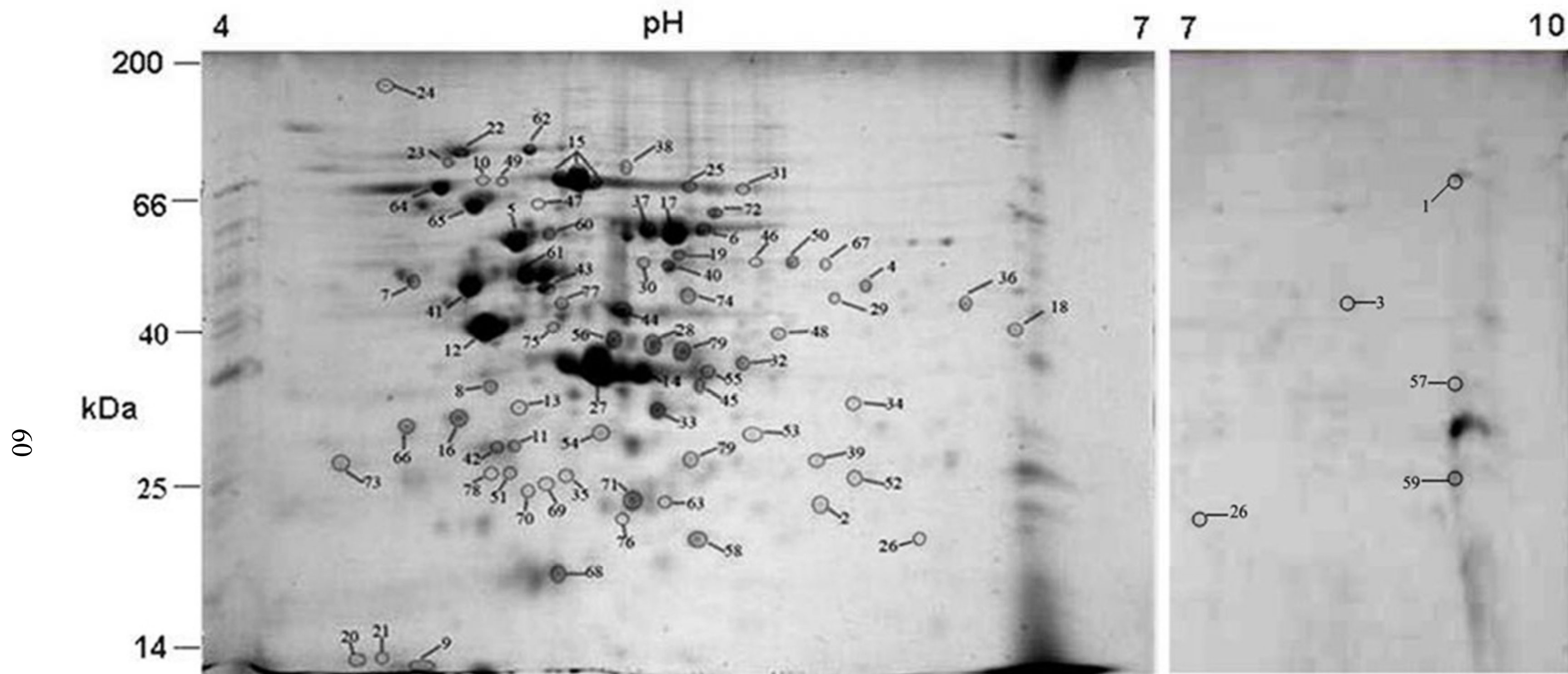

Figure 3.2 2-DE map of *L. monocytogenes* proteins solubilized by mutanolysin and ASB-14/Urea/Thiourea treatment.

82 proteins (81 spots) separated on pH 4-7 or pH 3-10 IPG strips and identified by MALDI-TOF/TOF-MS are indicated on the gels. Circled spots are listed in Table 3.1.

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CHAPTER IV

PROTEIN EXPRESSION BY *LISTERIA MONOCYTOGENES* GROWN ON A READY-TO-EAT MEAT MATRIX

Abstract

Listeria monocytogenes is a deadly foodborne pathogen that can survive and grow in extreme environments including various food preservation and packaging conditions. Recently, several foodborne outbreaks have been related to the consumption of *L. monocytogenes* contaminated ready-to-eat (RTE) meat products particularly sliced turkey meat. A number of studies have suggested that environmental conditions can alter bacterial protein expression and consequently pathogenicity, thereby allowing efficient growth within a host and adaptation to different host cells. However, little is known about whether the growth of *L. monocytogenes* on a RTE meat matrix has an impact on the bacterium's pathogenic capabilities. In this report, we examined the characteristic protein expression of *L. monocytogenes* grown on RTE sliced turkey meat, using *L. monocytogenes* grown on brain-heart-infusion agar as a control. Total protein fractions of *L. monocytogenes* from both growth conditions were extracted and compared by two-dimensional gel electrophoresis. Seventy-seven proteins expressed by turkey meat-grown *L. monocytogenes* were identified by MALDI-TOF/TOF mass spectrometry analysis. The identified proteins include proteins known to be involved in virulence and stress adaptation such as ClpB, ClpC, ClpP, and surface antigen. This is the first report

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describing the proteome expressed by *L. monocytogenes* grown on a meat matrix. Our results suggest that certain proteins that are expressed by RTE meat-grown *L. monocytogenes* may contribute to the virulence of the bacterium.

Introduction

Listeria monocytogenes is a facultative gram-positive foodborne bacterium that is ubiquitous in the environment. It can cause listeriosis, a severe invasive illness, which leads to infections such as encephalitis, septicemia, and meningitis in newborns and immunocompromised individuals, or miscarriages and stillbirths in pregnant women (Ramaswamy et al., 2007; Vazquez-Boland et al., 2001). The primary means of *L. monocytogenes* infection is through consumption of contaminated food. As a psychrotroph and facultative anaerobe, *L. monocytogenes* can multiply at refrigeration temperatures and survive various food packaging conditions such as those involving air or vacuum atmospheres. Moreover, *L. monocytogenes* has the ability to multiply at a wide range of pHs and salt concentrations. These characteristics make it of particular concern in ready-to-eat (RTE) foods, especially processed meat products, which have long refrigerated shelf lives and can be consumed without being reheated or cooked after purchase (FAO/WHO, 2000; ILSI RSI, 2005; Johnson, Doyle, and Cassens, 1990; Kathariou, 2002).

A number of major listeriosis outbreaks have been linked to *L. monocytogenes*-contaminated RTE meat products. In 1998-99, a multistate outbreak of listeriosis in the United States was attributed to contaminated RTE meats (meat frankfurters and chicken and turkey deli meat) and resulted in 14 fatalities and 4 miscarriages or stillbirths

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(Kathariou, Graves, Buchrieser, Glaser, Siletzky, and Swaminathan, 2006; Mead et al., 2006). Another multistate outbreak of listeriosis, causing 4 fatalities and 3 miscarriages or stillbirths, occurred in the year 2000 and was linked to the ingestion of contaminated RTE turkey deli meat (Olsen et al., 2005). RTE turkey deli meat was also implicated in 2001 and 2002 outbreaks of *L. monocytogenes* infection, the latter of which resulted in 8 fatalities and 3 fetal deaths (Frye et al., 2002; Gottlieb et al., 2006; Kathariou et al., 2006). Interestingly, several studies have found that RTE poultry products, particularly turkey deli meat, provide an exceptionally favorable environment for growth of *L. monocytogenes*, even during refrigerated storage (Glass and Doyle, 1989; Lianou, Geornaras, Kendall, Scanga, and Sofos, 2007; Lin et al., 2006).

The survival and growth of *L. monocytogenes* on a food matrix such as RTE turkey meat indicate the bacterium's ability to undergo changes in protein expression and consequent physiological changes that allow it to adapt to the stresses of a RTE meat environment and that can potentially contribute to its ability to mount a successful infection (FAO/WHO, 2000; Roberts and Wiedmann, 2003; Vazquez-Boland et al., 2001). That is to say, *L. monocytogenes* adapted to a RTE-meat matrix may express certain stress and virulence proteins that contribute to the bacterium's ability to enter host cells, multiply, and spread from cell to cell. Although a number of stress and virulence proteins that play a role in *L. monocytogenes* pathogenesis have been identified (Kathariou, 2002; Popowska and Markiewicz, 2004; Roberts et al., 2003; Vazquez-Boland et al., 2001), no information is available on protein expression induced in *L. monocytogenes* grown on a meat matrix. Such data can be valuable in understanding how the environmental signals encountered by *L. monocytogenes* while growing on a

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meat matrix affect not only its ability to survive and grow on the matrix, but also the pathogen's potential to cause disease.

The majority of human listeriosis cases, particularly outbreak cases, have been associated with serotype 4b of *L. monocytogenes* (FDA/FSIS, 2003; Ramaswamy et al., 2007; Vazquez-Boland et al., 2001). In order to understand the impact of a RTE-meat growth matrix on stress and virulence protein expression by *L. monocytogenes*, we used two-dimensional gel electrophoresis (2-DE) to examine the characteristic protein expression of *L. monocytogenes* serotype 4b grown on RTE turkey deli meat, using the same strain grown on nutrient-rich medium as a control. The proteome expressed by *L. monocytogenes* grown on a RTE-meat matrix is described in this report.

Materials and Methods

Preparation of Inoculum

L. monocytogenes serotype 4b strain F2365 was maintained in Brain Heart Infusion (BHI) broth with 20% glycerol at -80°C and resuscitated before use by two successive inoculations into BHI broth followed by incubation with shaking at 37°C for 16-18 h after each inoculation ($A_{600} \approx 1.2$).

Inoculation of Meat and Medium

Commercial packages of RTE smoked turkey breast deli meat slices (sodium content: 253mg or 1.2% per slice) were purchased and kept at 4°C until used in an experiment and were not held beyond the use-by date. BHI agar plates were used as a control matrix.

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Individual turkey slices were aseptically removed from the original packaging and each transferred into a separate sterile stomacher bag. Turkey slices and agar plates were then equilibrated to the desired experimental temperature of 15°C for 30 min prior to inoculation. After temperature equilibration, the surface of each turkey slice was inoculated with 1 ml of the *L. monocytogenes* inoculum, spread evenly on both sides. Likewise, each BHI agar plate was inoculated with 1 ml of the inoculum. The turkey slices and agar plates were then incubated at 15°C for 5 days in a BOD10 refrigerated incubator (Thermo Fisher Scientific-Revco, Asheville, NC).

Cell Harvest and Protein Extraction

After the 5 day incubation period, 40 ml of phosphate buffered saline (PBS) (pH 7.4) were added to each turkey slice bag. The bag was placed in a shaker for 5 min at 4°C, after which the suspension was collected and *L. monocytogenes* cells were harvested by centrifugation at 2,900 × g for 15 min at 4°C. For agar-grown *L. monocytogenes*, the contents of each Petri dish were emptied into 10 mL of PBS and thoroughly mixed. *L. monocytogenes* cells were harvested by centrifugation at 2,900 × g for 15 min at 4°C. The bacterial pellets were collected for protein extraction.

Total protein extracts of turkey meat-grown and agar-grown *L. monocytogenes* were prepared using a Partial Bacterial Proteome Extraction Kit (P-PEK) (catalog # 539780, Calbiochem, San Diego, CA), according to the manufacturer's instructions (*http://wolfson.huji.ac.il/purification/PDF/Protein_Expression_Extraction/NOVAGEN_ParitalBactProtExtrKit.pdf*). The kit is designed for a serial sample preparation of total

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protein mixtures using reagent mixtures with increasing solubilization strength. Briefly, *L. monocytogenes* cells recovered from turkey slices and agar plates were enumerated by dilution plating on BHI agar (data not shown). Appropriate amounts of harvested cells were washed twice in Tris-buffered sucrose, after which the washed cell pellet was frozen at -20°C for 10 min. A protease inhibitor cocktail was then added to the cell pellet and the most soluble proteins were extracted by suspending the cells in Extraction Reagent 1 containing Tris followed by sonication on ice for four 10 sec intervals. Benzonase® was added to the sample to effectively remove nucleic acids, and the sample was incubated at 4°C for 15 min with gentle agitation to achieve maximum protein solubilization. The cell extract was clarified by centrifugation at 20,000 x g for 10 min at 4°C. The supernatant was transferred to a fresh microcentrifuge tube and designated as Fraction 1. Proteins of intermediate solubility were extracted by suspending the cell pellet in Extraction Reagent 2 containing urea, detergent, and DTT. Benzonase® was added to the extraction solution, and the sample was incubated at room temperature under gentle agitation for 15 min. The sample was clarified by centrifugation at 20,000 x g for 10 min at 10°C. The supernatant was transferred to a fresh microcentrifuge tube and designated as Fraction 2. Proteins of low solubility were extracted by suspending the cell pellet in Extraction Reagent 3 containing urea, thiourea, solfobetain, non-ionic detergent, and DTT. The sample was incubated at room temperature under gentle agitation for 15 min. The sample was clarified by centrifugation at 20,000 x g for 10 min at 10°C. The supernatant was transferred to a fresh microcentrifuge tube and designated as Fraction 3. Proteins that were otherwise insoluble were extracted by suspending the final cell pellet in SDS-Buffer. The sample was heated to 95°C for 5 min and quickly cooled to room

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temperature on ice. The sample was clarified by centrifugation at 20,000 x g for 30 min at 10°C. The supernatant was transferred to a fresh microcentrifuge tube and designated as Fraction 4.

Protein concentrations for all fractions were determined using a 2-D Quant Kit (Catalog # 80-6483-56, Amersham Biosciences, Piscataway, NJ). The procedure works by quantitatively precipitating proteins while leaving interfering substances, such as detergents, reductants, and chaotropes used in sample preparation, in solution. After quantification, the required amount of protein from each fraction for subsequent 2-DE analysis was transferred to fresh microcentrifuge tubes and treated with a ReadyPrep® 2-D cleanup kit (Catalog # 163-2130, Bio-Rad, Hercules, CA) to remove substances from the extraction process that interfere with the first dimension isoelectric focusing (IEF) stage of 2-DE. The resulting pellets were resuspended in IEF solubilization buffer containing 7 M urea, 2 M thiourea, 1 % ASB-14 (amidosulfobetaine-14), 50 mM DTT, and 0.2 % 100× Bio-lyte 3/10 ampholyte. The fractions were clarified by centrifugation at 16,000 × g for 5 min, after which they were ready to be loaded for IEF.

Two-Dimensional Electrophoresis

In the first dimension, IEF was performed using a PROTEAN® IEF cell (Bio-Rad, Hercules, CA). Equal amounts of protein from each fraction of turkey meat-grown and agar-grown *L. monocytogenes* were loaded onto the IEF focusing tray with ReadyStrip® IPG strips (11cm, pH 4-7 or pH 3-10) and rehydrated overnight (12 h) at 23° C and 50 V. IEF was carried out at as follows: 250 V for 15 min, followed by voltage ramping, linear mode, to 8000 V for 2.5 h, and final focusing at 8000 V for 35,000 volt-hours. The

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current was limited to 50 μA per IPG strip, and the temperature was kept at 23° C for all focusing steps. Following IEF, the IPG strips were stored at -80ºC. Prior to electrophoresis in the second dimension, IPG strips were thawed and incubated for 30 min in 5 mL of equilibration solution containing 6 M urea, 2 % SDS, 20 % glycerol, 25 % 1.5 M Tris pH 6.8, 5 % 2-mercaptoethanol, and 0.002 % bromophenol blue. Lastly, the IPG strips were positioned on top of 10.5%-14% Criterion® precast gels (Bio-Rad, Hercules, CA) and sealed in place with 1% agarose. The second dimension was performed in a Criterion Dodeca® Cell (Bio-Rad, Hercules, CA). Gels were run at 100 mA until the marker dye reached the bottom of the gel. Following electrophoresis, the gels were stained using Coomassie Blue R-250. Gel images were acquired using a ChemiImager® 5500 imaging system (Alpha Innotech, San Leandro, CA). Three independent replicates were performed with each replicate started from an independent bacterial cultivation.

Protein Identification

Gel images were compared and protein spots were counted using PDQuest 2-D Analysis Software (Bio-Rad, Hercules, CA). To confirm the consistency and reproducibility among replicate gels, all the spots were counted and compared by the software and further confirmed by visual inspection. For protein identification by mass spectrometry, the spots of interest, i.e. unique spots and certain landmark spots present on turkey meat-grown and agar-grown *L. monocytogenes* gels, were robotically excised from the 2-D gels by a Proteome Works Spot Cutter (Bio-Rad, Hercules, CA). In-gel trypsin digestion was performed using the ProPrep robotic digester/spotter (Genomic Solutions,

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Ann Arbor, MI). The method used included disulfide bond reduction and alkylation with DTT (dithiotreitol) and iodoacetamide, respectively. The resulting peptide mix was desalted with C18 ZipTips (Millipore) and spotted on a MALDI plate in a solution of 70% acetonitrile, 0.1% trifluoroacetic acid, and 5mg/ml matrix (alfa-cyano-4-hydroxycinnamic acid).

Mass spectra were collected on an ABI 4700 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Foster City, CA), and protein identification (ID) was performed using the Result Dependent Analysis (RDA) of ABI GPS Explorer software, version 3.5 (Applied Biosystems), using the MASCOT (Matrix Science, Boston, MA) algorithm. Peptide mass fingerprinting (PMF) data obtained from the initial MS scan served for preliminary protein identification and to select precursors for further MS/MS analysis. If the initial MS scan resulted in PMF protein identification of a Confidence Interval (C.I.%) value equal to or higher than 90%, the three most intensive peptides corresponding to the identified protein were selected for fragmentation and their MS/MS spectra were collected in RDA1 analysis. If the initial MS scan (PMF) failed to yield preliminary identification (C.I.% value less than 90%), the seventeen most intensive ions of particular MS spectra were selected for subsequent MS/MS analysis called RDA2. The spectral data from all three scans (PMF, RDA1, and RDA2) was pooled in a combined analysis and matched against the *Listeria* taxonomy protein database of the National Center for Biotechnology Information (NCBI), as of May 2006. To check for contamination by turkey meat proteins, data was also matched against the *Gallus* (chicken) protein database. The turkey protein database was found not to include

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many proteins at the time of analysis, therefore the protein database of a closely related bird was used. Only proteins with a combined Protein Score C.I.% equal to or higher than 95% were considered as positively identified. The parameters of the mass spectrometry analysis were set as follows: MS (precursor-ion) peak filtering- mass range: 800 - 4000 m/z, minimum S/N=10; RDA1 precursor selection type: PMF precursors, PMF precursors selection: Protein Score C.I.% > 90, top protein confirmation with maximal number of precursors/protein= 3, minimum S/N = 10; RDA2 precursor selection type: spot based precursors with minimum S/N = 10, peak sorting order & MS/MS acquisition order: strongest precursors first, maximum precursors/spot=17; MS/MS (fragment-ion) peak filtering and database search: minimum S/N= 3, precursor tolerance 75 ppm, peptide charges 1^+, monoisotopic, MS/MS fragment tolerance 0.2 Da, variable modifications: carbamidomethyl (cysteine), oxidation (methionine).

Results

2-DE maps with exceptional protein resolution, as determined by streaking and sharpness of protein spots, were attained consistently over replicate gels of proteins expressed by *L. monocytogenes* grown on RTE-turkey-meat or on BHI agar at 15°C for 5 days. Protein spots uniquely expressed by turkey meat-grown *L. monocytogenes* or by agar-grown *L. monocytogenes* were selected and identified using MALDI-TOF/TOF-MS. Thirty-four proteins corresponding to 44 spots uniquely expressed by *L. monocytogenes* grown on a RTE-turkey-meat matrix were identified (Table 4.1; Fig. 4.1); whereas, 16 proteins corresponding to 20 spots uniquely expressed by *L. monocytogenes* grown on a BHI agar matrix were identified (Table 4.2; Fig. 4.2). Of the unique protein spots

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excised from turkey meat-grown *L. monocytogenes*, 102 spots matched to chicken proteins, and were likely contaminants from turkey meat. Certain protein spots that were present in the same location on both turkey meat-grown and agar-grown *L. monocytogenes* gels were excised and identified as fixed markers, or landmarks. In total, 43 landmark proteins corresponding to 160 spots were identified as being expressed by both turkey meat-grown and agar-grown *L. monocytogenes* (Table 4.3; Fig. 4.1 and Fig. 4.2).

The cellular role categories of the identified proteins were determined according to The Institute for Genomic Research (TIGR) Comprehensive Microbial Resource (CMR) online database (*http://cmr.tigr.org/tigr-scripts/CMR/CmrHomePage.cgi*). The molecular functions and biological processes were determined according to Gene Ontology (GO) 'molecular function' and 'biological process' terms from the Universal Protein Resource (UniProt), which is a comprehensive repository of information on proteins (*http://www.pir.uniprot.org/*). The cellular role categories, molecular functions, and biological processes of all the identified proteins are listed in the tables, along with gene names, descriptions, molecular weights, and p*I*s.

The majority of the 34 proteins uniquely expressed by *L. monocytogenes* grown on a turkey-meat matrix were found to have catalytic functions in a variety of cellular processes (Table 4.1). Ten of these proteins are involved in energy metabolism, including amino acid and amine metabolism, biosynthesis and degradation of polysaccharides, electron transport, fermentation, glycolysis/gluconeogenesis, pentose phosphate pathway, pyruvate dehydrogenase, and sugar metabolism. Four of the proteins are involved in

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biosynthetic and degradative processes of fatty acid and phopholipid metabolism, and 1 is involved in central intermediary metabolism. Of the remaining proteins, 4 are involved in transcription, 3 are involved in protein synthesis, and one each is involved in cell envelope biosynthesis, cell division, purine ribonucleotide biosynthesis, pyridine nucleotide biosynthesis, and proteolysis. Six are hypothetical proteins or of unknown cellular roles. Interestingly, a single stress response protein, Chaperone protein ClpB-lmof2365_2239, was uniquely expressed by turkey meat-grown *L. monocytogenes*.

The 16 proteins that were not expressed by *L. monocytogenes* grown on a turkey-meat matrix, i.e. proteins uniquely expressed by *L. monocytogenes* grown on BHI agar, were found to have catalytic functions as well as roles in transport and stress response (Table 4.2). Specifically, BHI agar-grown *L. monocytogenes* uniquely expressed 3 stress response proteins: DNA mismatch repair protein MutL- lmof2365_1423 and OsmC/Ohr family proteins- lmof2365_0924 and lmof2365_2232. Of the remaining proteins, 2 are involved in protein synthesis and one each is involved in transport and binding, energy metabolism related to substrate-specific transmembrane transport, proteolysis, cysteine biosynthetic process, and pyridoxine biosynthetic process. Six are hypothetical proteins or of unknown cellular roles.

The 43 proteins that were identified as landmarks, i.e. expressed by both turkey meat-grown and agar-grown *L. monocytogenes,* were found to have a wide range of functions (Table 4.3). The landmark proteins include 4 stress response proteins: ClpC ATPase- lmof2365_0244, cold-shock domain family protein- lmof2365_1381, universal stress protein family- lmof2365_1602, and ATP-dependent Clp protease, proteolytic subunit

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ClpP- lmof2365_2441. Moreover, surface antigen- lmof2365_1407, a basic membrane protein that is antigenic in nature, was expressed by both turkey meat-grown and BHI agar-grown *L. monocytogenes*. The remaining landmark proteins have functions in amino acid biosynthesis, detoxification, transcription, transport and binding, protein synthesis, proteolysis, protein repair, regulation, purine and thiamine biosynthesis, as well as DNA, energy, and fatty acid and phospholipid metabolism. Some of the landmark proteins are hypothetical proteins or have unknown cellular roles.

In summary, 34 proteins uniquely expressed by *L. monocytogenes* grown on a RTE-turkey-meat matrix, 16 proteins uniquely expressed by *L. monocytogenes* grown on BHI agar, and 43 landmark proteins expressed by both turkey meat-grown and agar-grown *L. monocytogenes* were identified in this study. *L .monocytogenes* grown on a RTE-turkey-meat matrix uniquely expressed proteins involved in general cellular processes along with a single protein involved in stress response. Among landmark protein spots identified from turkey meat-grown *L. monocytogenes* and agar-grown *L. monocytogenes*, 4 stress adaptation proteins as well as a surface antigen protein were found to be expressed under both growth conditions. However, 3 stress adaptation proteins were found to be uniquely expressed by agar-grown *L. monocytogenes* and thus are missing from the proteome expressed by *L. monocytogenes* grown on a turkey-meat matrix.

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Discussion

To gain a complete understanding of the pathogenesis of *L. monocytogenes*, pathogen, host, and food matrix effects have to be considered. Yet, one of the priority knowledge gaps according to recent reports is the impact of food type, or food matrix, on the ability of *L. monocytogenes* to cause disease (FAO/WHO, 2000; ILSI RSI, 2005). In this study, we sought to understand the impact of a RTE-meat growth matrix on stress and virulence protein expression by *L. monocytogenes*. Our main goal was to determine if *L. monocytogenes* grown on a RTE meat environment expresses stress adaptation and virulence proteins that not only allow it to survive and grow on the meat matrix, but that may potentially play a role in the bacterium's ability to mount a successful infection. To our knowledge, this is the first report describing the proteome expressed by *L. monocytogenes* grown on a meat matrix.

RTE foods are "high risk" for listeriosis (ILSI RSI, 2005), particularly RTE-turkey-deli meat, which supports the growth of *L. monocytogenes* exceptionally well and has been linked to a number of listeriosis outbreaks (Frye et al., 2002; Glass et al., 1989; Gottlieb et al., 2006; Kathariou et al., 2006; Lianou et al., 2007; Lin et al., 2006; Mead et al., 2006; Olsen et al., 2005). We were able to identify 34 proteins uniquely expressed by *L. monocytogenes* grown on RTE turkey meat slices at 15°C for 5 days using 2-DE and MALDI-TOF/TOF-MS (Table 4.1; Fig. 4.1). The majority of these proteins are involved in energy metabolism, protein synthesis, and cellular processes such as transcription, cell division, proteolysis, and cell wall biogenesis. This indicates that *L. monocytogenes* has to upregulate certain metabolic proteins in order to grow efficiently on a RTE meat matrix at 15°C. Indeed, it is known that survival under stress involves an

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adaptive response mediated by a set of conserved proteins that are upregulated *in vitro* upon exposure to any situation in which bacterial growth is arrested. These proteins may be chaperones that assist in the proper refolding or assembly of stress-damaged proteins or proteases that degrade damaged proteins, ensuring that essential physiological pathways function correctly in stressed cells (Vazquez-Boland et al., 2001). The main stresses encountered by *L. monocytogenes* in our experiment are likely salt stress, as each turkey meat slice had a sodium content of 253mg or 1.2%, and cold stress. *L. monocytogenes* proteins that show high induction after salt stress have previously been reported (Duche, Tremoulet, Glaser, and Labadie, 2002), and among these, only translation elongation factor Tu, EF-TU- lmof2365_2632 was found to be uniquely expressed by turkey meat-grown *L. monocytogenes* in our study. EF-TU most likely plays an essential role in protein folding and protein renaturation in stressed *L. monocytogenes* cells (Caldas, Yaagoubi, Richarme, 1998; Duche et al., 2002).

To acclimatize to cold temperature, *L. monocytogenes* also expresses certain proteins at elevated levels (Liu, Graham, Bigelow, Morse, and Wilkinson, 2002). Among the proteins uniquely expressed by turkey meat-grown *L. monocytogenes* in our study, the genes for thioredoxin-disulfide reductase, trxB- lmof2365_2451 and chaperone protein ClpB- lmof2365_2239 have previously been reported to show increased RNA expression levels during sustained growth of *L. monocytogene*s at 10°C compared to 37°C (Liu et al., 2002). TrxB is involved in the generation of precursor metabolites and energy for electron transport. Increased levels of transcripts encoding trxB at low temperatures suggest that *L. monocytogenes* experiences increased oxidative stress at such temperatures (Liu et al., 2002). ClpB is the only protein uniquely expressed by turkey

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meat-grown *L. monocytogenes* that is part of a characterized stress response system. The Clp (caseinolytic protein) complex is a stress-induced multi-chaperone system that is not only involved in general stress tolerance of *L. monocytogenes* but also in virulence (Kreft and Vazquez-Boland, 2001). Clp proteins play a central role in protein folding and are therefore important factors for efficient growth and cell fitness. A recent study shows that ClpB plays a role in *L. monocytogenes* virulence as well as a role in induced thermotolerance, allowing increased resistance of *L. monocytogenes* to lethal temperatures (Chastanet, Derre, Nair, and Msadek, 2004). The study examined the virulence of a *L. monocytogenes* ΔclpB mutant in a murine model and showed that the mutant displays a significant decrease (100-fold) in virulence. It was found that the role for ClpB in virulence is most likely due to its chaperone activity rather than to a regulatory role in virulence gene expression. The same study also demonstrates that ClpB is required for induced thermotolerance of *L. monocytogenes*, which allows for better survival of lethal conditions when cells have been exposed to nonlethal stress. Moreover, the study established that ClpB is not involved with general adaptation to cold stress in *L. monocytogenes*. These findings along with the unique expression of ClpB by *L. monocytogenes* grown on a RTE meat matrix in our study suggest that the role of ClpB in facilitating *L. monocytogenes* survival on RTE meat at low temperatures and the protein's subsequent role in human infection should be investigated further.

Along with proteins uniquely expressed by turkey meat-grown *L. monocytogenes*, we identified 43 landmark proteins expressed by both turkey meat-grown and agar-grown *L. monocytogenes* (Table 4.3; Fig. 4.1 and Fig. 4.2). Although we did not attempt to identify all the protein spots present on both turkey meat-grown and agar-grown *L.*

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monocytogenes gels, identifying certain landmark protein spots made it possible for us to have fixed markers on the gels for comparison and allowed us to get an idea of proteins that were expressed by *L. monocytogenes* under both growth conditions. As expected, the landmark proteins were found to have a wide range of functions in a number of metabolic processes. Among the landmark proteins, 4 stress response proteins were identified: universal stress protein- lmof2365_1602, cold-shock domain family protein- lmof2365_1381, ClpC ATPase- lmof2365_0244, and ATP-dependent Clp protease, proteolytic subunit ClpP- lmof2365_2441. Moreover, surface antigen- lmof2365_1407, a basic membrane protein that is antigenic in nature, was also identified. Universal stress protein and cold-shock domain family protein are both involved in *L. monocytogenes* adaptation to atypical conditions and have DNA binding functions (*http://www.pir.uniprot.org/*). It is expected that such general stress response proteins would be expressed by both turkey meat-grown and agar-grown *L. monocytogenes* at 15°C. Interestingly, two Clp family proteins, ClpC ATPase and ClpP, were also expressed by *L. monocytogenes* under both growth conditions. As mentioned previously, Clp proteins are not only involved in general stress tolerance of *L. monocytogenes* but also in virulence (Kreft et al., 2001). ClpC ATPase of *L. monocytogenes* is a general stress protein involved in intracellular growth and *in vivo* survival of the pathogen in host tissues (Rouquette et al., 1996). The ClpC ATPase of *L. monocytogenes* has been shown to be required for promoting early bacterial escape from the phagosome of macrophages (Rouquette, Chastellier, Nair, and Berche, 1998). Moreover, the protein is required for adhesion and invasion of *L. monocytogenes* and modulates the expression of *L. monocytogenes* virulence factors InlA, InlB, and ActA (Nair, Milohanic, Berche, 2000).

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Likewise, the stress-induced protease ClpP is required for virulence of *L. monocytogenes*, being essential for intracellular survival and replication of the pathogen as well as for modulating the expression of listeriolysin O (LLO), a major immunodominant virulence factor promoting intracellular growth (Gaillot, Pellegrini, Bregenholt, Nair, and Berche, 2000). ClpP has been proposed as a potential target for modulating the presentation of protective antigens such as LLO and thereby the immune response against *L. monocytogenes* (Gaillot, Bregenholt, Jaubert, Di Santo, and Berche, 2001). ClpP has also been reported to be induced during *L. monocytogenes* growth at low temperatures (Liu et al., 2002), while 3 landmark proteins, cysteine synthase A, CysK- lmof2365_0234, pyruvate dehydrogenase alpha subunit, PdhA- lmof2365_1073, and inosine-5'-monophosphate dehydrogenase, GuaB- lmof2365_2746, have been reported to show high induction after salt stress (Duche et al., 2002). On the whole, the landmark proteins identified in our study indicate that *L. monocytogenes* grown on RTE turkey meat or on agar at 15°C is capable of expressing certain stress and virulence associated proteins under both growth conditions. It is quite likely that these proteins are induced at varying rates between the growth conditions; however that possibility was not examined in the current study.

Certain proteins that are expressed by *L. monocytogenes* grown on BHI agar at 15°C for 5 days were found to not be expressed by *L. monocytogenes* grown on RTE turkey meat under the same conditions. In total, we identified 16 proteins uniquely expressed by agar-grown *L. monocytogenes* (Table 4.2; Fig. 4.2). These proteins have catalytic functions as well as roles in transport and stress response. Specifically, BHI agar-grown *L. monocytogenes* uniquely expressed 3 stress response proteins: DNA

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mismatch repair protein MutL- lmof2365_1423 and OsmC/Ohr family proteins-lmof2365_0924 and lmof2365_2232. MutL is an ATP binding-ATPase involved in response to DNA damage stimulus, and the OsmC/Ohr family proteins are involved in response to stress, however their exact functions are unknown (*http://www.pir.uniprot.org/*). The remaining identified proteins with known cellular roles are involved in protein synthesis and degradation, transport and binding, energy metabolism, or biosynthetic processes. These results indicate that although the protein profile of *L. monocytogenes* growing on RTE meat encompasses proteins important for stress adaptation, efficient growth, and overall cell fitness, the bacterium may not be able to express certain metabolic proteins while growing on RTE meat that it can express while growing on nutrient rich media under the same conditions.

The results presented in this report are part of ongoing research to understand the interactions of *Listeria* with its food environment that influence its ability cause disease. While the cold and salt stress proteins of *L. monocytogenes* have previously been reported (Duche et al., 2002; Liu et al., 2002), this is the first study to use a proteomic approach to evaluate these stress factors on an actual food matrix, RTE turkey deli meat, which is an important source of human listeriosis outbreaks. Consequently, an initial database of proteins that *L. monocytogenes* expresses when it grows on a RTE meat matrix has been established. However, certain limitations to our study have to be taken into account. First, with regard to the 2-DE process, protein spots that were uniquely identified may actually be present on gels from both growth conditions, but in an undetectable amount on gels from one condition. Also, it is possible that the same protein may have migrated differently on gels from different growth conditions due to

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post-translational modifications. A shotgun proteomics approach combined with peptide labeling for relative protein quantitation can be used in future work to verify total protein expression and determine variation in individual protein quantity between different growth conditions. Second, *L. monocytogenes* was grown at 15°C, which is higher than typical refrigeration temperature. A recent study shows that there is no appreciable difference in recovery of *L. monocytogenes* from RTE wiener packages maintained at 4°C versus 10°C (Wallace, Call, Porto, Cocoma, and Luchansky, 2003). Nevertheless, we cannot exclude the possibility that protein expression by *L. monocytogenes* growing on a RTE meat matrix may be affected by a lower incubation temperature. In conclusion, our data indicate that *L. monocytogenes* expresses proteins involved in a number of metabolic processes along with known stress and virulence proteins when growing on a RTE turkey meat matrix. More studies are needed to determine if the adaptation of *L. monocytogenes* to a RTE meat environment facilitates the bacterium's ability to mount a successful infection.

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Table 4.1 MALDI-TOF/TOF-MS identified proteins uniquely expressed by *L. monocytogenes* grown on ready-to-eat turkey deli meat slices at 15°C for 5 days

Spot	Gene	Description	M_r	p*I*	Cellular Role Category	Molecular Functions / Biological Process
0210	lmof2365_0210	ribose-phosphate pyrophosphokinase	35047	5.83	Purines, pyrimidines, nucleosides, and nucleotides: Purine ribonucleotide biosynthesis	catalytic activity > transferase activity / purine ribonucleotide biosynthetic process
0292	lmof2365_0292	hydrolase, haloacid dehalogenase-like family	30318	4.79	Unknown function: Enzymes of unknown specificity	catalytic activity > hydrolase activity / metabolic process resulting in cell growth
0312	lmof2365_0312	Serine protease	52850	4.34	Protein fate: Degradation of proteins, peptides, and glycopeptides	catalytic activity > hydrolase activity / proteolysis
0363	lmof2365_0363	Probable transaldolase 2	23551	5.14	Energy metabolism: Pentose phosphate pathway	catalytic activity > transferase activity / pentose-phosphate shunt
0375	lmof2365_0375	putative long-chain acyl-CoA synthetase	58091	6.18	Fatty acid and phospholipid metabolism: Degradation	catalytic activity > ligase activity / metabolic process resulting in cell growth
0385	lmof2365_0385	conserved hypothetical protein	26679	4.64	Hypothetical proteins: Conserved	Unknown
0550	lmof2365_0550	Glycosyl hydrolase, family 4	48990	4.78	Energy metabolism: Biosynthesis and degradation of polysaccharides	catalytic activity > hydrolase activity / carbohydrate metabolic process
0568	lmof2365_0568	tagatose 1,6-diphosphate aldolase	37674	4.94	Energy metabolism: Biosynthesis and degradation of polysaccharides	catalytic activity > lyase activity / cellular carbohydrate catabolic process

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Table 4.1 (continued)

0582	lmof2365_0582	CBS domain protein	23532	7.01	Unknown function: General	Unknown
0604	lmof2365_0604	transcriptional regulator, GntR family	28041	6.61	Regulatory functions: DNA interactions	transcription regulator activity > DNA binding / regulation of transcription, DNA-dependent
0654	lmof2365_0654	lipase/acylhydrolase, putative	26583	7.12	Unknown function: Enzymes of unknown specificity	catalytic activity > hydrolase activity / lipid metabolic process
0802	lmof2365_0802	FMN-dependent NADH-azoreductase 2	23065	5.07	Fatty acid and phospholipid metabolism: Other	catalytic activity > hydrolase activity / coenzyme biosynthetic process
0827	lmof2365_0827	carbonic anhydrase	27219	4.83	Central intermediary metabolism: Other	catalytic activity > lyase activity / one-carbon compound metabolic process
0884	lmof2365_0884	ATP-dependent RNA helicase DeaD	57259	9.43	Transcription: Other	catalytic activity > helicase activity / cell growth and/or maintenance
1075	lmof2365_1075	dihydrolipoamide acetyltransferase	58318	4.75	Energy metabolism: Pyruvate dehydrogenase	catalytic activity > transferase activity / pyruvate metabolic process
1348	lmof2365_1348	polyribonucleotide nucleotidyltransferase	79492	5.23	Transcription: Degradation of RNA	catalytic activity > transferase activity / mRNA catabolic process
1442	lmof2365_1442	conserved hypothetical protein	32210	7.77	Hypothetical proteins: Conserved	Unknown
1580	lmof2365_1580	threonyl-tRNA synthetase	73177	5.24	Protein synthesis: tRNA aminoacylation	catalytic activity > ligase activity / threonyl-tRNA aminoacylation
1608	lmof2365_1608	inorganic polyphosphate/ATP-NAD kinase	30479	6.54	Biosynthesis of cofactors, prosthetic groups, and carriers: Pyridine nucleotides	catalytic activity > transferase activity / cofactor, pyridine nucleotide, biosynthetic process
1836	lmof2365_1836	malonyl CoA-acyl-carrier protein transacylase	32883	4.83	Fatty acid and phospholipid metabolism: Biosynthesis	catalytic activity > transferase activity / fatty acid biosynthetic process

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Table 4.1 (continued)

1837	lmof2365_1837	fatty acid/phospholipid synthesis protein PlsX	36384	6.93	Fatty acid and phospholipid metabolism: Biosynthesis	catalytic activity / lipid biosynthetic process
1946	lmof2365_1946	formate acetyltransferase	86195	5.22	Energy metabolism: Fermentation	catalytic activity > transferase activity / generation of precursor metabolites and energy > fermentation
1951	lmof2365_1951	TPR domain protein	47662	4.23	Unknown function: General	binding > ligand
2064	lmof2365_2064	cell division protein FtsZ	41392	4.81	Cellular processes: Cell division	catalytic activity > hydrolase activity / cell division > cytokinesis
2143	lmof2365_2143	mannose-6-phosphate isomerase, class I	35254	4.94	Energy metabolism: Sugars	catalytic activity > isomerase activity / carbohydrate metabolic process
2151	lmof2365_2151	phosphoglucosamine mutase	48399	4.72	Cell envelope: Biosynthesis and degradation of murein sacculus and peptidoglycan	catalytic activity > isomerase activity / lipid biosynthetic process and peptidoglycan-based cell wall biogenesis
2231	lmof2365_2231	tryptophanyl-tRNA synthetase	36939	6.49	Protein synthesis: tRNA aminoacylation	catalytic activity > ligase activity / amino acid metabolic process > tryptophanyl-tRNA aminoacylation
2239	lmof2365_2239	Chaperone protein clpB	97525	4.73	Protein fate: Protein folding and stabilization	catalytic activity > hydrolase activity and protein binding / protein refolding Note: Part of a stress-induced multi-chaperone system.
2334	lmof2365_2334	glutamate decarboxylase beta	53550	5.11	Energy metabolism: Amino acids and amines	catalytic activity > lyase activity / glutamate decarboxylation to succinate
2428	lmof2365_2428	enolase (2-phosphoglycerate dehydratase)	46457	4.7	Energy metabolism: Glycolysis/gluconeo-genesis	catalytic activity > lyase activity / glycolysis

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Table 4.1 (continued)

2451	lmof2365_2451	Thioredoxin-disulfide reductase, trxB	34173	4.51	Energy metabolism: Electron transport	catalytic activity > oxidoreductase activity / generation of precursor metabolites and energy > electron transport
2632	lmof2365_2632	translation elongation factor Tu, EF-TU	43314	4.81	Protein synthesis: Translation factors	nucleic acid binding > translation elongation factor activity / translation > translational elongation
2640	lmof2365_2640	transketolase	72166	4.95	Energy metabolism: Pentose phosphate pathway	catalytic activity > transferase activity / pentose-phosphate shunt
2843	lmof2365_2843	RNA-binding protein	23049	6.99	Transcription: Other	nucleic acid binding > RNA binding

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Table 4.2 MALDI-TOF/TOF-MS identified proteins uniquely expressed by *L. monocytogenes* grown on Brain Heart Infusion agar at 15°C for 5 days

Spot	Gene	Description	M_r	p*I*	Cellular Role Category	Molecular Functions / Biological Process
0185	lmof2365_0185	conserved hypothetical protein	47475	5.02	Hypothetical proteins: Conserved	Unknown
0811	lmof2365_0811	conserved hypothetical protein	23342	5.11	Hypothetical proteins: Conserved	catalytic activity > oxidoreductase activity / lysine biosynthetic process via diaminopimelate
0924	lmof2365_0924	OsmC/Ohr family protein	13970	5.13	Unknown function: General	response to stimulus > response to stress
1249	lmof2365_1249	phosphoesterase family protein	19773	5.13	Unknown function: Enzymes of unknown specificity	catalytic activity > hydrolase activity / metabolic process resulting in cell growth
1408	lmof2365_1408	ABC transporter, ATP-binding protein	56699	6.13	Transport and binding proteins: Unknown substrate	ATP binding > ATPase activity, coupled to transmembrane movement of substances / establishment of localization > transport
1412	lmof2365_1412	peptidase, M16 family	48770	4.79	Protein fate: Degradation of proteins, peptides, and glycopeptides	catalytic activity > hydrolase activity / proteolysis
1423	lmof2365_1423	DNA mismatch repair protein MutL	68048	5.33	DNA metabolism: DNA replication, recombination, and repair	ATP binding -ATPase activity / response to endogenous stimulus > response to stress > response to DNA damage stimulus
1453	lmof2365_1453	Metallo-beta-lactamase family protein	61929	6.05	Unknown function: Enzymes of unknown specificity	catalytic activity > hydrolase activity / metabolic process resulting in cell growth

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Table 4.2 (continued)

1664	lmof2365_1664	conserved hypothetical protein	15747	4.92	Hypothetical proteins: Conserved	Unknown
1850	lmof2365_1850	sun protein	49876	6.45	Protein synthesis: tRNA and rRNA base modification	RNA binding and catalytic activity > transferase activity / rRNA processing
2133	lmof2365_2133	pyridoxine biosynthesis protein	31652	5.24	Biosynthesis of cofactors, prosthetic groups, and carriers: Pyridoxine	catalytic activity > lyase activity / coenzyme, vitamin B6-pyridoxal phosphate, biosynthetic process
2232	lmof2365_2232	OsmC/Ohr family protein	14494	4.8	Unknown function: General	response to stimulus > response to stress
2384	lmof2365_2384	cysteine desulfurase, SufD subfamily	44676	5.08	Biosynthesis of cofactors, prosthetic groups, and carriers: Other	catalytic activity >transferase activity and cofactor binding / cysteine metabolic process
2502	lmof2365_2502	ATP synthase subunit beta	51608	4.46	Energy metabolism: ATP-proton motive force interconversion	substrate-specific transmembrane transporter activity > hydrogen ion transporting ATP synthase activity, rotational mechanism / cofactor, ATP, biosynthetic process > ATP synthesis coupled proton transport
2604	lmof2365_2604	50S ribosomal protein L4	22589	10.03	Protein synthesis: Ribosomal proteins: synthesis and modification	structural molecule activity >structural constituent of ribosome / translation
2671	lmof2365_2671	conserved hypothetical protein	11813	4.76	Hypothetical proteins: Conserved	Unknown

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Table 4.3 MALDI-TOF/TOF-MS identified landmark proteins expressed by both *L. monocytogenes* grown on ready-to-eat turkey deli meat slices and *L. monocytogenes* grown on Brain Heart Infusion agar at 15°C for 5 days

Spot	**Gene**	**Description**	M_r	**p*I***	**Cellular Role Category**	**Molecular Functions / Biological Process**
0053	lmof2365_0053	ribosomal protein S6	11499	5.08	Protein synthesis: Ribosomal proteins: synthesis and modification	structural molecule activity > structural constituent of ribosome / translation
0234	lmof2365_0234	cysteine synthase A, CysK	32183	5.32	Amino acid biosynthesis: Serine family	catalytic activity > lyase activity / cysteine biosynthetic process from serine
0244	lmof2365_0244	ClpC ATPase	91149	7.02	Cellular processes: Adaptations to atypical conditions	catalytic activity > hydrolase activity > protein, nucelotide, ATP binding / response to endogenous stimulus > DNA repair
0253	lmof2365_0253	RNA methyltransferase, TrmH family, group 3	28007	6.54	Protein synthesis: tRNA and rRNA base modification	catalytic activity > transferase activity / RNA methylation
0274	lmof2365_0274	DNA-directed RNA polymerase beta subunit	132521	5.05	Transcription: DNA-dependent RNA polymerase	catalytic activity > transferase activity / transcription
0275	lmof2365_0275	DNA-directed RNA polymerase beta' chain (RNAP beta' subunit) (Transcriptase beta' chain) (RNA polymerase beta' subunit)	134670	8.42	Transcription: DNA-dependent RNA polymerase	catalytic activity > transferase activity / transcription

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Table 4.3 (continued)

0364	lmof2365_0364	oxidoreductase, short-chain dehydrogenase/reductase family	27244	5.27	Unknown function: Enzymes of unknown specificity	catalytic activity > oxidoreductase activity / metabolism resulting in cell growth
0696	lmof2365_0696	phosphomethylpyrimidine kinase	28844	5.36	Biosynthesis of cofactors, prosthetic groups, and carriers: Thiamine	catalytic activity > transferase activity / water-soluble vitamin, thiamine, biosynthetic process
1023	lmof2365_1023	phosphocarrier protein HPr	9397	4.81	Transport and binding proteins: Carbohydrates, organic alcohols, and acids AND Signal transduction: PTS	catalytic activity > transferase activity / cell communication > signal transduction AND establishment of localization > transport > PTS
1048	lmof2365_1048	metallo-beta-lactamase family protein	61520	6.18	Unknown function: Enzymes of unknown specificity	catalytic activity > hydrolase activity / metabolic process resulting in cell growth
1049	lmof2365_1049	conserved hypothetical protein	8292	4.51	Hypothetical proteins: Conserved	Unknown
1073	lmof2365_1073	pyruvate dehydrogenase complex, E1 component, pyruvate dehydrogenase alpha subunit, PdhA	41242	5.91	Energy metabolism: Pyruvate dehydrogenase	catalytic activity > oxidoreductase activity / pyruvate metabolic process
1074	lmof2365_1074	pyruvate dehydrogenase complex, E1 component, pyruvate dehydrogenase beta subunit	35275	4.79	Energy metabolism: Pyruvate dehydrogenase	catalytic activity > oxidoreductase activity / pyruvate metabolic process
1293	lmof2365_1293	DNA topoisomerase I	79381	9.17	DNA metabolism: DNA replication, recombination, and repair	DNA binding / DNA topological change

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Table 4.3 (continued)

1310	lmof2365_1310	glycerol-3-phosphate dehydrogenase, aerobic	63213	6.32	Energy metabolism: Other	catalytic activity > oxidoreductase activity / glycerol metabolic process
1381	lmof2365_1381	cold-shock domain family protein	7261	4.45	Cellular processes: Adaptations to atypical conditions	binding > DNA binding / regulation of transcription, DNA-dependent AND response to abiotic stimulus > response to cold
1407	lmof2365_1407	surface antigen	38391	5.02	Cell envelope: Surface structures	binding > lipid binding Note: A basic membrane protein, antigenic in nature
1458	lmof2365_1458	superoxide dismutase, Mn	22601	5.23	Cellular processes: Detoxification	catalytic activity > oxidoreductase activity / superoxide metabolic process
1477	lmof2365_1477	glycyl-tRNA synthetase, beta subunit	78452	4.91	Protein synthesis: tRNA aminoacylation	catalytic activity > ligase activity / amino acid metabolic process > glycyl-tRNA aminoacylation
1592	lmof2365_1592	pyruvate kinase	62577	5.39	Energy metabolism: Glycolysis/gluconeogenesis	catalytic activity > transferase activity / glycolysis
1602	lmof2365_1602	universal stress protein family	16856	4.98	Cellular processes: Adaptations to atypical conditions	DNA binding / response to stimulus > response to stress
1662	lmof2365_1662	aconitate hydratase 1	98292	4.88	Energy metabolism: TCA cycle	catalytic activity > lyase activity / TCA cycle
1679	lmof2365_1679	30S ribosomal protein S2	28356	5.86	Protein synthesis: Ribosomal proteins: synthesis and modification	structural molecule activity > structural constituent of ribosome / translation

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Table 4.3 (continued)

1735	lmof2365_1735	aminopeptidase	44931	4.69	Protein fate: Degradation of proteins, peptides, and glycopeptides	catalytic activity > hydrolase activity / proteolysis
1750	lmof2365_1750	oxidoreductase, Gfo/Idh/MocA family	36748	4.97	Unknown function: Enzymes of unknown specificity	catalytic activity > oxidoreductase activity / metabolic process resulting in cell growth
1871	lmof2365_1871	ribosomal large subunit pseudouridine synthase, RluD subfamily	34010	6.35	Protein synthesis: tRNA and rRNA base modification	catalytic activity > isomerase activity / RNA modification
1888	lmof2365_1888	peptide methionine sulfoxide reductase msrA (Protein-methionine-S-oxide reductase) (Peptide Met(O) reductase)	19934	5.41	Protein fate: Protein modification and repair	catalytic activity > oxidoreductase activity / protein repair
1957	lmof2365_1957	chorismate synthase	42161	6.59	Amino acid biosynthesis: Aromatic amino acid family	catalytic activity > lyase activity / aromatic amino acid family biosynthetic process
2044	lmof2365_2044	isoleucyl-tRNA synthetase	103812	5.18	Protein synthesis: tRNA aminoacylation	catalytic activity > ligase activity / isoleucyl-tRNA aminoacylation
2126	lmof2365_2126	class II aldolase/adducin domain protein	24241	5.42	Unknown function: General	catalytic activity > metal ion binding
2155	lmof2365_2155	glycosyl transferase, family 65	86703	4.96	Energy metabolism: Biosynthesis and degradation of polysaccharides	catalytic activity > hydrolase activity / carbohydrate metabolic process

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Table 4.3 (continued)

2225	lmof2365_2225	oligopeptide ABC transporter, ATP-binding protein	36597	6.85	Transport and binding proteins: Amino acids, peptides and amines	catalytic activity > hydrolase activity > ATP binding / establishment of localization > transport > oligopeptide transport
2249	lmof2365_2249	HIT family protein	16124	5.21	Unknown function: General	involved in cell-cycle regulation
2363	lmof2365_2363	pyridine nucleotide-disulfide oxidoreductase family protein	44029	5.84	Unknown function: Enzymes of unknown specificity	catalytic activity > disulfide oxidoreductase activity / generation of precursor metabolites and energy > electron transport
2441	lmof2365_2441	ATP-dependent Clp protease, proteolytic subunit ClpP	21605	4.94	Protein fate: Degradation of proteins, peptides, and glycopeptides	catalytic activity > hydrolase activity / ATP-dependent proteolysis
2484	lmof2365_2484	ribosomal subunit interface protein	21620	5.25	Protein synthesis: Translation factors	Unknown
2488	lmof2365_2488	DNA-binding response regulator DegU	25799	6.31	Regulatory functions: DNA interactions AND Regulatory functions: Protein interactions AND Signal transduction: Two-component systems	catalytic activity > transferase activity / cellular process > cell communication > signal transduction AND regulation of transcription, DNA-dependent
2528	lmof2365_2528	fructose-biphosphate aldolase, putative	30048	5.2	Energy metabolism: Glycolysis/gluconeogenesis	catalytic activity > lyase activity / glycolysis
2633	lmof2365_2633	elongation factor EF-2	76801	4.85	Protein synthesis: Translation factors	nucleic acid binding > translation factor activity / translational elongation

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Table 4.3 (continued)

2675	lmof2365_2675	dihydroxyacetone kinase, Dak2 subunit, putative	21499	4.97	Fatty acid and phospholipid metabolism: Biosynthesis	catalytic activity > transferase activity / glycerol metabolic process
2743	lmof2365_2743	hydrolase, CocE/NonD family	62731	4.98	Unknown function: Enzymes of unknown specificity	catalytic activity > hydrolase activity / proteolysis
2746	lmof2365_2746	inosine-5'-monophosphate dehydrogenase, GuaB	52522	6.06	Purines, pyrimidines, nucleosides, and nucleotides: Purine ribonucleotide biosynthesis	catalytic activity > oxidoreductase activity / purine ribonucleotide biosynthetic process
2780	lmof2365_2780	DNA-binding protein	28898	7.67	Unknown function: General	sequence specific DNA binding

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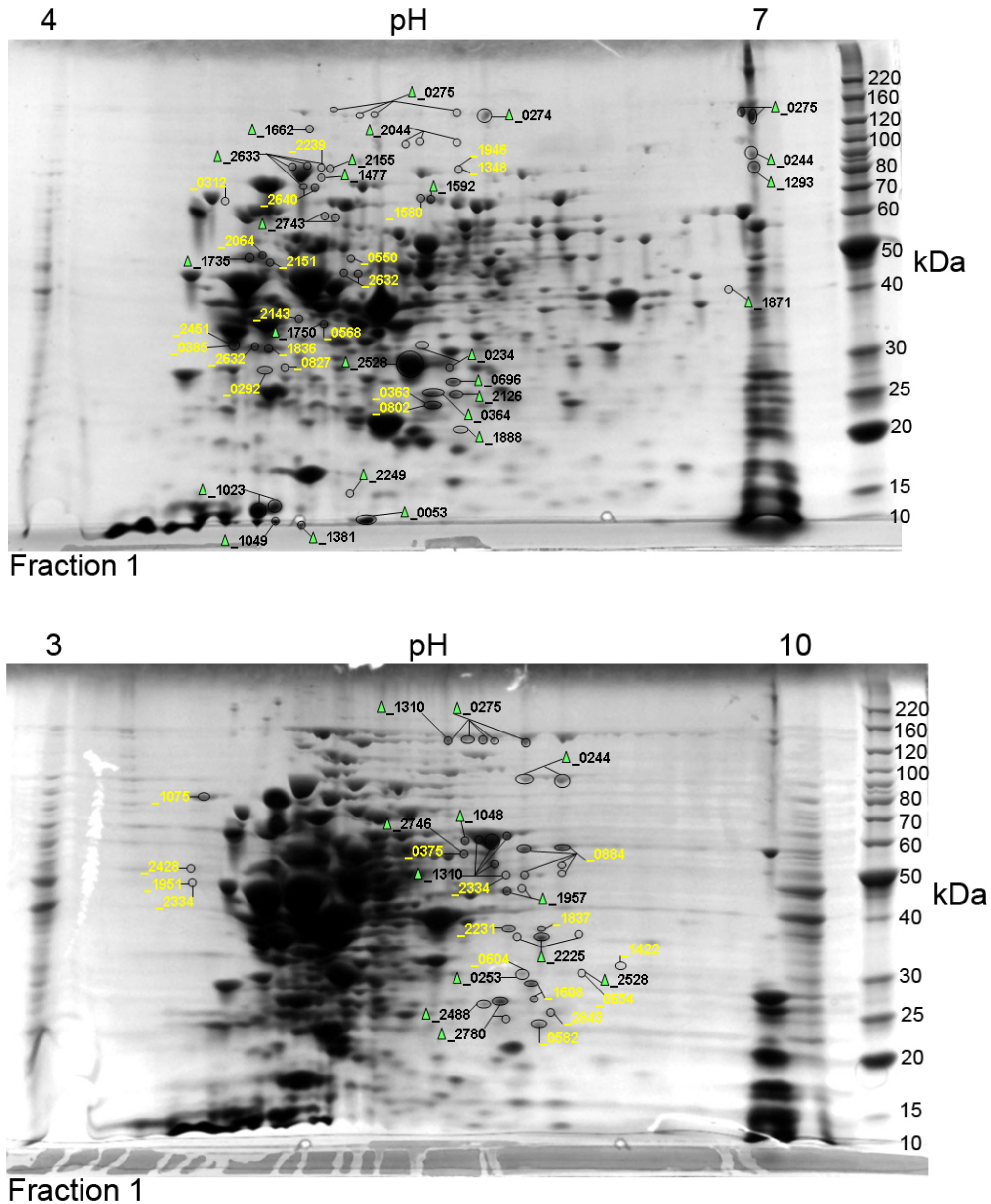

Figure 4.1 2-DE maps of proteins expressed by *L. monocytogenes* grown on ready-to-eat turkey deli meat slices at 15°C for 5 days.

Four protein fractions based on varying protein solubility were obtained using P-PEK, and each fraction was separated on pH 4-7 and pH 3-10 IPG strips. Protein spots of interest identified by MALDI-TOF/TOF-MS after 2-DE are indicated on the gels. No proteins were identified on Fraction 3 pH4-7, Fraction 4 pH4-7, and Fraction 4 pH 3-10 gels. Uniquely expressed proteins are indicated in yellow. Landmark proteins are indicated by a colored triangle (△). Marked unique spots are listed in Table 4.1 and landmark spots are listed in Table 4.3. (2-DE maps continue on next two pages)

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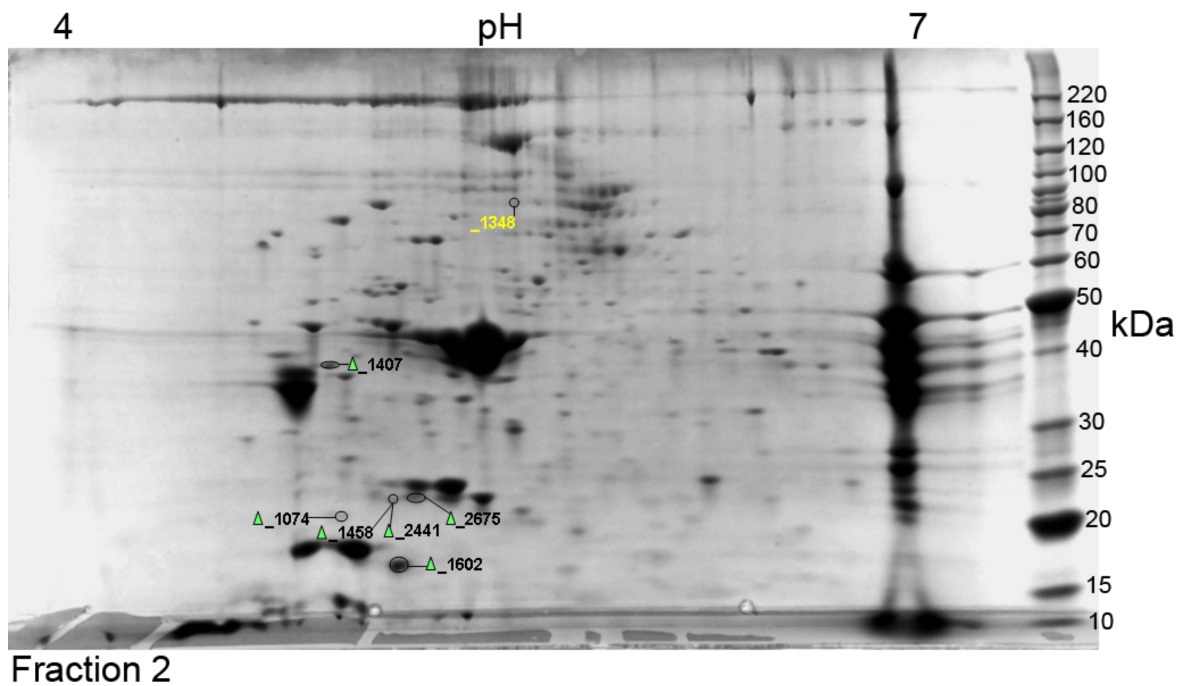

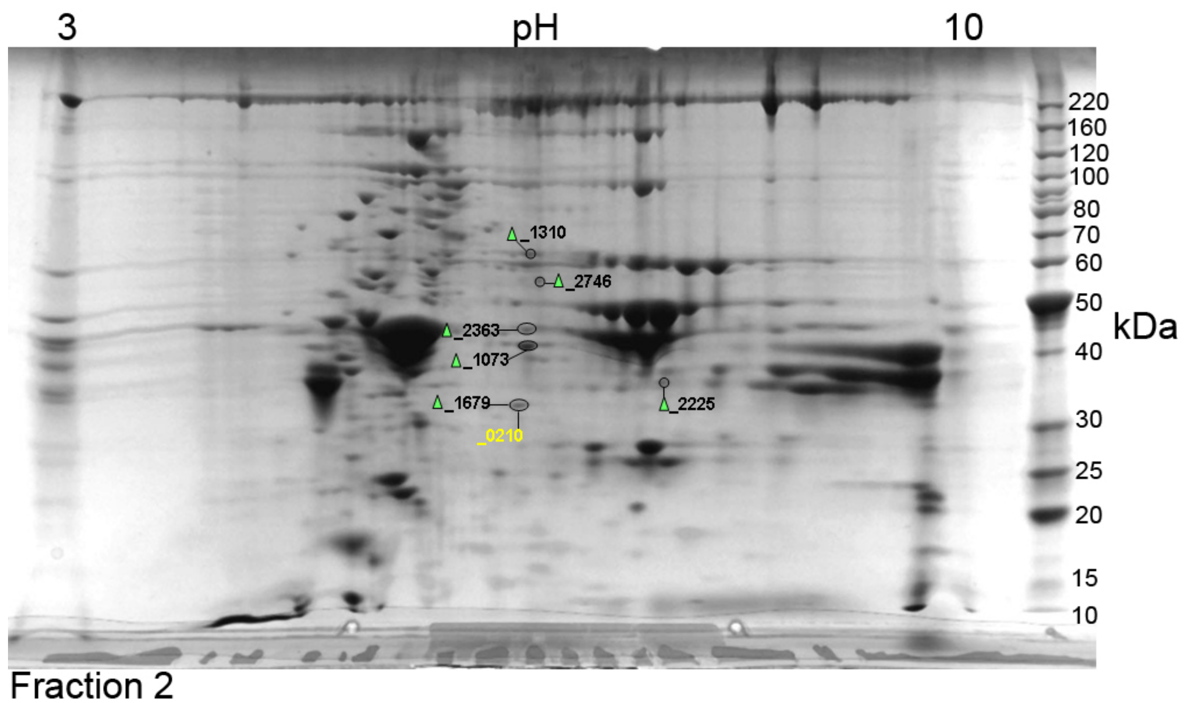

Figure 4.1 (continued)

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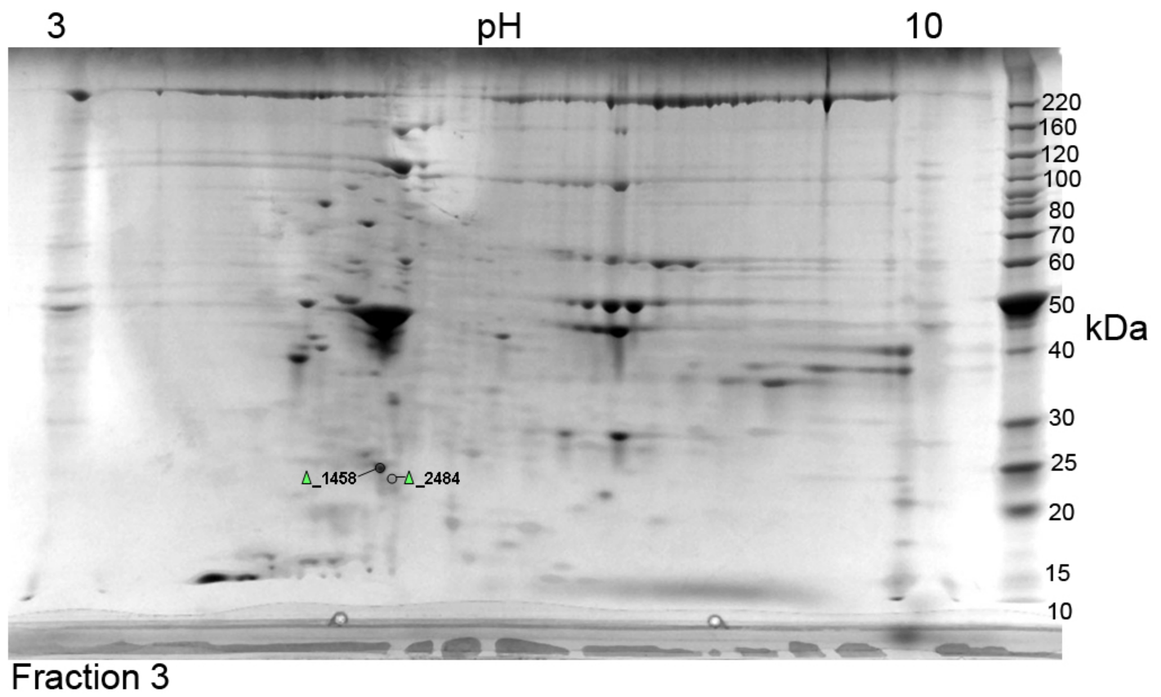

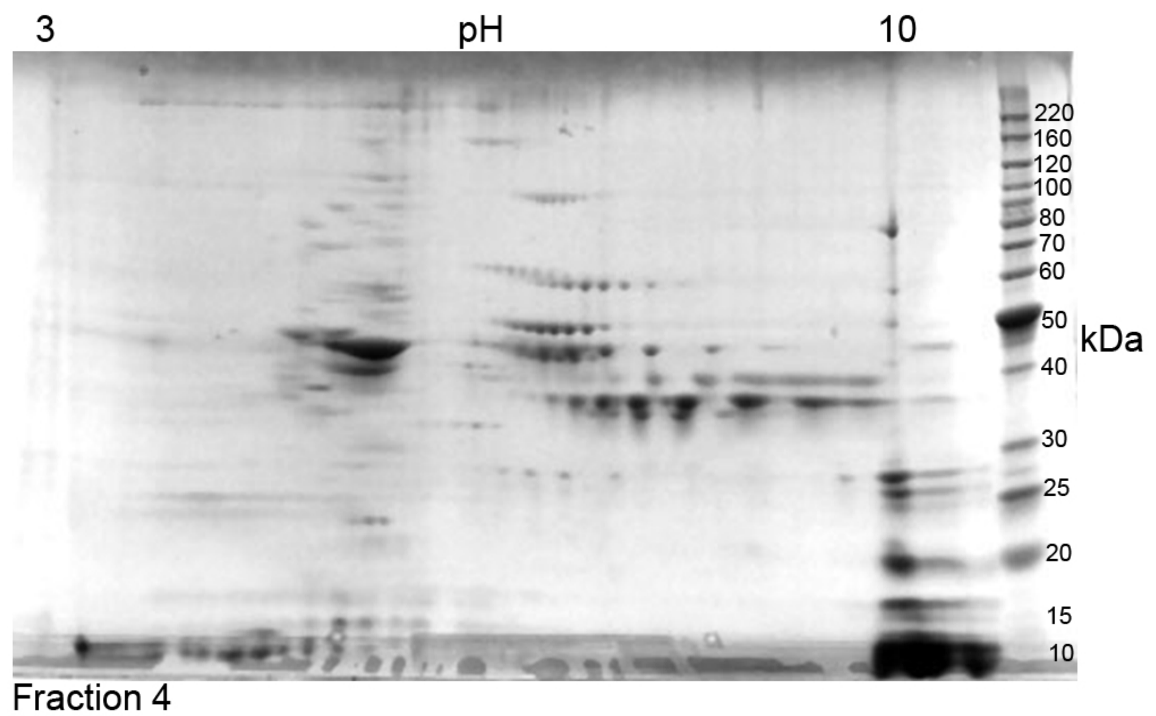

Figure 4.1 (continued)

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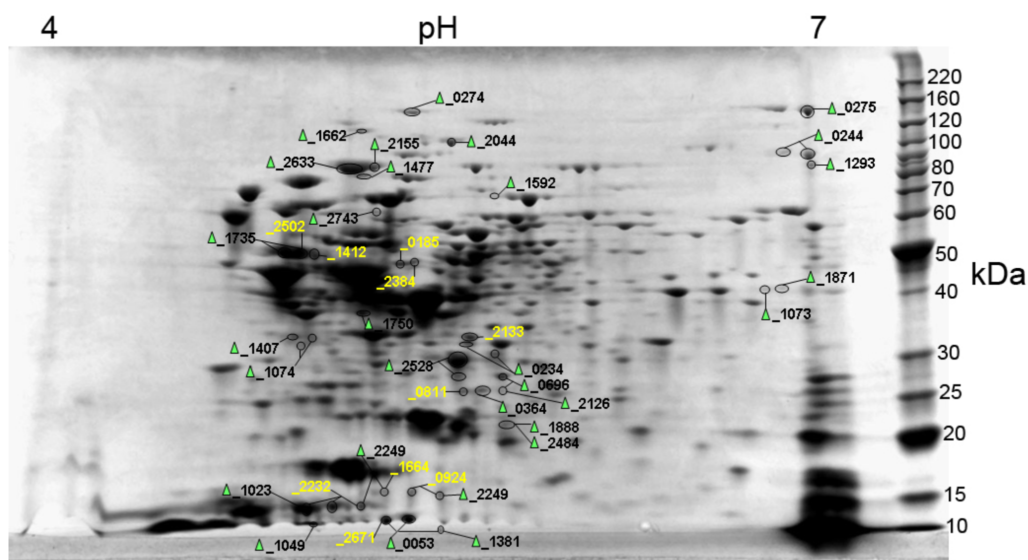

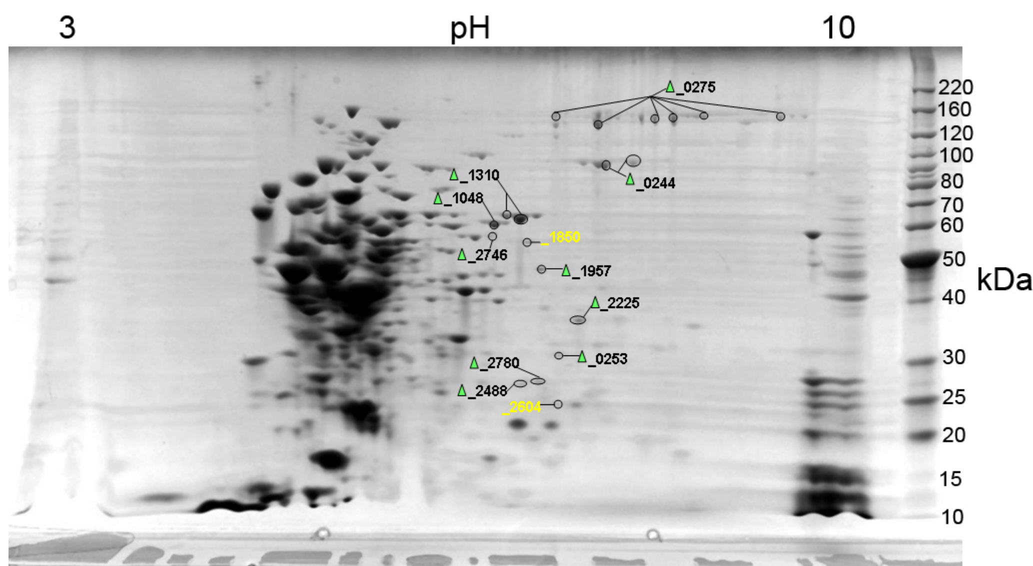

Figure 4.2 2-DE maps of proteins expressed by *L. monocytogenes* grown on Brain Heart Infusion agar at 15°C for 5 days.

Four protein fractions based on varying protein solubility were obtained using P-PEK, and each fraction was separated on pH 4-7 and pH 3-10 IPG strips. Protein spots of interest identified by MALDI-TOF/TOF-MS after 2-DE are indicated on the gels. No proteins were identified on Fraction 3 pH4-7 and Fraction 4 pH4-7 gels. Uniquely expressed proteins are indicated in yellow. Landmark proteins are indicated by a colored triangle (△). Marked unique spots are listed in Table 4.2 and landmark spots are listed in Table 4.3. (2-DE maps continue on next two pages)

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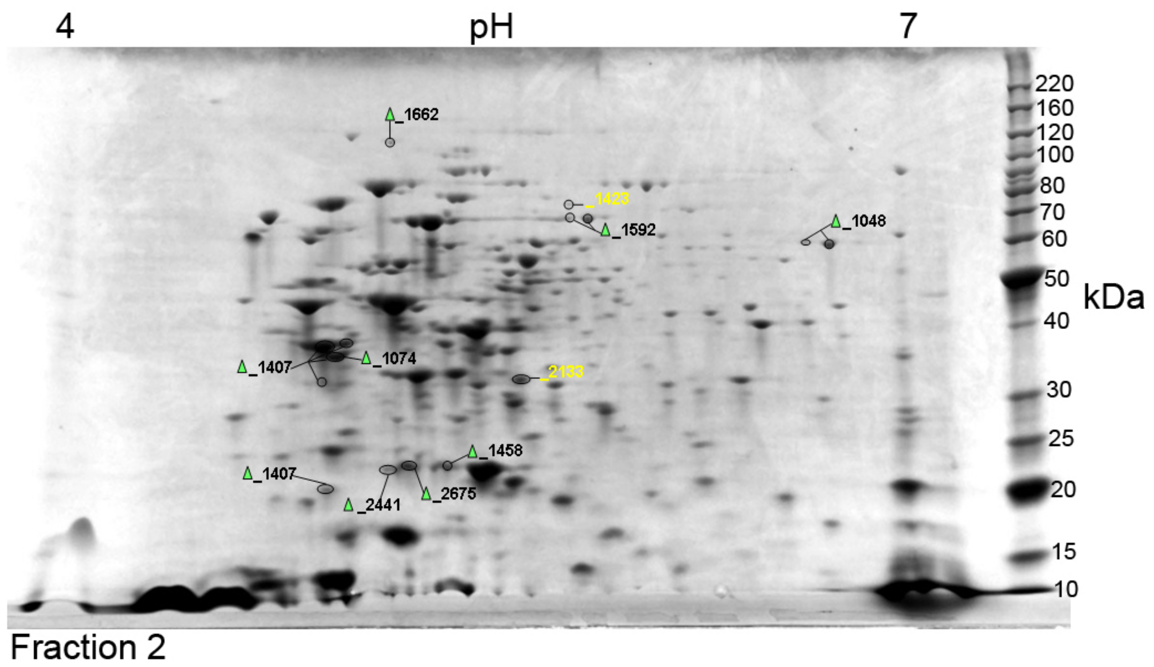

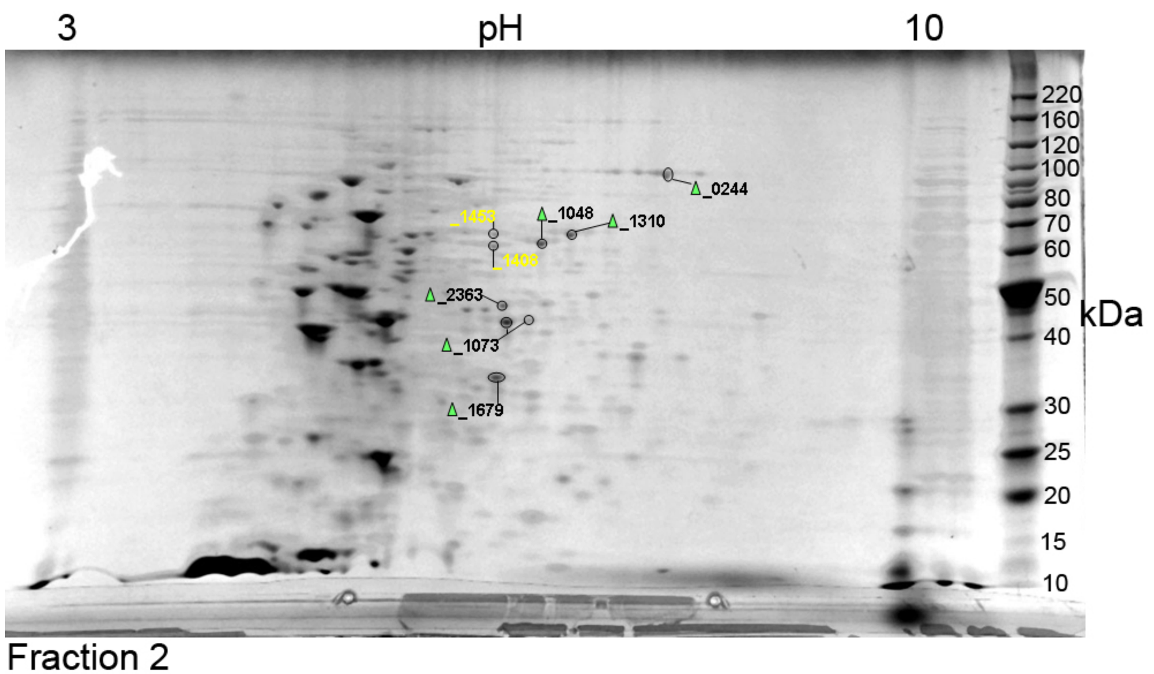

Figure 4.2 (continued)

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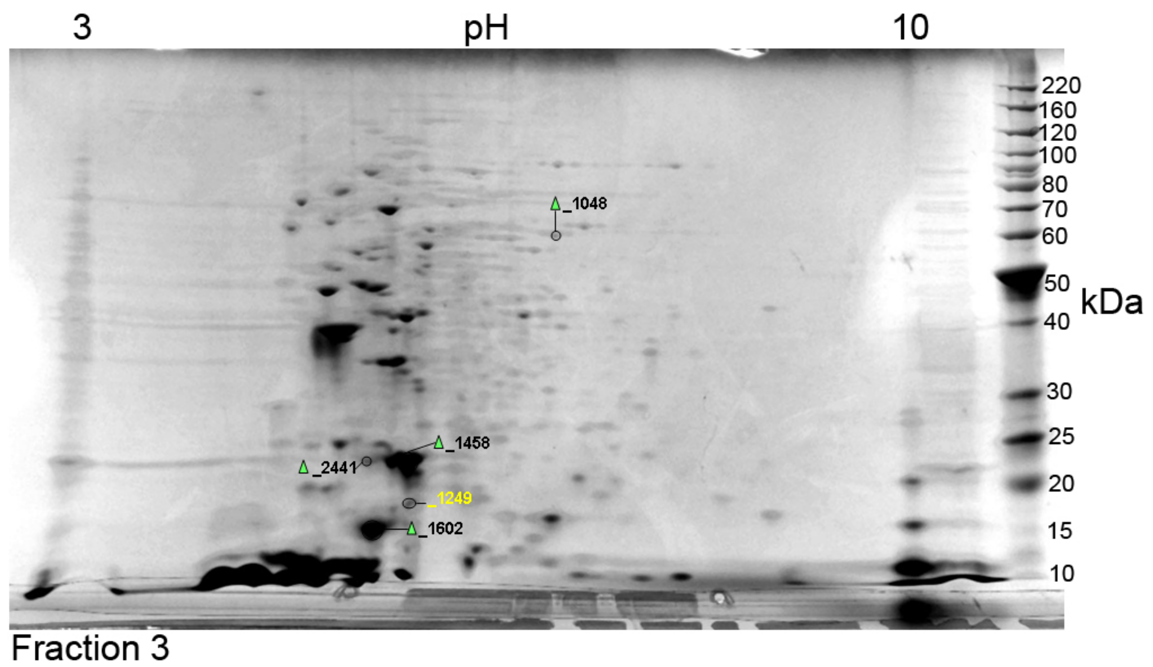

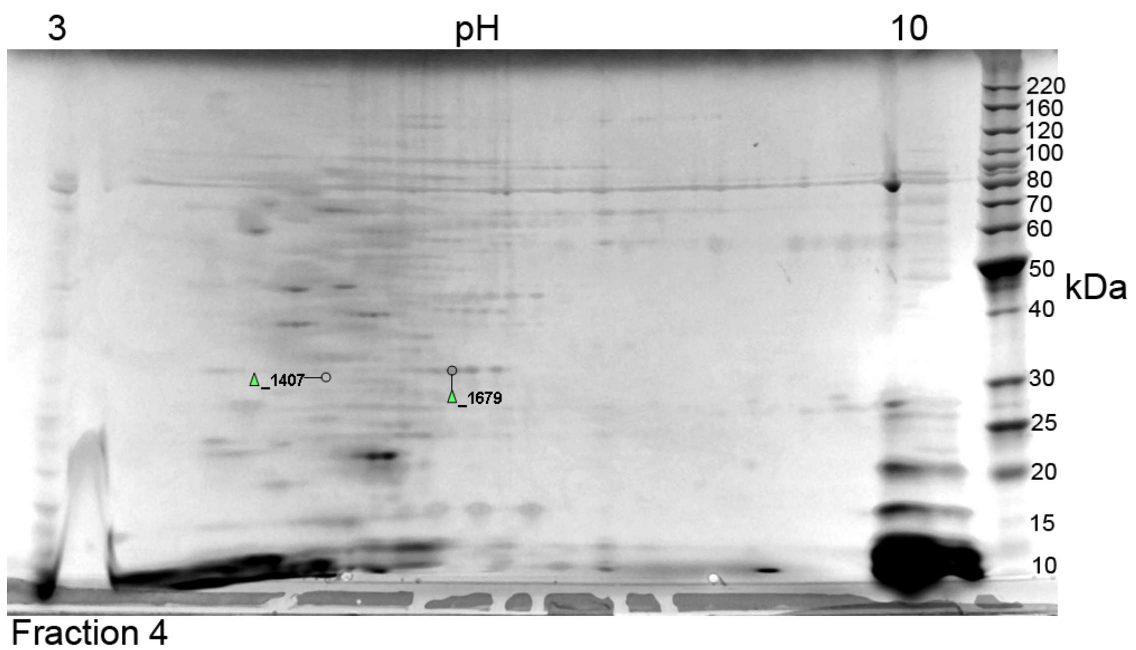

Figure 4.2 (continued)

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CHAPTER V

CONCLUSIONS

Proteins are the principle executors of most activities in a living cell. It is therefore essential to conduct proteomic analyses to gain insight into the complex biological processes of an organism under normal and abnormal conditions or to understand the difference in biological states and reactions between different groups of the same organism. Proteomic analyses of *Listeria monocytogenes* are underway to gain further insight into the physiology and pathogenesis of the bacterium. In the presented studies, two advances in *L. monocytogenes* proteomics were made. First, a method for improved 2-DE analysis of *Listeria monocytogenes* surface proteins was developed. The surface proteins of *L. monocytogenes*, and other gram-positive bacteria, play a vital role in bacterial virulence yet they cannot be easily compared by 2-DE, a central technique for proteomic analyses. The method described in this report will allow for improved analytical and comparative studies of surface proteins from *Listeria,* and possibly other Gram-positive bacteria, using 2-DE proteomic analysis. However, additional improvements in surface protein solubilization techniques for 2-DE are still required in order to isolate more defined proteomes. Second, protein expression by *L. monocytogenes* grown on a ready-to-eat (RTE) meat matrix was investigated for the first time. Various stress-related proteins and potential virulence factors were found to be expressed by RTE turkey meat-grown *L.monocytogenes*. The database of proteins

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obtained allows for greater understanding of the interactions of *Listeria* with its food environment that influence its ability cause disease and will aid future investigations on the subject.

Both the studies presented can be enhanced by using a shotgun proteomics approach combined with peptide labeling for relative protein quantitation. This would allow a comparison of individual surface protein quantity between different strains of *L. monocytogenes* or pathogenic and non-pathogenic *Listeria* species. Variation in individual protein quantity caused by growth of *L. monocytogenes* under different environmental conditions could also be determined using this combined approach.

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