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Characterization of novel virulence factors of *Listeria monocytogenes* and their roles in pathogenesis

By

Ting Zhang

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

Mississippi State, Mississippi

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Characterization of novel virulence factors of *Listeria monocytogenes* and their roles in pathogenesis

By

Ting Zhang

Approved:

Chinling Wang
Associate Professor
Basic Sciences
(Director of committee)

Keun Seok Seo
Assistant Research Professor
Basic Sciences
(Committee member)

Stephen B. Pruett
Professor
Basic Sciences
(Committee member)

Lesya M. Pinchuk
Associate Professor
Basic Sciences
(Committee member)

G. Todd Pharr
Associate Professor
Basic Sciences
(Committee member)

Larry Hanson
Professor
Basic Sciences
(Graduate Coordinator)

Kent H. Hoblet
Professor and Dean
College of Veterinary Medicine

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Name: Ting Zhang

Date of Degree: August 17, 2013

Institution: Mississippi State University

Major Field: Veterinary Medical Science

Major Professor: Chinling Wang

Title of Study: Characterization of novel virulence factors of *Listeria monocytogenes* and their roles in pathogenesis

Pages in Study: 99

Candidate for Degree of Doctorate of Philosophy

The pathogenicity of food-borne intracellular bacterium *Listeria monocytogenes* is greatly associated with its abilities to invade non-phagocytic cells, counteract the host innate immune system, resist bactericidal antibiotic-mediated killing, and breaking the physical barriers. In the last 30 years of research on *L. monocytogenes*, several virulence factors, such as Listeriolysin O (LLO), InlA, InlB, ActA, PI-PLC, and PC-PLC have already been characterized as important players that help this bacterium to achieve the key stage of infection. There are approximately 3,000 open reading frames in *Listeria's* genome; however, only few virulence factors are functionally characterized. Thus, it is important to identify new virulence factors and understand how new virulence factors in *Listeria* help this opportunistic pathogen to counteract the host innate immune system, resist antibiotic-mediated killing, colonize vital organs, and finally successfully develop life-threatening listeriosis.

In this study, in-frame deletion mutagenesis was used to generate the deletion mutants of novel listerial virulence factors and a series of biochemical, *in vitro* and *in vivo* experiments were conducted to characterize the roles of these virulence factors

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during the infection process. In the first part of this study, an AlkD-like protein (Adlp, LmoF2365_0220) was identified and the protein is associated with oxidant tolerance and aminoglycoside antibiotic resistance. In the second part of this study, a new internalin-like protein (LmoH7858_0369) was shown to be involved in invasion of Hep-G2 cells and organ colonization in mice. The third part of this study showed that listeriolysin O (LLO) mediates cytotoxicity on brain endothelial cells, suggesting that LLO may contribute to the invasion of the central nervous system by *L. monocytogenes*.

In summary, we identified and characterized two novel virulence factors, Adlp and LmoH7858_0369 that contributed to bacterial infection and revealed a new invasion mechanism of CNS cells that is mediated by LLO. Results from these studies provide a better understanding on the pathogenicity of *L. monocytogenes* and can be used as therapeutical targets or vaccine candidates

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DEDICATION

I dedicate this dedication to my parents Dehong Zhang and Fengyan He, who bring me to this world; and to my wife Yang Lu for her love and encouragement.

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LIST OF ABBREVIATIONS

Blood brain barrier: BBB

Central nervous system: CNS

Colony forming unit: CFU

Human brain microvascular endothelial cells: HBMECs

Interferon γ: INF-γ

Interleukin: IL

Leucine rich repeats: LRRs

Listeria pathogenicity island: LIPI

Listeriolysin O: LLO

Metallopreotease: MPL

Methyl methanesulfanate: MMS

Monocyte chemotactic protein: MCP

Phosphatidylcholine-specific Phospholipase C: PC-PLC

Phosphatidylinositol-Specific Phospholipase C: PI-PLC

Positive regulatory factor A: PrfA

Post infection: PI

Reactive oxygen species: ROS

Standard deviation: SD

Standard error of mean: SEM

Tumor necrosis factor α: TNF-α

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CHAPTER I

INTRODUCTION

A series of epidemic outbreaks of listeriosis in humans in North America that were associated with consumption of vegetables, fruits (2011) and dairy products (2012) raised the public concern about food contaminated with *Listeria monocytogenes*, which is one of the most important food-borne pathogens for the food industry. As an opportunistic pathogen, this bacterium affects neonates, pregnant women, elderly people and immune-compromised individuals through life threatening bacteremia and central nervous system infection. Virulence factors of *L. monocytogenes* enable the bacterium to establish a successful infection in hosts. Thus, it is important to unveil the molecular mechanisms of how virulence factors in *L. monocytogenes* contribute to counteract against host denfense mechanism, antibiotic resistance, organ colonization, and invasion of physical barriers.

In the past 30 years, the virulence determinants of *L. monocytogenes* have been widely studied. Despite several known invasion proteins and toxins that have been documented, a vast number of virulence factors that are associated with the key stages of infection remain largely unknown. In this study, we characterized the virulence factors and elucidated the molecular mechanisms of how these factors facilitated *L. monocytogenes* to counteract host innate immune response, resist animoglycoside antibiotics, invade non-phagocytic cells, and cross blood-brain barrier (BBB).

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The specific objectives of this study include:

1. Characterization of proteins in *L. monocytogenes* that are involved in counteracting the host innate immune responses and resistance to bactericidal antibiotic-mediated killing;
2. Characterization of listerial surface proteins that mediate the adhesion and invasion of non-phagocytic cells by *L. monocytogenes;*
3. Characterization of secreted proteins in *L. monocytogenes* that are involved in CNS infection.

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CHAPTER II

LITERATURE REVIEW

Pathophysiology of *Listeria monocytogenes* infection

Overview

Listeria monocytogenes is a gram-positive, intracellular bacterium found in many environmental niches and a variety of ready-to-eat food products (Welshimer and Donker-Voet 1971; Weis and Seeliger 1975; McLauchlin, Saunders et al. 1988; Farber and Peterkin 1991; Swaminathan and Gerner-Smidt 2007). As the pathogen responsible for "listeriosis," *L. monocytogenes* was first discovered in 1926 through the detection of the increased number of monocytes in the peripheral bloodstream of infected lab animals such as rabbits and guinea pigs (E. G. D. Murray 1926). In1929, *L. monocytogenes* was found to infect humans (Nyfelt 1929). Since 1980, outbreaks in Canada, Europe, and the United States, such as those in the United States associated with cantaloupe (2011) and cheese (2012), make *L. monocytogenes* an important, food-borne pathogen (Schlech, Lavigne et al. 1983; Swaminathan and Gerner-Smidt 2007; Cartwright, Jackson et al. 2012; Lomonaco, Verghese et al. 2013). Although the incidence of listeriosis is low, with approximately 4-7 cases per million people in United States (Gellin, Broome et al. 1991; Tappero, Schuchat et al. 1995), listeriosis is a life-threatening disease with a mortality rate of 20-30%, even when antibiotic treatment is administered early (Vazquez-Boland, Kuhn et al. 2001). As a representative intracellular bacterium, *L. monocytogenes* is one of

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the most extensively studied bacterial pathogens, making it an excellent model for the investigation of the innate and adaptive immune responses, as well as the molecular mechanisms that contribute to intracellular parasitism (Shen, Tato et al. 1998; Vazquez-Boland, Kuhn et al. 2001; Pamer 2004; Hamon, Bierne et al. 2006; Cossart 2011).

Clinical manifestation

L. monocytogenes causes gastroenteritis in healthy individuals (Aureli, Fiorucci et al. 2000; Ooi and Lorber 2005). However, *L. monocytogenes* severely affects immunocompromised individuals (patients with diabetes, chronic liver disease, patients receiving immunosuppressive therapy or chemotherapy, patients with AIDS, et. al) by causing sepsis and invasive central nervous system diseases, such as meningitis and brainstem encephalitis (Gellin, Broome et al. 1991; Lorber 1997; Wing and Gregory 2002). Pregnant women comprise another population that is extremely vulnerable to *Listeria* infection. Due to an impaired cell-mediated immune response during pregnancy (Weinberg 1984), the infection risk of pregnant women by *L. monocytogenes* is significantly increased compared to general population (Mylonakis, Paliou et al. 2002). Once bacteremia occurs in the maternal body, *L. monocytogenes* is able to disseminate through a route of fetalplacental interface (Bakardjiev, Stacy et al. 2005). Surprisingly, *L. monocytogenes* is able to traffic back from the fetus to the maternal body, and thus uses the placenta as a mean to maintain a persistent infection (Bakardjiev, Theriot et al. 2006). A *L. monocytogenes* infection is devastating to the fetus (Mylonakis, Paliou et al. 2002), causing a clinical syndrome known as granulomatosis infantiseptica (Klatt, Pavlova et al. 1986). In neonatal infections, the most frequent manifestation is bacterial meningitis (Schuchat, Swaminathan et al. 1991; Lorber 1997).

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Pathogenesis of infection

Among the 13 serotypes of *L. monocytogenes*, serotypes 1/2a, 1/2b, and 4b cause most cases of infection, and serotype 4b accounts for 50% of infections worldwide (Farber and Peterkin 1991). Consumption of contaminated food is believed to be the major route for *Listeria* infection (Farber and Peterkin 1991). Experiments using non-human primates and mice infected by oral inoculation indicate that a very high dose is required: around 10^9 bacteria for oral ingestion (Audurier, Pardon et al. 1980; Farber, Daley et al. 1991).

The gastrointestinal tract is believed to be the primary site by which *L. monocytogenes* gains entry into the host. The exact molecular mechanisms for crossing the intestinal barrier and the precise anatomical invasion site are still under debate, and two models are proposed. Early studies using mice and rats indicate that translocation through phagocytic M cells on Peyer's patches could be the preferred route for *L. monocytogenes* infection (Marco, Prats et al. 1992; Marco, Altimira et al. 1997; Jensen, Harty et al. 1998; Pron, Boumaila et al. 1998; Lecuit, Vandormael-Pournin et al. 2001). Data from a rat ligated ileal loop model suggest that crossing the intestinal barrier is a passive process because the translocation rate is independent of virulence factors such as InlA, InlB, hemolysin, and ActA (Pron, Boumaila et al. 1998). Alternatively, an early study using guinea pigs as an animal model showed that *L. monocytogenes* preferentially localized to the apical membrane of the intestinal villi (Racz, Tenner et al. 1972). Additional supports for this second hypothesis come from recent studies using polarized MDCK cells and rabbit ileal loops, showing that *L. monocytogenes* prefers to target the cell extrusion zone at the tips of intestinal villi, which are defined as multicellular

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junctions (MCJ) (Pentecost, Otto et al. 2006; Pentecost, Kumaran et al. 2010). Recent work in mice confirms the process of MCJ formation, which involves a redistribution of apical junction complex proteins (Marchiando, Shen et al. 2011). In addition, *L. monocytogenes* is able to efficiently cross the intestinal barrier and reach lamina propria during cell extrusion by preferentially targeting goblet cells and other neighboring cells that secrete mucus (Nikitas, Deschamps et al. 2011).

After crossing the intestinal barrier, *L. monocytogenes* disseminates via the lymph or blood (Marco, Prats et al. 1992). Once in the bloodstream, most bacterial cells quickly localize to liver and spleen. In a mouse model using an intravenous inoculation route, 90% of the bacteria are engulfed by hepatic Kupffer cells in the liver within 10 minutes (Mackaness 1962). The remaining 10% are endocytosed by marginal zone macrophages and dendritic cells in the white pulp of spleen (Conlan 1996). Inside the Kuffer cell, 90% of the engulfed bacteria are killed, but around 10% of the bacteria can escape from the phagosome and move into the cytosol (de Chastellier and Berche 1994). The surviving *Listeria* infect the liver parenchyma by either cell-to-cell spread or direct invasion of hepatocytes (Dramsi, Biswas et al. 1995; Drevets 1999). Within the liver parenchyma, *L. monocytogenes* replicates, leading to a bacterial burden which peaks in 2-3 days before cells are destroyed by the adaptive immune response (Pamer 2004). Infection of Kupffer cells and replication in hepatocytes by *L. monocytogenes* will trigger the innate immune system, and later adaptive immunity (Gregory and Wing 1990; Rogers, Callery et al. 1996), which controls the infection and eliminates the bacteria from the organs. However, in immunocompromised individuals the infection may not be controlled by adaptive immune responses. Thus, unlimited replication of bacteria occurs in hepatocytes, leading

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to bacteremia and a possible central nervous system (CNS) infection (See details in chapter 4).

Molecular virulence determinants involving in invasion

Listerial surface proteins

Besides being passively engulfed by phagocytic cells, *L. monocytogenes* can invade a wide range of non-phagocytic mammalian cells, including epithelial cells (Gaillard, Berche et al. 1987), endothelial cells (Drevets, Sawyer et al. 1995; Greiffenberg, Goebel et al. 2000), hepatocytes (Dramsi, Biswas et al. 1995), and fibroblasts (Kuhn, Kathariou et al. 1988). Bacterial surface proteins are required for adherence and entry of *L. monocytogenes* to non-phagocytic cells. In *L. monocytogenes*, surface proteins are anchored to bacterial cell wall in multiple ways. First, they may be covalently linked to the peptidoglycan (PG). Alternatively, surface proteins may be non-covalently attached to lipoteichoic acid or PG by glycine tryptophan (GW), lysM, and WxL domains (Bierne and Cossart 2007). In the case of ActA, anchoring of the protein to cell membrane is accomplished by inserting its hydrophobic segment on C-terminal region to the cell membrane, while other lipoproteins use *N*-terminal covalent lipidation with a conserved “lipobox” (Kocks, Gouin et al. 1992; Sutcliffe and Harrington 2002). Deletion of a peptidase involved in lipoprotein maturation significantly decreases the virulence of *L. monocytogenes* (Reglier-Poupet, Frehel et al. 2003). As substrates of sortase A, one large set of surface proteins are linked covalently to PG via a “LPXTG” motif and a positively charged tail known as a sorting signal (Ton-That, Marraffini et al. 2004).

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The internalin family

Internalins, a large group of bacterial proteins found in *Listeria*, are characterized by the presence of leucine-rich repeat (LRR) domains (Marino, Braun et al. 2000). The LRR domains are generally 20-29 residues in length and have a conserved consensus sequence (Kobe and Kajava 2001). These motifs are involved in protein-protein interactions that associate internalins with immunomodulatory proteins of plants and innate immune response proteins in mammals (Kobe and Kajava 2001). Besides *Listeria*, LRR-containing proteins are also found in other bacterial pathogens, such as YopM in *Yersina pestis* (Boland, Havaux et al. 1998) and IpaH in *Shigella flexneri* (Hartman, Venkatesan et al. 1990). The *L. monocytogenes* genome contains a large number of internalin genes. For instance, *L. monocytogenes* 1/2a serotype EGD-e has 25 internalins, and serotype 4b F2365 and H7858 have 25 and 26 internalins, respectively (Bierne, Sabet et al. 2007). The number of internalins in *L. monocytogenes* is the highest among all the complete genome sequence data available for gram-positive bacteria (Bierne, Sabet et al. 2007).

Two proteins in the internalin family, InlA and InlB, have been extensively studied *in vitro.* In1A and In1B have roles in penetrating polarized epithelial cells (Gaillard, Berche et al. 1991) and several non-phagocytic cells (endothelial cells, hepatocytes, and fibroblasts), respectively (Kuhn, Kathariou et al. 1988; Dramsi, Biswas et al. 1995; Greiffenberg, Goebel et al. 1998; Parida, Domann et al. 1998). InlA and InlB are recognized as the only two molecules among the invasion proteins that are capable of utilizing cellular machinery to perform receptor mediated endocytosis (Pizarro-Cerda and Cossart 2006). InlA has a LLR domain and an inter-repeat domain on its *N*-terminus,

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both of which are important for epithelial cell invasion (Lecuit, Ohayon et al. 1997). Crystal structure data indicate that the central region of the LRR motif of InlA is able to bind to the *N*-terminus of the human E-Cadherin receptor (Schubert, Urbanke et al. 2002). This ligand-receptor interaction triggers signal transduction and cytoskeleton rearrangements, resulting in uptake of the bacterium into epithelial cells (Hamon, Bierne et al. 2006). On the other hand, In1B has a more complicated interaction with its receptors. Both the LRR region and the GW domain on InlB are recruited to interact with its main signaling receptor, hepatocyte growth factor receptor (c-Met) (Shen, Naujokas et al. 2000; Niemann, Jager et al. 2007). Two other proteins, the receptor for complement component C1q (gC1qR) (Braun, Ghebrehiwet et al. 2000) and glycosaminoglycans (Jonquieres, Pizarro-Cerda et al. 2001), are considered as co-receptors of InlB and are functional during invasion.

Due to the receptor specificity in different animal species, the role of InlA in intestinal barrier penetration cannot be properly investigated in normal mice using an oral inoculation route (Lecuit 2007). Instead, in an *in vivo* model using human E-cadherin knock-in mice, InlA was shown to be involved in crossing the intestinal barrier (Lecuit, Vandormael-Pournin et al. 2001). Because E-cadherin is relatively concealed in a buried lateral location, it was unclear how In1A could gain access; however, several recent publications reported that a cell extrusion event in the intestinal epithelium, along with mucus secretion from goblet cells, allows E-cadherin exposure to In1A (Pentecost, Otto et al. 2006; Nikitas, Deschamps et al. 2011). Since E-cadherin is present in the placenta, InlA/E-cadherin interaction is also necessary for fetal-placental barrier infection (Lecuit, Nelson et al. 2004). *In vivo* experiments suggest that InlB is involved in bacterial

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replication in the liver and spleen (Khelef, Lecuit et al. 2006), and a recent study reported a cooperative role of InlA and InlB in crossing the fetal-placental barrier (Disson, Grayo et al. 2008).

Besides InlA and InlB, no internalin molecules in *L. monocytogenes* have been clearly documented for activating host cellular machinery to achieve bacterial entry. However, the deletion of InlH or InlJ impairs virulence in mice (Schubert, Gobel et al. 2001; Sabet, Lecuit et al. 2005). InlJ is only expressed in the late stage of infection and its expression cannot be detected *in vitro* (Sabet, Toledo-Arana et al. 2008). InlC, the only soluble internalin protein (Engelbrecht, Chun et al. 1996; Domann, Zechel et al. 1997), may play a role with InlA in invading non-phagocytic cells (Bergmann, Raffelsbauer et al. 2002). In1C is able to interact with IκB kinase in the host cell to interfere with the innate immune response (Gouin, Adib-Conquy et al. 2010). Lmo2026, an internalin-like molecule, is thought to be involved in *Listeria* crossing the blood-brain barrier (BBB) (Autret, Dubail et al. 2001). Interestingly, a recent study reports that InlK is able to interact with host cell ribonucleoprotein MVP to avoid autophagy (Dortet, Mostowy et al. 2011).

<u>Other virulence factors involved in invasion</u>

Among those "LPXTG" proteins without a LRR domain, only one protein, Vip, has been implicated in invasion of Caco-2 cells and mouse fibroblast L2071 cells (Cabanes, Sousa et al. 2005). Gp96, an ER resident chaperone, is the cellular receptor for Vip (Cabanes, Sousa et al. 2005). Among non-covalently bound proteins, the members of the autolysin family of proteins (Ami, Auto, p60) play important roles in adhesion and/ or invasion of *L. monocytogenes* to non-phagocytic cells (Kuhn and Goebel 1989; Cabanes,

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Dussurget et al. 2004; Milohanic, Jonquieres et al. 2004). Interestingly, listerolysin O (LLO, see Chapter 4), one of most extensively studied toxins, is involved in *L. monocytogenes* invasion of human hepatocytes via the influx of extracellular Ca^{2+} (Dramsi and Cossart 2003). A more recent report indicates that LLO is able to facilitate the entry of *L. monocytogenes* to HepG-2 cells through activation of the endocytic cell machinery (Vadia, Arnett et al. 2011).

<u>Lectin-carbohydrate interactions involved in *L. monocytogenes* invasion</u>

Lectin receptors and their carbohydrate ligands play a critical role in pathogen molecular pattern recognition and signaling, which initiate either phagocytosis or the cytokine response (Geijtenbeek and Gringhuis 2009). Interestingly, this response may also play a role in helping *L. monocytogenes* adhere or enter non-phagocytic host cells. For example, alpha-D-galactose from a virulent strain of *L. monocytogenes* was proposed to interact with its lectin receptor (alpha-D-galactose receptor) on HepG-2 cells, promoting adhesion and invasion (Cowart, Lashmet et al. 1990). This observation was confirmed by a second study showing that inactivation of GtcA, a protein involves in the glycosylation of teichoic acids (TA), significantly decreased the adherence and invasion of *L. monocytogenes* to HepG-2 cells (Autret, Dubail et al. 2001). Similarly, a lectin-carbohydrate interaction facilitates the invasion of Caco-2 cells (Facinelli, Giovanetti et al. 1998). Interestingly, infection of murine dendritic cells (DCs) by *L. monocytogenes* is associated with a glycosylated receptor and can be blocked with galactose, suggesting that a lectin-carbohydrate interaction is involved during infection process (Guzman, Rohde et al. 1995). Additionally, on the surface of *L. monocytogenes*, lectin-like components are present. These components are able to bind to D-glucosamine, L-

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fucosylamine, or para-amino-phenyl-alpha-D-mannopyrannoside residues on neoglycoproteins of host cells (Cottin, Loiseau et al. 1990).

Host innate immnue responses to *L. monocytogenes* infection

In the early stage of *L. monocytogenes* infection, innate immunity plays a critical role in controlling the bacterial burden in a non-specific manner. Innate immunity also controls the time taken by the adaptive immune system to clear the infection. Once in the bloodstream, most *L. monocytogenes* are quickly taken by resident macrophages in liver and spleen (Mackaness 1962; Conlan 1996). In severe combined immunodeficient mouse, macrophages and splenocytes stimulated with *L. monocytogenes* produce IL-12 and tumor necrosis factor-α (TNF-α) (Tripp, Wolf et al. 1993). Although not required for developing adaptive immunity, TNF-α contributes to the early resistance of a *L. monocytogenes* infection by enhancing bactericidal function of the macrophage (Endres, Luz et al. 1997). TNF-α, together with IL-12, synergistically activates nature killer (NK) cells to secrete interferon-gamma (IFN-γ) (Tripp, Wolf et al. 1993), which is another cytokine important to initial resistance to *L. monocytogenes* infection. IFN-γ knockout mice are severely impaired in their resistance to *L. monocytogenes* infection (Harty and Bevan 1995). Similar results were observed using other approaches: depletion of IFN-γ by monoclonal antibody and inactivation of IFN-γ receptor (Buchmeier and Schreiber 1985; Huang, Hendriks et al. 1993). Early elimination of NK cells, which are major sources for IFN-γ production, also impairs the clearance of *L. monocytogenes* infection (Dunn and North 1991).

In vitro and *in vivo* experiments show that IL-6, a neutrophil chemoattractant, is induced after inoculation with *L. monocytogenes* (Nakane, Numata et al. 1992; Gregory,

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Wing et al. 1998). Depletion of Kupffer cells in mice decreases IL-6 levels, indicating that Kupffer cells are the major source for IL-6 production in liver (Gregory, Wing et al. 1998). Loss of IL-6 results in an inability to form neutrophilia in peripheral blood; IL-6 deficient mice are extremely susceptible to *L. monocytogenes* infection (Dalrymple, Lucian et al. 1995). Histopathology analysis showed that neutrophils were quickly recruited to the infection sites in the liver and spleen (Mandel and Cheers 1980). Depletion of neutrophils by specific monoclonal antibodies significantly impairs the resistance of mice to *L. monocytogenes* infection (Rogers and Unanue 1993; Czuprynski, Brown et al. 1994). Regarding bacterial burden, neutrophils play an important role in controlling the growth of bacteria in both the spleen and liver (Rogers and Unanue 1993; Czuprynski, Brown et al. 1994), although neutrophils are more effective at controlling bacterial numbers in the liver (Conlan and North 1994). Interestingly, *L. monocytogenes* ΔInlH mutant strain enhances the production of IL-6 in infected tissue, indicating the role of InlH in balancing IL6 expression and thus evading host innate immune responses (Personnic, Bruck et al. 2010).

In addition to macrophages, neutrophils, and NK cells, monocytes are also recruited early during infection. Monocyte chemotactic protein-1 (MCP-1), a major chemoattractant for recruiting monocytes, is induced in the spleen in the early stage (<12 hours) of *L. monocytogenes* infection (Serbina, Kuziel et al. 2003). Mice lacking the CCR2 chemokine receptor, which responds to MCP-1, have a weak resistance to *L. monocytogenes* infection (Kurihara, Warr et al. 1997). One remarkable finding in both the *Listeria* and immunology fields is the discovery of TNF/iNOS-producing Tip-DCs (Serbina, Salazar-Mather et al. 2003). Interestingly, a recent study indicated that traffic of

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monocytes to hepatic infection sites was independent of chemokines, but directly dependent on intracellular adhesion molecule-1 (ICAM-1) expression in hepatocytes (Shi, Velazquez et al. 2010). Although not functional in T-cell priming, Tip-DCs are a major source for TNF and iNOS during *L. monocytogenes* infection and play an important role in controlling the bacterial burden (Serbina, Salazar-Mather et al. 2003). Activation of NK cells is beneficial for infection control, and interestingly, *L. monocytogenes* infected DCs, as well as IL-18, are required to activate NK cells (Humann and Lenz 2010).

Despite the fact that exact mechanisms of how recruited granulocytes and local macrophages kill bacteria are not clearly elucidated, production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) are believed to be paramount contributors for bacteria clearance (Shiloh, MacMicking et al. 1999). Activated macrophages, together with Tip-DCs and neutrophils, produce heavy doses of ROS and RNS, including hydrogen peroxide (H_2O_2) and nitric oxide (NO).

Immune evasion and antioxidant responses of listeria

Innate immune evasion

L. monocytogenes is able to escape from the vacuole with the help of LLO (Hamon, Ribet et al. 2012). Once in the cytosol, *L. monocytogenes* immediately faces autophagy, a host cytosolic innate immune surveillance system that is able to destroy bacteria through a double membrane autophagosome (Amano, Nakagawa et al. 2006). *L. monocytogenes* has developed sophisticated mechanisms to avoid being cleared by autophagy. By employing ActA as well as bacterial phospholipases, *L. monocytogenes* is able to evade autophagy efficiently (Birmingham, Canadien et al. 2007). A recent study

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indicates that InlK is able to recruit cellular protein MVP to encounter the host autophagy system (Dortet, Mostowy et al. 2011). As the only soluble internalin, InlC is able to interact with IκB kinase in the host cell and interfere with the innate immune response (Gouin, Adib-Conquy et al. 2010). Activation of macrophages by IFN-γ significantly limited phagosomal escape of *L. monocytogenes* (Portnoy, Schreiber et al. 1989; Shaughnessy and Swanson 2007), through a way of IFN-γ inducible renitence on phagosomal membrane (Davis, Gregorka et al. 2012). However, on the other side, *L. monocytogenes* ultilize host GILT, a γ-interferon-inducible thiol reductase on phagolysosome to activate the LLO and facilitate its phagosomal escape (Singh, Jamieson et al. 2008).

As an intracellular pathogen, evading the innate immune surveillance is critical for *L. monocytogenes* to survive in the host. To evade detection, *L. monocytogenes* successfully utilizes two enzymes: PgdA, an *N*-deacetylase of *N*-acetylglucosamine, and OatA, an *O*-acetyltransferase that is able to perform acetylation on muramic acid to modify PG (Boneca, Dussurget et al. 2007; Aubry, Goulard et al. 2011). Both *pgdA*-null and *oatA*-null mutants demonstrate significant attenuation phenotypes *in vivo* due to their impaired ability to limit innate immune responses (Boneca, Dussurget et al. 2007; Aubry, Goulard et al. 2011).

Antioxidant responses

To maintain genome integrity during infection, *L. monocytogenes* has developed several strategies to prevent damage from the oxidative burst. One representative method used by *L. monocytogenes* is to escape the vacuole using LLO (Portnoy, Jacks et al. 1988). A recent study indicated that LLO is able to suppress ROS production by

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macrophages (Lam, Fattouh et al. 2011), which allows the bacteria to escape from the phagosome and replicate in the cytosol. Additionally, *L. monocytogenes* is capable of counteracting host bactericidal activities through the antioxidant effect of bacterial superoxide dismutase (Archambaud, Nahori et al. 2006). Listeria is also thought to recover from DNA damage caused by ROS, since expression of SOS activator *recA* was induced when inside the macrophages (Chatterjee, Hossain et al. 2006).

The base excision repair (BER) pathway is a conserved multi-step pathway that plays a critical role in oxidized base lesion repair, initiated by recognition and removal of damaged bases by bi-functional glycosylases (Friedberg, Aguilera et al. 2006). In *E. coli*, oxidized pyrimidines are removed by DNA glycosylase EndoIII (Nth) and oxidized purines are fixed by MutM (Fpg) (Krokan, Standal et al. 1997). A recent report on the pathogen *Neisseria meningitidis* reveals a network of glycosylases for oxidative DNA damage repair (Nagorska, Silhan et al. 2012). Contribution of BER pathway on macrophage oxidative burst resistance is also found in other pathogens, such as *Mycobacterium smegmatis* and *Salmonella typhimurium* (Suvarnapunya, Lagasse et al. 2003; Kurthkoti, Kumar et al. 2008; Richardson, Soliven et al. 2009). Such oxidative repair systems have not been reported in *L. monocytogenes* yet.

Central nervous system infection

Pathophysiology of CNS infection by *L. monocytogenes*

Accompanied by life-threatening clinical manifestations such as meningitis, rhombencephalitis, and brain abscess, *L. monocytogenes* demonstrates a high ability to penetrate the CNS (Vazquez-Boland, Kuhn et al. 2001; Disson and Lecuit 2012). The route that *L. monocytogenes* exploits to cross the BBB is still under debate (Drevets,

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Leenen et al. 2004). Direct invasion through brain endothelial cells, trafficking with parasitized macrophages, and migration through cranial nerves are all considered as possible ways for *L. monocytogenes* to cause a CNS infection (Drevets, Sawyer et al. 1995; Antal, Loberg et al. 2001; Drevets, Leenen et al. 2004). Unlike other bacterial pathogens that prefer to cross the BBB through the choroid plexus via the blood and cerebrospinal fluid (CSF) (Quagliarello and Scheld 1992; Tunkel and Scheld 1993), *L. monocytogenes* preferentially chooses the hematogenous route (Cordy and Osebold 1959) and has a preference for the microvascular endothelium (Kirk 1993). Thus, the high rate (up to 24%) of rhombencephalitis observed in patients may be because *L. monocytogenes* is likely to target the rhombencephalon due to its richly distributed microvessels (Oevermann, Zurbriggen et al. 2010).

For an effective CNS infection, constant bacteremia is usually required. Data from a mouse experiment using intravascular infection routes indicates that no CNS infection is detectable with a low dose of bacteria, although a high bacterial burden was detected in liver and spleen and transient bacteremia occurred (Berche 1995). In contrast, when bacteremia was persistent, brain infection was clearly observed (Berche 1995). This observation is further confirmed by an experiment showing that a low-dose, mid-ear infection can successfully cause CNS infection in gerbils (Blanot, Joly et al. 1997). In addition, this conclusion is supported by the result that intracarotid inoculation in sheep can successfully cause CNS infection because this inoculation route avoids bacterial filtration by the liver and spleen (Akiyama, Asahi et al. 1957).

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Virulence factors involving in CNS infection

Virulence factors for direct invasion

InlA and InlB play critical roles in penetrating the intestinal (Lecuit, Vandormael-Pournin et al. 2001; Pentecost, Kumaran et al. 2010; Nikitas, Deschamps et al. 2011) and fetal-placental barriers (Lecuit, Nelson et al. 2004; Disson, Grayo et al. 2008), through either an interdependent or a conjugated way by interacting with their receptors E-cadherin and Met. These two receptors are found in both human brain macrovascular endothelial cells (HBMECs) (Rubin, Hall et al. 1991; Coyne, Kim et al. 2007), the key component of the BBB; and the basolateral pole of choroid plexus epithelial cells (Szmydynger-Chodobska, Pascale et al. 2007), the key for the blood and CSF barrier. Thus, InlA and InlB may also play a role in CNS infection by interacting with these receptors *in vivo*. The direct invasion of HBMECs by *L. monocytogenes* is InlB-dependent, as shown in several *in vitro* studies (Greiffenberg, Goebel et al. 1998; Greiffenberg, Goebel et al. 2000). Actin-based motility of *L. monocytogenes* is also observed in the cytosol of HBMECs (Greiffenberg, Goebel et al. 1998). A study using signature-tagged mutagenesis and a mouse intravascular infection model indicated that disruption of bacterial surface proteins GtcA, YtgP, and an internalin-like molecule Lmo2026 resulted in a decreased bacterial load in the brain (Autret, Dubail et al. 2001), although the bacterial number was decreased in the liver and spleen as well. Vip, a virulence factor that is associated with invasion of several organs, can interact with Gp96 (Cabanes, Sousa et al. 2005), which is abundant on the surface of brain microvessels (Liu, Mittal et al. 2011).

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Toxins involving in CNS infection

Besides direct invasion through cells or phagocyte trafficking, bacteria are also able to cross the BBB via disruption of tight junctions or by increasing the permeability of endothelial layers via bacterial toxins (Kim 2008). In *L. monocytogenes*, pore-forming toxin LLO, phospholipases Pl-PLC (PlcA) and PC-PLC (PlcB), and metalloprotease (Mpl) are responsible for a series of lytic events on the primary and secondary vacuoles, resulting in phagosome escape and cell-to-cell spread (Portnoy, Jacks et al. 1988; Smith, Marquis et al. 1995; Yeung, Zagorski et al. 2005). PLCs are well-known to break cell membranes via hydrolysis of phosopholipids and activation of signal transduction pathways (Goldfine, Johnston et al. 1993; Goebel and Kuhn 2000). Interestingly, a mouse experiment indicated that PlcB plays a very important role in CNS listeriosis (Schluter, Domann et al. 1998). PlcB mutants have a significantly decreased bacterial load in the brain compared to the parental wild type and InlAB deletion strains (Schluter, Domann et al. 1998). Limited dissemination of the mutant strain, as shown by histopathology data, may account for the attenuated virulence (Schluter, Domann et al. 1998).

LLO is known as the major toxin in *L. monocytogenes* and has a wide range of functions (Hamon, Ribet et al. 2012). LLO has also been reported for its ability to disrupt the function of the intestinal epithelial barrier by inducing chloride secretion (Richter, Gitter et al. 2009). LLO also helps *L. monocytogenes* to enter hepatocytes by forming extensions on the host cell membrane to capture bacteria (Vadia, Arnett et al. 2011). In the case of a CNS infection, the roles of LLO, which include disrupting the function of the BBB and facilitating penetration of brain endothelial cells, have not been confirmed. LLO is able to activate the NF-κB pathway in endothelial cells (Kayal, Lilienbaum et al.

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1999; Kayal, Lilienbaum et al. 2002), which induces expression of the adhesion molecules P-selectin, E-selectin, ICAM-1, and vascular cell adhesion molecule-1 (Krull, Nost et al. 1997; Wilson and Drevets 1998), as well as chemokines, such as IL-8 and MCP-1 secretion (Kayal, Lilienbaum et al. 1999). These events facilitate the adhesion of monocytes to endothelial cells and bypassing of the BBB, which may help *L. monocytogenes* spread to the brain. An *in vivo* experiment confirmed that macrophages infected with *L. monocytogenes* can successfully cause a brain infection (Drevets 1999; Drevets, Jelinek et al. 2001). Thus, it is possible that *L. monocytogenes* can use monocytes as a vector to spread throughout the host.

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CHAPTER III

AN ALKD-LIKE PROTEIN IN *L. MONOCYTOGENES* IS INVOLVED IN OXIDATIVE-STRESS TOLERANCE AND AMINOGLYCOSIDE RESISTANCE

Abstract

Counteracting the damages caused by exogenous and endogenous agents, such as alkylating compounds and reactive oxygen species (ROS) that are generated by host innate immune cells or antibiotics are essential for bacterial pathogens to survive. In this study, we identified an AlkD-like Protein (Adlp) in *L. monocytogenes* that is involved in resistance to oxidative stress, aminoglycoside antibiotics, and DNA alkylating compounds. By comparing to a F2365 wild type strain, a Δ*adlp L. monocytogenes* strain demonstrated impaired survivability when the bacteria were treated the alkylating reagent methyl methanesulfonate. Although structurally similar to AlkD, the alkylbase DNA glycosylase in *Bacillus cereus*, *adlp* deleted *L. monocytogenes* strain was more sensitive to the oxidative agent H_2O_2 and more susceptible to murine macrophage RAW 264.7. In addition, we showed that deletion of Adlp decreased the resistance of *L. monocytogenes* to aminoglycoside antibiotics, which are a typical choice for the treatment of *Listeria* infection. Our evidence demonstrates that Adlp plays a multifunctional role in resistance to aminoglycoside antibiotics, alkylation reagents, and oxidants induced by host-cellular oxidative response.

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Introduction

Bacterial pathogens are continuously challenged by a variety of environmental chemicals and host metabolites. Among the most potent threats accompanying the bacterial colonization process are the reactive oxygen species (ROS) generated by the host innate-immune cells. ROS causes DNA lesions in bacteria by forming base derivatives (Imlay and Linn 1988; Wallace 2002) including highly prevalent 7,8-dihydro-8-oxo-2'-deoxyguanosine (8-oxogaunine, or GO), a pre-mutagenic lesion on guanine (Cheng, Cahill et al. 1992), and 5,6-dihydroxydihydrothymine (thymine glycol), a lethal lesion that blocks DNA replication and RNA synthesis in bacteria (Clark and Beardsley 1986; Tornaletti, Maeda et al. 2001). Oxidants produced by bactericidal antibiotics, on the other hand, are another major source of insults to the integrity of the bacterial genome (Dwyer, Kohanski et al. 2009). Kohanski et al showed that three major categories of bactericidal antibiotics (β-lactam, aminoglycoside, and quinolone) are able to hijack bacterial metabolism to produce large amounts of hydroxyl radicals via a mechanism that alters the bacterial metabolic pathways, destroys iron-sulfur clusters, and activates Fenton's reaction, ultimately inducing double-stranded DNA breaks or base damages (Kohanski, Dwyer et al. 2007). Also, the oxidation of the guanine nucleotide pool by antibiotic induced ROS is another cause of bacterial cell death through the excessive formation of GO lesions in the bacterial genome (Foti, Devadoss et al. 2012).

The base-excision repair pathway is an evolutionarily conserved, and a multi-step repair system that plays a central role in counteracting oxidative lesions on DNA (Bjelland and Seeberg 2003). To initiate the base-repair process, DNA glycosylases cleave the *N*-glycosylic bond and release the damaged bases, and leave an

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apurinic/apyrimidinic (AP) site on the DNA backbone (Fortini, Pascucci et al. 2003). In the GO-repair system in *Escherichia coli*, MutM (Fpg), together with MutY and MutT as safeguards, preferentially repairs 8-oxoguanines (Michaels, Tchou et al. 1992); while DNA glycosylase EndoIII (Nth) removes oxidized pyrimidines (Krokan, Standal et al. 1997). A recent report on the pathogen *Neisseria meningitidis* revealed a network of glycosylases involved in the repair of oxidatively damaged bases (Nagorska, Silhan et al. 2012), indicating the functional redundancy and substrate overlap among the DNA glycosylases. The DNA glycosylases are widely reported to contribute to resistance to DNA damages caused by oxidative and nitrosative stress among bacterial pathogens including *Salmonella typhimurium* and *Helicobacter pylori (O'Rourke, Chevalier et al. 2003; Suvarnapunya, Lagasse et al. 2003; Richardson, Soliven et al. 2009).*

Listeria monocytogenes is a gram-positive, food-borne intracellular pathogen that causes severe symptoms (listerosis) in neonates, pregnant women, and immunocompromised individuals (Hamon, Bierne et al. 2006). The pathogenicity of this opportunistic bacterium is strongly associated with its ability to invade a broad spectrum of non-phagocytic cells (Gaillard, Berche et al. 1987; Dramsi, Biswas et al. 1995; Greiffenberg, Goebel et al. 2000) and its survival in macrophages (de Chastellier and Berche 1994).

L. monocytogenes serotype 4b F2365 strain possesses a set of oxidative-lesion-repair DNA glycosylases including the GO-repair system members, MutM, MutY, and the oxidized-pyrimidine DNA glycosylase, Nth, based on the sequence homology with other bacterial DNA glycosylases. Until now, however, the functions of these glycosylases have not been characterized.

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Recently, a novel alkylbase DNA glycosylase, AlkD, was discovered in *Bacillus cereus* and classified as a new family based on its unique protein architecture (Alseth, Rognes et al. 2006; Rubinson, Metz et al. 2008). AlkD forms a solenoid fold consisting of six HEAT-like repeats and plays a central role in *N*3- and *N*7-alkylated base repair (Rubinson and Eichman 2012; Rubinson, Gowda et al. 2012). In this study, we identified a protein that has a similar structure to AlkD, named AlkD-like protein (Adlp), in *L. monocytogenes* and investigated its function. To our surprise, we found that Adlp is involved in host-cellular oxidative burst tolerance, and resistance to aminoglycoside antibiotics and DNA alkylating agents. This is the first example of an AlkD-like protein playing a role in antioxidants tolerance and antibiotics resistance.

Materials and methods

Bacteria strains and culture conditions

Table 1 lists the wild-type, mutant, and complement *L. monocytogenes* serotype 4b F2365 strains. *L. monocytogenes* strains were cultured in BHI broth (Difco Laboratories, Detroit, MI) at 37°C. *E. coli* DH5α were cultured in Luria-Bertani (Difco) broth. To make the deletion mutant, transformed *L. monocytogenes* were selected on BHI agar plate supplemented with erythromycin (5 µg/ml) or tetracycline (10 µg/ml) when necessary. RAW 264.7 murine macrophage cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and 50U/ml penicillin/streptomycin. All cells were cultured at 37°C in a 5% CO2 incubator.

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***adlp* deletion mutagenesis**

pMAD, a temperature-sensitive shuttle plasmid, was used to generate the deletion mutant(Arnaud, Chastanet et al. 2004). From the genomic DNA of *L. monocytogenes* F2365, a 1.2 kb upstream region and a 1.3 kb downstream region flanking *adlp* were amplified by PCR using the primers listed in Table 2. PCR products were digested with restriction enzymes and cloned in tandem into a pMAD_tet plasmid (Table 1). The recombinant plasmid was introduced into *L. monocytogenes* by electroporation at 2.5 KV, 400 Ω, and 25 μF. Deletion of *adlp* was conducted by allelic exchange. (4) The transformed bacteria were incubated at 43°C for the first integration and in antibiotic-free BHI media at 30°C for the second recombination. Mutants were selected and confirmed with PCR using the primers harboring the deleted region of *adlp* (Table 2).

Growth curve of *L. monocytogenes*

Three strains (Δ*adlp*, wild-type, and complement) of *L. monocytogenes* were cultured in BHI broth shaken at 180 rpm for 16 hours at 37°C. The cultures were then diluted 1000 times and 50μl diluted bacteria was re-inoculated into 5 ml fresh BHI broth. Bacteria were incubated at 37°C in the shaker and bacterial numbers were counted at the specified time points by plating.

MMS assays

L. monocytogenes cells in mid-exponential growth phase were harvested by centrifugation and washed three times with phosphate-buffered saline (PBS). Approximately 10^6 cells were incubated with the indicated concentrations of MMS (Sigma-Aldrich Co., St. Louis, MO) for 1 hour at room temperature. The numbers of

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recovered bacteria were counted by serially plating. We calculated the percentages of surviving bacteria by comparing the numbers of bacteria (cfu) recovered from the MMS solution to numbers of bacteria (cfu) recovered from the PBS solution.

H_2O_2 sensitivity assay

L. monocytogenes cells were prepared as described above. Bacteria were incubated with the indicated concentrations of H_2O_2 (Sigma-Aldrich Co) for 10 minutes at room temperature, and then *Micrococcus lysodeikticus* catalase (1,000 U/ml) (Sigma-Aldrich Co.) was added to inactivate the remaining H_2O_2. We calculated the percentages of surviving bacteria by comparing the numbers of bacteria (cfu) recovered from the H_2O_2 solution with the numbers of bacteria (cfu) recovered from the PBS solution.

Macrophage survival assay

Murine macrophage RAW 264.7 cells were seeded onto 24-well plates and incubated at 37°C in a 5% CO2 incubator. Bacteria were prepared as described above. RAW 264.7 cells were infected with bacteria at a multiplicity of infection of 10:1 for 60 minutes. After 1 hour of infection, cells were incubated with fresh cell-culture medium supplemented with 50 µg/ml gentamicin to kill the extracellular bacteria. Cells were washed with PBS three times at the indicated times and then lysed with 0.2% Triton X-100. The amount of intracellular bacteria was quantified in cell lysates by serial dilution and plating.

Antibiotic survival assay

Bacterial cells (10^7) from overnight cultures were inoculated into BHI broth supplemented with the indicated concentrations of antibiotics. After 4 hours of incubation

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in a 37°C shaker, bacterial numbers were enumerated. For the timed-killing curve, bacteria were incubated in BHI broth supplemented with 0.5 µg/ml gentamicin and 1µg/ml ciprofloxacin, respectively, in a 37°C shaker for the 4 hours. Bacteria were serially diluted and bacterial numbers were counted by plating. We calculated the percentage of surviving bacteria by comparing the numbers of bacteria (cfu) recovered after antibiotic exposure to the numbers of bacteria (cfu) at time zero.

Results

The *adlp* locus is flanking the *Listeria* pathogenicity island-1 (LIPI-1).

According to the complete genome sequence data available for *L. monocytogenes* F2365, the *adlp* locus, previously designated "*vclA*", is located in the downstream flanking region of the virulence cluster locus (*vcl*, now also known as *Listeria* pathogenicity island 1 [LIPI-1]). The *adlp* locus is evolutionarily conserved among the six species in the *Listeria* genus and located between the housekeeping genes *vclB* and *ldh-1* (lactate dehydrogenase) (Figure3.1A). *adlp*, together with *vclB* and *ldh-1,* directly flank LIPI-1 on the downstream side.

Adlp is structurally similar to AlkD in *B. cereus.*

The National Center for Biotechnology Information conserved-domain search tool indicated that *Listeria* Adlp contains a putative AlkD-like domain (residues 20-216) (Figure3.1B): a newly identified protein architecture in the family of DNA glycosylases. We predicted the protein structure of Adlp using the SwissModel server (Schwede, Kopp et al. 2003) with *B. cereus* hypothetical protein (protein ID: 1T06 A) as a template. A structural homology search with the DALI server (Holm and Sander 1993) indicated that

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Adlp is structurally similar to AlkD, an alkylbase DNA glycosylase in *B. cereus* (Protein ID: 3BVS), a *B. cereus* hypothetical protein (Protein ID: 1T06 A), and a predicted alkylbase-repair enzyme in *Enterococcus faecalis* (Protein ID: 2B6C)

A cartoon representation of our Listeria Adlp homology model and the published *B. cereus* AlkD structure are shown in Figure 3.1C. Adlp possesses a solenoid superhelical structure consisting of 13 alpha helices; thus, Adlp is similar to AlkD in its overall structure and protein fold. We found differences in the C-terminus regions: AlkD has an extra helical tail (α14) and Adlp has a long loop linking α12 and α13 (Figure 3.1C). In Adlp, except for α2, the 12 α-helices form the HEAT-like motifs: six pairs of antiparallel α-α repeats in tandem (Figure 3.1 C, D). A concave area on the surface of Adlp is formed by α3, α5, α7, α9, α11, and α13. Similar to AlkD, the helices that form the concave surface are rich in Lysine and Arginine residues (Figure 3.1D). The Delphi-calculated surface electrostatic potential (Rocchia, Sridharan et al. 2002) (Figure 3.1E) showed an extremely electropositive concave Adlp surface: a typical feature for accommodating DNA. Due to a series of bulky aromatic residues, especially the Phe106 and Tyr171 in the center of the concave area, Adlp has a shallow cleft (Figure 3.1D, Figure 3.2), which is also a conserved feature of AlkD orthology.

The role of Adlp in alkylating-compound resistance.

To study the role of Adlp, we constructed an *adlp*-deletion mutant in *L. monocytogenes* (Table 3.1) using a pMAD shuttle-vector system (Arnaud, Chastanet et al. 2004). We confirmed the deletion of the *adlp* locus by polymerase chain reaction (PCR) using primers harboring the deletion region (Figure 3.3A). We used gene *plcB* as an internal quality control. To ensure that the deletion of *adlp* did not cause growth

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defects, we compared the growth kinetics of the Δ*adlp* strain, the parental strain, and the complement strain by serial plating on BHI plate. We observed no significant differences in the growth rates of all three strains (Figure 3.3B), indicating that the deletion of *adlp* did not affect the growth of the bacteria.

Due to the structural similarity between AlkD and Adlp, we hypothesized that the deletion of *adlp* enhanced the sensitivity of bacteria treated with DNA alkylating compounds. This possibility was tested by the survivability of three *L. monocytogenes* F2365 strains (Δ*adlp*, wild-type, and complement) in solutions with different concentrations of the alkylating reagent methyl methanesulfanate (MMS) for 1 hour. As predicted, compared to the wild-type and complement strains, the Δ*adlp* strain showed significantly higher sensitivity to the treatment with 20mM and 40mM MMS ($p<0.01$ and $p<0.05$, respectively, Figure 3.4). By contrast, we found no significant differences in low-concentration (10mM) or high-concentration (60mM) MMS solution treatment (Figure 3.4), suggesting a tolerable dose and a lethal dose for *L. monocytogenes* F2365, respectively. These data show that Adlp plays a role in MMS resistance in *L. monocytogenes*.

A lack of Adlp impairs oxidative-stress resistance in *L. monocytogenes.*

To assess the role of Adlp in oxidative stress condition, we compared the dose-dependent and time-dependent sensitivities to the oxidative reagent H_2O_2 of the three *L. monocytogenes* F2365 strains (Figure 3.5A, B). After 10 minutes of exposure to a range of H_2O_2 concentrations (0.5 - 30 mM), the Δ*adlp* strain had approximately 20-30% decreased survivability compared with the parental and complement strains (Figure 3.5A). Interestingly, increasing the concentration of H_2O_2 did not have much effect on the

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overall survival rates of the three strains (Figure 3.5A); The survival rate in the highest concentration of H_2O_2 (30mM) was not significantly different from that in the lowest concentration of H_2O_2 (0.5mM). We also investigated the survivability of the three strains in 10mM H_2O_2 at multiple time points. In addition to the early exposure (10-minute) results, the Δ*adlp* strain was considerably more sensitive to H_2O_2 after 1 hour of exposure (Figure 3.5B). After 90 minutes of exposure, all three strains were killed (Figure 3.5B).

Host immune cells produce oxidants such as ROS following exposure to pathogens; these oxidants are one of the major threats to *L. monocytogenes* (de Chastellier and Berche 1994; Inoue, Itagaki et al. 1995; Zenewicz and Shen 2007). To assess the role of Adlp in counteracting host-cellular oxidative burst, we further evaluated the survivability of the three strains in RAW 264.7 murine macrophages. We infected the RAW 264.7 cells with the three strains at equal multiplicities of infection and counted intracellular bacteria. Compared with the parental and complement strains, the Δ*adlp* strain exhibited decreased survival at all observed time points (Figure 3.5C). Especially at 12 and 16 hours post-infection, the intracellular bacteria number of the Δ*adlp* strain was significantly lower than that of the parental strain (Figure 3.5C).

Contribution of Adlp to antibiotic resistance.

To examine the role of Adlp in antibiotic-induced oxidative-stress resistance, we tested the survivability of the three strains in the presence of three categories of antibiotics: β-lactam (ampicillin), aminoglycoside (gentamicin), and quinolone (ciprofloxacin). We performed a timed-killing study using 0.5 μg/ml gentamicin. Starting from 2 hours of exposure, the Δ*adlp* strain had a significantly lower recovery rate than the parental and complement strains (Figure 3.6A). After 4 hours exposure, we observed

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a difference of one order of magnitude in the recovery rates between the Δ*adlp* and parental strains (Figure 3.6A). We observed similar results with streptomycin, another drug in the aminoglycoside category (Figure 3.7). In both timed-killing study and dose-dependent killing study, the mutant strain is more sensitive to streptomycin comparing to parent strain (Figure 3.7). We found a more striking survival defect in the Δ*adlp* strain after 4 hours shaking at 37°C and subsequent overnight incubation at room temperature in brain-heart infusion (BHI) broth with 0.5 μg/ml gentamicin; while we observed robust growth of both the wild-type and complement strains, the Δ*adlp* strain completely lost its growth capability (Figure 3.6C).

To examine the dose dependent effect of gentamicin, three *L. monocytogenes* strains were exposed to 0.5, 0.75, and 1 μg/ml gentamicin for 4 hours. The survival rate of the Δ*adlp* strain was approximately one order of magnitude lower than those of the parental strain in 0.5 and 0.75 μg/ml gentamicin (Figure 3.6B). We also tested the survivability of the three *L. monocytogenes* strains in the presence of a quinolone (ciprofloxacin) and a β-lactam (ampicillin). In response to ciprofloxacin, the recovery rate of the Adlp mutant was significant lower than the recovery rates of the wild-type, although the difference was very moderate compared with the gentamicin results (Figure 3.6D). By contrast, we observed no significant difference between the Δ*adlp* strain and the other two strains in their recovery rates following treatment with ampicillin (Figure 3.6E).

Discussion

Based on the sequence homology comparison with other bacterial DNA repair enzymes, *L. monocytogenes* has a set of oxidative-repair DNA glycosylases in its genome

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including the GO repair system; MutM and MutY, which are essential for 8-oxoG repair; and the oxo-pyrimidine glycosylase Nth. In *N. meningitides*, these enzymes form a network and show functional redundancy; and thus no single-deletion mutant of these DNA glycosylases demonstrated a striking sensitivity to oxidative stress (Nagorska, Silhan et al. 2012). Our study demonstrated that Adlp is involved in oxidative-stress resistance. The Δ*adlp* strain, however, was only moderately more sensitive, about 20~30% difference in the case of H_2O_2 treatment, than its parental strain to oxidative reagents (Figure 3.5A and 3.5B). We speculate that there might be other glycosylases, such as MutM or Nth, backing up Adlp. Double- or triple-deletion of glycosylates are necessary to prove the specific role of each glycosylase in oxidative stress resistance. Interestingly, in the time dependent H_2O_2 killing experiment, a time dependent "two-mode" killing pattern was observed, which indicated that the mutant strain had two sensitive time zones (at 10 minutes and after 60 minutes) and a resistance intermediate time zone (around 30 minutes) (Figure 3.5B) to H_2O_2 killing. This "two mode" killing by H_2O_2 was also observed in *E.coli* in a dose dependent manner (Imlay and Linn 1986) and therefore it is possible that a network of glycosylases could exist in *L. monocytogenes*.

Facing oxidative stress from host macrophages is one of the major challenges that *L. monocytogenes* must overcome during its lifecycle (de Chastellier and Berche 1994). *L. monocytogenes* has several strategies to prevent damage from host oxidative burst, including escape from vacuoles by listeriolysin O and the antioxidant effects of bacterial superoxide dismutase (Portnoy, Jacks et al. 1988; Archambaud, Nahori et al. 2006). Our data suggest that Adlp plays a role in helping *L. monocytogenes* to survive in macrophages, which provides an additional insight of how *L. monocytogenes* can

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counteract the host-cellular oxidative burst. Similarly, induction of a DNA repair system candidate, such as SOS activator, RecA, following DNA damage caused by reactive oxidants was documented when *L. monocytogenes* is inside macrophages (Chatterjee, Hossain et al. 2006).

Antibiotic-induced oxidative stress contributes to cell death among many bacteria (Goswami, Mangoli et al. 2006; Bizzini, Zhao et al. 2009; Wang and Zhao 2009). Regardless of the interactions between antibiotics and specific targets, bactericidal antibiotics are suggested to induce bacterial cell death through a common mechanism of enhancing the production of intracellular hydroxyl radicals on *E. coli* (Kohanski, Dwyer et al. 2007; Dwyer, Kohanski et al. 2009), although this theory was questioned by recent works on *E.coli* (Keren, Wu et al. 2013; Liu and Imlay 2013). Our data show that Adlp mutant of *L. monocytogenes* was sensitive to killing by aminoglycoside antibiotics, but not to β-lactam (ampicillin), or quinolone (ciprofloxacin) (Figure 3.6, Figure 3.7), indicating antibiotic-induced reactive oxygen species mediated killing might not be uniformly working on *L. monocytogenes*. A recent study on *L. monocytogenes* showing that the oxidative-stress mutants Δ*sod* and Δ*fir* did not have reduced survival in the presence of three categories of antibiotics (Feld 2012). Unlike Δ*sod* and Δ*fir* strain, our Adlp mutant is sensitive to animoglycosides. Indeed, many different models were proposed to explain the mechanism of animoglycoside induced cell death other than drug-target interaction, including mistranslation of membrane proteins and subsequently activation of the two component system (Kohanski, Dwyer et al. 2008). Our data suggest that the loss of functional Adlp significantly affects the survivability of *L. monocytogenes*

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in the presence of listeriacidal antibiotics, which may provide new insights for antimicrobial drug design.

Table 3.1 Bacterial strains and plasmids

Bacteria strains / Plasmids	**Description**	**Reference**
Strains		
Listeria monocytogenes F2365	Wild type	this study
Δ*adlp*	*Lmof2365_0220* deletion mutant, Tet^r	this study
Δ*adlp* + pMAD_*adlp*	Complement strain of Δ*adlp*, Tet^r, Erm^r	this study
E.coli DH5α	Host for molecular cloning	Invitrogen
Plasmids		
pMAD	Shuttle vector for constructing deletion mutant , Erm^r	(Arnaud, Chastanet et al. 2004)
pMAD_tet	pMAD vector containing *Tet* cassette, Tet^r, Erm^r	this study
pMAD_*adlp*	pMAD_tet vector containing *adlp* flanking regions, Tet^r, Erm^r	this study

Table 3.2 Primer list for deletion mutagenesis

Primers	5'-3' sequence
Adlp upstream Forward	GCGCGGATCCGCAGTTTGTTAGGTACCCTT
Adlp upstream Reverse	GCGCGTCGACAAGTAGTAAGAGGTATGATA
Adlp downstream Forward	GCGCCTCGAGGCATGCTTTTAGGATTTGAG
Adlp downstream Reverse	GCGCAGATCTTCAGGACGTTCAACGCTTCA
Adlp confirm Forward	TAGCATCCGGAACTTTACACGC
Adlp confirm Reverse	CCGAGCTACAAGCCTTAGAAAAC
plcB Forward	FTGGCTGATTACCGAGAAGGG
plcB Reverse	ATTATTGGCGTGCATAGGTTGA

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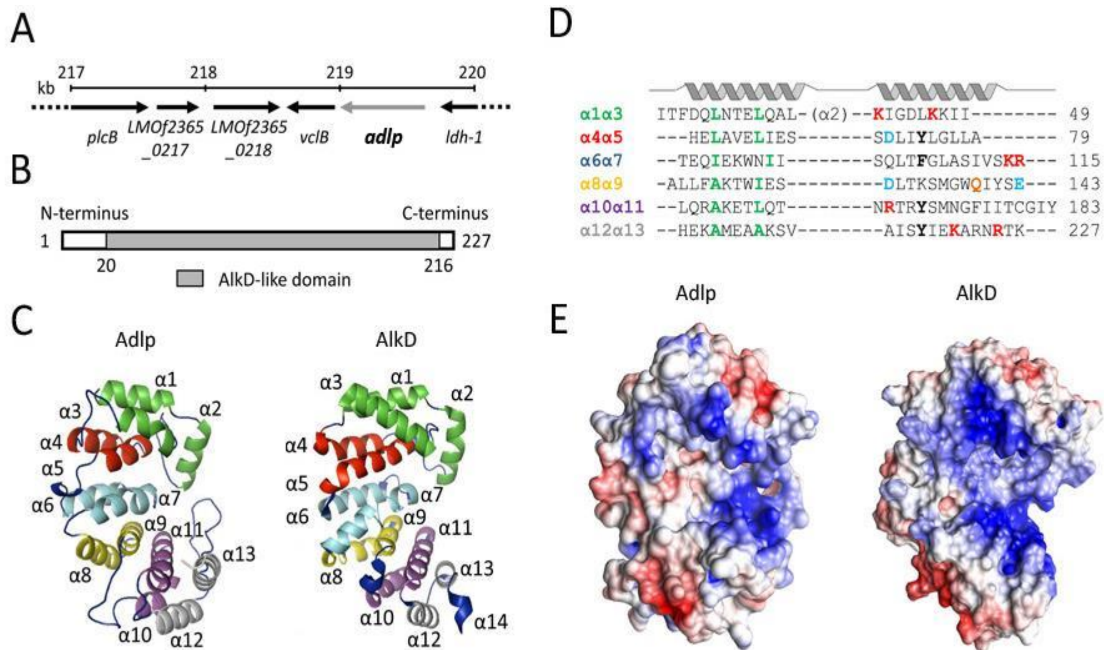

Figure 3.1 *L. monocytogenes* Adlp is structurally similar to *B.cereus* AlkD.

(A) Genomic organization of the adlp locus in L. monocytogenes strain F2365. The map was generated from the complete-genome sequence of L. monocytogenes strain F2365. adlp is located between the housekeeping genes ldh-1 and vclB, and flanks the downstream region of Listeria pathogenic island 1 (LPI-1). (B) Domain architecture of Adlp. An AlkD-like domain is located from aa 20 to aa 216 (grey box) (C) Predicated three-dimensional structure of Adlp and published structure of B. cereus AlkD (Protein ID: 3BVS). Alpha helices are marked in order and six pairs of anti-parallel HEAT-like repeats are shown with six different colors. (D) Structure-based sequence alignment of HEAT motifs in ADLP. Positively charged residues that contribute to the electropositive concave surface are highlighted in red, aromatic residues are in bold, and acidic residues are in blue; a glutamine residue that has a potent role in catalytic activity is marked in orange. Conserved residues that maintain the structure of alpha helices are shown in green. (E) Solvent-accessible protein surface with the same orientation of the models in (c) is colored by electrostatic potential (red, negative; blue, positive; −5 to 5 kBT). Potentials were calculated with Delphi (Rocchia, Sridharan et al. 2002) and images demonstrated with PyMOL (http://pymol.org/) and Chimera (http://www.cgl.ucsf.edu/chimera/).

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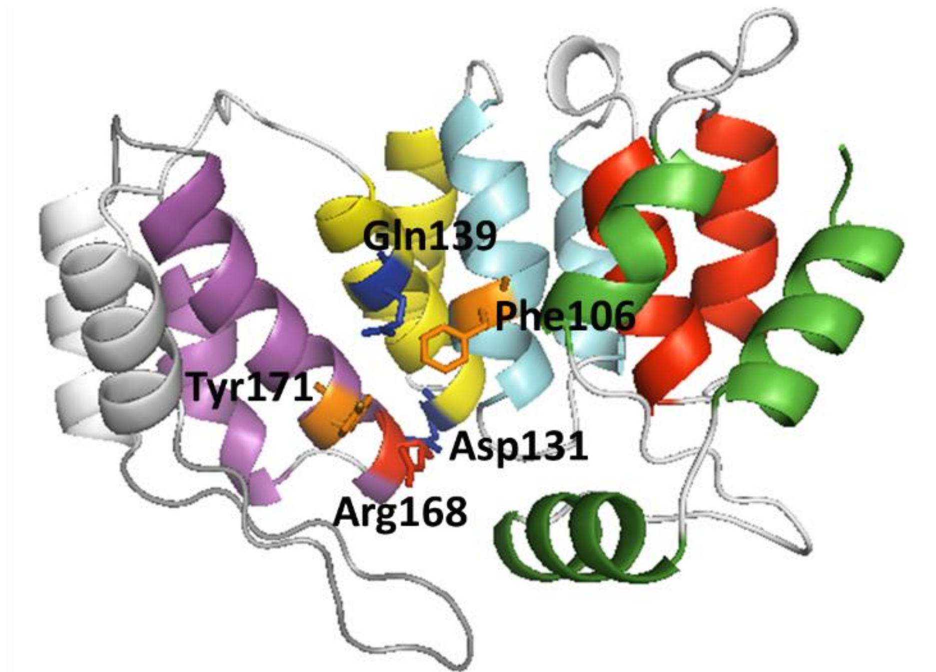

Figure 3.2 Predicated catalytic pocket of Adlp

We predicted the catalytic pocket and essential residues for catalysis activity. Phe106 and Tyr171 in the center of the cleft could be potent in stacking damaged bases and/or stabilizing the DNA backbone. Arg168, Asp131, and Gln139 could be potent essential residues for catalytic activity.

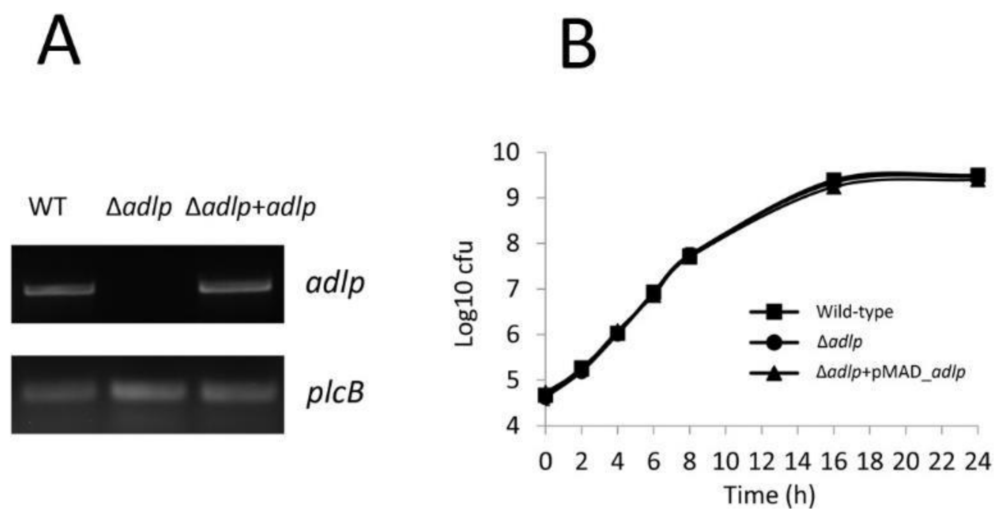

Figure 3.3 Confirmation of Adlp-deletion mutants and growth kinetics of *L. monocytogenes*.

(A) PCR to confirm the deletion of ADLP using primers harboring the deletion region. Genomic DNA from each strain were extracted and used as template; *plcB* was used as an internal quality control. (B) Growth of three strains (Δ*adlp*, wild type, and complement strains) of *L. monocytogenes* in BHI at 37°C. Data are the mean and standard error of the mean (SEM) of three independent measures.

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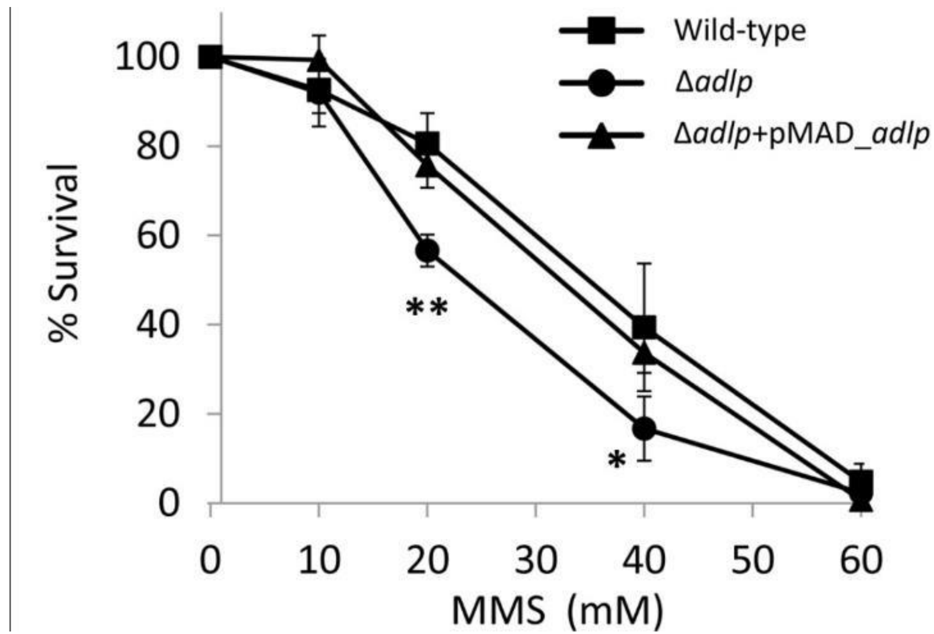

Figure 3.4 Adlp is associated with resistance to DNA-alkylating compounds.

Three strains (Δ*adlp*, wild type, and complement strains) of *L. monocytogenes* were incubated with 10, 20, 40, and 60mM of MMS or PBS at room temperature for 1 hour and measured survival rates. The survival percentages were calculated by comparing the recovered bacteria (cfu) from MMS with recovered bacteria (cfu) from PBS. Data are mean and SEM of at least three independent experiments. (N≥3; * indicates $p<0.05$, ** $p<0.01$, ANOVA)

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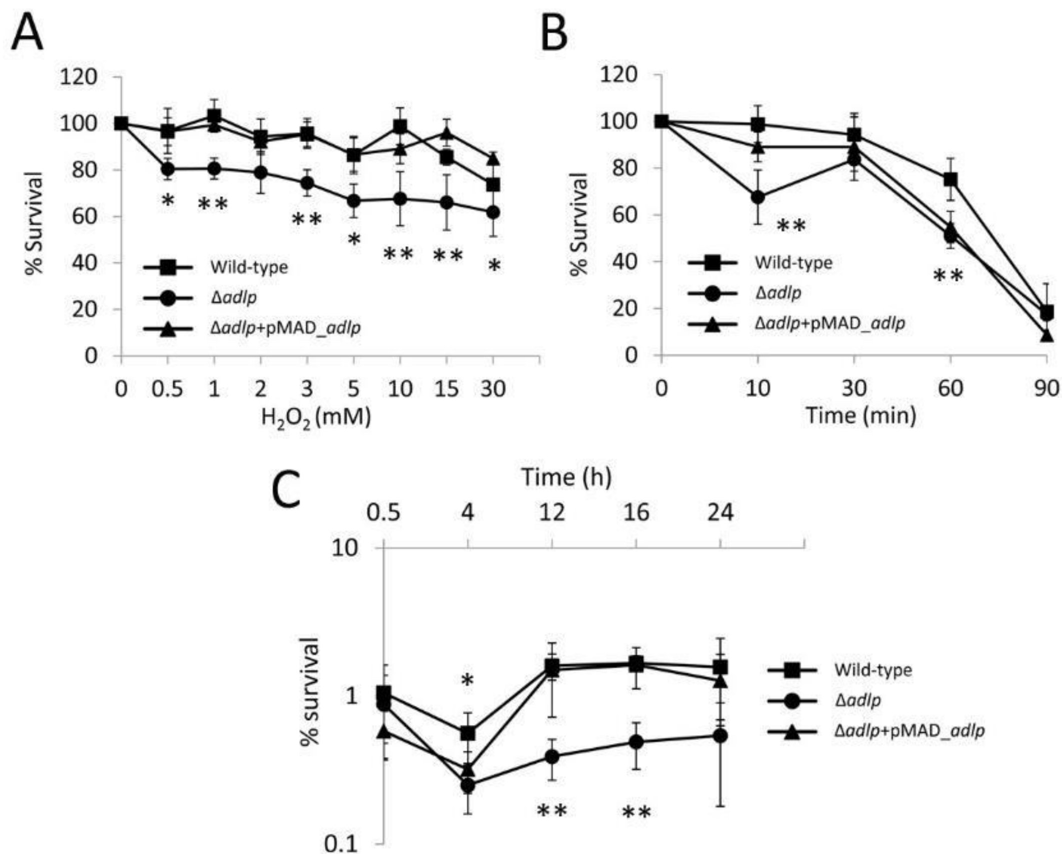

Figure 3.5 Role of Adlp in the oxidative stress response

(A) Three strains of bacteria were treated with the indicated concentrations of H_2O_2 or PBS at room temperature for 10 minutes and measured survival rates. Survival percentages were calculated by comparing the recovered bacteria (cfu) from H_2O_2 to the recovered bacteria (cfu) from PBS. (B) Three strains of bacteria were incubated with 10mM H_2O_2 or PBS at room temperature for the indicated time and measured survival rates. (C) Three strains of bacteria were incubated with RAW 264.7 cells at a 10:1 multiplicity of infection and allowed them to replicate in RAW 264.7 cells for the indicated time. We calculated the survivability as the ratio of recovered bacteria to inoculated bacteria. Data are mean and SEM of three independent experiments. (N=3; * indicates $p<0.05$, ** $p<0.01$, ANOVA)

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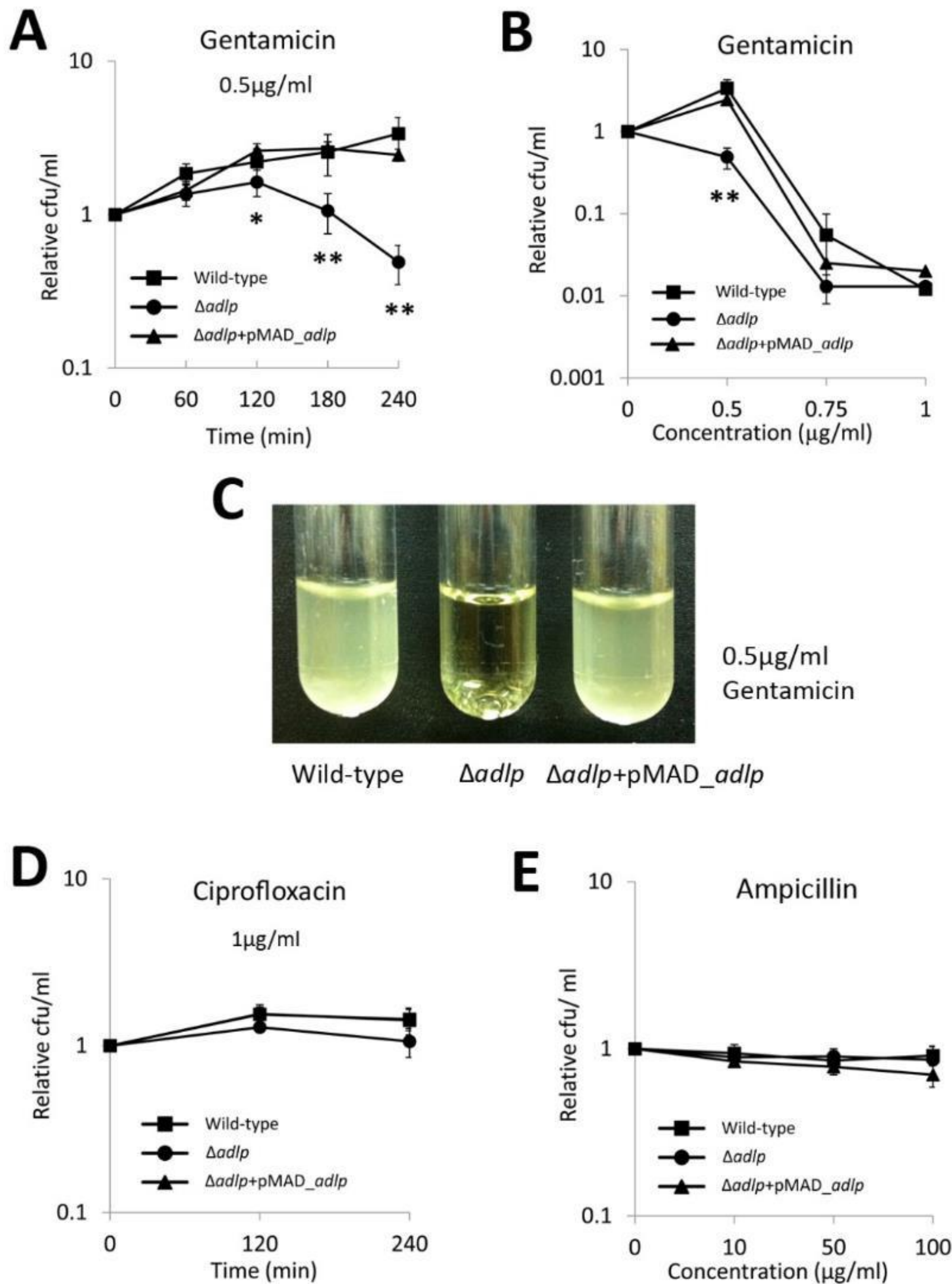

Figure 3.6 Contribution of Adlp to bactericidal antibiotic resistance.

(A, B) Bacteria number changes following exposure to gentamicin. Three strains of bacteria were exposed to to 0.5 µg/ml gentamicin for the indicated time (A) or to gentamicin at the indicated concentration for 4 hours (B). Data are mean and SEM of three independent experiments. (N=3; * indicates $p<0.05$, ** $p<0.01$, ANOVA) (C) Three strains of bacteria were exposed to 0.5 µg/ml of gentamicin, shaken at 37°C for 4 hours, and then left at room temperature for 16 hours. (D, E) Bacteria number changes following exposure to ciprofloxacin and ampicillin. Three strains of bacteria were exposed to 1 µg/ml ciprofloxacin for the indicated time (D) or to the indicated concentration of ampicillin for 4 hours (E). Data are mean and SEM of three independent experiments. (N=3)

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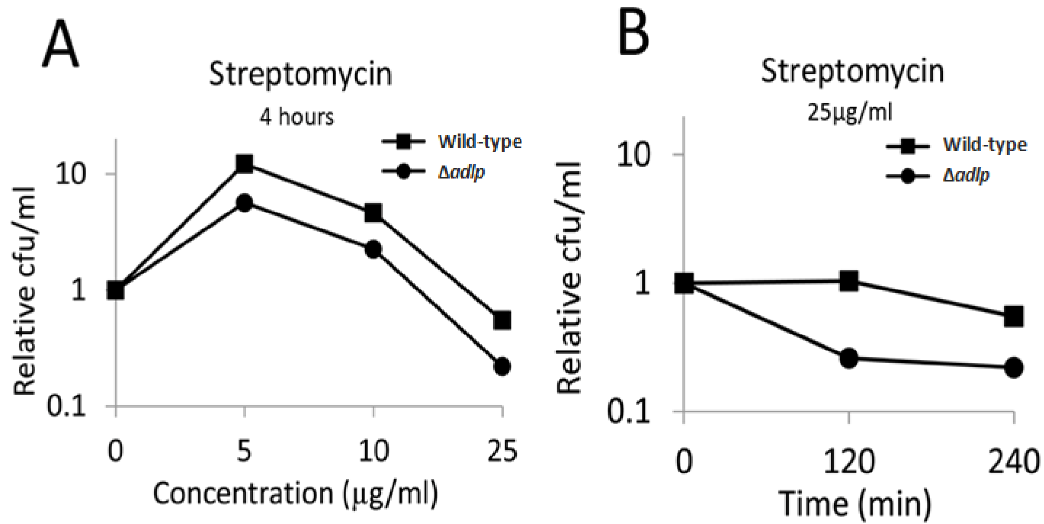

Figure 3.7 Contribution of Adlp to streptomycin resistance.

(A, B) Bacteria number changes following exposure to streptomycin. We exposed Δ*adlp* and the parental strain to 25 μg/ml streptomycin for the indicated time (A) or to streptomycin at the indicated concentration for 4 hours (B). Data represent mean of one independent experiment of triplicates. (N=1, n=3)

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CHAPTER IV

CHARACTERIZATION OF LISTERIAL SURFACE PROTEINS THAT ARE INVOLVED IN ADHESION AND INVAISON OF *L. MONOCYTOGENES* TO NON-PHAGOCYTIC CELLS

Abstract

Listeria monocytogenes possesses the highest number of leucine rich repeats (LRRs) motifs in internalins than other gram positive bacteria. To understand the functions of largely uncharacterized internalin molecules, we generated seven in-frame deletion mutants targeting LRR motifs in *L. monocytogenes* H7858 strain and tested for their virulence. Among seven mutants, H7858Δ0369 and H7858Δ2546 showed significant impaired invasiveness to Hep-G2 cells. We further tested the virulence of H7858Δ0369 and H7858Δ2546 strains in the intravascular sepsis model using BALB/c mice. Interestingly, H7858Δ0369 strain showed reduction in colonization to various tissues, bacteremia formation, and invasion to the brain compared to the wild type. Host immune responses to listerial intravascular infection were measured by quantitative real-time PCR. Result showed that transcript levels of proinflammatory cytokines including IL-1, IL-6, IL-12, TNF-α, and IFN-γ and chemokines including MCP-1 and MCP-3 that induced by H7858Δ0369 infection was significantly lower than those by parental wild type strain. These results suggest that lmoH7858_0369, an internalin-like protein

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modulates host immune responses to the listerial infection, and thus might be a novel virulence factor of *L. monocytogenes*.

Introduction

Listeria monocytogenes is an important gram-positive intracellular bacterium that is responsible for the disease known as "listeriosis". As a food-borne pathogen, it is able to cause gastroenteritis in healthy individuals (Aureli, Fiorucci et al. 2000; Ooi and Lorber 2005), septicemia, and invasive diseases in the central nervous system, such as meningitis and encephalitis in immunocompromised patients (Lorber 1997; Wing and Gregory 2002), as well as fetal-maternal infection (Mylonakis, Paliou et al. 2002). Listeriosis is a life threatening disease; even with early antibiotic treatment, it still reaches a 20~30% mortality rate (Vazquez-Boland, Kuhn et al. 2001). *L. monocytogenes* is well known for its capability of crossing physical barriers in hosts, including the intestinal barrier, the blood-brain barrier and the fetal-placental barrier (Hamon, Bierne et al. 2006). *L. monocytogenes* is capable of crossing the intestinal barrier and disseminating into blood via lymphatic/blood route (Marco, Prats et al. 1992). In a mouse model, within minutes after intravenous injection, about 90% of bacteria were captured by Kupffer cells in the liver (Mackaness 1962), and the other 10% were trapped in the spleen (Conlan 1996). If the infection is not controlled by the host immune system, *L. monocytogenes* could re-enter the bloodstream to form bacteremia, and reach a secondary organ such as the brain or placenta (Berche 1995; Bakardjiev, Theriot et al. 2006).

In order to generate a cross-barrier infection, *L. monocytogenes* is able to enter a wide range of non-phagocytic cells, including epithelial cells (Gaillard, Berche et al. 1987), hepatocytes (Dramsi, Biswas et al. 1995), endothelial cells (Drevets, Sawyer et al.

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1995), and fibroblasts (Kuhn, Kathariou et al. 1988). Bacterial virulence factors play a critical role in entering these non-phagocytic cells. Until now, a serial of virulence factors are reported to be involved in bacterial invasion, including the extensively studied inlA and inlB, which utilize host cellular machineries to perform receptor mediated endocytosis (Pizarro-Cerda and Cossart 2006). Leucine-rich repeats (LRRs) motifs are generally 20-29 conserved residues with a consensus sequence that is important for protein-protein interaction. LRRs also play a pivotal role in host innate immune responses and bacterial pathogenesis (Hartman, Venkatesan et al. 1990; Boland, Havaux et al. 1998; Kobe and Kajava 2001). In *L. monocytogenes*, a large of group of proteins containing LRRs are classified as the internalin family (Marino, Braun et al. 2000). Interestingly, *L. monocytogenes* has more LRR motifs than any other gram positive bacterium (Bierne, Sabet et al. 2007). Among these internalin proteins, InlA and InlB are extensively studied for their role of entering non-phagocytic cells. InlA and InlB direct the invasion of *L. monocytogenes* to different types of cells using E-cadherin and c-Met as their receptors, respectively (Kuhn, Kathariou et al. 1988; Gaillard, Berche et al. 1991; Dramsi, Biswas et al. 1995; Greiffenberg, Goebel et al. 2000; Pizarro-Cerda and Cossart 2006). Other internalins are also involved in listerial pathogenesis. For instance, deletion of InlH and InlJ significantly impaired virulence of *L. monocytogenes* in mice (Raffelsbauer, Bubert et al. 1998; Sabet, Lecuit et al. 2005; Sabet, Toledo-Arana et al. 2008). InlC, the only soluble internalin protein, may play a conjugated role with InlA in entering non-phagocytic cells (Bergmann, Raffelsbauer et al. 2002); meanwhile, it is able to interfere innate immune responses through interacting with IκB (Gouin, Adib-Conquy et al. 2010). Lmo2026, an internalin-like molecule is essential for CNS infection (Autret,

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Dubail et al. 2001). In addition, a recent study indicated that InlK, another internalin-like molecule, plays a critical role in avoiding autophagy by interacting with host ribonucleoprotein MVP (Dortet, Mostowy et al. 2011). Although with a similar structure in the leucine rich repeats domain, the functions of the majority of the LRR-motif containing proteins remains unknown.

In this study, we investigated the role of seven genes of *L. monocytogenes* H7858 strain that contain LRRs motifs. We found that LmoH7858_0369, a serotype 4b specific protein that is absent in non-pathogenic species *L. innocua*, is involved in pathogenesis as a novel virulence factor.

Materials and methods

Bacterial strains and cell culture conditions

Listeria strains used in this study are listed in Table 1. We cultured *L. monocytogenes* in BHI broth (Difco Laboratories, Detroit, MI) and *E. coli* DH5α in Luria-Bertani (Difco) broth at 37°C. To select *Listeria* LRR mutants, the constructed and transformed *L. monocytogenes* was plated on BHI agar plates containing erythromycin (5 μg/ml) or tetracycline (10 μg/ml). Caco-2 and Hep-G2 cells were grown in RPMI-1640 medium (Invitrogen, Gland Island, NY) supplemented with 20% and 10% fetal bovine serum (Invitrogen), respectively. All cells were supplemented with 50U/ml Penicillin and 50μg/ml streptomycin and cultured at 37°C in a 5% CO2 incubator.

Invasion and adhesion assays

Hep-G2 and Caco-2 cells were seeded in 24 well plates and incubated at 37 °C in a 5% CO_2 incubator. *L. monocytogenes* was grown in BHI to reach in mid-log phase and

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then collected by centrifugation. The bacterial pellet was washed twice with PBS and resuspended in RPMI 1640 medium. The cells were infected with bacteria at multiplicity of infection of 50:1 and incubated for 1 hour. For the adhesion assay, the cells were washed five times with PBS, and lysed with 0.25% Triton X-100. The cell lysates were performed with 10-fold serial dilution and plated on BHI agar to count the viable bacteria. For the invasion assay, the infected cells were further incubated in medium containing gentamicin (100 μg/ml) for 1 hour to kill the extracellular bacteria. After incubation, the cells were washed, lysed, diluted, plated, and counted the same as the adhesion assay.

Construction of Internalin mutants

From the genomic DNA of *L. monocytogenes* H7858 strain, we amplified the upstream and the downstream of target genes flanking regions using PCR with the primers listed in Table 3. The PCR products were digested with BamHI/SalI and XhoI/BglII, respectively, and cloned in tandem into a pMAD_tet plasmid, a temperature-sensitive shuttle vector. Then, the recombinant plasmid was introduced into *L. monocytogenes* using electroporation at 2.5 KV, 400 Ω, and 25 μF. The deletion of target genes was completed by allelic exchange. The transformed bacteria were incubated at 43°C for the first integration and in antibiotic-free BHI media at 30°C for the second recombination. Mutants were selected and confirmed using PCR with the primers specific for the deleted region of target genes.

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Infection of mice and organ distribution

Four groups (twelve mice per group, divided into two cages with 6 mice per cage) of 10 to 12 week old female BALB/c mice were injected intravenously with PBS, *L. monocytogenes* H7858 wild type, H7858Δ0369, or H7858Δ2546 strains at a dose of 6 x10^3 viable bacteria. Six mice in each group were euthanized at 24 h and 72 h post infection, respectively. Blood was collected with or without heparin (100U/ ml). Spleen, liver, and brain of the mice were collected and homogenized in PBS. All samples were serially diluted and plated on BHI agar, and incubated at 37°C for 48 h. The colony forming units were recorded.

Quantitative Real-time PCR

Liver and spleen from mice were immediately placed in TRIZOL (Invitrogen) and the total RNA was extracted by following manufacturer's instructions. The RNA was treated with RNase-free DNase (Qiagen, Valencia, CA) to remove the genomic DNA, and the RNA was further purified using RNeasy mini kit (Qiagen) following the manufacturer's instructions. One microgram of RNA was used to convert to cDNA using a reverse transcription kit (Applied Biosystems, Foster City, CA). The concentration of cDNA was measured with Nanodrop ND1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE) and approximately 100ng cDNA was used as the template in a 25ul volume reaction system using primers listed in table 4. Quantitative real-time PCR is performed using SYBR Green Master kit (ABI) under condition: 95°C for 10 min, followed with 95°C 30 seconds, 58°C 30 seconds, and 72°C 30 seconds for 40 cycles on Mx3005P™ Real Time PCR system (Stratagene, La Jolla, CA). β-actin was

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used as internal control and $2^{\triangle\triangle C_t}$ method was used to calculate the relative mRNA expression (Yuan, Reed et al. 2006).

Statistical Analysis

Student t-test and Mann-Whitney tests were used to compare means between two groups. One way ANOVA with Tukey's multiple comparison tests were used to compare the data from three or more groups.

Results

Invasiveness of different *L. monocytogenes* strains/isolates.

In order to evaluate adherence and invasion abilities of various strains/isolates and serotypes to Caco-2 cells and Hep-G2 cells, we tested 11 human-origin isolates and a rabbit-origin EGD strain (Table 4.1). Among these 12 strains/ isolates, significant differences of adherence and invasion rates were observed on both Caco-2 cells and Hep-G2 cells (Figure 4.1A, B, Invasion rates on Hep-G2, ANOVA, $p<0.0001$; Adherence rates on Hep-G2, ANOVA $p=0.0018$; Invasion rates on Caco-2, ANOVA, $p<0.0001$; and Adherence rates on Caco-2, ANOVA, $p<0.0001$). Interestingly, great variations of invasiveness were observed between the strains within the same serotype (Figure 1A, B). For instance, EGD strain showed significantly higher invasiveness than all three of the other isolates under 1/2a serotype on Caco-2 cells (Figure 4.1B, Tukey's test, $p<0.05$).

Two completely sequenced serotype 4b strains, F2365 and H7858, were used to compare their ability to invade epithelial cells. A cheese isolate F2365 that is related to listeriosis outbreak, was less invasive with both Caco-2 and Hep-G2 cells than a meat isolate H7858 strain that is associated with a multistate outbreak in the USA (Figure 4.2A

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and 4.2B, Tukey's test, *p*<0.001). Completed sequence information indicated that these two strains have similar genomic organization (Nelson, Fouts et al. 2004). A previous report indicated that several authentic point mutants were detected on the genome of F2365, including a point mutation on the invasion protein InlB (Nelson, Fouts et al. 2004), which leads to a premature stop codon. In order to test if the absence of InlB in F2365 is accounted for the attenuated phenotype, we made a InlB deletion mutant using H7858 as a parental strain and evaluated its invasiveness and adhesion ability. H7858ΔInlB strain demonstrated a significantly decreased (*p*<0.001, Tukey's test) invasion rate with both Caco-2 cells and Hep-G2 cells when compared to the H7858 parental strain (Figure 4.2A and 4.2B). The H7858ΔInlB reached the similar invasion rate (no significant difference, Tukey's test) as F2365 strain (Figures 4.2A and 4.2B).

Identification of novel internalin-like protein that involves in Hep-G2 invasion.

The study of Nelson, K. E et al. indicated that *L. monocytogenes* H7858 strain has 26 genes containing LRR motif (Nelson, Fouts et al. 2004). In order to evaluate the role of these genes in virulence, we selected 7 uncharacterized candidate genes with LRRs motif (Table 4.2) and performed in-frame deletion mutagenesis. These mutants were further tested for their adherence and invasiveness abilities to Caco-2 cells and Hep-G2 cells. No significant differences (ANOVA-Tukey's test) were observed on adherence rates among seven mutants and the parental strain on both Caca-2 and Hep-G2 (Figures 4.3A and 4.3C). Apparently, these seven proteins containing LRR motif were not associated with adherence to these epithelial cells. Except the InlB mutant, there was no significant difference on invasion rates between the wild type and other mutants on Caco-2 cells (Figure 4.3B, ANOVA-Tukey's test). Two mutant strains LmoH7858Δ0369 and

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LmoH7858Δ2546 lost the ability to invade Hep-G2 cells (Figure 4.3D, $p<0.001$, ANOVA-Tukey's test). Remarkably, *LmoH7858_0369* is a serotype 4b specific gene that is found in the genome of 4b strains such as H7858 and F2365, but the gene was absent in 1/2a serotype EGD-e strain, and non-pathogenic strain *L. innocua* (Table 4.2). These data indicated that *LmoH7858_0369* and *LmoH7858_2546* were involved in entry to Hep-G2 cells. The deletion of InlB in H7858 strain also significantly decreased entry of *L. monocytogenes* to Hep-G2 cells when compared to the parent strain (Figure 4.3D, $p<0.001$, ANOVA-Tukey's Test).

LmoH7858_0369 is required for host colonization.

To investigate the role of LmoH7858Δ0369 and LmoH7858Δ2546 in pathogenesis, we tested these two mutants in BALB/c mice. The mice were injected intravascularly (*i.v.*) with 6 $\times 10^3$ viable bacteria per strain. The bacterial number of LmoH7858Δ0369 strain was significantly different from the parent strain in the liver (Figure 4.4A) at 24 h or 72 h post infection as well as in spleen 72 h post infection (Figure 4.4B). Interestingly, LmoH7858Δ0369 was not detected in blood and brain 24 or 72 h post infection. The results indicate that LmoH7858Δ0369 strain lost the ability to grow *in vivo*. LmoH7858Δ2546 strain was not different from the parent strain in liver and spleen (Figure 4.4A and 4.4B) although this mutant did lose its ability to invade to Hep-G2 cells. When we injected mice with the H7858 parent strain or the LmoH7858Δ2546 strain, bacteria were detected in brain and blood 72 h post infection (Figure 4.4C and 4.4D).

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LmoH7858Δ0369 mutant loses its ability to induce cytokines.

In order to investigate the responses of cytokines and chemokines to *L. monocytogenes* infection in mice, we tested transcript levels of cytokines and chemokines in liver and spleen 24 or 72 h PI using real-time quantitative PCR. IL-1β, IL-6, IL-10, IL-12, MCP-1, MCP-3, TNF-α, and INF-γ in spleen were induced by the wild type or LmoH7858Δ0369 strain (Figure 4.5A) 24 h PI. However, there was no significant difference in the transcript levels of these cytokines/chemokines induced by wild type and mutant strain (Figure 4.5A). Notably, at 72 h PI, transcript levels of these cytokines/chemokines induced by LmoH7858Δ0369 were significantly lower than of those induced by the parent strain (Figure 4.5A). Interestingly, compared to 24 h PI, IL-10 mRNA level was elevated at 72 h PI while the transcript levels of other proinflammatory cytokines such as TNF-α and IL-12 were significantly decreased (Figure 4.5A). In liver, the mean transcript levels of IL-1β, IL-6, IL-10, MCP-1, MCP-3, TNF-α and INF-γ were also significantly higher than uninfected at 24 h PI, although slightly lower overall induction fold was observed comparing to spleen (Figure 4.5B). At 72 h PI, mRNA level of IL-6 and INF-γ induced by LmoH7858Δ0369 strain were significantly lower than that induced by H7858 wild type (Figure 4.5B).

Discussion

L. monocytogenes serotype 4b strains or isolates cause more than 50% of human listeriosis worldwide, and thus are considered more adapted to mammalian hosts than other serotypes (Farber and Peterkin 1991; Kathariou 2003). Two complete sequenced 4b strains, F2365 and H7858 used in this study are responsible for the listeriosis outbreak of

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1985 in California and a multistate outbreak of 1998-1999 in the USA, respectively (Mascola, Lieb et al. 1988; 1998). Interestingly, we found that the invasion rate of F2365 is significantly lower than H7858 strain but similar with H7858ΔInlB strain (Figure 4.2A, B), indicating the low pathogenic phenotype of F2365. F2365 belongs to the epidemic clone I (ECI), which is a large cluster of 4b strains that involve in several large outbreaks (Kathariou 2002). However, F2365 has point mutations on around 20 genes including *InlB*, (Nelson, Fouts et al. 2004), which may account for its virulence attenuation. Our results show F2365 strain demonstrated lower invasion rate on Caco-2 comparing to other genetically related 4b strains, and the results are consistent with the study of Nightingale et al. (Nightingale, Milillo et al. 2007). This type of virulence attenuation that induced by point mutations on key virulence factors were also reported on low virulent *L. monocytogenes* strains that were isolated from raw food (Temoin, Roche et al. 2008; Lopez, Navas et al. 2013).

Despite the structural similarity within LRR repeats, which are known as the "internalin-LRR-prototype motif" (Bierne, Sabet et al. 2007), proteins with LRRs in *L. monocytogenes* have diverse functions and play their roles in bacterial pathogenesis in multiple ways, which cover cell invasion, innate immune modulation, and counteracting host autophagy (Cossart 2011). In our study, deletion of *LMOh7858_0367, LMOh7858_0394, LMOh7858_0798,* and *LMOh7858_0574*

LRRs gene in *L. monocytogenes* did not affect their invasiveness into host cells, which indicated that either functional redundancy of the internalin proteins exists or these LRRs proteins bear other functions than invasion. Interestingly, the LmoH7858_Δ0369 strain showed decreased invasiveness to Hep-G2 cells but not to Caco-2 cells, indicating

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that there might be a cell type-specific cellular receptor mediating the invasion process, which has been shown with InlA, InlB, and other virulence factors such as vip (Cabanes, Sousa et al. 2005; Pizarro-Cerda and Cossart 2006).

The critical role of LmoH7858_0369 to the virulence of *L. monocytogenes* H7858 strain is revealed by cell invasiveness, mouse organ bacterial loading, as well as cytokine responses in mice. Our study suggested that LmoH7858_0369 is one of the virulence factors that contributes to pathogenesis in hosts. In our study, bacteremia was not found at 24 h PI, which is consistent with previous study using EGD strain as inoculums (Berche 1995). However, we did observed bacteremia formation at 72 h PI using H7858 parent strain and LmoH7858Δ2546 strain while a similar dose of EGD strain did not induce bacteremia at 72 h PI (Berche 1995), which suggests that H7858 strain might be more pathogenic than the EGD strain in the mouse model. Interestingly, bacteremia is not observed in mice infected with LmoH7858Δ0369 at 72 h PI indicating that bacteremia is associated with the bacterial load in liver and spleen. Further observation on brain infection strongly suggested that the success of CNS invasion depends on the formation of bacteremia, which is consistent with the previous report (Berche 1995). The failure of the LmoH7858_Δ0369 to invade the CNS suggests that the gene may be associated with CNS invasion or just due to a lower bacterial load in liver and spleen.

L. monocytogenes is known to induce cytokine (IL-1β, IL-6, IL-10, IL-12, MIP-1α, MCP-1, TNFα, and INFγ) expression rapidly and dramatically in infected mice (Nakane, Numata et al. 1992; Holub, Cheng et al. 2009; Stavru, Archambaud et al. 2011). In our study, robust induction of cytokine expression was observed at 24 h post infection in both liver and spleen. Interestingly, induction of cytokine transcript levels by

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LmoH7858Δ0369 strain were lower than those of wild type strain in spleen at 72 h PI, which is consistent with previous study indicating that cytokine production is correlated with bacterial load in organs (Kim, Reilly et al. 2001). Induction of proinflammatory cytokines INF-γ and TNF-α is essential for restriction of bacterial replication and early control of *L. monocytogenes* infection (Pamer 2004). In our study, INF-γ and TNF-α were both robustly induced at 24 h PI, however, the transcript levels of TNF-α were back to normal in both liver and spleen at 72 h post infection, which is consistent with a previous report indicating that TNF-α mRNA reaches a peak at 24 h PI and then gradually decreases in murine listeriosis model (Poston and Kurlander 1992). IL-6 controls the innate immune response to *L. monocytogenes* infection through classical IL-6 signaling (Hoge, Yan et al. 2012) and plays a critical role in *L. monocytogenes* clearance (Kopf, Baumann et al. 1994; Dalrymple, Lucian et al. 1995) at least partially by modulating neutrophil function (Dalrymple, Lucian et al. 1995; Mocci, Dalrymple et al. 1997). A previous study indicated that the ΔInlH mutant strain enhances the production of IL-6 in infected tissue, indicating the role of InlH in balancing IL6 expression and thus evading host innate immune response (Personnic, Bruck et al. 2010). In our study, IL-6 induced by LmoH7858Δ0369 strain is lower in both liver and spleen than the wild type at 72 h post infection. However, IL-6 mRNA expression in liver induced by the LmoH7858Δ0369 strain at 24 h post infection was statistically higher than that induced by the wild type (Figure 4.5B). Thus, whether LmoH7858Δ0369 has a function similar to InlH, which is playing a role on modulating IL-6 response, needs to be further investigated.

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In summary, our study identified a novel 4b specific internalin in H7858 strain that is required for hepatocytes invasion. An attenuated phenotype was observed in LmoH7858Δ0369 strain, particularly in mouse organ colonization. These findings reveal the role of LmoH7858_0369 as one of critical factor that contributes to virulence of *L. monocytogenes*.

Table 4.1 Strains used in LRRs study

Strain	Serotype/description	Resource
H7858	4b	Frankfurter
F2365	4b	Jalisco cheese
PRL-NW3556	4b	Human
PRL-NW3555	4b	Human
PRL-NW1625	1/2b	Human
PRL-NW3324	1/2b	Human
PRL-NW3664	1/2b	Human
PRL-NW1598	1/2b	Human
PRL-NW1608	1/2a	Human
PRL-NW1610	1/2a	Human
PRL-NW1622	1/2a	Human
EGD	1/2a	Rabbits
H7858_Δ0367	*H7858_0367* deletion mutant, Tet^r	this study
H7858_Δ0369	*H7858_0369* deletion mutant, Tet^r	this study
H7858_Δ0394	*H7858_0394* deletion mutant, Tet^r	this study
H7858_ΔInlB	*H7858_0499* deletion mutant, Tet^r	this study
H7858_Δ0574	*H7858_0574* deletion mutant, Tet^r	this study
H7858_Δ0798	*H7858_0798* deletion mutant, Tet^r	this study
H7858_Δ2546	*H7858_2546* deletion mutant, Tet^r	this study

Table 4.2 Comparison of deletion candidates between different strains of *L. monocytogenes* and *L. innocua*

H7858	F2365	EGD-e	Clip11262
LMOh7858_0367	*LMOf2365_0347*	*Lmo0331*	*lin0354*
LMOh7858_0369	*LMOf2365_0349*	---	---
LMOh7858_0394	*LMOf2365_0374*	---	*lin0372*
LMOh7858_0499	*LMOf2365_0472*	*inlB (lmo0435)*	--
LMOh7858_0574	*LMOf2365_0543*	*Lmo0514*	*lin0514*
LMOh7858_0798	*LMOf2365_0768*	*Lmo0732*	*lin0741*
LMOh7858_2546	*LMOf2365_2370*	*Lmo2396*	*lin2459*

Comparison was performed using NCBI BLAST program

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Table 4.3 Primer list for deletion mutagenesis

LMOh7858_0367 upstream forward	GCGCggatccTATCGAAGGCTGCTCAGTGG
LMOh7858_0367 upstream reverse	GCGCgtcgacACTAAGCGTGGCAGAATCAG
LMOh7858_0367 downstream forward	GCGCctcgagTACCATTGGTATCGGTAGGCT
LMOh7858_0367 downstream reverse	GCGCagatctGAGAGCTACTTTAGGCTCTAAC
LMOh7858_0369 upstream forward	GCGCggatccTACCATTGGTATCGGTAGGCT
LMOh7858_0369 upstream reverse	GCGCgtcgacGAGAGCTACTTTAGGCTCTAAC
LMOh7858_0369 downstream forward	GCGCctcgagATTACCAGCTACAGGCGATGA
LMOh7858_0369 downstream reverse	GCGCagatctGCAAGATTGTCGTCTGGAAAC
LMOh7858_0394 upstream forward	GCGCggatccCCAAGATGCAGGACTTCCTGT
LMOh7858_0394 upstream reverse	GCGCgtcgacCTGTCGCTAAAGCATCATCG
LMOh7858_0394 downstream forward	GCGCctcgagCCACATCGTACTACCAGCAAC
LMOh7858_0394 downstream reverse	GCGCagatct TTCCTTCGTACTTCCGAATATAA
LMOh7858_0574 upstream forward	GCGCggatcc GGCGAATATTTCTGCCATTTGC
LMOh7858_0574 upstream reverse	GCGCgtcgacCACTTGCTTGCTTGAGCAGAC
LMOh7858_0574 downstream forward	GCGCctcgagGGTGATACTAATAAGAGCACCT
LMOh7858_0574 downstream reverse	GCGCagatctATTCAGATGGTAGTACGACAGA
LMOh7858_InlB upstream forward	GCGCggatccGGTAGTTACGCAGAACCTGAT
LMOh7858_InlB upstream reverse	GCGCgtcgac ATATTTCCTTCTTGGGTTGTGC
LMOh7858_InlB downstream forward	GCGCctcgag ATTGTCGATCGAACAGCCAC
LMOh7858_InlB downstream reverse	GCGCagatct TTGTTACGCAAGAAAGTGTGC
LMOh7858_0798 upstream forward	GCGCggatccGATGAGGAATCATTCTGTTGCA
LMOh7858_0798 upstream reverse	GCGCgtcgacAATGTTAAATCCACACCGACCT
LMOh7858_0798 downstream forward	GCGCctcgagTGGGATAGTGTGCTGTATAGT
LMOh7858_0798 downstream reverse	GCGCagatctCGGTACGTAGAATCAATTGTCT
LMOh7858_2546 upstream forward :	GCGCggatccGGTTGTACCCGTGATTTCAC
LMOh7858_2546 upstream reverse	GCGCgtcgacACTACATCAGTTGATGCTGCC
LMOh7858_2546 downstream forward	GCGCctcgagCTGGTGATAATCAACAAGATAG
LMOh7858_2546 downstream reverse	GCGCagatctCGTACACATTGACGATGGAG

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Table 4.4 Primers for real-time PCR

Gene	Sequence	Reference
β-Actin F	CGTGCGTGACATCAAAGAGAA	(Yamakawa, Kojima et al. 2011)
β-Actin R	TGGATGCCACAGGATTCCAT	
IL-1 β F	CATCCAGCTTCAAATCTCGCAG	(Salguero Palacios, Roderfeld et al. 2008)
IL-1 β R	CACACACCAGCAGGTTATCATC	
IL-6 F	ACGGCCTTCCCTACTTCACA	(Yamakawa, Kojima et al. 2011)
IL-6 R	CATTTCCACGATTTCCCAGA	
IL-10 F	GGTTGCCAAGCCTTATCGGA	(Becher, Durell et al. 2002)
IL-10 R	ACCTGCTCCACTGCCTTGCT	
IL-12 F	CAGCTCGCAGCAAAGCAA	(Holscher, Atkinson et al. 2001)
IL-12 R	GACGCCATTCCACATGTCACT	
TNFα F	GCCTCTTCTCATTCCTGCTTG	(Yamakawa, Kojima et al. 2011)
TNFα R	CTGATGAGAGGGAGGCCATT	
INFγ F	TCAAGTGGCATAGATGTGGAAGAA	(Wuttge, Zhou et al. 2004)
INFγ R	TGGCTCTGCAGGATTTTCATG	
CCL-2 (MCP-1) F	TCTTCCTCCACCACCATGC	(Baran, Opalek et al. 2007)
CCL-2 (MCP-1) R	TCATTGGGATCATCTTCGTGG	
CCL-7 (MCP-3) F	TCTGCCACGCTTCTGTGCCT	(Bry, Whitsett et al. 2007)
CCL-7 (MCP-3) R	GCTCTTGAGATTCCTCTTGGGGAT	

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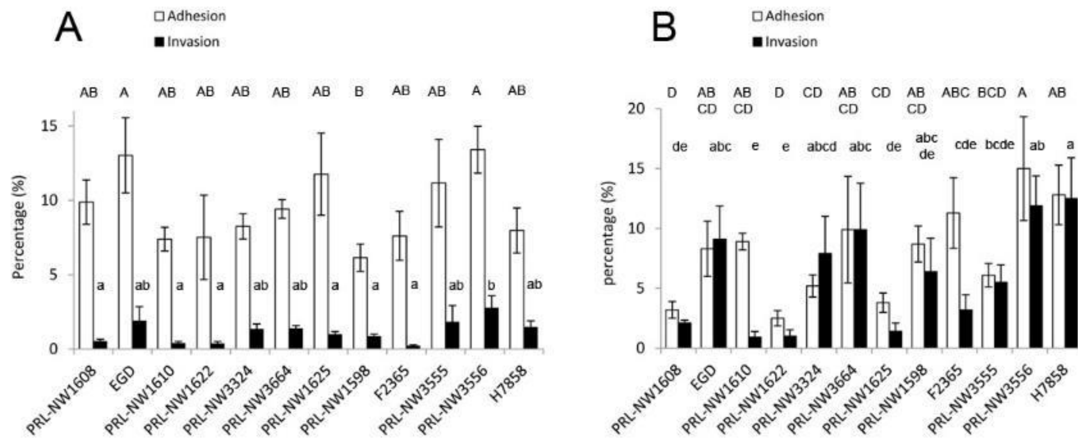

Figure 4.1 Adhesion and invasion of *L. monocytogenes* to Hep-G2 and Caco-2 cells

Adhesion and invasion rates of 12 *L. monocytogenes* strains/ isolates including three serotypes 1/2a, 1/2b, and 4b (four strains/isolates per serotype) were assessed on Hep-G2 cells (A) and Caco-2 cells (B). For adhesion assay, cells were infected with bacteria at M.O.I. of 50:1 for 1 hour. For invasion assay, cells were further incubated with medium containing 100µg/ml gentamicin for 1 hour to kill the extracellular bacteria. Data are mean and SEM of three biological replicates with two technical replicates for each cell line. (N=3) Analysis of variances (ANOVA) with Tukey's multiple comparisons posttests were used to calculate the *p* value. Different letters represent statistical difference ($p<0.05$)

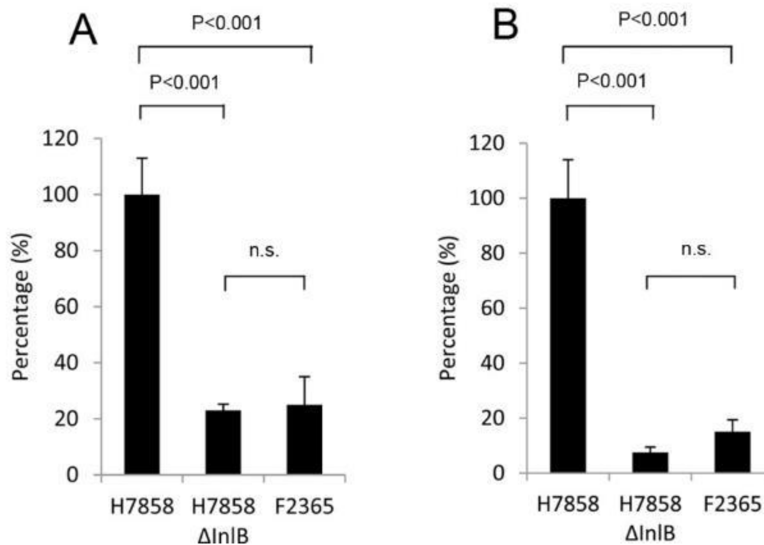

Figure 4.2 Role of InlB in *L. monocytogenes* on invasion of host cells

Invasion of *L. monocytogenes* H7858, H7858ΔinlB, and F2365 strain to Caco-2 cells (A) and Hep-G2 cells (B). Cells were infected with bacteria at M.O.I. of 50:1 for 1 hour, and incubated with medium containing 100µg/ml gentamicin for 1 hour to kill the extracellular bacteria. Invasion rates of all strains are given relative values to the H7858 strain. Data are mean and SEM of three biological replicates with two technical replicates for each cell line. (N=3) Analysis of variances (ANOVA) with Tukey's multiple comparisons posttests were used to calculate the *p* value.

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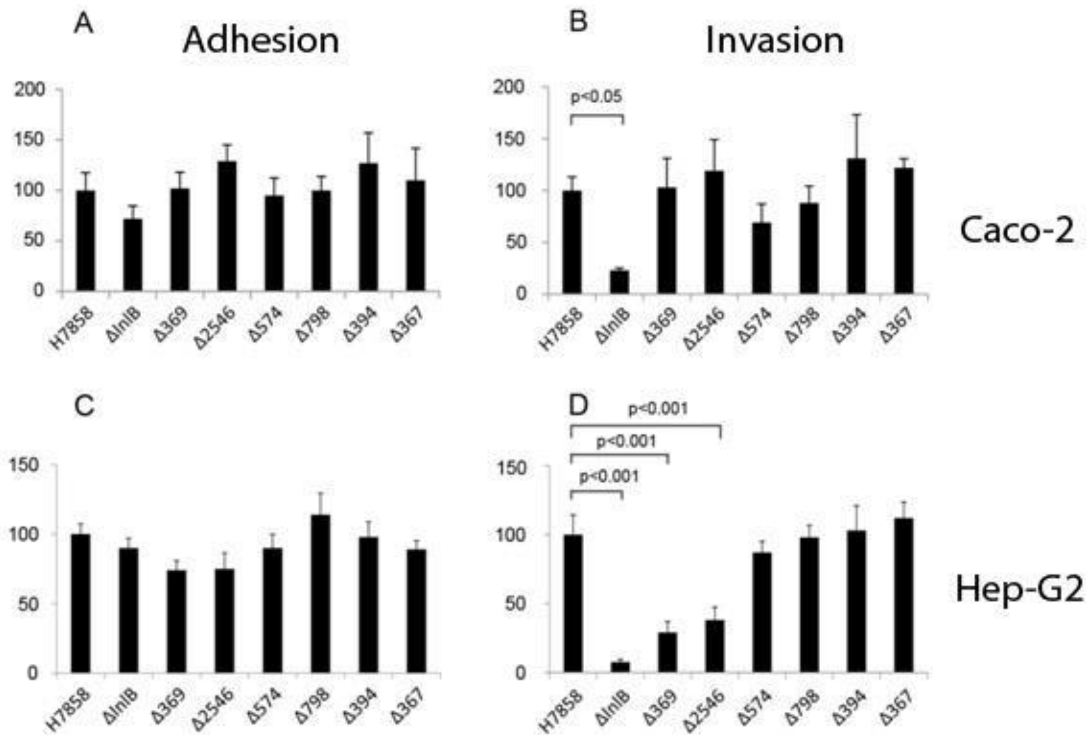

Figure 4.3 Adherence and invasiveness of H7858 wild type and LRRs deletion mutants

Adhesion and invasion of H7858 wild type strain and 7 LRRs deletion mutants were assessed on Hep-G2 cells and Caco-2 cells. Caco-2 and Hep-G2 cell monolayers were infected with *L. monocytogenes* wild type and mutant strains at M.O.I. of 50:1. Adhesion and invasion rates to Caco-2 cells (A: adhesion, and B: invasion) and Hep-G2 Cells (C: adhesion, and D: invasion) are given relative values to adhesion and invasion rate of H7858 wild type, respectively. Data are mean and SEM of three biological replicates with two technical replicates for each cell line. (N=3) Analysis of variances (ANOVA) with Tukey's multiple comparisons posttests were used to calculate the p value.

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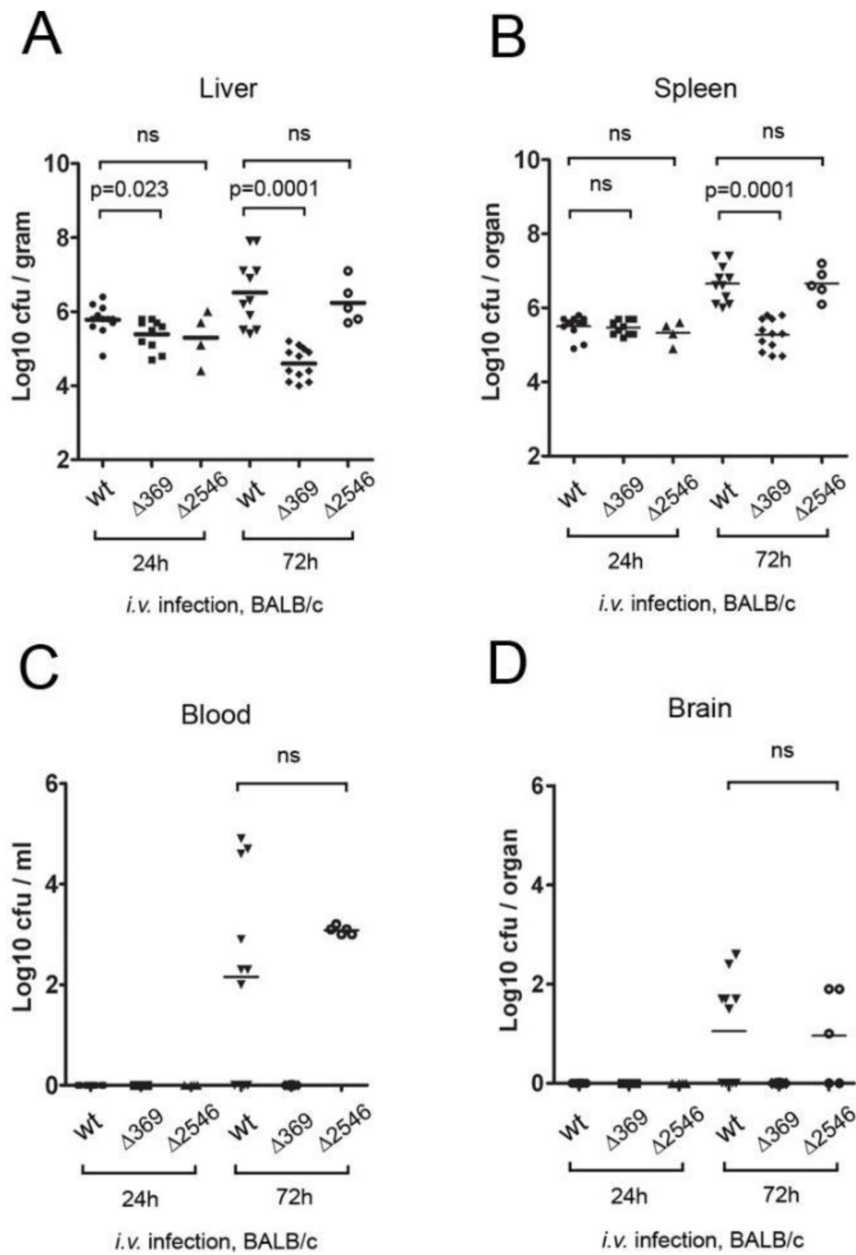

Figure 4.4 Virulence of LmoH7858Δ0369 strain in mice

Ten to twelve weeks old BALB/c mice were infected intravenously with PBS or 6 x10^3 *L. monocytogenes* H7858 wild type, LmoH7858Δ0369, and LmoH7858Δ2546 strains, respectively. At 24 h and 72 h post infection, bacterial counts in spleen (A), liver (B), blood (C), and brain (D) were calculated. Data are results from two independent experiments (N= 10 to 12) for H7858 parent strain and LmoH7858Δ0369 strain, and one independent experiment (N= 4-5) for LmoH7858Δ2546 strain. Mann-Whitney tests were used to calculate the *p* value.

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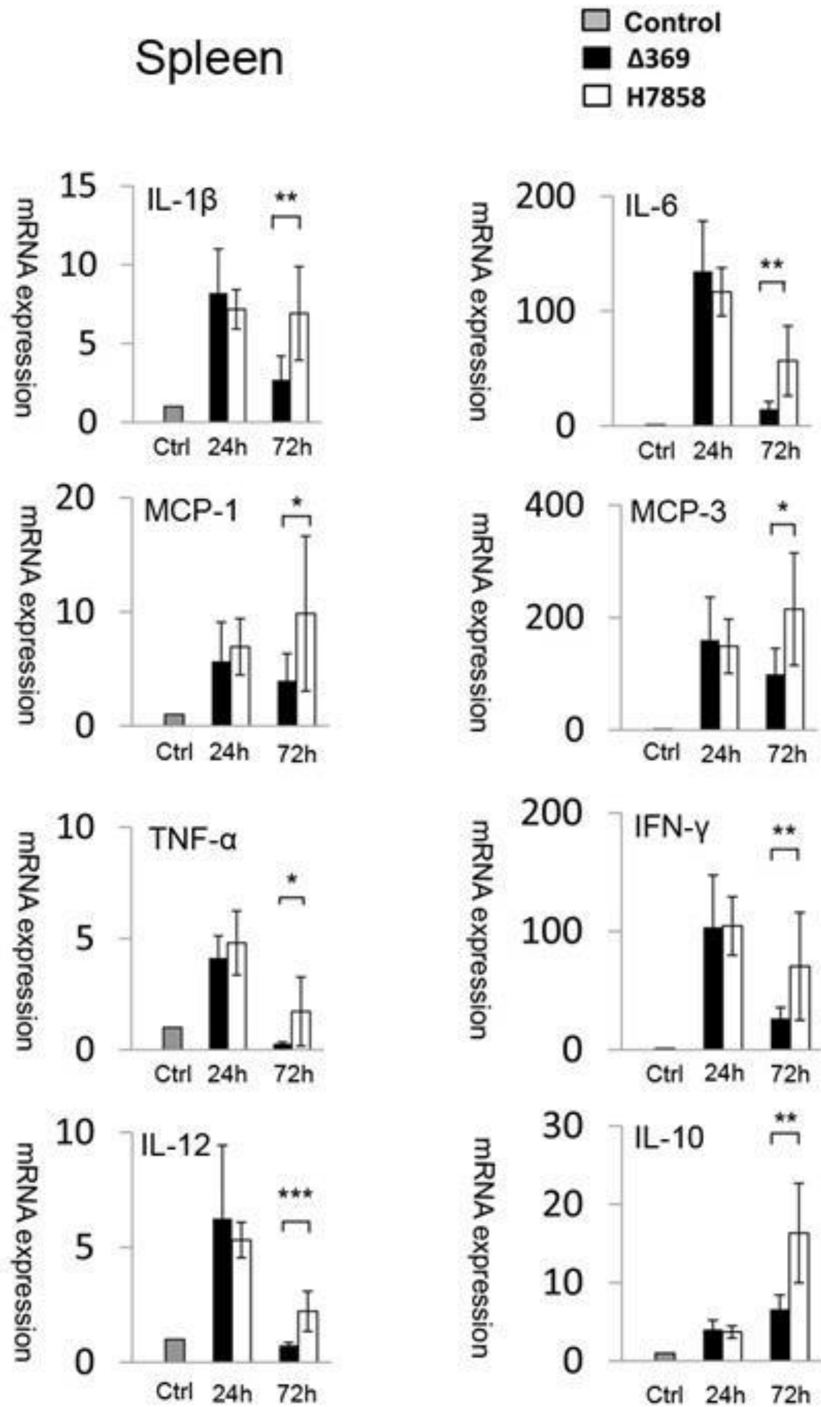

Figure 4.5 Cytokine and chemokine responses induced by ΔLmoH7858_0369 strain and H7858 wild type strain

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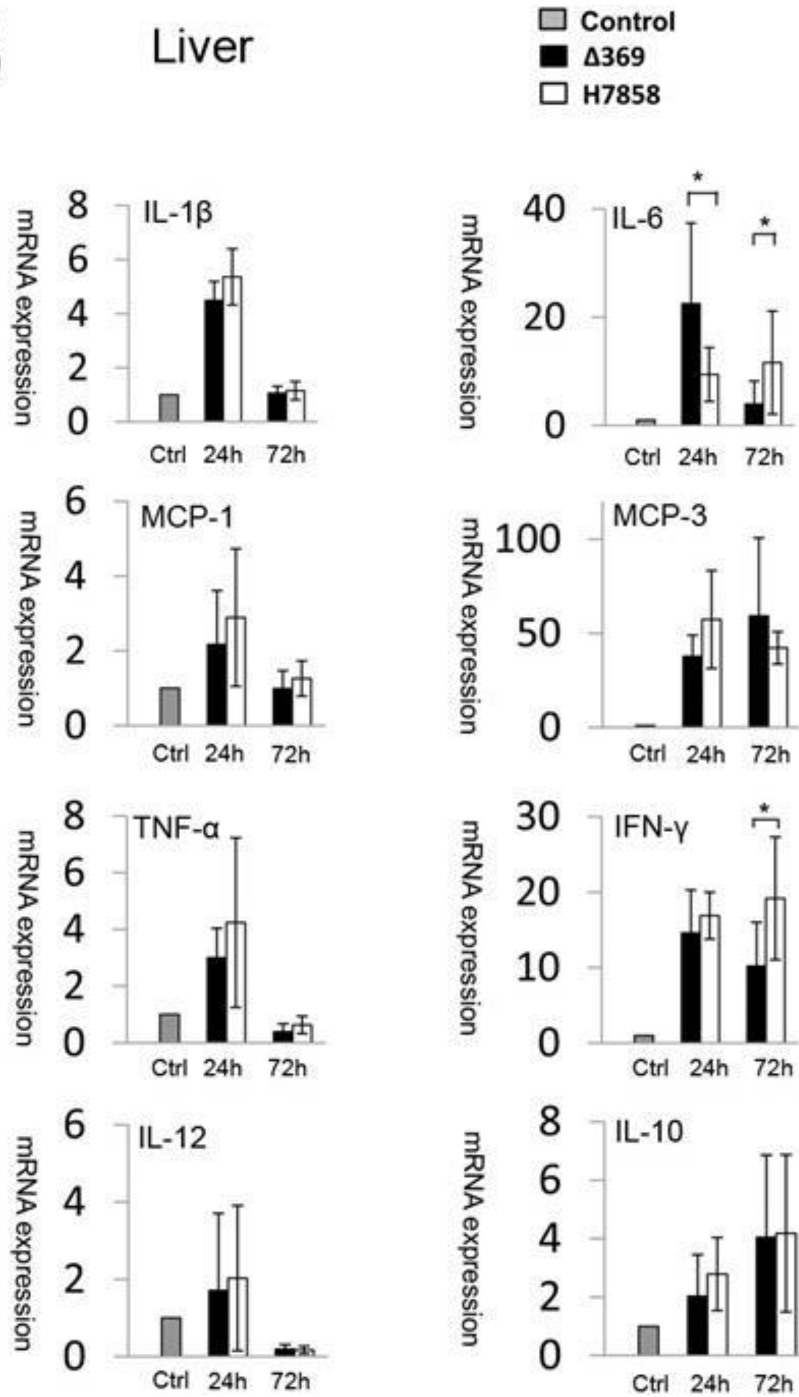

Figure 4.5 (continued)

Transcripts level of cytokines and chemokines in spleen (A) and liver (B) of infected mice induced by ΔLmoH7858_0369 strain and H7858 wild type strain were evaluated using real-time quantitative PCR. The level of cytokine/chemokine transcripts were normalized with housekeeping gene β-actin and further compared to PBS injected mice as control. Data are mean and SD of results from 4-5 mice. (N=4-5) T-tests were used to calculate the p value. (* $p<0.05$, ** $p<0.01$, and *** $p<0.001$)

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CHAPTER V

CHARACTERIAZTION OF SECRETED PROTEINS IN *L. MONOCYTOGENES* THAT ARE INVOLVED IN CNS INFECTION

Abstract

Targeting and dissemination through brain microvascular endothelium is considered as one of the routes for *L. monocytogenes* to cause CNS infection. To date, the molecular mechanisms of how *L. monocytogenes* interact with brain endothelium and cross the blood brain barrier are not completely understood. Due to the low invasiveness of *L. monocytogenes* on human brain microvascular endothelial cells (HBMEC), we hypothesized that overcome of HBMECs layer by *L. monocytogenes* is mediated by other mechanism such as the cytotoxic effect of listerial toxins rather than direct invasion. In this study, secreted proteins were collected from bacterial culture supernatant using ethanol precipitation and their cytotoxicity effect on HBEMCs was evaluated using live/dead staining method. Interestingly, cytotoxicity was induced by supernatant from the EGD wild type strain but the supernatant from the isogenic Δ*prfA* strain did not induce cytotoxicity. Therefore, we further investigated the cytotoxicity effects of supernatants from the isogenic mutant strains (Δ*plcA*, Δ*mpl,* and Δ*hly*) that are incapable of producing the bacterial products (PlcA, Mpl, and LLO, respectively) that are under regulation of prfA. Using fluorescent microscopy and flow cytometry, supernatants from mutant strain Δ*hly* did not induce the cytotoxicity on HBMEC but Δ*plcA* and Δ*mpl* did.

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The results suggest that LLO mediated the cytotoxicity on brain endothelial cells may contribute to the invasion of CNS by *L. monocytogenes.*

Introduction

Listeria monocytogenes is a gram positive pathogen that causes invasive diseases in the central nervous system of both human and ruminants, leading to clinical manifests such as: meningitis, meningoencephalitis, brain abscesses and rhombencephalitis (Vazquez-Boland, Kuhn et al. 2001; Oevermann, Zurbriggen et al. 2010; Disson and Lecuit 2012). Although, the incidence of bacterial meningitis is low, *L. monocytogenes* is one of the top five causative agents of bacterial meningitis and it has the highest fatality rate (18.1%) among the five pathogens (Thigpen, Whitney et al.). There are three major routes that allow *L. monocytogenes* to cross the blood-brain barrier (BBB), including a neural retrograde migration route, invasion of brain endothelium, and through trafficking of infected monocytes which is known as the “Trojan horse” mechanism (Drevets, Leenen et al. 2004; Drevets and Bronze 2008; Oevermann, Zurbriggen et al. 2010). Brain microvascular endothelial cells are closely joined with each other via tight junctions, which are the key component of the blood-brain barrier (Disson and Lecuit 2012). In an electron microscopy study, *L. monocytogenes* adhered to the luminal side of the vascular endothelium in the central nervous system (Kirk 1993). Perivascular lesions were observed in histology slides from *L. monocytogenes* infected brain of ruminants, although bacteria were not found around the lesion (Jungi, Pfister et al. 1997). *In vitro* studies showed that *L. monocytogenes* were able to directly invade both SV40 large-T antigen transformed immortal human brain microvascular endothelial cells (Greiffenberg, Goebel et al. 1998; Greiffenberg, Goebel et al. 2000), and a primary culture of human

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brain microvascular endothelial cells (HBMEC) (Wilson and Drevets 1998). However, a later study showed that antibodies present in human serum severely blocked the entry of *L. monocytogenes* to HBMECs (Hertzig, Weber et al. 2003), indicating direct invasion through endothelial cells to cause CNS infection was hindered by the presence of antibody.

Bacterial virulence products, such as listeriolysin O (LLO), Phosphatidylinositol-Specific Phospholipase C (PI-PLC), Phosphatidylcholine-specific Phospholipase C (PC-PLC), and metallopreotease (Mpl) that are under regulation of the positive regulatory transcription factor A (PrfA), play a critical role in host cell survival and cell to cell spread. LLO, a cholesterol-dependent pore-forming toxin, plays multifunctional roles during the infection process of *L. monocytogenes* (Kayal and Charbit 2006; Schnupf and Portnoy 2007; Hamon, Ribet et al. 2012). It is critical for *L. monocytogenes* to escape from the primary vacuole (Portnoy, Jacks et al. 1988; Schnupf and Portnoy 2007), and is essential for immune modulation (Hamon, Ribet et al. 2012). In addition, LLO is responsible for a novel form of hepatocyte invasion via inducing the formation of membrane extensions (Dramsi and Cossart 2003; Vadia, Arnett et al. 2011). LLO exhibits strong apoptogenic function, which induces cell death in T cells and dendritc cells (DC) (Guzman, Domann et al. 1996; Carrero, Calderon et al. 2004). Interestingly, studies showed that direct infection of cells by *L. monocytogenes* and bacterial culture supernatants from an LLO positive strains could both induce cytotoxic effects on human PBMC (Stachowiak, Lyzniak et al. ; Glomski, Decatur et al. 2003), although less virulence was observed in mice with those strains that had stronger cytotoxicity effects (Glomski, Decatur et al. 2003). LLO was also reported for disrupting intestinal barrier

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function by inducing secretion of chloride in colon epithelial cells (Richter, Gitter et al. 2009).

The two phospholipases PI-PLC and PC-PLC facilitate the vacuolar escape activity of LLO and play a significant role in cell to cell spread (Vazquez-Boland, Kocks et al. 1992; Camilli, Tilney et al. 1993; Smith, Marquis et al. 1995). Meanwhile, PC-PLC and Mpl, a metalloprotease that is required for functional maturation of PC-PLC, mediate vacuolar escape in some cultured human cells where the role of LLO is dispensable (Marquis, Doshi et al. 1995; Grundling, Gonzalez et al. 2003; Yeung, Zagorski et al. 2005). Interestingly, a PC-PLC constitutively activated *L. monocytogenes* strain increased membrane permeability of host cells during intracellular growth, indicating the role of PC-PLC in damaging host cell membranes (Yeung, Na et al. 2007). In addition, an *in vivo* experiment using the intracerebrally *(i.c.)* infection route indicated that PC-PLC are essential for bacterial spread in brain of mice (Schluter, Domann et al. 1998)

In this study, we found HBMECs were weakly permissive to all 12 tested *L. monocytogenes* strains/isolates. We examined the cytotoxicity effects of bacterial culture supernatants on HBMECs. We found that supernatants from Δ*hly* strain lack the cytotoxicity but Δ*plcA* and Δ*mpl* mutant strains retain cytotoxic effects on HBMECs. These studies revealed a new invasion mechanism of HBMECs that may be exploited by *L. monocytogenes* to cause CNS infection.

Materials and methods

Bacteria strains and cell culture conditions

Listeria strains used in this study were listed in Table 1. *L. monocytogenes* EGD strain and isogenic muntants: Δ*mpl*, Δ*plcA*, and Δ*prfA* strains were kindly provided by

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Dr. Michael Kuhn in the Lehrstuhl für Mikrobiologie, Theodor-Boveri-Institut für Biowissenschaften der Universität Würzburg, Germany. *L. monocytogenes* strains were cultured in BHI broth (Difco Laboratories, Detroit, MI) at 37°C. *E. coli* DH5α were cultured in Luria-Bertani (Difco) broth. To make the deletion mutant, transformed *L. monocytogenes* were selected on BHI agar plates containing erythromycin (5 μg/ml) or tetracycline (10 μg/ml). Human brain microvascular endothelial cells (HBMECs) were purchased from Cell Systems Corporation (CSC, Kirkland, WA) and cultured in CSC complete medium supplemented with cell culture boost (CSC, Kirkland, WA). Cells were cultured at 37°C in a 5% CO2 incubator.

Invasion assay adhesion assay

Primary HBMECs were purchased from Cell Systems Corporation (CSC, Kirkland, WA) and were seeded to 24-well plates and incubated in a 37 °C 5% CO_2 incubator. Mid-log phase (OD_{600nm} 0.4~0.5) *L. monocytogenes* were collected by centrifugation, washed twice with PBS and resuspended with CSC minimum medium without serum(CSC, Kirkland, WA). Cells were infected with each *L. monocytogenes* strain/isolate at multiplicity of infection of 50:1 for 1 hour. For adhesion assay, infected cells were washed five times with PBS, and then lysed with 0.25% Triton X-100. Cell lysates were diluted and plated on BHI agar for colony count. For invasion assay, infected cells were further incubated with CSC complete medium containing gentamicin (100 μg/ml) for 1 hour to kill the extracellular bacteria. Cells were washed three times with PBS and then lysed with 0.25% Triton X-100. Cell lysates were serial diluted and plated on BHI agar to count the viable bacteria.

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LLO deletion mutagenesis

pMAD, a temperature-sensitive shuttle plasmid, was used to generate the deletion LLO mutant. From the genomic DNA of *L. monocytogenes* H7858 strain, the upstream and the downstream regions flanking *hly* gene were amplified using PCR with the primers listed in Table 2. PCR products were digested with BamHI/SalI and XhoI/BglII, respectively, and cloned in tandem into a pMAD_tet plasmid. The recombinant plasmid was introduced into *L. monocytogenes* by electroporation at 2.5 KV, 400 Ω, and 25 µF. Deletion of the *hly* gene was conducted by allelic exchange. Transformed bacteria were incubated at 43°C for 48 hours for the first integration and colonies were inoculated into antibiotic-free BHI media at 30°C and passage three times (1 day per passage) for the second recombination. Deletion mutants were selected with BHI agar plates containing erythromycin (5 µg/ml) or tetracycline (10 µg/ml) and confirmed with PCR using the primers harboring the deleted region of *hly* genes.

Supernatant of bacterial culture medium

L. monocytogenes EGD wild type and isogenic mutant strains were inoculated to BHI broth and cultured in 37°C for 16 hours, respectively. Bacterial cultures were centrifuged at 20,000g for 10 minutes. The resulting supernatants were mixed with ethanol (vol/vol at 1/4) and centrifuged at 20,000g to pellet the proteins. The pellets were washed three times with 80% ethanol, air dried and resuspended with sterile water. The resuspended supernatant fluids (10 times concentrated, 5 ml of bacterial culture supernatant to 0.5 ml of final solution in sterile water) were sterilized by filtration and used for the cytotoxicity test.

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Live/dead cell analysis by fluorescent microscopy

HBMECs were seeded into 24-well plates. For the cytotoxicity test, 0.1 ml of concentrated bacterial culture supernatants from *L. monocytogenes* EGD wild type strain, isogenic Δ*prfA*, Δ*hly*, Δ*plcA* or Δ*mpl* strain was added to HBMECs with 0.3 ml of CSC minimum medium. Cells were incubated at 37°C in a 5% CO_2 incubator for 20 minutes. For fluorescent microscopic analysis, 0.4 μM calcein AM and 2 μM EthD-1 working solution were added to the cells by following to the manufacturer's instructions (Invitrogen, Gland Island, CA). After 20 minutes incubation at room temperature, labeled samples were examined under a fluorescence microscope (Nickon, Tokyo, Japan).

Flow cytometry

To investigate the cytotoxicity effect, HBMECs were incubated with the previously prepared bacterial supernatant for 20 min and the cells were dissociated with trypsin and pelleted by centrifugation at 400 g. The cells were washed one time with PBS, and then 0.4 μM calcein AM and 2 mM ethidium homodimer-1 working solution (Invitrogen, Gland Island, CA) was added and incubated for 20 min at room temperature. Cells were pelleted and washed one time with PBS containing 5mM EDTA to remove the free dye and resuspended in PBS containing 0.2% BSA. Samples were analyzed with a BD flow cytometer (BD Biosciences, San Jose, CA) to determine the percentage of dead and live cells.

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Results

The permissiveness of human brain microvascular endothelial cells (HBMECs) to *L. monocytogenes.*

In order to access the adherence and invasion rates of various serotypes and strains/isolates of *L. monocytogenes*, we tested the adherence and entry of clinical isolates and reference strains (Table 5.1) to HBMECs primary cell culture. Adherence rates of 12 *L. monocytogenes* strains/isolates were reached to 0.5-1% to initial inoculums (Figure 5.1); however, to our surprise, the entry rates of all 12 strains were below 0.01%, which was more than 100 times lower than the adherence rates.

Cytotoxicity effect of extracellular proteins was regulated by prfA.

To investigate the involvement of extracellular proteins on HBMECs' cell membrane integrity, we tested cytotoxicity effects of supernatants from *L. monocytogenes* EGD wild type and isogenic mutant Δ*prfA*. Interestingly, while strong cytotoxicity was observed using the supernatant from the WT, HBMECs did not show cytopathic effect in presence of the supernatant from Δ*prfA* strain (Figure 5.3). The investigation of the proportion of damage versus intact cell membranes in the population was further evaluated using flow cytometry. By comparing to the control group, the mean fluorescent intensity (MFI) of the calcein AM channel, which reflects the membrane integrity of HBMECs, was not changed by the supernatant from Δ*prfA* strain, while MFI of calcein AM was dramatically decreased when the cells were incubated with supernatant from the wild type (Figure 5.5, A,B,C). These results indicated that prfA regulated extracellular proteins of *L. monocytogenes* that had cytotoxic effects on HBMECs.

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LLO mediated the cytotoxicity effect on HBMECs.

In order to determine the specific proteins that contributed to the cytotoxicity effect on HBMECs, we investigated the cytotoxicity effects of supernatants from isogenic mutant strains (Δ*plcA,* Δ*mpl,* and Δ*hly*) that are incapable of producing the bacterial products (PlcA, Mpl, and LLO, respectively) that are under regulation of prfA (Figure 5.2). Interestingly, all mutant strains except the Δ*hly* strain retained a strong cytotoxic effect on HBMEC (Figure 5.4). These results were further confirmed by flow cytometry (Figure 5.5D, 5.5C, and 5.5E), showing that HBMEC treated with supernatants from Δ*plcA* and Δ*mpl* strains had dramatically reduced MFI of calcein AM. HBMEC treated with supernatants from Δ*hly* strain showed a similar MFI of calcein AM when compared to that of the control group (Figure 5.5D, 5.5C, and 5.5E). These data indicated that LLO, a major extracellular protein of *L. monocytogenes* that encoded by *hly* gene, contributes to the cytotoxic effect on HBMECs.

Discussion

Breaking the blood-brain barrier (BBB) by the cytotoxic effects of bacterial/parasitic toxins/soluble factors was considered as a mechanism of causing CNS infection. For instance, pertussis toxin from *Bordetella pertussis* could induce a transient increase in permeability of HBMECs, indicating the role of toxin on disrupting the function of the physical barrier (Kugler, Bocker et al. 2007). Meanwhile, supernatant from *Plasmodium falciparum* infected erythrocytes are able to decrease the integrity of a monolayer of HBMECs (Tripathi, Sullivan et al. 2007). In *L. monocytogenes*, a similar mechanism was observed; for instance, LLO could disrupt intestinal barrier function by inducing secretion of chloride in colon epithelial cells (Richter, Gitter et al. 2009). Our

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study showed that LLO is capable of mediating cell lytic effect on HBMECs, indicating the probable role of LLO in CNS infection via disrupting the endothelial cells of the BBB *in vivo*. It was shown that bacteria can be found in the area around perivascular cuffs when cattle, goats and sheep were infected with *L. monocytogenes* (Jungi, Pfister et al. 1997). It was also been suggested that a transient change of endothelial cell permeability and function may be enough for bacteria to pass the endothelial layers (Kugler, Bocker et al. 2007). Thus, the contribution of LLO mediated cytotoxicity effects or dysfunction effect on brain endothelial cells is a possible mechanism for *L. monocytogenes* to cause CNS infection.

Direct invasion of brain microvascular endothelial cells by *L. monocytogenes* was documented *in vitro* and *in vivo* (Kirk 1993; Greiffenberg, Goebel et al. 1998; Wilson and Drevets 1998; Greiffenberg, Goebel et al. 2000). However, our study showed that all *L. monocytogenes* strains tested demonstrated very weak invasiveness to HBMECs in contrast to their robust adherence ability. To address the differences in invasiveness of *L. monocytogenes* to HBMEC between our studies and results from other groups, different cell sources and methodologies were found. In our study, primary cultures of HBMECs were used while SV40 large-T antigen transformed immortal HBMECs were used by the studies of Greiffenberg et. al and Hertzig et. al (Greiffenberg, Goebel et al. 1998; Greiffenberg, Goebel et al. 2000; Hertzig, Weber et al. 2003). Although Wilson et al and our study used HBMEC primary cells from the same source, a centrifugation step was used in the study by Wilson's group in order to ehance the loading of the bacteria to cells (Wilson and Drevets 1998). Despite the observation of Herzi et al. (Hertzig, Weber et al. 2003) that antibodies present in human serum severely blocked the entry of *L.*

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monocytogenes to HBMECs, direct invasion through endothelial cells is a limited and rather difficult route for CNS infection without support from contributing factors such as host immune status. This is supported by the fact that numerous bacteria were found in the brain of IgM deficient mice but less bacteria were found in the spleen (Ochsenbein, Fehr et al. 1999). Macrophage trafficking that spreads *L. monocytogenes* from infected phagocytes to endothelial cells is an efficient mechanism to cause CNS infection because this route can avoid exposure of bacteria to serum (Drevets, Sawyer et al. 1995; Greiffenberg, Goebel et al. 1998; Drevets, Jelinek et al. 2001). Alternative ways of crossing brain endothelial cells, such as breaking the endothelial layer through cytotoxic effects of listerial toxins should be considered rather than direct invasion. However, animal experiments are needed for further confirmation.

Ultrastructural analysis in a mouse model using the intracerebral (*i.c.*) infection route indicated that intracellular *L. monocytogenes* were found in macrophages, granulocytes, plexus epithelial cells, ependymal cells, and neurons of the brain (Schluter, Chahoud et al. 1996). The virulence factors involved in invasion of these target cells and the detailed CNS infection mechanism remains unclear. An epidemiology study investigating the expression of full length InlA appeared in clinical strains vs food isolates. Clinical isolates from patients with CNS infection expressed more full-length InlA (98%) comparing to those strains that cause bacteremia (93%), (Jacquet, Doumith et al. 2004), implicating that InlA may be involved in CNS infection. Interestingly, a study using intracerebrally (*i.c.*) infection route in mice reveal that PC-PLC, rather than InlA or InlB, is important for dissemination of *L. monocytogenes* in brain (Schluter, Domann et al. 1998). In addition, signature-tagged mutagenesis (STM) identified gtcA, a

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gene involved in cell wall components, showed attenuated brain infection phenotype (Autret, Dubail et al. 2001), although with the same gene mutants, attenuated liver and spleen colonization was observed (Autret, Raynaud et al. 2003). Our data suggested that LLO might be another candidate virulence factor for *L. monocytogenes* to cause CNS infection through LLO mediated cytotoxicity effects or dysfunction effect on brain endothelial cells, and is worthy of further *in vivo* studies.

Table 5.1 Strains used in toxin study

Strain	Serotype/description	Resource
H7858	4b	Human
F2365	4b	Human
PRL-NW3556	4b	Human
PRL-NW3555	4b	Human
PRL-NW1625	1/2b	Human
PRL-NW3324	1/2b	Human
PRL-NW3664	1/2b	Human
PRL-NW1598	1/2b	Human
PRL-NW1608	1/2a	Human
PRL-NW1610	1/2a	Human
PRL-NW1622	1/2a	Human
EGD	1/2a	Rabbits
EGD_ΔPrfA	EGD *prfA* deletion mutant, Erm^r	(Chakraborty, Leimeister-Wachter et al. 1992)
EGD_ΔMpl	EGD *mpl* deletion mutant,	(Böckmann R and Goebel W, unpublished)
EGD_ΔPlcA	EGD *plcA* deletion mutant, Erm^r	Domann, Ph.D Thesis, Universität Würzburg, 1992
EGD_ΔLLO	EGD *hly* deletion mutant, Tet^r	this study

Table 5.2 Primer list for deletion mutagenesis

LmoLLO upstream forward	GCGC<u>ggatcc</u>TATCGAAGGCTGCTCAGTGG
LmoLLO upstream reverse	GCGC<u>gtcgac</u>ACTAAGCGTGGCAGAATCAG
LmoLLO downstream forward	GCGC<u>ctcgag</u>TACCATTGGTATCGGTAGGCT
LmoLLO downstream reverse	GCGC<u>agatct</u>GAGAGCTACTTTAGGCTCTAAC

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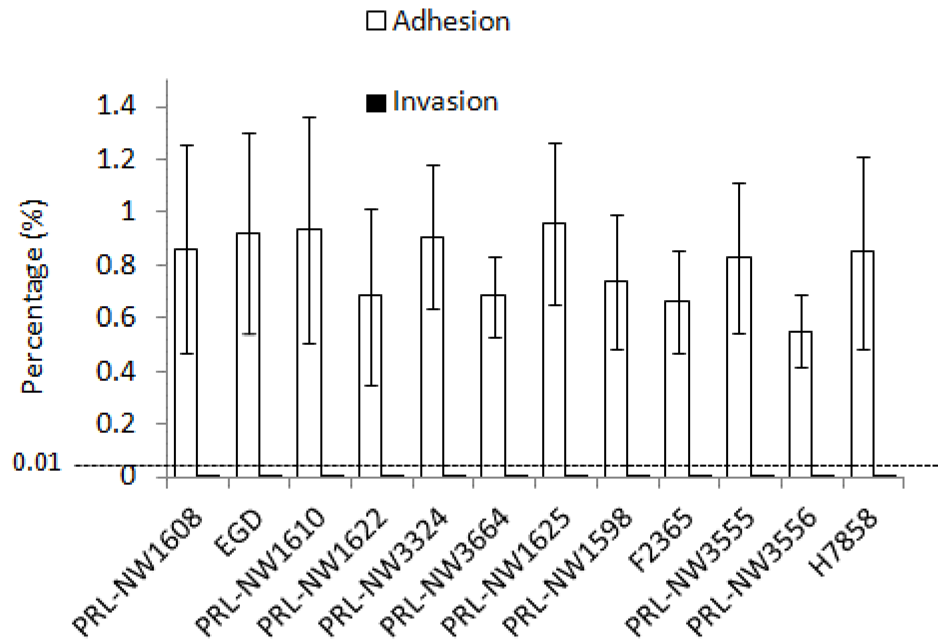

Figure 5.1 Adhesion and entry of *L. monocytogenes* strains/isolates with three different serotypes to HBMECs

Adhesion and entry rates of 12 *L. monocytogenes* including three serotypes 1/2a, 1/2b, and 4b (four strains/isolates per serotype) were assessed on HBMECs. For adhesion assay, cells were infected with bacteria at M.O.I. of 50:1 for 1 hour; for invasion assay, cells were further incubated with medium containing 100µg/ml gentamicin for 1 hour to kill the extracellular bacteria. Serially diluted cell lysates were cultured and the colony forming unit was calculated. Data represent mean and SEM of three biological independent experiments. (N=3)

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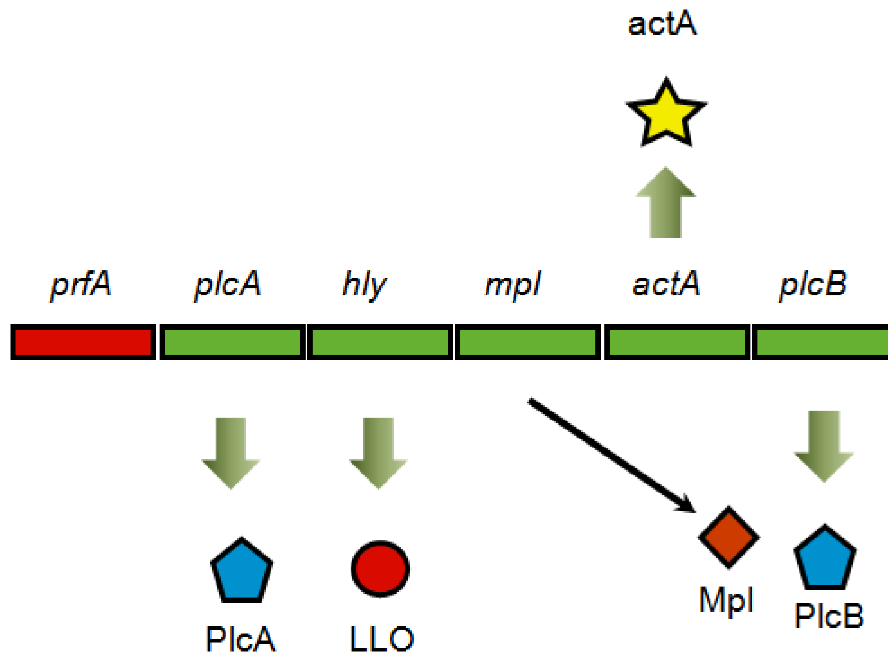

Figure 5.2 Genes encoding bacterial toxins within Listeria Pathogenicity Island 1(LIPI-1) are under regulation of PrfA.

Genes (*plcA, hly, mpl, and plcB*) that encode bacterial products (PlcA, LLO, Mpl, and PlcB, respectively) of *L. monocytogenes* are located within LIPI-1. The transcription of these genes is under regulation of a positive transcriptional regulator PrfA.

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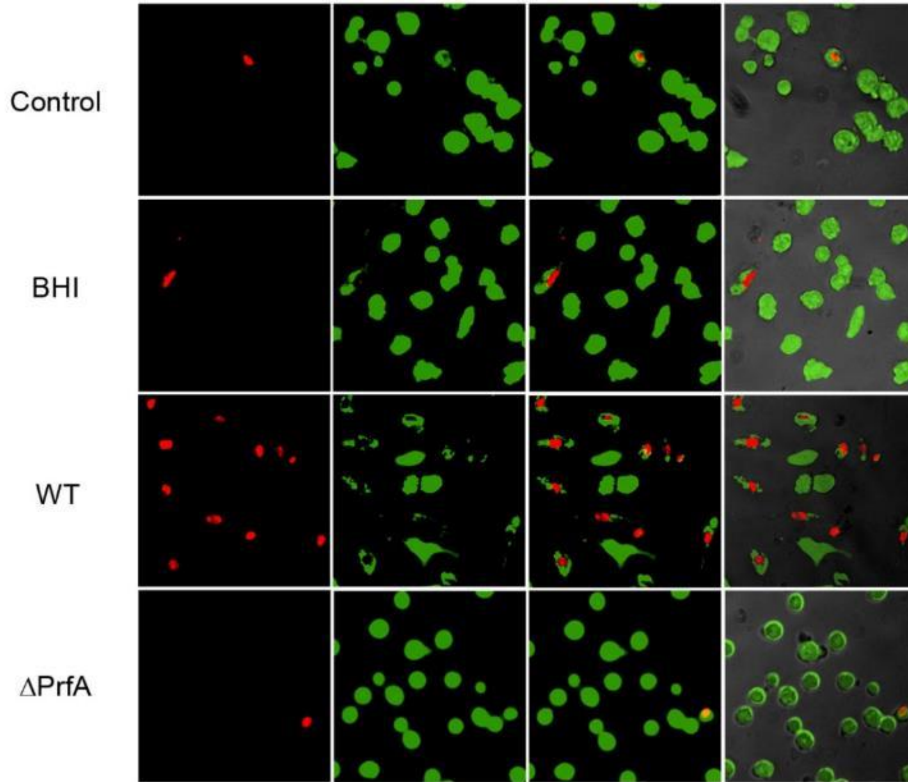

Figure 5.3 The cytotoxic effect of supernatant from Δ*prfA* strain you on HBMECs

Cytotoxicity effects on HBMECs were accessed using cell culture medium (control), BHI media (BHI), and supernatants from EGD wild type (WT) and Δ*prfA* strain (ΔprfA). HBMECs were incubated with cell culture medium, BHI medium and concentrated bacterial culture supernatant at 37°C in a 5% CO2 incubator for 20 minutes. Cells were labeled with calcein AM and EthD-1 to visualize cell membranes and nuclei. Images are representative results of two biological replicates and with two technical replicates.

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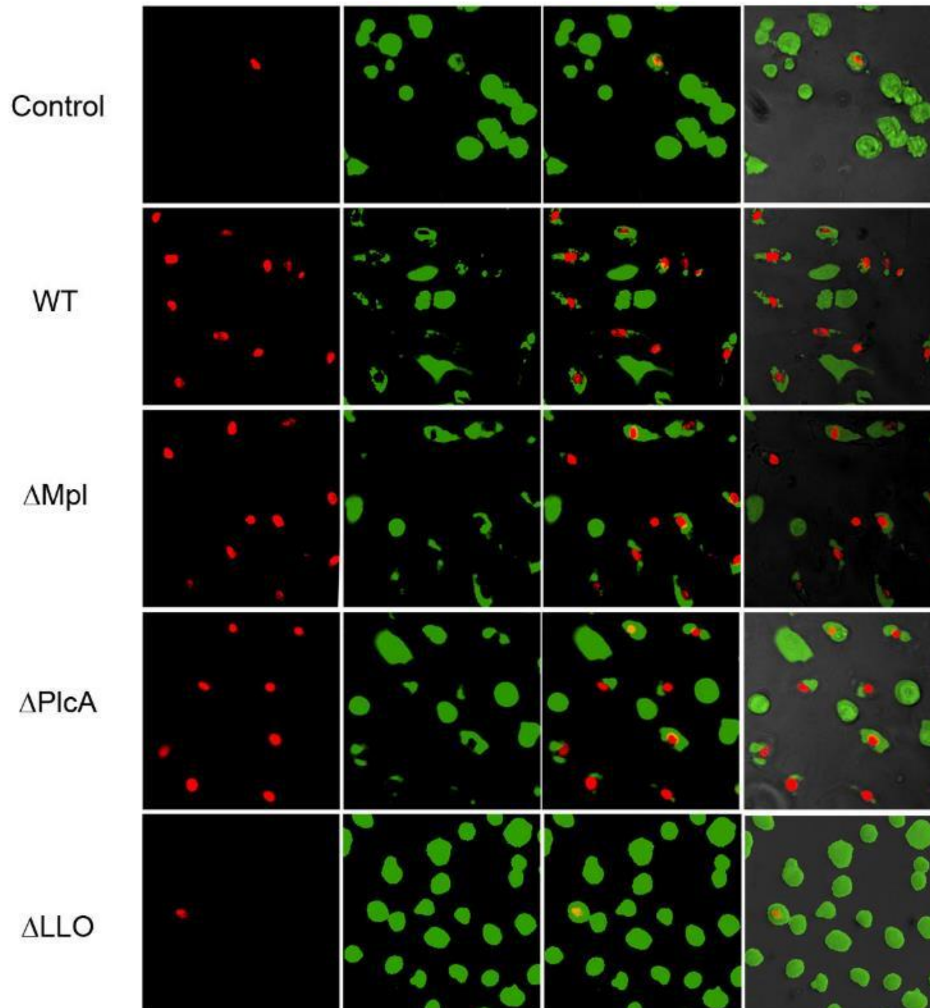

Figure 5.4 LLO is the major contributor to cytotoxicity on HBMECs

Cytotoxicity effects on HBMECs were accessed using cell culture medium (control), supernatant from EGD wild type (WT), Δ*plcA* (ΔPlcA), Δ*mpl* (ΔMpl), and Δ*hly* (ΔLLO) strains. HBMECs were incubated with cell culture medium and concentrated bacterial culture supernatant at 37°C in a 5% CO2 incubator for 20 minutes. Cells were labeled with calcein AM and EthD-1 to visualize cell membranes and nuclei. Two independent experiments with duplicates for each treatment were used.

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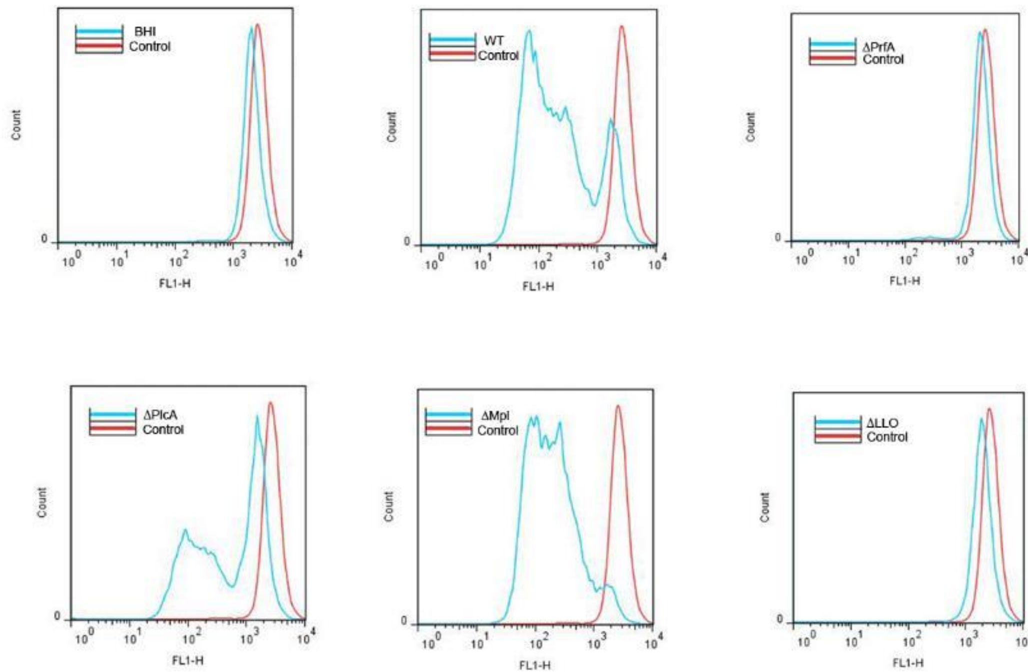

Figure 5.5 Flow cytometry analysis of cytotoxic effects on HBMEC infected with *L. monocytogenes* mutant strains.

Cytotoxic effects on HBMECs were accessed using cell culture medium (control), BHI medium, supernatants from EGD wild type, Δ*plcA* strain, Δ*plcA* strain, Δ*mpl* strain, and Δ*hly* strain. HBMECs were incubated with cell culture medium, BHI medium, and 2.5 times concentrated bacterial culture supernatant at 37°C in a 5% CO2 incubator for 20 minutes. To label cell membrance and nuclie, cells incubated with calcein AM and EthD-1 for 20 minute and were analyzed using flow cytometry. Results are representatives of two biological replicates.

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CHAPTER VI

CONCLUSIONS

In this dissertation, we characterized virulence factors in *L. monocytogenes* that enable the bacterium to counteract host innate immune system, resist bactericidal antibiotics, invade non-phagocytic cells, and break physical barriers.

In the first part, we identified an AlkD-like protein (Adlp) in *L. monocytogenes* that is involved in resistance to oxidative stress, aminoglycoside antibiotics, and DNA alkylating compounds. We showed that deletion of Adlp significantly impaired the survivability after treating with alkylating reagent methyl methanesulfonate. Remarkably, *adlp* deleted *L. monocytogenes* strain was more sensitive to both the oxidative agent H_2O_2 and murine macrophage RAW 264.7. Finally, we showed that deletion of Adlp also decreased the resistance of *L. monocytogenes* to aminoglycoside antibiotics, which are a typical choice for treatment of *Listeria* infection.

In the second part, we generated seven in-frame deletion mutants targeting LRRs motif in *L. monocytogenes* H7858 strain and evaluated the mutants for virulence. Among seven mutants, H7858Δ0369 and H7858Δ2546 displayed a significant impairment in the invasiveness of Hep-G2 cells. We further tested the virulence of the H7858Δ0369 and H7858Δ2546 strains *in vivo* using BALB/c mice via intravenous injection. H7858Δ0369 strain showed significant defects in host organ colonization, bacteremia formation, and invasion of the brain when compared to the wild type. Host immune responses to listerial

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intravascular infection were measured by quantitative real-time PCR. Result showed that transcript levels of proinflammatory cytokines including IL-1, IL-6, IL-12, TNF-α, and IFN-γ and chemokines including MCP-1 and MCP-3 that induced by H7858Δ0369 infection was significantly lower than those by parental wild type three days post infection.

In third study, cytotoxicity effects induced by supernatant from the EGD wild type strain were observed on HBMECs, but the supernatant from the isogenic Δ*prfA* strain was not. Therefore, we further investigated the cytotoxic effects of supernatants from the isogenic mutant strains (Δ*plcA*, Δ*mpl,* and Δ*hly*) that are incapable of producing the bacterial products (PlcA, Mpl, and LLO, respectively) that are under regulation of prfA using a viability staining method. Using fluorescent microscopy and flow cytometry, supernatants from mutant strains, the Δ*plcA* and the Δ*mpl* did have a cytotoxic effect on HBMEC but the Δ*hly* mutant did not.

In summary, we identified and characterized two novel virulence factors, Adlp and LmoH7858_0369 that contributed to bacterial infection and revealed a new invasion mechanism of CNS cells that is mediated by LLO. This information provides a better understanding of the key virulence components of *L. monocytogenes* as well as their contribution to pathogenicity.

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