Identification of chitinase as a novel virulence factor for *Listeria monocytogenes*

BY

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THESIS

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<td>ActA</td>
<td>Actin-assembly inducing protein</td>
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<tr>
<td>AIDS</td>
<td>Acquired immune deficiency syndrome</td>
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<tr>
<td>AMCase</td>
<td>Acidic mammalian chitinase</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
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<tr>
<td>BCIP-NBT</td>
<td>5-bromo-4chloro-3-indolylphosphate-nitroblue tetrazolium</td>
</tr>
<tr>
<td>BHI</td>
<td>Brain heart infusion</td>
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<tr>
<td>CBP</td>
<td>cyclic AMP responsive element binding protein-binding protein</td>
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<tr>
<td>CD3</td>
<td>Cluster of differentiation</td>
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<tr>
<td>CFU</td>
<td>Colony forming unit</td>
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<tr>
<td>CI</td>
<td>Competitive index</td>
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<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>erm(R)</td>
<td>erythromycin resistant</td>
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<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>HMG-I(Y)</td>
<td>High mobility group-I (Y)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1 beta</td>
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<tr>
<td>IL-4</td>
<td>Interleukin -4</td>
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<tr>
<td>Abbreviation</td>
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<td>IL-6</td>
<td>Interleukin-6</td>
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<td>IL-10</td>
<td>Interleukin-10</td>
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<tr>
<td>IL-12</td>
<td>Interleukin-12</td>
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<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
</tr>
<tr>
<td>InlA</td>
<td>Internalin A</td>
</tr>
<tr>
<td>InlB</td>
<td>Internalin B</td>
</tr>
<tr>
<td>IRF-1</td>
<td>Interferon regulatory factor 1</td>
</tr>
<tr>
<td>LB</td>
<td>Luria broth</td>
</tr>
<tr>
<td>LLO</td>
<td>Listeriolysin O</td>
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<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear factor-kappa B</td>
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<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOS2</td>
<td>Nitric oxide synthase 2</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PBST</td>
<td>Phosphate buffered saline with Tween</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PrfA</td>
<td>Positive regulatory factor A</td>
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<tr>
<td>RNI</td>
<td>Reactive nitrogen intermediate</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
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SDS          Sodium dodecyl sulphate
SDS-PAGE     Sodium dodecyl-sulphate  polyacrylamide gel electrophoresis
STAT-α       Signal transducer and activator of transcription alpha
TGF-β        Tumor growth factor beta
TiP DC       TNF and iNOS producing dendritic cells
TNF          Tumor necrosis factor
TRAF         Tumor necrosis factor receptor associated factor
**SUMMARY**

*Listeria monocytogenes* is a food borne Gram positive pathogen that causes the disease listeriosis in humans and animals. *L. monocytogenes* has been the responsible for a number of food-borne outbreaks of listeriosis among humans. The disease caused by this bacterium can become very severe in immunocompromised people, often resulting in deaths.

*L. monocytogenes* is ubiquitous in nature, and is found in diverse ecological niches such as soil, water and natural vegetation. However, when this free living bacterium enters a mammalian host it often causes disease, and is therefore regarded as an environmental pathogen. Environmental pathogens are those microorganisms that spend substantial parts of their life cycles outside their hosts, but can cause disease when they are inside the host. An important question about environmental pathogens is whether they are able to use molecules that are required for their survival outside the host, as virulence factors when they are inside their hosts. This hypothesis has come into the focus with the emergence of chitinases and chitin binding proteins as virulence factors in two other environmental pathogens, *Legionella pneumophila* and *Vibrio cholerae*. Chitinases are enzymes that hydrolyze chitin, which is a linear polymer of N-acetylglucosamine residues linked by (β1, 4) glycosidic bonds. Chitin is present in cell walls of algae and fungi; and in the exoskeletons of insects, mollusks, crustaceans and is the second most abundant organic compound on the earth. Bacterial chitinases and chitin binding proteins are
supposed to be used for nutrient acquisition by utilizing chitin as a readily available source of carbon and nitrogen. However, with the works done on *L. pneumophila* and *V. cholerae*, it is evident that these pathogenic bacteria use chitinases and chitin binding proteins to enhance their survival in mammalian hosts as well.

The goal of this thesis is to find out whether *L. monocytogenes* uses its chitinase and chitin binding proteins for its survival inside animal models of infection. The thesis has two main parts. The first part will examine whether mutant strains of *L. monocytogenes* lacking chitinase and/or chitin binding proteins are attenuated in in vitro and in vivo models of infection. The second part of the thesis will examine the probable mechanisms by which chitinases of *L. monocytogenes* can be used by the bacterium to enhance its survival in the host.
Chapter 1

Introduction of *Listeria monocytogenes* as an environmental pathogen and of chitinase as a potential virulence factor

1.1 Environmental pathogens

Environmental pathogens are microorganisms that spend a substantial part of their lifecycles outside of human hosts, but when introduced to humans cause disease. These pathogens are ubiquitous in nature and are found in diverse environmental niches such as soil, water, air, vegetation, food etc. Due to their widespread occurrence over the earth, this group of pathogens is very difficult to monitor and control. Environmental pathogens pose major threats to the human population, and include bacteria such as *Listeria monocytogenes* [1], [2], *Vibrio cholerae* [3], *Legionella pneumophila* [4], *Klebsiella pneumoniae*, viruses such as the West Nile virus[5], protozoa such as *Giardia lamblia*[6] and many other pathogenic microorganisms. Most of the major epidemics in human history have been caused by environmental pathogens, including food and water borne diseases such as cholera [7,8] and typhoid fever [9]. Therefore, in developing and underdeveloped countries with poor sanitary conditions, environmental pathogens continue to be a major health concern. Because of their ubiquitous occurrence in diverse environmental niches and ability to cause infections in humans, environmental pathogens are health concerns in developed countries as well.
Environmental pathogens have two distinct lifestyles – one outside of the host, and one inside of the host. In order to understand how environmental pathogens transform from free living environmental organisms to disease causing pathogens, it is important to understand their life styles in both of these environmental niches. However, research on most environmental pathogens has tended to focus on identification of gene products that are important for survival of the pathogen inside the host only. These approaches often fail to identify gene products that are important for the pathogen and which serve roles for the pathogen both inside the host as well as outside the host. Some important questions regarding environmental pathogens include (1) how are they able to maintain genes required for causing disease when living in the outside environment, and, (2) can these pathogens utilize genes needed for survival in the outside environment as virulence factors when the organisms are present inside the host?

1.2 *Listeria monocytogenes* as a model environmental pathogen

The Gram positive bacterium *L. monocytogenes* serves as a model environmental pathogen. *L. monocytogenes* is ubiquitously found in nature [10]. It has been isolated from diverse niches such as soil [2], water [11], vegetation [12] and marine environments [13]. It has also been isolated from silage [14], sewage [15] and slaughter house waste. Other than humans, this bacterium has been isolated from cattle [16], sheep, goats [17] and poultry [18]. *L. monocytogenes* has been found to infect other mammals such as rabbit, gerbils, hedge-hog and
fox as well [19-22]. In soil it is found as free living bacteria and, thought to grow as a saprophyte, on decaying vegetation [24]. However, when this free living bacterium enters a mammalian host it often causes serious disease, termed listeriosis.

*L. monocytogenes* is primarily a food borne pathogen and spreads through the consumption of contaminated food [23]. It has caused numerous food-borne human outbreaks [25, 26], as well as outbreaks in animals such as cattle and sheep that have been fed with silage contaminated with this bacterium [157]. Most of the outbreaks of listeriosis in humans have resulted from consumption of food materials such as cole slaw, unpasteurized cheese, pasteurized milk, butter, meat and seafood products that have been contaminated with this bacterium. Most of these epidemics are associated with raw, uncooked and ready-to-eat food materials [27-31]. *L. monocytogenes* has been isolated from a variety of aquatic organisms such as fish, shrimps, crabs, lobsters, as well as, seafood products such as smoked fish, cooked crab, cooked shrimp, mussels and shellfish [32-35]. Some of the largest and the most expensive recalls in the food processing industry have been due to contamination of processed food by *L. monocytogenes*. Thus, this bacterium is a major concern to the food industry. The United States Food and Drug Administration (FDA) have established a zero tolerance policy for *L. monocytogenes* in processed food.
Figure 1. *Listeria monocytogenes* is a food borne environmental pathogen. *Listeria monocytogenes* is a free living bacterium that is found in diverse environments such as soil, water and natural vegetation. It also grows in artificial settings such as food processing plants and ready to eat food materials. It infects humans by consumption of contaminated food. Inside the host, *L. monocytogenes* replicates intracellularly and spreads from cell to cell to infect different organs.
*L. monocytogenes* possesses a number of attributes that contribute to its success as a food-borne pathogen. The bacterium grows in a wide range of temperature, pH and salt concentrations [36-39]. It has a remarkable ability to grow in low temperatures that allows it to grow in refrigerated food [40, 41]. Thus, this bacterium has a competitive advantage over many other food borne pathogens that do not grow at low temperature. Although it does not produce endospores, *L. monocytogenes* is remarkably resistant to environmental stress [42]. It can also form biofilms that allow it to grow on different surfaces that come into contact with food, such as in food processing plants, or in food containers [43-46].

### 1.3 Listeriosis – an overview of the disease caused by *L. monocytogenes*

The disease caused by *L. monocytogenes* is termed listeriosis (Figure 2). In healthy adult individuals, the disease is restricted to mild gastroenteritis, fever and nausea. However, it becomes very severe in immunocompromised people such as the elderly, neonates, people taking immunosuppressive drugs and patients of Acquired Immuno Deficiency Syndrome (AIDS) [47-50]. Under conditions that lead to suppression of cell mediated immunity, listeriosis can result in systemic bacterial spread, causing severe septicemia, and often death. Listeriosis has a high mortality rate among humans, and therefore continues to be a serious threat to the population.
Figure 2. Disease caused by *L. monocytogenes*. Listeriosis, the disease caused by *L. monocytogenes* results primarily from the consumption of contaminated food products. In healthy immunocompetent people the disease is restricted to mild gastroenteritis and nausea. In people with a weak immune system such as the elderly, infants, pregnant women and AIDS patients, the disease often results in systemic bacterial spread. Liver and spleen are the primary target organs. In the invasive form, the bacteria can cross the blood-brain barrier and spread to the central nervous system causing often fatal meningitis. In pregnant women the bacteria can cross the placenta to infect the fetus often resulting in stillbirths and abortions. This illustration is an adaptation from [156].
In humans, listeriosis presents with a variety of clinical syndromes. In adults, the disease includes infection of the central nervous system and bacteremia. In many cases endocarditis has also been associated with listeriosis [51]. Some *L. monocytogenes* strains have been found to have an enhanced capacity to cause cardiac infections [52]. Several case reports have described acute hepatitis caused by *L. monocytogenes* [53, 54]. In these cases, liver biopsy reveals microabscesses and sometimes granulomas. Some of the predisposing factors for hepatitis caused by *L. monocytogenes* are diabetes mellitus, liver transplantation, cirrhosis and alcoholism.

Neonates are highly susceptible to listeriosis. In pregnant women, *L. monocytogenes* can cross the placenta and infect the fetus, often resulting in stillbirth and abortions. *L. monocytogenes* is a major cause of neonatal meningitis [55,56]. Other manifestations of neonatal listeriosis are respiratory distress, rash, purulent conjunctivitis and pneumonia. Most neonatal deaths because of listeriosis appear to occur due to pneumonia and respiratory failure. The bacteria can be readily isolated from the cerebro spinal fluid (CSF), placenta gastric aspirate, blood and skin of the new born.

**1.4 *Listeria monocytogenes* pathogenesis**

*L. monocytogenes* is an intracellular pathogen that can infect and invade many different types of mammalian species and host cells [57, 58]. The natural route of infection is through the intestine after consumption of contaminated food. In many cases, intravenous infection of mice
is routinely used to study *L. monocytogenes* pathogenesis [59] as this method of delivery skips intestinal translocation and proceeds right to the systemic form of disease. After entry into the blood, most of the bacteria are trafficked to the liver and the spleen by macrophages. In the liver, the bacteria are taken up by resident macrophages known as Kupffer cells [60, 61]. The Kupffer cells become activated and secrete proinflammatory cytokines such as interleukin 6 (IL-6), IL-1β, IL-12 and tumor necrosis factor (TNF) that help in the recruitment and activation of macrophages and neutrophils [62, 63]. Most of the bacteria are killed by neutrophils within the first 6 hours of infection. However the remaining bacteria spread to the liver hepatocyte cells and replicate exponentially for the next 72 hours [64, 65]. Hepatocytes are the primary sites of intracellular replication of *L. monocytogenes*. As mentioned previously, macrophages and neutrophils are critically important in controlling the initial bacterial burden in vivo. However for complete clearance of *L. monocytogenes*, an adaptive immune response in the form of activated T cells is essential [66].

*L. monocytogenes* infects phagocytic and non-phagocytic cells by distinct mechanisms. Phagocytic cells such as macrophages and dendritic cells engulf the bacteria by the process of phagocytosis such that the bacteria are entrapped in phagocytic vesicles or phagosomes. In non-phagocytic cells, *L. monocytogenes* actively induces its own uptake into host cells. *L. monocytogenes* invades non-phagocytic cells with the help of its surface proteins internalin (InlA) [67] and internalin B (InlB) [68] that are recognized by specific receptors on the host cell
surface. InlA binds to human E cadherin, a tight junction protein that is expressed in most human epithelial cells [69]. E-cadherin is a calcium dependent glycoprotein that is critically important in formation of cell to cell junctions and in maintenance of tissue architecture [70]. In many mammalian cell types such as hepatocytes, and endothelial cells, the entry of *L. monocytogenes* is mediated by InlB [71,72]. The receptor for InlB is Met, a receptor tyrosine kinase that is highly expressed in hepatocytes and endothelial cells [73]. The physiological ligand for Met is hepatocyte growth factor (HGF). Met and HGF are important in development of liver, placenta and specific muscle tissues [74]. Binding of InlB with Met promotes entry of *L. monocytogenes* into cells that express the receptor. The internalins, InlA and InlB both contribute to *L. monocytogenes* internalization by a process similar to receptor mediated endocytosis such that the internalized bacteria are located within endosomes [75]. Inside the phagosome or endosome, *L. monocytogenes* secretes a cholesterol dependent cytolysin protein known as listeriolysin O (LLO) which becomes activated in the acidic environment of the membrane-bound compartment [76-78]. *L. monocytogenes* secretes LLO and two phospholipase enzymes, PlcA and PlcB, to lyse the phagosome/endosome [79-81]. Following membrane
Figure 3. The intracellular lifecycle of *L. monocytogenes*. *L. monocytogenes* invades host cells with the help of its surface proteins InlA and InlB in a process similar to receptor mediated endocytosis by which the bacteria are trapped inside endocytic vesicles called phagosomes. Inside the phagosomes, *L. monocytogenes* secretes listeriolysin O (LLO) and a phospholipase (PlcA) to lyse the phagosomal membrane and escape into the cytosol. *L. monocytogenes* replicates in the cytosol and initiates actin polymerization by its surface Act A protein that propels it towards neighboring host cells, and thereby resulting in intercellular spread of the bacteria. Upon entry into an adjacent cell, the bacteria are enclosed in a double membrane vesicle. *L. monocytogenes* lyases the double membrane with LLO, PlcA and an additional phospholipase, PlcB. The illustration is adapted from [158].
disruption, *L. monocytogenes* enters the cytosol and replicates intracellularly. In the cytosol, *L. monocytogenes* initiates host cell actin polymerization with its surface protein ActA. *L. monocytogenes* uses actin polymerization initiated by ActA to propel itself into neighboring cells [82, 83, 84, 158]. This results in intercellular spread of the bacteria without bacterial exposure to the extracellular milieu.

### 1.5 Virulence factors of *L. monocytogenes*

*L. monocytogenes* maintains an arsenal of virulence factors that are required for its survival and proliferation in mammalian hosts. As mentioned before, InlA, InlB, LLO, PlcA, PlcB and ActA play important roles in the invasion and proliferation of *L. monocytogenes* in mammalian cells and are therefore the major virulence factors of the bacterium. Another recently identified virulence factor for *L. monocytogenes* is PrsA2, a post-translocation chaperone protein of *L. monocytogenes*. PrsA2 is involved in proper folding and secretion of a number of virulence factors of *L. monocytogenes*, including LLO. Mutant strains that lack the *prsA2* gene are severely attenuated for virulence in mice which demonstrate that PrsA2 is necessary for *L. monocytogenes* survival and replication in vivo [85, 86, 87].

Expression of all of the above mentioned virulence factors is controlled by the transcription factor Positive Regulatory Factor A (PrfA) [88-92]. PrfA thus plays a critical role in *L. monocytogenes* pathogenesis by acting as the master regulator of virulence gene expression.
PrfA remains inactive when *L. monocytogenes* is located outside of mammalian hosts [93]. However, inside the host, PrfA becomes activated and induces the expression of numerous virulence factors of *L. monocytogenes*. Therefore, PrfA is the key factor that helps the bacterium to adjust to changes in its environment as it enters a host from an outside niche. Most of the gene products identified thus far as controlled by PrfA are tuned to promote bacterial survival inside the host, and are therefore, classified as virulence factors.

### 1.6 Chitinase as a novel virulence factor

As mentioned previously, environmental pathogens are equipped to survive both inside and outside of the host. Given the fact that they spend a substantial part of their life cycle outside the host, it is intriguing how they are able to maintain their repertoire of virulence factors that are required only when they are inside the host body. It is anticipated that it would be a metabolic burden to replicate and maintain distinct sets of genes for environments that may only rarely be encountered. Environmental pathogens may solve this problem by utilizing the same gene products for survival in both environments i.e, inside the host and outside the host. This possibility has come into focus with the emergence of chitinase and chitin binding proteins as virulence factors in some environmental pathogens.

Chitin is a linear polysaccharide of N-acetyl glucosamine residues linked by (\(\beta 1, 4\)) glycosidic bonds [94,95]. Chitin is abundant in nature, and it is the second most abundant
organic compound on the earth’s surface after cellulose. Chitin is present in cell walls of algae and fungi, in shells of crustaceans such as crabs and shrimps, and in exoskeletons of worms, mollusks and arthropods such as cockroaches [94, 95].

Massive amounts of chitin are synthesized in oceans, and then rapidly degraded by chitinases secreted by marine bacteria [94,96]. Chitin in its polymeric form is not present in mammals. However, N-acetyl glucosamine residues linked by (β1,4) glycosidic bonds are present in most mammalian systems in the form of glycoproteins and glycolipids [97-99]. Most mammalian cell surfaces contain glycoproteins consisting of N-acetylglucosamine [100-102]. Many of the key proteins involved in host immune responses such as T cell receptors, CD3, MHC proteins, immunoglobulins and cytokines are glycosylated with N-acetylglucosamine residues [103-105].

Chitinases are enzymes that degrade chitin by hydrolyzing the (β1, 4) glycosidic bonds between the N-acetylglucosamine residues. Chitinases are produced by a variety of organisms such as bacteria, protozoa, fungi, plants, crustaceans, insects and mammals including humans [106]. Chitinases belong to the group known as glycosyl hydrolases, a class of enzymes that hydrolyze carbohydrates. Based on amino acid sequences, glycosyl hydrolases have been classified into several families [107, 108]. Although mammals do not have chitin in its polymeric form, chitinases are produced by mammals. The exact function of the mammalian chitinases is still not known. However mammalian chitinases are produced in large quantities in
various inflammatory conditions and allergic diseases such as asthma. Humans produce two enzymatically active chitinases – chitotriosidase and acidic mammalian chitinase (AMCase) as well as three chitinase like proteins [109-113]. The chitinases and chitinase like proteins have been found to be associated with a number of diseases. Chitotriosidase is a biomarker for Gaucher’s disease [160]. It has also been associated with atherosclerosis, non alcoholic fatty liver disease and juvenile idiopathic arthritis [161-163]. The chitinase like protein YKL-40 is found in high levels in the serum in several diseases such as inflammatory bowel disease, liver fibrosis, rheumatoid arthritis and cancer [109, 165]. However, the reason for this association remains unclear.

Acidic mammalian chitinase (AMCase) has been found to be expressed at high levels in asthma, a chronic inflammatory lung disease that is characterized by hypersecretion of mucus resulting in airway obstruction [111]. Asthma is a manifestation of a hyperactive Th2 adaptive immune response mediated by the cytokines IL-4 and IL-13 [111, 114, 115, 159]. These cytokines stimulate activation of B cells and production of immunoglobulin E (IgE) which leads to mast cell degranulation [109, 164]. Experiments with mice have shown increased AMCase production in the lungs during Th2 responses, but not during Th1 responses and, the increase in
Figure 4. Structure of chitin. Chitin is a linear polymer of N-acetylglucosamine residues linked by (β1,4) glycosidic bonds. Chitin is found in fungal cell walls, exoskeletons of mollusks, insects and marine crustacean organisms, and is the second most abundant organic compound on earth. Chitinases are enzymes that breakdown chitin by hydrolyzing the (β1,4) glycosidic bonds. Illustration adapted from (http://academic.brooklyn.cuny.edu/biology/bio4fv/page/chitin.html).
AMCase has been found to be mediated by IL-13 [111]. It has also been reported that the chitinase like protein Ym2 is expressed in CD4+ Th2 lymphocytes in response to IL-4 and IL-13 [166]. Although chitinases and chitinase-like proteins have been found to be associated with inflammatory diseases resulting from exaggerated Th2 immune response, their exact contributions and molecular targets remain to be identified.

1.7 Bacterial chitinases and chitin binding proteins

Bacterial chitinases belong to the family 18 group of glycosyl hydrolases [167]. These chitinases are produced by many diverse bacterial species, both Gram positive and Gram negative. Chitinase and chitin binding proteins are thought to be important for bacterial nutrient acquisition in the environment by utilizing chitin as a readily available source of carbon and nitrogen. Chitinase and chitin binding proteins have been discovered in both pathogenic and non-pathogenic bacteria. Many environmental pathogens such as *L. pneumophila*, *V. cholerae*, *Vibrio vulnificus*, *Bacillus anthracis*, and *Burkholderia cepacia* [116-120] produce chitinases. Recently, chitinase and chitin binding proteins have been shown to contribute to the pathogenicity of some environmental pathogens such as *L. pneumophila* [116] and *V. cholerae* [121].

*L. pneumophila* is a Gram negative bacterium that causes Legionnaires’ disease, an often fatal pneumonia. This bacterium is found in fresh water, in protozoan hosts, in man-made aquatic
systems such as cooling towers and potable water system. Once inside mammalian hosts, it invades alveolar macrophages resulting in inflammation and tissue damage. Inside the host *L. pneumophila* secretes a number of effector molecules through its type II secretion pathway that promote its survival [122]. Among the effectors, it secretes a chitinase (ChiA), that promotes bacterial persistence in the lungs of infected mice. However, the exact mechanism by which ChiA promotes bacterial persistence is not known yet.

Most bacteria of the genus *Vibrio* harbor chitinase and chitin binding protein genes [123]. This is not surprising, given the fact that most species of this family are found in oceans where chitin is generated in massive amounts. *V. cholerae*, the causative agent of the diarrheal disease cholera in humans, contains chitinase and chitin binding protein genes. It has been shown that *V. cholerae* secretes a chitin binding protein, GbpA, which promotes persistence of the bacteria in the intestine in a mouse model of cholera [121]. This protein is secreted by the same pathway as the cholera toxin, and has been shown to bind to mucin proteins of the intestinal mucus layer [124]. Mucin consists of a group of heavily glycosylated proteins. This indicates that the chitin binding protein secreted by *V. cholerae* is able to bind to polysachharides that are similar to chitin, but not exactly chitin.
1.8 Chitinases and chitin binding proteins of *L. monocytogenes*

Little is known about the chitinase and chitin binding proteins of *L. monocytogenes*. Genome sequence analysis had shown that *L. monocytogenes* genome contains two chitinase genes – *lmo1883* or *chiA*, *lmo0105* or *chiB* - and one chitin binding protein gene (*lmo2467*). It has been shown that the chitinases ChiA and ChiB enable *L. monocytogenes* to hydrolyse chitin in vitro [125]. However, the function of the chitin binding protein (Lmo2467) remains to be elucidated.

The physiological role of the chitinases and chitin binding protein in *L. monocytogenes* is not very clear. Bacterial chitinases are thought to provide nutritional benefits in the environment where they can enable bacteria to utilize chitin, a readily available source of carbon and nitrogen. Although *L. monocytogenes* does not survive on chitin as the sole source of carbon, it has been shown that addition of chitin to a minimal medium improves the growth of *L. monocytogenes* [126].

It has been shown that expression of the chitinases genes, *chiA* and *chiB*, is dependent on the type of sugar molecules present in the environment. Neither of the chitinase genes is expressed in the presence of glucose in the medium, whereas the presence of chitin in the medium induces *chiA* and *chiB* expression. Moreover, expression of *chiA* is triggered by the presence of N-acetylglucosamine, the monomeric subunit of chitin, in the medium [127]. These facts suggest the existence of a catabolite repression system that may regulate the expression of the chitinase
genes of \textit{L. monocytogenes}. Recent work has shown that other factors are also functional in controlling chitinase expression in \textit{L. monocytogenes}. It has been demonstrated that during intracellular infection of macrophages by \textit{L. monocytogenes}, chiA expression is increased by 3 fold [128]. It has been also shown that \textit{chiA} expression is induced by PrfA [127]. As the majority of gene products identified thus far as being regulated by PrfA directly contribute to \textit{L. monocytogenes} virulence, it is possible that \textit{chiA} may have a role in pathogenesis as well.

1.9 Plan of the thesis

As described before, chitinase and chitin binding proteins have been recently recognized as virulence factors for the environmental pathogens \textit{L. pneumophila} and \textit{V. cholerae}. However, it is not known whether chitinases and chitin binding proteins might more generally be considered as are used as virulence factors for other environmental pathogens. The overall goal of this project was to address the possible roles of chitinases and chitin binding proteins in pathogenesis for other bacterial pathogens by investigating the function of these proteins in \textit{L. monocytogenes}.

Our findings indicate that chitinases of \textit{L. monocytogenes} function to promote \textit{L. monocytogenes} virulence by suppressing specific aspects of the host immune response. To our knowledge, this is the first demonstration of a bacterial chitinase functioning as an immune modulator. Chapter 2 will describe the materials and experimental methods that have been used in this study. Chapter 3 will describe studies that show that chitinases and chitin binding proteins of \textit{L. monocytogenes} contribute to bacterial virulence in an animal model of infection.
Chapter 4 will focus more directly on the role of ChiA in *L. monocytogenes* pathogenesis and show that ChiA enhances bacterial survival in the host by suppression of inducible nitric oxide synthase activity. Chapter 5 will discuss the future directions and perspectives on bacterial and mammalian chitinases and their linkages to human disease.
Figure 5. Chitinase and chitin binding protein genes of *L. monocytogenes*. *L. monocytogenes* genome contains two chitinase genes, *chiA* and *chiB*, and one chitin binding protein gene, *lmo2467* (black arrows). The adjacent genes for each of the chitinase or chitin binding protein genes are indicated as white arrows. The chitinase/ chitin binding protein genes are located well apart from one another in the *L. monocytogenes* chromosome, and are conserved in all species of *Listeria*. 
Chapter 2

Materials and Methods

2.1 Bacterial strains, plasmids and media

The bacterial strains used in this study are listed in Table 1. in the Appendix. *L. monocytogenes* 10403S was used as the parent strain for the construction of bacterial mutants. The host strains used to maintain recombinant plasmids were *Escherichia coli* alpha select (Bioline, Boston, MA), One Shot TOP10 (Invitrogen, Carlsbad, CA), and SM10 [177]. All the strains were grown in Luria-Bertani (LB) medium [176] (Invitrogen Corp., Carlsbad, CA), or brain heart infusion (BHI) medium (Difco Laboratories, Detroit, MI). The following antibiotic concentrations were used unless otherwise indicated: Carbenicillin 50 µg/ml; chloramphenicol, 10 and 5 µg/ml; and streptomycin, 200 µg/ml. The *L. monocytogenes* strains were grown overnight at 37°C without shaking for the in vitro assays and with shaking for the in vivo assays. To generate *L. monocytogenes* mutants by allelic exchange, the temperature sensitive shuttle plasmid pKSV7 [130] was used, and the integration plasmid pPL2 [131] was used for genetic complementation.
2.2 Construction of *L. monocytogenes* in-frame deletion mutants

*L. monocytogenes* chromosomal in-frame deletion mutants were generated by allelic exchange as previously described [129]. The plasmid vectors used for allelic exchange are listed in Table 2 in the Appendix. A two step cloning strategy was taken to construct recombinant plasmids for allelic exchange. Approximately 1 kb regions upstream and downstream of each gene were PCR amplified and inserted sequentially into the shuttle plasmid vector pKSV7 [130] to generate recombinant plasmids where upstream and downstream segments of the gene of interest were sequentially integrated in the pKSV7 vector. Primers designed to amplify regions 5’ of the target gene were designed to contain a *SacI* restriction site at the 5’ end and an *XbaI* site at the 3’ end. Similarly, primers designed to PCR amplify downstream regions had *XbaI* sites at the 5’ end and *HindIII* sites at the 3’ end. Primers used in this process have been listed in Table 3 in the Appendix. The recombinant plasmids were introduced into *L. monocytogenes* by electroporation. As the pKSV7 vector contains a chloramphenicol resistance gene cassette, transformed bacteria could be selected by plating the bacterial culture in bovine heart infusion (BHI) media containing chloramphenicol. The plasmid pKSV7 being temperature sensitive does not replicate at 40ºC. The plasmid containing strains of *L. monocytogenes* were grown at this non permissive temperature that does not allow plasmid replication in presence of drug selection to identify bacteria in which the plasmid has integrated into the *L. monocytogenes* chromosome at homologous regions. The plasmid integrant strains were then grown at temperature permissive of
Figure 6. Generation of recombinant plasmid for allelic exchange by two step cloning. An upstream fragment and a downstream fragment of the gene of interest are PCR amplified and sequentially inserted in the temperature sensitive shuttle plasmid vector pKSV7. The primers used for PCR contained the mentioned restriction enzyme cleavage sequences that allowed the PCR amplified fragments to be inserted in the vector. The recombinant plasmid was then introduced into \textit{L. monocytogenes} for allelic exchange.
plasmid replication (30°C) in the absence of chloramphenicol selection. Overnight grown bacterial cultures were diluted 1:1000 in fresh BHI media. This process was repeated 5 times. After 5 passages in media without antibiotic selection, candidate strains were identified by their inability to grow in media containing chloramphenicol. Deletion of the gene of interest was confirmed by PCR amplification of the appropriate chromosomal region and DNA sequencing. After generation of a single deletion mutant, the above procedure was repeated to generate double and triple chitinase/chitin binding protein deletion mutants.

2.3 Complementation of the mutant strains

The integration plasmid vector pPL2 [131] was used for genetic complementation of the in-frame deletion strains of L. monocytogenes. The gene of interest was PCR amplified and cloned into the pPL2 vector. The primers used in this process have been listed in Table 3 in the Appendix. Escherichia coli SM10 bacterial cells were used to harbor the recombinant plasmid. The recombinant plasmid was transferred from E. coli SM10 to L. monocytogenes in-frame chitinase deletion strains by conjugation. As the pPL2 vector contains a chloramphenicol resistance gene cassette, and the in-frame deletion strains were derived from the streptomycin resistant parental L. monocytogenes 10403S strain, the complemented strains could be selected by using a double antibiotic selection pressure of chloramphenicol and streptomycin. Thus, the ΔchiA strain could be complemented with chiA.
The ΔchiA+ chiAE163M strain was generated by transforming the ΔchiA strain with a plasmid containing the mutated chiA gene, with the point mutation E163M, integrated in the pPL2 vector. For competitive index assay, a wild type strain having an erythromycin resistance gene was used [132]. The bacterial cultures were grown in Bovine Heart Infusion (BHI) medium for the in vivo assays. For the chitinase and chitin binding assays, the bacteria were grown in Luria broth (LB) with 0.1 % N-acetylglucosamine.

2.4 Generation of a chiA active site mutant

The chiA active site mutant was generated by a point mutation where a glutamate residue (E) at position 163 of ChiA was replaced by a methionine (M) residue. It has been found that a similar point mutation in a chitinase from the bacterium Vibrio harveyi resulted in a mutant in which the chitinase activity was abrogated without affecting the protein folding [133]. The point mutation was carried out by the “Change – IT™ Multiple Mutation Site Directed Mutagenesis Kit” from the USB Corporation. The primer GGATTAGACATCGACTTAATGCAAAGTGCGATTACCGCGGGA was used to generate the required point mutation by PCR. The mutation was confirmed by sequencing the PCR product. The mutated chiA(chiAE163M) was cloned into the integrational vector pPL2 and introduced in the ΔchiA strain by electroporation.
2.5 Chitinase assay

The chitinase assay, as described [134] in was carried out with supernatants derived from overnight grown bacterial cultures. Briefly, the wild type and the in-frame chitinase/chitin binding protein deletion strains were grown overnight in LB at 30ºC. 200µl of the supernatant was incubated with 50µl of 0.5mM 4-nitrophenyl-N,N´-diacetyl- β- D chitobioside (Sigma N6133-5MG) and 250 µl of 50mM sodium phosphate buffer (pH 6.0) at 30ºC for 2 hours, after which, the reaction was stopped with 100µl of 0.1 M sodium carbonate. The absorbance of the reaction mixture at 405 nm was taken. The absorbance of the bacterial cultures at 600nm were also noted. The ratio of OD$_{405}$ / OD$_{600}$ indicated the chitinase activity per bacterial cell.

2.6 Chitin binding assay

The chitin binding assay was done as described in [121]. Briefly, the wild type, the ΔchiA and the ΔchiA + chiA E163M strains were grown overnight in 50 ml LB with 0.1 % N-acetylglucosamine at 30ºC. The culture supernatants were concentrated to 1 ml by Millipore Amplicon Ultra (10000 MWCO) filters that removed molecules smaller than 10kD from the supernatants. The concentrated supernatants were incubated with 50µl of phosphate buffered saline (PBS) washed chitin beads (New England Biolabs) for 1 hour at 4ºC. Then the mixtures were washed 5 times with PBS and separated in 10% SDS-PAGE. ChiA was detected by Western Blot using an anti-chiA antibody. The polyclonal anti-ChiA antibody, which was used
as the primary antibody for the Western blot, was raised in rabbits in the UIC-RRC facility. Briefly, proteins were transferred from the gel to polyvinylidene difluoride membrane at 30 V for 1 hour in Tris-glycine transfer buffer (25mM Tris, 192mM glycine, 20% methanol). The membrane was then kept in blocking buffer consisting of 0.05% Tween20 and 5% dried milk in PBS for 1 hour. Then the membrane was incubated overnight with the primary antibody at 1:2500 dilution in PBST (0.05% Tween20 in PBS) at 4°C with shaking. After incubation with primary antibody, the membrane was washed three times with PBST for 15 minutes each. Then it was incubated with a goat anti rabbit secondary antibody conjugated to alkaline phosphatase (Southern Biotech) at 1:2500 dilution in PBST buffer for 1.5 hours at room temperature with shaking. The membranes were washed three times with PBST. Proteins were detected by incubating the membrane with 1ml BCIP-NBT Plus (5-bromo-4chloro-3-indolylphosphate-nitroblue tetrazolium) (Southern Biotech) for 10 minutes at room temperature. Images of the blots were taken in Alphalmager 2200 (Alpha Innotech, San Leandro, CA).

2.7 Intracellular growth assay

Intracellular bacterial growth in mammalian host cells were done as previously described [85]. Briefly, monolayers of mammalian host cells were grown on glass coverslips and infected
Figure 7. Intracellular growth assay for *L. monocytogenes*. Intracellular growth of *L. monocytogenes* is measured by infecting monolayers of host cells grown on glass coverslips, in tissue culture dishes. After an hour of infection extracellular bacteria are killed by adding gentamicin, an antibiotic that cannot penetrate into the host cells, and thereby does not affect intracellular bacteria. At different time points, glass coverslips are removed and the host cells are lysed by vortexing the coverslips in sterile distilled water. The lysates are then plated on LB agar medium. The resulting number of CFUs represents the number of intracellular bacteria at each time point.
with *L. monocytogenes*. After 30 mins of infection, the medium was washed with PBS and after 1 hour, the antibiotic gentamicin was added at a concentration of 5µg/ml to kill the extracellular bacteria. At the indicated time points, coverslips were removed and host cells were lysed in 5 ml sterile water by vortexing. The lysates were then plated on LB agar media. The number of colonies represented the number of intracellular bacteria at each time point. This intracellular growth assay was performed with two different mammalian cell lines – J774 macrophage like cells and Caco2 intestinal epithelial cells. J774 macrophage like cells were infected at a multiplicity of infection (MOI) of 1, whereas Caco2 cells were infected at an MOI of one host cell to ten bacteria.

### 2.8 Cell to cell spread assay

Cell to cell spread of *L. monocytogenes* was assessed using the plaque assay as previously described [135]. Briefly, L2 fibroblast monolayers were grown on six-well tissue culture plates and infected with *L. monocytogenes* for 1 hour at a multiplicity of infection of 30:1. Gentamicin was added to single wells after infection (20 µg/ml) to kill extracellular bacteria. Plaque size was measured with a micrometer (Finescale, Orange, CA). At least ten plaques were measured for infection with each strain. Results were obtained from three independent experiments.
Figure 8. Cell-to-cell spread assay for *L. monocytogenes*. This figure represents the plaque assay that is done to check cell to cell spread of *L. monocytogenes*. L2 fibroblast cell monolayers are infected with *L. monocytogenes*. After an hour of infection, extracellular bacteria are killed with gentamicin. Intracellular bacterial proliferation and cell to cell spreading result in formation of clear zones, or plaques, on the host cell monolayer. The size of the plaque is dependent on the efficiency of the cell to cell spread by *L. monocytogenes*. The plaque diameters are therefore measured to assess the ability of intercellular spread.
2.9 Mouse intravenous infection assay

All animal experiments were performed in the Biological Resources Laboratory at the University of Illinois at Chicago using approved procedures. Bacterial cultures grown overnight in BHI at 37°C were diluted 1:20 (vol:vol) into fresh BHI broth and grown to an O.D$_{600}$ of 0.6 at 37°C with shaking. 1 ml of culture corresponding to approximately 6 x10$^8$ CFU/ml was diluted in PBS to a final concentration of 1x10$^5$ CFU/ml and bacterial numbers were confirmed by plating of diluted culture aliquots onto BHI agar. Eight to ten week old female ND4 Swiss Webster mice (Harlan, Madison, WI) were infected with 200µl of the bacterial suspension (2x10$^4$ CFU) by tail vein injection. After 72 hours of infection the animals were euthanized and the livers and spleens of the infected mice were harvested and homogenized. The homogenates were plated in BHI agar with 200 µg/ml streptomycin. The number of viable bacteria present in each organ was measured by counting the number of colonies formed after 24 hours incubation at 37°C. For NOS2 −/− mice, 2X10$^2$ CFU of bacteria were used to infect, whereas, for the corresponding wild type mice, C57BL6, an infectious dose of 2x10$^4$ CFU of bacteria was used.

2.10 Oral infection of mice

Oral infection of mice was carried out as described in [136]. Two point mutations (S192N and Y369S) were introduced in the Internalin A(InlA) protein of the wild type and the ΔchiA strains of L. monocytogenes. As described in [137], these mutations enable InlA to bind to
murine E-cadherin, the receptor of InlA, resulting in efficient and reproducible oral infections in mice. C57BL6 mice were orally inoculated with a dose of $1 \times 10^8$ CFUs of the *L. monocytogenes* strains. After 72 hours of infection, the liver and the spleen were harvested, homogenized and plated on BHI. The resulting number of colonies represented the number of bacteria present in each organ.

### 2.11 Competitive index assay

The competitive index assay was carried out as described in [138]. Briefly, a 1:1 mixture of the wild type and mutant strains for a total bacterial load of $2 \times 10^4$ CFUs was injected into Swiss Webster mice by tail vein injection. The wild type strain carried an erythromycin resistance gene. Prior to infection, a 1:1 mixture of the two strains was plated on BHI and BHI with erythromycin (1 µg/ml) to distinguish between the two strains. The mutant : wild type ratio ($C_{in}$) was calculated. 72 hours post infection, the livers and the spleens of the infected mice were harvested, homogenized and plated as mentioned above, and the mutant : wild type ratio ($C_{out}$) was calculated. The competitive index values were calculated by taking the ratio of $C_{out}$ and $C_{in}$ ($C_{out} / C_{in}$).
2.12 Identification of secreted ChiA by *L. monocytogenes*

Proteins secreted by *L. monocytogenes* were isolated from supernatants of bacterial culture. 50 ml LB medium containing 0.1% N-acetylglucosamine was inoculated with 2 ml of overnight grown strains of *L. monocytogenes*. After 5 hours of growth (OD$_{600}$ ~ 1.2), the culture supernatants were recovered. The supernatant proteins were precipitated by adding trichloroacetic acid to a final volume of 10%. The precipitated proteins were resuspended in 200µl of Laemmli Sample Buffer (Bio-Rad Laboratories) and separated by SDS polyacrylamide gel electrophoresis. ChiA was detected by Western blot using an anti-ChiA antibody.
Contribution of Chitinases to *Listeria monocytogenes* Pathogenesis

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### 3.1 Summary

*Listeria monocytogenes* secretes two chitinases (ChiA and ChiB) and one chitin binding protein (Lmo2467). Mutant strains lacking *chiA*, *chiB* or *lmo2467* exhibited normal growth in broth cultures and in cultured mammalian cells but were defective for growth in the livers and spleens of infected mice. Since mammals lack chitin, *L. monocytogenes* may have adapted chitinases to recognize alternative substrates to enhance pathogenesis.

### 3.2 Introduction

*Listeria monocytogenes* is a Gram-positive bacterium that is found as an inhabitant of soil, water and decaying vegetation, where the organism is believed to survive as a saprophyte [24]. Upon consumption of the bacterium by a susceptible host, *L. monocytogenes* adopts a pathogenic lifestyle that can lead to serious and sometimes fatal infections. *L. monocytogenes* is considered as a major food-borne pathogen and has been responsible for some of the largest and most
expensive food recalls in U.S. history. While significant attention has focused on the identification of *L. monocytogenes* gene products that specifically contribute to bacterial life within host cells, relatively less is known regarding the function of gene products that may be adapted to contribute to bacterial life both inside and outside the infected host.

Chitinases catalyze the hydrolysis of chitin, a linear polymer of N-acetylglucosamine residues linked by (β1,4) glycosidic bonds. Microbial chitinases have been associated with nutrient acquisition, as chitin is abundant in the environment and can serve as a source of carbon and nitrogen. Recently, chitinase and chitin binding proteins have been linked to bacterial virulence for two environmental pathogens, *Legionella pneumophila* and *Vibrio cholerae* [116, 121]. Although chitin is not synthesized by mammals, *L. pneumophila* chitinase has been implicated in enhancing bacterial colonization of the lungs of infected mice, while a chitin binding protein produced by *V. cholerae* has been implicated in intestinal colonization.

*L. monocytogenes* produces two chitinases encoded by *lmo1883* (*chiA*) and *lmo0105* (*chiB*) and one chitin binding protein encoded by *lmo2467*. *chiA* is a 1059 base pair (bp) gene that encodes a 352 amino acid protein that has a molecular mass of 37.9 kilo Daltons (kD), and *chiB*, a 2271bp gene, encodes a 756 amino acid protein with a molecular mass of 81.5 kD. Both of these proteins- ChiA and ChiB possess chitinolytic activity. It has been observed that mutants of *L. monocytogenes* lacking *chiA* or *chiB* have much reduced ability to hydrolyse chitin [125].
*lmo2467* encodes a hypothetical chitin binding protein that lacks the chitinase domain. The function of *lmo2467* is yet to be identified.

In this work, we have assessed the roles of the chitinases and the chitin binding proteins in the pathogenicity of *L. monocytogenes*. By using in-frame deletion mutants for each of the chitinase genes, we demonstrate that the chitinases ChiA and ChiB contribute to the pathogenesis of *L. monocytogenes* in vivo.

### 3.3 Results

**In-frame chitinase deletion strains of *L. monocytogenes* have reduced chitinase activity**

As described in Chapter 2, in-frame deletion mutants for each of the chitinase (Δ*chiA* and Δ*chiB*) and the chitin binding protein (Δ*lmo2467*) genes were generated by the process of allelic exchange. Briefly, an upstream and a downstream fragment of the gene of interest were sequentially cloned into the shuttle plasmid vector pKSV7. The recombinant plasmid was introduced into *L. monocytogenes*10403S after which, the in-frame deletion mutant strain was generated by homologous recombination. The in-frame deletion strains were characterized for their ability to grow in BHI media. Each of the in-frame deletion strains was found to resemble wild type *L. monocytogenes* with respect to bacterial growth in BHI broth culture (Figure 9). However, deletion of either *chiA* or *chiB* significantly reduced secreted chitinolytic activity of *L. monocytogenes*. 
monocytogenes (Figure 10). Deletion of lmo2467 did not have any effect on the chitinolytic activity of L. monocytogenes.

**Chitinases do not affect intracellular growth of L. monocytogenes or cell to cell spread**

Intracellular growth of L. monocytogenes was assessed in two mammalian cell lines. As described in Chapter 2, monolayers of mammalian cells were grown on glass coverslips and infected with L. monocytogenes. Extracellular bacteria were killed by the antibiotic gentamicin. At different time points the coverslips were removed, and the host cells lysed and plated in LB agar medium. The resulting number of colonies represented the number of bacteria present inside the host cells. In the human intestinal epithelial cell line Caco2, the chitinase and chitin binding protein knockout mutants showed similar intracellular growth as the wild type strain (Figure 11). Identical patterns of bacterial replication were also observed in the murine derived J774 macrophage like cell line using a similar approach (Figure 12). These observations show that the chitinases and chitin binding proteins do not influence the intracellular growth of L. monocytogenes.
Figure 9. Growth of chitinase and chitin binding protein deletion mutant strains of *L. monocytogenes* is not defective in broth culture. BHI broth was inoculated with wild-type (WT) and chitinase/chitin binding protein deletion strains (ΔchiA, ΔchiB and Δlmo2467) of *L. monocytogenes*. Growth of the bacterial strains was monitored by taking the OD_{600} values of the cultures at different time points over a period of 10 hours.
Figure 10. In-frame deletion strains for chitinase genes show reduced chitinolytic activity in vitro. Chitinase assay was done with supernatants of overnight grown cultures of wild type (WT) and chitinase/ chitin binding protein deletion (ΔchiA, ΔchiB and Δlmol2467) of L. monocytogenes. Culture supernatants were incubated with the substrate 4 nitrophenyl N,N´ – diacetyl- β- D chitobioside for 2 hours at 30°C. The reaction was then stopped by adding 1 M Na₂CO₃. OD₄₀₅ of the reaction mixture was measured to assess the enzymatic activity of the chitinases. OD₆₀₀ values were also taken in order to normalize the OD₄₀₅ values with respect to the bacterial population density of the cultures.
Figure 11. Chitinases and chitin binding proteins do not influence growth of *L. monocytogenes* in Caco2 intestinal epithelial cells. Caco2 cells were grown on glass coverslips and infected with wild-type (WT), and chitinase/chitin binding protein deletion (*ΔchiA*, *ΔchiB*, *Δlmo2467* and *ΔchiA ΔchiB Δlmo2467*) strains of *L. monocytogenes*. Extracellular bacteria were killed by adding gentamicin. At different time points coverslips were removed, host cells lysed and plated on LB agar media. The resulting number of CFUs of each strain for each time point has been plotted.
Figure 12. Chitinases and chitin binding proteins do not influence growth of *L. monocytogenes* in J774 macrophage like cells. J774 cells were grown on glass coverslips and infected with wild type and chitinase/chitin binding protein knock out strains (ΔchiA, ΔchiB, Δlmo2467 and ΔchiA ΔchiB Δlmo2467) of *L. monocytogenes*. Extracellular bacteria were killed by adding gentamicin. At different time points coverslips were removed, host cells lysed and plated on LB agar media. The resulting numbers of CFUs for each strain at each time point has been plotted.
Cell to cell spread of intracellular *L. monocytogenes* was measured by the plaque assay described in Chapter 2. The chitinase and chitin binding protein deletion mutants, Δ*chiA*, Δ*chiB* and Δ*lmo2467* resulted in plaque formation to the same efficiency and of the same diameter as those formed by the wild type strain (Figure 13). This indicates that chitinases and chitin binding proteins do not influence cell to cell spread of *L. monocytogenes*.

**Chitinase deletion strains are attenuated in mice**

The chitinase and chitin binding protein mutants were tested for virulence in a mouse model of infection. As described in Chapter 2, ND4 Swiss Webster mice were infected via tail vein injection. The bacterial burdens in the infected mice were assessed by plating homogenized livers and spleens of infected mice in BHI media. The Δ*lmo2467* mutant exhibited the most modest level of attenuation, with approximately 4-fold fewer bacteria recovered from the livers and 6-fold fewer bacteria recovered from the spleens of infected mice than from those infected with wild-type *L. monocytogenes*. The Δ*chiB* mutant exhibited more significant levels of attenuation, with approximately 8-fold and 14-fold fewer bacteria recovered from the livers and spleens, respectively. The most dramatic virulence defect was observed for the Δ*chiA* mutant. The absence of *chiA* reduced bacterial colonization of the liver and spleen more than 19-fold and 45-fold respectively. This defect could be fully compensated in the liver and almost fully compensated in the spleen by providing *chiA* in *trans* on the integrative plasmid vector pPL2.
Figure 13. Chitinases and chitin binding proteins do not influence intercellular spread of *L. monocytogenes*. Cell-to-cell spread of *L. monocytogenes* is assessed by the plaque assay in which monolayers of L2 fibroblast cells are infected with the wild type (WT) and chitinase/chitin binding protein deletion mutant (ΔchiA, ΔchiB, and Δlo2467) strains. Intercellular spread of bacteria results in formation of zone of clearances or plaques, whose diameters are measured to assess the bacterial cell to cell spread efficiency. Ten plaques were measured for infection with each strain. Sizes of plaques formed by the mutant strains have been compared to plaque sizes caused by wild-type *L. monocytogenes* (set as 100%).
Attempts to provide chiB in trans were not successful because it was not possible to recover stable L. monocytogenes plasmid integrants for reasons that are not apparent. These data indicate a significant role for the chitinase ChiA in L. monocytogenes virulence and suggest that additional contributions are made by chiB and lmo2467 during mouse infection.
Figure 14. Chitinase deletion strains of *L. monocytogenes* are attenuated for virulence in mice. Groups of Swiss Webster mice were intravenously infected with $2 \times 10^4$ CFUs of wild type (WT) and chitinase/chitin binding protein deletion ($\Delta chiA$, $\Delta chiB$ and $\Delta lmo2467$) strains of *L. monocytogenes*. A $\Delta chiA$ strain complemented with *chiA* in trans ($\Delta chiA + pPL2-chiA$) was also used to infect the mice. After 72 hours of infection livers and spleens of the infected mice were isolated, homogenized and plated on BHI media. The resulting numbers of CFUs represent the bacterial burden in the liver and in the spleen for the respective strains. Statistical significance was calculated using a 1-way ANOVA with Tukey’s multiple comparison test. (*, $P < 0.01$; **, $P < 0.001$; ***, $P < 0.0001$)
3.4 Discussion

The recently discovered roles of chitinases and chitin binding proteins in bacterial virulence suggested that these proteins may merit consideration as general virulence factors. Chitinases and chitin binding proteins have been implicated as virulence factors for *L. pneumophila* and *V. cholerae* [116, 121]. In *L. pneumophila*, the chitinase ChiA has been observed to be secreted by the bacterial Type II secretion system along with other virulence factors [116]. This chitinase contributes to bacterial persistence in the lungs of infected mice. However, the mechanism by which the enzyme enhances bacterial colonization of lung epithelium has not yet been established.

For the secreted chitin binding protein GbpA of *V. cholerae*, it has been reported that the protein serves to enhance bacterial colonization in the intestine through binding to mucin [124], which consists of glycoproteins rich in carbohydrates including N-acetylglucosamine [139]. It has been reported that a chitin binding protein expressed by the bacterium *Serratia marcescens* increased bacterial adhesion to colonic epithelial cells via binding to the chitinase 3-like1 (CHI3L1) protein expressed on the host cell surface [140].

For *L. monocytogenes*, neither the chitinases ChiA and ChiB, nor the chitin binding protein Lmo2467 appeared to have any influence on bacterial invasion or replication within tissue culture cell lines. The chitinases and the chitin binding protein did not have any influence on
intercellular spread of the bacterium as well. These experiments indicate that none of the gene products has any appreciable role in host cell adhesion and invasion in these in vitro models of infection. However, the proteins clearly contributed to virulence via bloodstream infection of mice. Since the in vitro experimental set up does not exactly recreate the in vivo system, the roles of the chitinase and chitin binding proteins in vivo remain unclear. It remains possible that one or more of the secreted proteins recognize glycoproteins or carbohydrate moieties present on host cells and this recognition somehow enhance bacterial uptake in other cell types in vivo.

Although chitin in its polymeric form is absent inside mammals, molecules consisting of terminal N-acetylglucosamine residues, are present on the surfaces of many mammalian cells [168]. These chitin like molecules may be the target(s) of the chitinase and chitin binding proteins of *L. monocytogenes*.

It remains possible that chitinases could facilitate nutrient acquisition as chitinases be used to cleave N-acetylglucosamine containing oligosaccharides as sources of carbon and nitrogen. Although the chitinase deletion strains did not have any difficulty to grow and replicate inside mammalian cells, the situation may be different in vivo. It has been observed that expression of *L. monocytogenes* chitinase genes is repressed by the presence of glucose in the medium. On the other hand, expression of the chitinase genes is induced by chitin and/or N-acetylglucosamine [127]. Therefore, *L. monocytogenes* may utilize its chitinases for nutrient
acquisition in the host depending on the availability of carbon sources to stimulate chitinase expression.

Chitinases may be utilized for other purposes such as evasion of host immune response as well. It has been reported that the expression of chiA is induced within infected macrophages [128]. Although the ΔchiA strain was found to grow as efficiently as wild-type L. monocytogenes inside J774 macrophages, ChiA may influence other functions of macrophages. Macrophages are very important constituents of the mammalian immune system [141,169, 170]. As part of the innate immune system macrophages take part in pathogen clearance by actively killing microorganisms [141, 170]. Macrophages also secrete pro-inflammatory cytokines that activate other immune cells, and help in the induction of adaptive immune response that result in pathogen clearance [169]. This raises the alternative possibility that ChiA and/or other chitin binding proteins may serve to modulate the host immune responses by hydrolyzing carbohydrate moieties from glycoproteins that play important roles in the host immune response. As many of the effector molecules involved in pathogen recognition and immune signaling are glycoproteins containing terminal N-acetylglucosamine residues [103, 104, 105], enzymatic cleavage of the carbohydrate moieties might prevent a proper immune response required for pathogen clearance. L. monocytogenes may therefore utilize ChiA for modulating the host immune response.
ChiA production has been shown to be induced by the *L. monocytogenes* central virulence regulator PrfA [127]. As described in Chapter 1, PrfA is a transcription factor that becomes activated when *L. monocytogenes* enters a mammalian host [93], and induces the expression of the major virulence factors such as LLO, InlA, InlB, PlcA, PlcB and ActA [88-92]. This indicates that similar to the other PrfA controlled virulence factors, ChiA is also specifically upregulated inside mammalian hosts. However, it is not clear how PrfA controls the expression of ChiA. As there is no recognizable PrfA binding site within the promoter region of *chiA* it seems unlikely that PrfA directly binds to *chiA* promoter to enhance its transcription. Alternatively, a PrfA activated protein may function to induce transcription of *chiA*. PrfA is not known to induce the expression of *chiB* or *lmo2467*. This indicates although they have similar functions, chitinase and chitin binding protein genes of *L. monocytogenes* are differently regulated. This raises the possibility that *L. monocytogenes* may utilize its two chitinases for different environmental conditions with ChiB more active outside the host, and ChiA being effective inside the host. However, as the ΔchiB strain was defective to some extent for survival inside the host, ChiB activity inside the host cannot be ruled out. It is possible that *chiB* expression inside the host is controlled by some transcription factor other than PrfA.

The findings documented in this chapter shows that *L. monocytogenes* utilizes its chitinases ChiA and ChiB for its survival in a mammalian model of infection, with ChiA being the more important of the two. However, the mechanism(s) by which ChiA provides survival benefit to *L.
*monocytogenes* remains to be elucidated. Elucidation of the role of chitinases and chitin binding proteins in *L. monocytogenes* pathogenesis should provide insight into how bacterial factors that contribute to survival in the outside environment can be exploited for additional roles within an infected mammalian host.
Chapter 4

ChiA chitinase of the environmental pathogen *Listeria monocytogenes* enhances virulence through modulation of host immunity

4.1 Abstract

Chitinases and chitin binding proteins have been demonstrated to contribute to the pathogenesis of some environmental bacterial pathogens including *Listeria monocytogenes*. Chitinase deletion strains of *L. monocytogenes* have been found to be attenuated for virulence in murine models of infection, although they displayed no visible defects for invasion, intracellular growth or cell-to-cell spread in tissue culture models of infection. The most significant levels of attenuation for strains lacking a single chitin-associated gene product were observed for strains containing an in-frame deletion of *chiA*. Here we demonstrate that the virulence defect of the Δ*chiA* strain, which can be rescued by co-infection with wild type *L. monocytogenes*, is apparent after more than 48 hours of infection. ChiA does not appear to contribute to the intestinal colonization or translocation of *L. monocytogenes* across the intestinal epithelium. PrsA2, a post translocation chaperone protein of *L. monocytogenes* that helps folding and secretion of some of its major virulence factors, is also required for the secretion of ChiA in vitro. Our study also indicates that the enzymatic activity of ChiA is required for *L. monocytogenes* to achieve full
virulence in mice. Our work indicates that *L. monocytogenes* uses ChiA to suppress expression of inducible nitric oxide synthase (iNOS), which is an essential component of mammalian innate immune response against pathogenic bacteria including *L. monocytogenes*. This is the first demonstration of a bacterial chitinase being utilized for modulation of the host innate immune response.

4.2 Introduction

Environmental pathogens have been responsible for some of the most devastating epidemics in the history of mankind. This group of pathogens is found in diverse natural habitats such as soil, water and natural vegetation. However, when these organisms enter a mammalian host they cause diseases. *L. monocytogenes* is a food-borne environmental pathogen that has been responsible for a large number of food-borne outbreaks of listeriosis among humans [1, 25, 26, 27, 28]. Some of the most expensive recalls in the food processing industry have been due to contamination of processed food by *L. monocytogenes*.

*L. monocytogenes* is ubiquitous in nature [10] and is thought to lead a saprophytic lifestyle in soil, living predominantly on decaying vegetation [24]. As an environmental pathogen, *L. monocytogenes* has two distinct life cycles – one outside the host and one inside the host - and it must balance bacterial fitness between these varied habitats [1]. Similar to most environmental pathogens, much of the research on *L. monocytogenes* has focused on gene products that
specifically promote survival of the bacterium inside the host. However, less is known about gene products that might potentially help the bacteria to survive outside the host, and yet be adaptable for life inside the host as well.

Recently, chitinase has been shown to function as a virulence factor for a number of environmental pathogens including \textit{Legionella pneumophila}, \textit{Vibrio cholerae} \cite{116, 121} and \textit{L. monocytogenes}. Bacterial chitinases belong to the class of family 18 glycosyl hydrolase enzymes \cite{167} that catalyze the hydrolysis of chitin, which is a linear polysaccharide consisting of N-acetylglucosamine residues linked by (β1,4) glycosidic linkages. Chitin is abundant in nature, being present in fungal cell walls, exoskeletons of mollusks, arthropods and different crustacean organisms \cite{94, 95}. Therefore, chitinases are thought to help in bacterial nutrient acquisition through the degradation of chitin as a source of carbon and nitrogen \cite{95, 106}. However, with the emergence of chitinase and chitin binding proteins as virulence factors in \textit{L. pneumophila} \cite{116}, \textit{V. cholerae} \cite{121} and \textit{L. monocytogenes}, it has become evident that these enzymes may play roles in mammalian disease as well.

\textit{L. monocytogenes} has two chitinase genes, \texttt{chiA} and \texttt{chiB}, and one chitin binding protein gene (\texttt{lmo2467}). In frame chitinase and chitin binding protein deletion strains of \textit{L. monocytogenes} have been found to be significantly attenuated in infected mice. The most significant attenuation in virulence was observed with the Δ\texttt{chiA} strain. It has been known from tissue culture models
of infection that expression of the *chiA* gene is specifically upregulated inside macrophages [128]. It has also been shown that *chiA* expression is upregulated by PrfA [127], the transcription factor that also upregulates the expression of all the major virulence factors of *L. monocytogenes* [88-92].

Since chitin in its polymeric form is absent inside mammals, it is intriguing to find a bacterial chitinase to be involved in the process of causing disease in a mammalian host. However, there have been examples of mammalian chitinases being produced in association with some inflammatory diseases such as asthma [109, 111, 113]. Since many of the key molecules involved in immune response are glycosylated with N-acetylglucosamine residues linked by (β1,4) glycosidic bonds [99, 102, 104], it is possible that one or more glycosylated immune signaling or regulatory molecules may serve as a potential target of chitinases.

In this study we have addressed the question how ChiA affects the virulence of *L. monocytogenes* in mice. Here, we have shown that the bacterial survival defect in vivo associated with the loss of ChiA is apparent more than 48 hours after infection which indicates that the ΔchiA strain is not defective in the initial colonization of the target organs. We have also shown that this survival defect of the ΔchiA strain in mice can be rescued by a co-infection with wild type *L. monocytogenes*. Our data also shows that ChiA produced by *L. monocytogenes* is instrumental in suppressing the expression of inducible nitric oxide (iNOS) that is known to play
an essential role in bacterial clearance by the host [141]. ChiA therefore, appears to promote *L. monocytogenes* survival in vivo by modulating the host immune response. This is the first demonstration of a pathogenic bacterium utilizing its chitinase to modulate host innate immune response. Altogether, our study shows that *L. monocytogenes* secretes ChiA in vivo which promotes bacterial survival by using its chitinolytic activity on some unidentified target(s) in the host that results in the modulation of the host immune response.

### 4.3 Results

**ChiA does not appear to affect bacterial translocation across the intestinal epithelium**

It has been shown that *V. cholerae* utilizes its chitin binding protein, GbpA, to bind to the mucin component of the intestinal mucus layer [124], which consists of proteins with carbohydrate modifications, especially N-acetylglucosamine. Since the natural route of *L. monocytogenes* infection is through the intestine, the role of ChiA in crossing the intestinal barrier was assessed by using an oral model of infection. Internalin A (InlA), a surface protein of *L. monocytogenes* [67] contributes to bacterial invasion of the intestinal epithelium and, binds to human E-cadherin [69], a tight junction protein expressed in most epithelial cells [70]. While InlA does not efficiently recognize murine E-cadherin, a murine-adapted version of InlA (InlA S192N, Y376S) has been described which increases its binding affinity for murine E-cadherin [137], thereby rendering *L. monocytogenes* capable of causing infection in mice through the oral
route. The *inlA* S192N, Y376S mutations were introduced in the wild type and ΔchiA strains, and the mutated strains were subsequently used for oral infections. It was found that the bacterial burdens of the ΔchiA strain were similar to that of the wild-type strain after 24 hours of infection, and approximately ten fold lower than the wild type strain both in the liver and in the spleen after 72 hours of infection (Figure 15). However, this difference was comparable to difference in bacterial burdens observed for mice infected via the tail vein. ChiA therefore, does not appear to contribute any significant role to the translocation of the intestinal barrier.
Figure 15. ChiA deletion strain is attenuated for virulence during oral infection in mice. C57BL6 mice were orally infected with 1X10⁸ CFUs of wild-type (WT) and ΔchiA strains of *L. monocytogenes*. After 24 and 72 hours respectively, livers and spleens of infected mice were isolated, homogenized and plated on BHI medium. The resulting number of colonies represented the bacterial burdens of each strain in each of the organs. Statistical significance was calculated using a Student’s T-test ( **, P < 0.001; ***, P < 0.0001)
Chitinase activity of ChiA is required for full virulence of *L. monocytogenes* in vivo

Based on bioinformatic analysis of the amino acid sequence of ChiA, it was observed that the protein contains a canonical chitinase active site (Figure 16), consisting of nine amino acids (FDGLDIDDDLE). The glutamic acid residue at the end is conserved in all bacterial chitinases, and acts as a proton donor in the chitin hydrolysis reaction. It has been reported that in *Vibrio harveyi* chitinase, a point mutation of this glutamic acid to methionine abolishes its chitinolytic activity without changing the conformation of the protein. Therefore, an active site mutation (E163M) was generated in *chiA* of *L. monocytogenes* in which this conserved glutamic acid residue was replaced by a methionine residue by site directed mutagenesis. The Δ*chiA* mutant was complemented with this *chiAE163M* gene and subsequent in vitro and in vivo assays were carried out to assess protein activity. Expression of the active site mutant and wild-type protein was confirmed by Western blot analysis of the supernatant of overnight grown bacterial cultures using antibody directed against ChiA (Figure 17). While the synthesis of both the wild type and the ChiAE163M proteins were confirmed, secreted E163M was found to migrate in SDS-PAGE at a slightly lower molecular weight than the wild type ChiA protein. Therefore, the amino acid substitution leads to a change in protein mobility through SDS-PAGE gels, or alternatively that the protein is proteolytically cleaved. Despite the apparent size difference, both wild type ChiA and the ChiA E163M mutant were fully capable of binding chitin beads (Figure 18). While
ChiA E163M bound chitin, strains containing the mutant chiA allele exhibited no chitinase activity in bacterial supernatants (Figure 19).
**Figure 16. Schematic diagram of ChiA.** This cartoon diagram represents ChiA, the 352 amino acid long chitinase protein of *L. monocytogenes*. The enzyme active site consisting of nine amino acids from position 155 to 163 has also been indicated by the black box. The glutamic acid residue (E) at position 163 is conserved in all bacterial chitinases discovered so far and is critically important for the enzyme function.
Figure 17. Detection of ChiA in supernatants of L. monocytogenes culture by western blot. Wild-type (WT), ΔchiA, ΔchiA complemented with chiA having a point mutation in its active site (ΔchiA+ chiAE163M) and ΔchiA complemented with wild type chiA strains of L. monocytogenes, were grown upto OD_{600} ~1.2 in Luria Bertani (LB) broth containing 0.1% N-acetylglucosamine at 30°C. Culture supernatants were separated and the supernatant proteins were precipitated by adding trichloroacetic acid (10% by vol). The supernatant proteins were then separated by SDS PAGE in 10% polyacrylamide gel. Before loading to gels, sample volumes were adjusted to reflect equivalent bacterial density by normalizing to OD_{600}. ChiA was identified by Western Blot by using an antibody against ChiA.
Figure 18. Chitin binding activity of ChiA secreted by wild type and mutant strains of *L. monocytogenes*. Wild-type (WT), ΔchiA, ΔchiA complemented with chiA having a point mutation in its active site (ΔchiA+ chiA E163M) and ΔchiA complemented with wild type chiA strains of *L. monocytogenes*, were grown overnight in Luria Bertani (LB) broth containing 0.1% N-acetylglucosamine at 30°C. The supernatants were incubated with chitin beads for an hour. After incubation the beads were washed with PBS and were subjected to SDS PAGE. Before loading to gels, sample volumes were adjusted to reflect equivalent bacterial density by normalizing to OD$_{600}$. ChiA was identified by Western blot using an anti-ChiA antibody.
Figure 19. *L. monocytogenes* mutant harboring ChiA active site mutant (ΔchiA+ chiA_E163M) show reduced chitinolytic activity in vitro. Chitinase assay was done with supernatants of overnight grown cultures of wild-type (WT), ΔchiA, ΔchiA complemented with chiA having a point mutation in its active site(ΔchiA+ chiA_E163M) and ΔchiA complemented with wild type chiA strains of *L. monocytogenes* (ΔchiA+ chiA). Culture supernatants were incubated with the substrate 4 nitrophenyl N,N´ – diacetyl- β- D chitobioside for 2 hours at 30°C. The reaction was then stopped by adding 1 M Na₂CO₃. OD₄₀₅ of the reaction mixture was measured to assess the enzymatic activity of the chitinases. OD₆₀₀ values were also taken in order to normalize the OD₄₀₅ values with respect to the bacterial population density of the cultures.
When tested for virulence in mice, the ΔchiA + chiAE163M strain showed a similar reduction in bacterial virulence based on bacterial burdens in liver and spleen to strains lacking chiA (Figure 20). The chiA active site mutant had more than six fold reduction in bacterial numbers in the liver and spleen of infected mice after 72 hours of infection. It thus appears that the chitinase activity, and not just chitin binding of ChiA, contributes to the enhanced virulence of L. monocytogenes in vivo.
Figure 20. *L. monocytogenes* strain harboring active site mutant ChiA is attenuated for virulence in mice. Swiss Webster mice were infected with 2X10^4 CFUs of wild type (WT) and ChiA active site mutant (ΔchiA+chiAE163M) strains of *L. monocytogenes*. Bacterial burdens in livers and spleens of infected mice were measured by plating homogenized organs on BHI medium. Statistical significance was calculated using a student’s T-test (***, P < 0.0001)
The post translocation secretion chaperone PrsA2 is required for ChiA stability

PrsA2 is a recently identified post translocation chaperone protein produced by *L. monocytogenes* that is required for the stability and activity of multiple secreted virulence factors. It is involved in proper folding and secretion of listeriolysin O (LLO), a cholesterol dependent cytolysin that is critically important for *L. monocytogenes* pathogenesis, and for the full activity of the phospholipase PlcB, that contributes to cell-to-cell spread [85, 86]. Previously, proteomic analysis had indicated that ChiA was one of several proteins that appear to require PrsA2 for its secretion [87]. To further investigate the potential role of PrsA2 in the regulation of ChiA stability and/or activity, supernatant proteins from the wild type and the ΔprsA2 strains of *L. monocytogenes* were isolated, and the presence of ChiA was checked by Western blot analysis. Compared to the levels of ChiA expressed by wild type *L. monocytogenes*, much lower amounts of ChiA were detected in the supernatant of the ΔprsA2 strain (Figure 21). In contrast, when the ΔprsA2 strain was complemented with PrsA2 in trans, increased levels of ChiA were detected in the supernatant fraction. These results suggest that PrsA2 is involved in the production and secretion of ChiA by *L. monocytogenes*. The ChiA that was present in the supernatant of the ΔprsA2 strain failed to bind chitin beads (Figure 22), suggesting that PrsA2 is required for ChiA stability and activity.
Figure 21. Secretion of ChiA by *L. monocytogenes* is dependent on the post translocation chaperone protein PrsA2. Wild-type (WT), mutant lacking PrsA2 (ΔprsA2) and ΔprsA2 complemented with PrsA2 were grown overnight in LB broth containing 0.1% N-acetylglucosamine at 30°C. The supernatant proteins were precipitated with trichoroacetic acid and subjected to SDS PAGE. Before loading to gels, sample volumes were adjusted to reflect equivalent bacterial density by normalizing to OD$_{600}$. ChiA was identified by Western Blot using anti-ChiA antibody.
Figure 22. ChiA produced by *L. monocytogenes* mutant lacking PrsA2 fails to bind chitin. Wild-type (WT), mutant lacking PrsA2 (ΔprsA2) and ΔprsA2 complemented with PrsA2 were grown overnight in LB broth containing 0.1% N-acetylglucosamine at 30°C. The supernatants were incubated with chitin beads for an hour. After incubation the beads were washed with PBS and were subjected to SDS PAGE. Before loading to gels, sample volumes were adjusted to reflect equivalent bacterial density by normalizing to OD_{600}. ChiA was identified by Western blot using an anti-ChiA antibody.
ΔchiA growth defect in mice appears late after infection

As previously described, the ΔchiA strain of *L. monocytogenes* was found to be defective in colonizing the liver and the spleen of infected mice 3 days after infection by tail vein injection. However, it was not determined whether this growth defect in vivo starts right after infection, or at later stages of infection. To determine the time point from which the virulence defect becomes apparent, a time course assay was done. In this assay Swiss Webster mice were infected with either the wild type *L. monocytogenes* 10403S strain or the ΔchiA mutant. After 24, 48, 72 and 96 hour time points, livers and spleens were harvested from the infected mice, homogenized and plated on BHI medium to determine bacterial numbers. The number of colony forming units of the wild type and the ΔchiA strains were similar after 24 hrs and 48 hrs of infection. However, after 72 hrs of infection, the numbers of the ΔchiA strain were nine fold lower in the spleen and thirteen fold lower in the liver than the wild type. The virulence defect of the ΔchiA strain was even more pronounced after 96 hrs of infection (Figure 23), with the mutant strain being more rapidly cleared from infected organs in comparison to mice infected with the wild type strain. The bacterial burdens of the ΔchiA strain in the liver and the spleen were more than thirty fold less than the wild type strain. This result shows that the ΔchiA strain is not defective in the initial colonization of the target organs, but it is probably more rapidly eliminated from the host than the wild type strain.
Figure 23. ChiA deletion strain of *L. monocytogenes* is cleared more rapidly than the wild-type strain from infected mice. Swiss Webster mice were infected with 2X10⁴ CFUs of wild-type (WT) and ChiA deletion (ΔchiA) strains of *L. monocytogenes*. After 24, 48, 72 and 96 hours respectively livers and spleens of infected mice were isolated, homogenized and plated on BHI medium. The resulting number of CFUs represent the bacterial burden in each organ at each time point. Statistical significance was calculated using a student’s T-test (***, P < 0.0001).
The virulence defect of the ΔchiA strain in vivo can be complemented in trans by wild type

*L. monocytogenes*

As ChiA is a secreted gene product, thus it seemed possible that whatever function was missing from the ΔchiA mutant strain in vivo might be trans complemented via co-infection with the wild type strain. A competitive index analysis was thus carried out in which, Swiss Webster mice were injected with a mixture of equal proportions of the ΔchiA mutant and a wild type strain containing an erythromycin resistance gene cassette. The organs were harvested 72 hours post infection, and plated on media with or without erythromycin. The competitive index (CI) was calculated by taking the ratio of the number of CFUs of the ΔchiA strain to that of the wild-type strain isolated from the liver and the spleen of the infected mice. A (CI) value close to 1 indicated that the number of the ΔchiA strain was approximately the same as the wild type (Figure 24). The absolute values of the CFUs of each strain show that the survival defect of the ΔchiA strain was rescued by the presence of the wild-type strain (Figure 25), indicating that ChiA secreted by the wild type was capable of restoring the ΔchiA defect when supplied in trans. This result would seem to suggest that ChiA function is required for some modification of the host environment and/or immune response that promotes *L. monocytogenes* replication.
Figure 24. $\Delta chiA$ strain of *L. monocytogenes* is complemented by the wild-type strain in vivo. Swiss Webster mice were infected with a mixture of equal proportions of a reference wild-type *L. monocytogenes* strain containing an erythromycin resistance gene cassette [WTerm(R)], and the $\Delta chiA$ mutant. After 72 hours of infection, livers and spleens of infected mice were isolated, homogenized and plated on BHI media with or without erythromycin. The competitive index values were calculated by taking the ratio of the resulting number of CFUs of the $\Delta chiA$ mutant to that of the WTerm(R). As a control the same experiment was done with a mixture of the reference strain and wild-type *L. monocytogenes* (WT) that had been used in previous experiments.
Figure 25. Co-infection with wild-type *L. monocytogenes* rescues the survival defect of the Δ*chiA* mutant. Swiss Webster mice were infected with a mixture of equal proportions of a reference wild-type *L. monocytogenes* strain containing an erythromycin resistance gene cassette [WTerm(R)] and the Δ*chiA* mutant. After 72 hours of infection, livers and spleens of infected mice were isolated, homogenized and plated on BHI media with or without erythromycin. The resulting number of CFUs represent the bacterial burden of each strain in the target organs.
Modulation of the host immune response by ChiA

As the ΔchiA mutant is more rapidly cleared from a mammalian host than wild-type L. monocytogenes it is possible that ChiA is used by L. monocytogenes to modulate the host immune response and thereby evade clearance from the host. To test this possibility, Swiss Webster mice were infected with wild-type and ΔchiA strains of L. monocytogenes by tail vein injection. After 48 hours of infection livers of infected mice were isolated, and mRNA levels of several molecules that are important in mammalian immune response against L. monocytogenes were measured by real time RT-PCR. The mRNA levels of the molecules of interest were normalized with respect to the mRNA levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Although tumor necrosis factor (TNF) and interleukin-1 beta (IL-1β) were similarly induced during infection with wild type and ΔchiA strains of L. monocytogenes (Figure 27), it was observed that the level of inducible nitric oxide synthase mRNA (NOS2) was significantly higher in mice infected with the ΔchiA strain than in mice infected with wild-type L. monocytogenes (Figure 26). This suggests that ChiA secreted by L. monocytogenes is instrumental in suppressing the induction of inducible nitric oxide synthase which plays a very important role in the clearance of many pathogenic bacteria including L. monocytogenes from the host, and is an essential component of the host innate immunity [141, 142]. This shows that L. monocytogenes utilizes ChiA to modulate the host innate immune response against it.
Figure 26. Suppression of inducible nitric oxide synthase (NOS2) in mice by ChiA of *L. monocytogenes*. Swiss Webster mice were intravenously infected with wild-type (WT) and ΔchiA strains of *L. monocytogenes*. After 48 hours of infection livers of infected mice were homogenized and treated with trizol to extract mRNA. Induction of NOS2 mRNA was measured by RT-PCR. As a control, levels of NOS2mRNA were also measured from livers of uninfected mice. The mRNA levels of NOS2 were normalized with respect to GAPDH mRNA levels. Statistical significance was measured by student’s T-test (**, P < 0.001). Experiment done in collaboration with Dr. Benjamin Gantner.
Figure 27. Induction of the cytokines TNF and IL-1β after *L. monocytogenes* infection in vivo is not affected by ChiA. Swiss Webster mice were intravenously infected with wild-type (WT) and ΔchiA strains of *L. monocytogenes*. After 48 hours of infection livers of infected mice were homogenized and treated with trizol to extract mRNA. Induction of TNF-α and IL-1β was assessed by measuring the levels of the respective mRNAs by RT-PCR. As a control, mRNA levels of tnfα and il1β were also measured from livers of uninfected mice. mRNA levels of tnfα and il1β were normalized with respect to the levels of *GAPDH* mRNA.
ΔchiA mutant strain is fully virulent in iNOS deficient mouse

As increased levels of iNOS were observed in mice infected with *L. monocytogenes* strains lacking ChiA, it was evident that ChiA promotes survival of the bacterium in the host by down-regulating inos expression. To test this hypothesis, NOS2 knock-out mice were infected with wild-type and ΔchiA strains of *L. monocytogenes*. It was observed that the bacterial burden of the ΔchiA strain was similar to that of wild type *L. monocytogenes* in livers and spleens of the infected iNOS knock-out mice (Figure 28). In contrast, in wild type C57BL6 mice, the bacterial burden of the ΔchiA strain was significantly lower than that of wild type *L. monocytogenes* both in the liver and the spleen.
Wild type mice

NOS2 knock-out mice

Figure 28. ΔchiA mutant strain of *L. monocytogenes* is fully virulent in iNOS deficient mouse. Wild-type and NOS2 knock-out strains of C57BL6 mice were separately intravenously infected with wild-type (WT) and ΔchiA strains of *L. monocytogenes*. After 72 hours of infection livers and spleens of infected mice were isolated, homogenized and plated on BHI medium. The resulting number of CFUs represented the bacterial burden of each *L. monocytogenes* strain in the organs. Statistical significance was calculated using student’s T-test (***, P < 0.0001).
4.4 Discussion

Environmental pathogens are equipped to survive both outside and inside of the host body. Since they spend a substantial part of their life cycles outside the host, it is intriguing how they are able to maintain their repertoire of virulence factors that are required only when they are present inside the host body. It may also be a metabolic burden to replicate and maintain separate sets of genes for different environments. Environmental pathogens may solve this problem by utilizing the same gene products for survival in both the environments, i.e, inside the host and outside the host. This possibility has come to the focus with the emergence of chitinases and chitin binding proteins as virulence factors for environmental pathogens. Bacterial chitinases and chitin binding proteins were thought to be required for bacterial survival in the environment by providing nutritional benefits [95, 106]. However, from works done on chitinases and chitin binding proteins of the bacterial pathogens *L. pneumophila* [116], *V. cholerae* [121] and *L. monocytogenes* it is evident that these proteins enhance bacterial survival inside the host as well.

In this work we have provided evidence that the chitinase ChiA secreted by *L. monocytogenes* modulates the host immune response by suppressing the production of inducible nitric oxide synthase, an enzyme that plays a very important role in pathogen clearance [141,147].
Our experiments with the point mutation in the active site of ChiA show that the chitinolytic activity of the enzyme is required for the bacteria to attain full virulence in mice. It has been shown in *Vibrio harveyi*, that, a change of the conserved glutamate (E) residue of its chitinase active site to methionine (M) rendered the protein enzymatically inactive without disrupting the conformation [133]. A similar point mutation (E163M) in ChiA of *L. monocytogenes* abrogated its chitinolytic activity. However, it was still able to bind chitin, as evident from the chitin bead binding experiments. An *L. monocytogenes* strain harboring the mutated version of ChiA (ChiAE163M) was found in significantly lower numbers in the livers and spleens of infected mice, which strongly suggested that chitinolytic activity of ChiA is required inside the host, and binding alone to its potential target(s) in mammals is not sufficient for *L. monocytogenes* to attain full virulence in mice.

Given that humans and other mammals do not produce chitin, the target of the *L. monocytogenes* ChiA enzyme remains unclear. While it is possible that chitin or organisms producing chitin may be found within the human gastrointestinal tract or within oral and nasal passages, *L. monocytogenes* ChiA must play a role in mammalian pathogenesis that is distinct from the hydrolysis of microbe or arthropod synthesized chitin. The virulence defect of *L. monocytogenes ΔchiA* mutants was evident following intravenous inoculation and bacterial replication within the liver and spleen, organs not anticipated to encounter or harbor exogenous sources of chitin. Molecules similar to chitin are, however, abundant both inside and outside of
mammalian cells. N- and O- glycans and glycosaminoglycans containing N- acetylglucosamine linked by (β1,4) glycosidic bonds are present on glycoproteins and glycolipids present on the cell surfaces of a variety of mammalian cell [172, 173]. As previously reported, mucin is rich in N-acetylglucosamine; and this property of mucin contributes to *V. cholerae* colonization of the intestine via the chitin binding protein GbpA [124]. Many key molecules involved in mammalian immune responses and signal transduction cascades are glycoproteins containing N-acetyl glucosamine residues [102-105]. Thus, despite the absence of endogenously synthesized chitin, mammals produce a variety of glycosylated proteins and lipids that could represent potential targets for chitin binding proteins and chitinases. The ability of secreted ChiA to rescue the defect of *L. monocytogenes* ΔchiA mutant strains in mixed infections is consistent with ChiA modification of a target that influences the host environment and/or the host response to infection. As described in Chapter 3, ΔchiA mutants have been previously shown to have no detectable defects in host adherence or invasion, vacuole escape, intracellular replication or cell-to-cell spread. From the time course assay of infection, it is evident that the ΔchiA strain is not defective in the initial colonization of the target organs. However, compared to wild type *L. monocytogenes*, the ΔchiA strain clearly has a survival disadvantage in vivo as the infection progresses which indicates that the ΔchiA strain may be cleared by the host immune response more rapidly than wild type *L. monocytogenes*. The fact that mixed infections of ΔchiA mutants with wild type *L. monocytogenes* can restore ΔchiA growth in liver and spleen suggests that the
wild type strain is capable of modifying the host response to infection in a way that benefits the ΔchiA mutant.

The possible immunomodulatory role of ChiA becomes evident from the suppression of expression of inducible nitric oxide synthase (iNOS) in vivo. iNOS, encoded by the gene NOS2, is an enzyme whose expression is highly upregulated in mammalian cells such as macrophages, neutrophils and hepatocytes [143-145] after infection by L. monocytogenes and other pathogenic bacteria [142, 146]. iNOS catalyses the synthesis of nitric oxide (NO) from arginine, which is an essential part of the host defense against bacterial infection [147]. The importance of iNOS in immunity against L. monocytogenes is evident from the increased susceptibility of iNOS^{−/−} mice to the bacteria. The bacterial burden in the organs of iNOS^{−/−} mice is about 100 fold higher than that in wild-type mice [175]. Therefore, a suppression of iNOS expression will dampen the host immune response against the infectious agent resulting in delayed bacterial clearance. From our studies it is therefore evident that L. monocytogenes utilizes its chitinase ChiA to either suppress the transcription of NOS2 or destabilize the NOS2 mRNA, which results in delayed clearance of the bacteria from the host. This is the first demonstration of a bacterial chitinase functioning to modulate host innate immunity

The mechanism by which ChiA suppresses the expression of NOS2 in vivo is not clear. Regulation of iNOS expression is a highly complex process [148]. NOS2 transcription is
induced during infection by pathogenic bacteria such as *L. monocytogenes* \[141, 142, 146\]. Immediately after *L. monocytogenes* infection, proinflammatory cytokines such as TNF, IL-1β and IFN-γ are secreted by macrophages and other cells involved in innate immune response \[60-63\]. These proinflammatory cytokines trigger iNOS expression \[147\]. Binding of TNF, IL-1β and IFN-γ to their respective receptors result in activation of several transcription factors such as nuclear factor kappa B (NF-κB), mitogen activated protein kinases (MAPK) and TRAF \[148\]. About fifteen transcription factors have been discovered to be involved in upregulation of *NOS2* transcription. As most cytokines are glycoproteins, and a large number of glycosylated transcription factors containing O-linked N-acetylglucosamine residues being discovered \[104, 151, 152\], ChiA can interfere with the glycosylation status of one or more of these molecules, which may result in a suppressed iNOS expression.

Little is known about the regulation of ChiA production and secretion in *L. monocytogenes*. Earlier studies had shown that the chitinases, ChiA and ChiB are not produced by *L. monocytogenes* in the presence of glucose in the medium \[127\]. On the other hand, expression of the chitinases by *L. monocytogenes* is induced by chitin. It has also been shown that expression of ChiA is induced by N-acetyl glucosamine \[127\]. These studies indicate that chitinases and chitin binding protein genes of *L. monocytogenes* are under the control of some kind of catabolite repression mechanism. These studies also show that when a readily usable sugar such as glucose is available in the environment, expression of the chitinases of *L.
*L. monocytogenes* remain turned off. The presence of chitin or its monomeric subunit N-acetylglucosamine in the medium provides a signal that leads to expression of the chitinase gene(s). Recently, a small RNA molecule (LhrA) has been shown to be involved in post-transcriptional regulation of *chiA* expression [174]. But factors involved in stability and secretion of ChiA have not been studied so far. Our present work shows that PrsA2, a post translocation chaperone protein of *L. monocytogenes* is required for the stability and secretion of the chitinase ChiA.

Interestingly PrsA2, a PrfA controlled protein, is involved in the folding and secretion of some of the major virulence factors of *L. monocytogenes*, and a ΔprsA2 strain is severely attenuated for virulence in mice [85, 86, 87]. Therefore the chaperone PrsA2 is probably tuned up to be expressed inside the host, and is dedicated to proper folding of proteins that are required for bacterial survival in the host. It has been shown that the *chiA* gene expression is upregulated when *L. monocytogenes* is present inside macrophages [128]. It has also been shown that *chiA* expression is upregulated by PrfA [127], the master regulator of all the major virulence factors of *L. monocytogenes* [89-92]. The induction of ChiA by PrfA could therefore be mediated by PrsA2.

Overall, this study shows that environmental pathogens like *L. monocytogenes* have evolved to utilize proteins, which are assumed to be important for survival outside the host, as machinery
to survive inside the host as well. This is true for many other environmental pathogens as well. Hence, in future studies with this kind of bacterial pathogens it will be necessary to include many of the so-called ‘environmental’ proteins as virulence factors. These may serve as novel therapeutic targets as well. As the exact molecular target(s) of ChiA inside the mammalian host remains elusive, it will be interesting to identify the target of ChiA in the host in future.
Chapter 5

Conclusions and Future Perspectives

5.1 Introduction

This dissertation has investigated the roles of chitinase and chitin binding proteins as virulence factors in the environmental pathogen *Listeria monocytogenes*. This work has implications for both studies of environmental pathogens, and for understanding mechanisms underlying bacterial pathogenesis in general.

5.2 Do environmental pathogens use same gene products to survive both inside and outside of a host?

This dissertation work has focused on some novel areas of research involving *L. monocytogenes* as a model environmental pathogen. As discussed in Chapter 1, environmental pathogens are those microorganisms that occupy different ecological niches outside a host, but can cause disease when they are present inside a host. These pathogens are therefore well adapted to survive and proliferate in environments that are very different from one another. Since these microorganisms encounter a mammalian host only occasionally, it is an interesting question as to how they maintain their arsenal of virulence factors if these factors are required only when the microbes are present inside a host. It would be metabolically demanding to
maintain and express separate repertoires of genes for separate environmental conditions. Environmental pathogens may solve this problem by utilizing the same set of molecules for adapting to different environments. Bacterial chitinase and chitin binding proteins may represent such molecules that promote bacterial survival both inside and outside of a host body. Chitin, a polysaccharide consisting of N-acetylglucosamine subunits, is abundant in the environment outside the host and is a readily available source of carbon and nitrogen [94-96]. Therefore, bacterial chitinases and chitin binding proteins that facilitate chitin degradation and utilization, are useful for nutrient acquisition by bacteria [95]. As shown by studies of *L. pneumophila*, *V. cholerae* [116, 121], and now *L. monocytogenes*, it is evident that bacterial chitinases and chitin binding proteins provide survival benefits to the bacteria inside the host as well. Thus chitinases and chitin binding proteins may represent a previously unrecognized class of bacterial gene products that have dual and probably distinct functions inside and outside the host. With the discovery of chitinases and chitin binding proteins as molecules that promote bacterial survival inside hosts, the approach to identify virulence factors of environmental pathogens should consider the role of molecules that are required for survival outside the host as well. With the discovery of chitinase and chitin binding proteins promoting bacterial survival in a host, virulence factors should probably be defined from a broader point of view.
5.3 Do chitinase and chitin binding protein function as virulence factors for other environmental pathogens?

Although chitinases and chitin binding proteins have been implicated to have roles in the pathogenicity of *L. pneumophila*, *V. cholerae* [116, 121] and *L. monocytogenes*, not much is known about their potential contribution as virulence factors in other pathogenic bacteria. It is not clear whether chitinases and chitin binding proteins of pathogenic bacteria in general contribute to their virulence, or whether the role of chitinases and chitin binding proteins as virulence factors is restricted to subsets of bacterial species. As a large number of pathogenic bacteria such as *Bacillus anthracis*, *Vibrio vulnificus*, *Burkholderia cepacia* [118-120] etc. express chitinases, it would be interesting to investigate their roles in pathogenesis. Although it may still be premature to generalize bacterial chitinases and/or chitin binding proteins as virulence factors, this work combined with previous reports of *L. pneumophila* and *V. cholerae* [116, 121] chitinases and their roles in bacterial virulence suggests that these secreted proteins may represent a previously unrecognized group of bacterial virulence factors.

5.4 What are the probable target(s) of bacterial chitinases inside a mammalian host?

It is quite fascinating to consider chitinase as a bacterial virulence factor in mammalian hosts, given that chitin is not present in mammals. As mentioned previously, chitin is a linear polymer of N-acetylglucosamine residues linked by (β1,4) glycosidic bonds [95, 106]. Chitinases are
enzymes that breakdown chitin into smaller subunits by hydrolyzing the (β1,4) glycosidic bonds between the N-acetylglucosamine residues. As discussed in the previous chapters, although chitin itself is not present in mammals, carbohydrate moieties similar to chitin are present in mammalian systems [98-102]. N-acetylglucosamine residues linked by (β1,4) glycosidic bonds are essential components of most mammalian glycoproteins. Most eukaryotic cell surfaces feature glycoproteins that contain N-acetylglucosamine [100, 101]. Many of key molecules of the mammalian immune response such as cytokines, chemokines, MHC molecules etc are glycoproteins that contain N-acetylglucosamine residues [103-105]. Therefore, molecules that mimic chitin are present in mammals, and hence can act as potential substrates for chitinases and chitin binding proteins. A very good example of this possibility is the binding of GbpA, a chitin binding protein secreted by *V. cholerae*, to mucin proteins of murine intestine [124]. Mucin consists of heavily glycosylated proteins that constitute the mucus layer of mammalian intestine. This demonstrates that although chitin in its polymeric form is absent in mammalian intestine, there are molecules that are similar to chitin, which GbpA recognizes as its substrate [124].

Within the lungs of infected animals, ChiA, a chitinase secreted by *L. pneumophila* promotes bacterial persistence [116]. The molecular target(s) of ChiA in the lungs has not been identified. However, these data strongly suggest that there are unidentified molecule(s) present in the lung which mimic chitin in molecular structure and therefore serve as substrates for ChiA.
This dissertation work showed that chitinase and chitin binding proteins secreted by *L. monocytogenes* contribute to bacterial virulence in mice. In-frame chitinase deletion mutants were found to be more rapidly cleared from livers and spleens of infected mice in comparison to the wild type strain. Although at this point of time it is highly speculative to identify the exact molecular target(s) of the chitinases of *L. monocytogenes* inside a mammalian host, some possibilities as to the mechanisms by which the chitinases benefit *L. monocytogenes* survival inside the host can be suggested.

5.5 How does chitinase produced by *L. monocytogenes* help the bacterium to survive in a mammalian host?

Compared to wild type *L. monocytogenes*, in-frame deletions in the chitinase genes (*chiA* and *chiB*) and chitin binding protein gene (*lmo2467*) attenuate bacterial virulence in mice. As shown in Chapter 2, the *lmo2467* deletion mutant was only slightly attenuated compared to the chitinase deletion strains. The most significant attenuation was observed with the Δ*chiA* strain. The triple deletion strain with all the chitinase and chitin binding protein genes deleted was attenuated in mice more or less to the same extent as the Δ*chiA* strain suggesting that ChiA makes the most significant contribution to *L. monocytogenes* virulence. The modest reduction in virulence observed for strains lacking *lmo2467* in comparison to strains lacking either *chiA* or *chiB* suggested that chitin binding activity alone was less important for enhancing *L.
monocytogenes survival in mice and chitinolytic activity is important for *L. monocytogenes* to survive in vivo. This hypothesis is corroborated by the survival defect of the *L. monocytogenes* strain that expresses a ChiA with abrogation of its chitinase activity because of a point mutation in the active site. These data suggest that the hydrolysis by chitinase of the (β1,4) glycosidic bonds between N-acetylglucosamine residues of some yet as unknown host target serves to enhance *L. monocytogenes* replication within an infected mammalian host.

A possible role for chitinase/chitin binding proteins could be bacterial attachment to and potentially modification of sugar moieties on eukaryotic cell surfaces that might serve to promote bacterial uptake and infection. However, as shown in Chapter 3, mutants lacking the chitinase and/or chitin binding protein could infect and invade cultured mammalian cells as well as their wild type counterpart. There were no defects observed for bacterial intracellular replication, and the mutants were equally adept as wild type *L. monocytogenes* in cell to cell spread. Therefore the survival defect of the chitinase deletion strains in vivo does not appear to be due to inability to infect host cells, nor due to a defect in intracellular growth or intercellular spread. This observation is also supported by data from the time course assay of infection, in that the ΔchiA strain was not found to be defective in intestinal translocation or in the initial colonization of the target organs. However, with increased time points after infection, the ΔchiA strain appeared to be cleared more rapidly than wild type *L. monocytogenes* suggesting that the host immune system was more effective in recognition and/or elimination of the mutant strain.
These data suggest an immunomodulatory role for ChiA in mice. Work done in this dissertation project indicates that ChiA influences the levels of molecules that are important for an effective immune response in vivo.

To survive inside a mammalian host, a successful pathogenic bacterium must overcome the host immune response. Much work has focused on the characterization of host immune response against *L. monocytogenes* [59-66, 141, 169, 170]. Indeed, much of our current knowledge on mammalian immune system has been gathered from innate and adaptive immune responses observed in experiments using *L. monocytogenes* as a model infectious agent in mice [59]. Although oral route is the natural route of *L. monocytogenes* infection, intravenous infection in mice has been routinely used to study bacterial growth and colonization of the organs, as well as the host immune response. Immediately after infection via the intravenous or the oral route, most of the bacteria are trafficked to the liver and the spleen where they are taken up by resident macrophages [60, 61]. *L. monocytogenes* elicits secretion of proinflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-12 and IFN-γ by macrophages and other cell types [63, 141] that lead to activation of the adaptive immune response in the form of CD4+ and CD8+ T cells [141, 170]. Activated T cells are necessary for bacterial clearance and are essential in providing protective immunity against *L. monocytogenes* [66]. Interestingly, all of these interlinked processes that are essential to eliminate a bacterial pathogen from the host are mediated by glycoproteins [104].
Protein glycosylation plays a very important role in recognition and clearance of foreign antigens. Most of the important molecules involved in immune response are glycosylated [104]. Glycan chains provide protection to the proteins involved in immune response from protease cleavage. Glycan chains also play important roles in the formation of T-cell synapses in which T cells and antigen presenting cells (APC) are aligned in close proximity and thereby promoting antigen presentation to T cells [103, 104, 105]. T cell receptors and major histocompatibility complex (MHC) that are crucial for an effective T cell response are glycoproteins that consist of N-acetylglucosamine residues [104]. Other critically important mediators of an effective immune response are cytokines. Cytokines represent a group of proteins that function as ligands which initiate signal transduction events after binding to specific receptor molecules present in mammalian cell surfaces, and most of the cytokines are glycosylated proteins. The signal transduction events triggered by cytokines are critically important in the context of an inflammatory response against a pathogen and lead to activation of effector cells that lead to pathogen clearance. Glycosylation is important for cytokine stability, and their ability to bind to specific receptors [104]. Therefore, interfering with the glycosylation status of these molecules of the immune system may be a way by which bacterial pathogens evade immune response. Bacterial enzymes that hydrolyze (β1,4) glycosidic bonds between sugar molecules, such as ChiA, can thus be instrumental in modifying the glycosylation status of glycoproteins, such as cytokines, that are important in host immune response. Although the induction of TNF and IL-
After in vivo infection with *L. monocytogenes* remains unaffected by ChiA, the effect of ChiA on other chemokines and cytokines involved in immune response against *L. monocytogenes* remain to be tested.

The effect of ChiA on iNOS levels in vivo is highly interesting. iNOS is an enzyme that is responsible for synthesis of nitric oxide (NO) from arginine in a variety of mammalian cells such as macrophages, neutrophils and hepatocytes. NO plays important roles in various physiological processes such as control of blood pressure, regulation of vascular resistance, cytotoxic killing of tumor cells and control of intracellular parasites microbial pathogens [147]. Induction of NO during immune response against pathogenic microorganisms is well known and is one of the ways by which proliferation of pathogens is controlled by innate immunity [142]. The role of NO in controlling *L. monocytogenes* infection is well documented [141, 146], and is an essential component of the innate immunity against *L. monocytogenes* due to its bactericidal properties. NO being a small water and lipid soluble molecule can easily diffuse through biological membranes, and react with reactive oxygen species, such as superoxide radical (O$_2^\cdot$), producing reactive nitrogen intermediates (RNI) such as NO$_2$, NO$_2^-$, NO$_3^-$, S-nitrosothiols, and peroxynitrite (OONO$^-$) [142, 147]. The RNIs are toxic to bacteria because of their ability to damage DNA and several moieties in proteins such as iron-sulfur clusters, tyrosyl radicals, hemes and thioethers, and therefore restrict bacterial growth by their bactericidal properties [149]. It has been shown that NO produced by macrophages and neutrophils plays an important
part in controlling *L. monocytogenes* infection [147]. In mice, iNOS expression and consequent NO production is induced rapidly after infection with *L. monocytogenes*. However, as shown by others, after 96 hours of *L. monocytogenes* infection, there is a sharp decline in iNOS levels in vivo [146]. TNF and iNOS producing dendritic cells (TiP DCs) are major sources of nitric oxide in the spleen and are essential in the immune response against *L. monocytogenes* [141, 150].

Work in this dissertation shows that the chitinase ChiA secreted by *L. monocytogenes* is functional in bringing down the level of iNOS in mice after 48 hours of infection. Since NO plays a crucial role in controlling *L. monocytogenes* proliferation in vivo, ChiA probably helps the bacterium to survive by bringing down the levels of iNOS and thereby reducing the levels of NO that is toxic to the pathogen.

### 5.6 Possible relation between iNOS and ChiA

In mammals, expression of iNOS is tightly regulated mainly at the level of transcription, although control mechanisms at various post-transcriptional and translational levels also exist [148]. Although regulation of iNOS expression is highly cell-specific and organism specific, some general trends have been discovered. Following infection by pathogenic microorganisms, expression of iNOS is triggered by a variety of pro-inflammatory cytokines such as TNF, IL-1β and IFN-γ. Interferon gamma (IFN-γ) is very important in inducing the expression of iNOS. IFN-γ alone has been shown to stimulate iNOS expression [147]. Other cytokines such as TNF
and IL-1 have been shown to induce the production of iNOS synergistically with IFN-γ. As discussed briefly in Chapter 4, induction of iNOS expression by pro-inflammatory cytokines is mediated by various transcription factors such as NF-κB, signal transducer and activator of transcription (STAT-α), MAPK and interferon regulatory factor (IRF-1) [148]. Other factors such as the scaffolding protein HMG-I(Y) and CBP interact with IRF-1 and NF-κB to form multi-subunit complexes that promotes NOS2 transcription [148]. Thus it will be an interesting field of research to study whether bacterial chitinase ChiA can interfere with any of the transcription factors involved in the transcription of NOS2. In recent years, many transcription factors with terminal N-acetylglucosamine residues have been discovered, and similar to phosphorylation, N-acetylglycosylation is also being considered as a mechanism of signal transduction [151, 152]. Therefore modification of the N-acetylglucosamine residues by chitinases such as ChiA of L. monocytogenes may lead to impaired transcription of genes such as NOS2.

The expression of iNOS have been found to be suppressed by the cytokines transforming growth factor beta (TGF β), IL-4 and IL-10 [147]. Suppression of iNOS by IL-4 is particularly interesting in the context of L. monocytogenes infection. It has been observed that IL-4 suppresses iNOS expression which is induced early during infection by L. monocytogenes [153]. IFN-γ and IL-4, which are hallmarks of Th1 and Th2 adaptive immune responses respectively, act antagonistically to one another to regulate the expression of NO [147, 154]. Therefore,
suppression of iNOS expression takes place as the T cell response becomes skewed towards the Th2 pathway. The Th2 pathway, triggered mainly by IL-4 [155], can prevent NO production by iNOS [147], and lead to suppression of the proinflammatory Th1 response which is needed for bacterial clearance. Although no direct correlation between bacterial chitinases and induction of Th2 pathways have been discovered yet, mammalian chitinases are thought to be involved in the Th2 immune responses [109, 110, 111, 114, 115]. As discussed in Chapters 1 and 3, mammalian chitinases and chitinase like proteins are produced in large quantities during asthma and allergic conditions [111, 112 113], which are largely manifestations of a Th2 immune response. Thus, it will be an interesting field of research to study whether ChiA of L. monocytogenes has a role in stimulating IL-4 and other mediators that tend to skew the host immune response towards the Th2 pathway.

5.7 Concluding remarks

This dissertation has described some novel findings that increase our knowledge on L. monocytogenes as an environmental pathogen and how it utilizes chitinase and chitin binding proteins which are thought to provide nutritional benefits in its natural habitats such as soil, as tools to survive in the host as well. Thus, this work broadens our general knowledge about environmental pathogens and bacterial virulence factors in general. For environmental pathogens the gene products required for survival outside the host are generally neglected in the
context of studying bacterial pathogenesis and host response. This dissertation has showed that more research on these gene products is necessary to have a better understanding of environmental pathogens. These types of works may lead to generation of novel therapeutic measures against environmental pathogens which are big problems to mankind. This work has further opened the avenues of research on possible ways by which pathogenic bacteria utilize chitinases to modulate the host immune response.
### APPENDIX

**Table 1. Bacterial strains used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Designation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TOP10</td>
<td><em>E. coli</em> host strain used to maintain pPL2 plasmid</td>
<td></td>
<td>[85]</td>
</tr>
<tr>
<td>SM10</td>
<td><em>E. coli</em> host strain used to maintain pPL2 plasmid</td>
<td></td>
<td>[177]</td>
</tr>
<tr>
<td>NF-L100</td>
<td>Wild type <em>L. monocytogenes</em> 10403S parent strain</td>
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<td></td>
</tr>
<tr>
<td>NF-L1472</td>
<td><em>chiB</em> gene deleted in NF-L100</td>
<td>Δ<em>chiB</em></td>
<td>This work</td>
</tr>
<tr>
<td>NF-L1473</td>
<td><em>lmo2467</em> gene deleted in NF-L100</td>
<td>Δ<em>lmo2467</em></td>
<td>This work</td>
</tr>
<tr>
<td>NF-L1593</td>
<td><em>chiA</em> gene deleted in NF-L100</td>
<td>Δ<em>chiA</em></td>
<td>This work</td>
</tr>
<tr>
<td>NF-L1651</td>
<td>10403S with Δ<em>prsA2::erm</em></td>
<td>Δ<em>prsA2</em></td>
<td>[85]</td>
</tr>
<tr>
<td>NF-L1656</td>
<td>NF-L1651 with integrated pPL2-<em>prsA2</em></td>
<td>Δ<em>prsA2</em>+pPL2-<em>prsA2</em></td>
<td>[85]</td>
</tr>
<tr>
<td>NF-L1772</td>
<td>10403S with <em>inlA</em>&lt;sub&gt;S192,Y376&lt;/sub&gt;</td>
<td>InlAm</td>
<td>[137]</td>
</tr>
<tr>
<td>NF-L1801</td>
<td>NF-L1593 with integrated pPL2-<em>chiA</em></td>
<td>Δ<em>chiA</em>+<em>chiA</em></td>
<td>This work</td>
</tr>
<tr>
<td>NF-L 1941</td>
<td>NF-L1593 with integrated pPL2-<em>chiA</em>E163M</td>
<td>Δ<em>chiA</em>+<em>chiA</em>E163M</td>
<td>This work</td>
</tr>
<tr>
<td>NF-L 1980</td>
<td>NF-L1593 with <em>inlA</em>&lt;sub&gt;S192,Y376&lt;/sub&gt;</td>
<td>Δ<em>chiA</em> InlAm&lt;sup&gt;m&lt;/sup&gt;</td>
<td>This work</td>
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<tr>
<td>DP-L3903</td>
<td>10403S with erythromycin resistance gene</td>
<td>WT&lt;sup&gt;ermR&lt;/sup&gt;</td>
<td>[132]</td>
</tr>
<tr>
<td>Plasmid</td>
<td>Purpose</td>
<td>Reference</td>
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<tr>
<td>------------------</td>
<td>----------------------------------------------</td>
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<tr>
<td>pKSV7</td>
<td>Allelic exchange in <em>L. monocytogenes</em></td>
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<tr>
<td>pKSV7-0105usds</td>
<td>Generation of ΔchiB</td>
<td>This work</td>
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<td>pKSV7-2467usds</td>
<td>Generation of Δlmo2467</td>
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<tr>
<td>pKSV7-1883usds</td>
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<td>This work</td>
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<tr>
<td>pKSV7-InlAm</td>
<td>Generation of NF-L1772, NF-L1980</td>
<td>[137, this work]</td>
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<td>pPL2</td>
<td>Complementation of <em>L. monocytogenes</em> mutants</td>
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<tr>
<td>pPL2-chiA</td>
<td>Generation of ΔchiA+chiA</td>
<td>This work</td>
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<tr>
<td>pPL2-chiAE163M</td>
<td>Generation of ΔchiA+chiAE163M</td>
<td>This work</td>
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<tr>
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<td>Sequence, 5’ to 3’</td>
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<td>lmo0105 ds-s</td>
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<tr>
<td>lmo0105 ds-as</td>
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<td>lmo1883ds-as</td>
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<td>lmo2467us-as</td>
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<td>lmo2467 ds-s</td>
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<td>pPL21883ds</td>
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</table>
REFERENCES:


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