Factors of the Bacterial Pathogen *Listeria monocytogenes* which Influence Niche Acquisition within Hosts

BY

PHILLIP D. MCMULLEN
B.A Microbiology, Molecular Biology
Miami University, Oxford, OH, 2008

THESIS

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Thesis Committee:

Dr. Nancy Freitag, Committee Chair and Research Advisor
Dr. Michael Federle, Medicinal Chemistry and Pharmacognosy
Dr. William Walden, Microbiology and Immunology
Dr. Deepak Shukla, Microbiology and Immunology
Dr. Marcelo Bonini, Pharmacology and Pathology

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LIST OF ABBREVIATIONS

1D-SDS PAGE	One-dimensional sodium dodecyl-sulfate polyacrylamide gel electrophoresis
ActA	Actin-assembling inducing protein precursor
AIDS	Acquired Immune Deficiency Syndrome
AMPK	AMP-activated protein Kinase
Arp	Actin Related Protein
ATCC	American Type Culture Collection
BHI	Brain-heart Infusion
BME	Beta-mercaptoethanol
BRL	Biological Resources Laboratory
BSA	Bovine Serum Albumin
C3bi	Complement receptor type 3
c-AMP	Cyclic Adenosine monophosphate
c-Met	Hepatic Growth Factor Receptor/Scatter Factor Receptor
CHEAP TORCHES	Chicken Pox, Hepatitis, Enteroviruses, AIDS, Parvovirus, Toxoplasma, Other, Rubella, Cytomegalovirus, Herpes, Everything Sexually transmitted
CFU	Colony Forming Unit
CNS	Central Nervous System
Crp	Cyclic Adenosine Monophosphate receptor protein
CSF-1	Colony stimulating factor 1
DC	Dendritic Cells
DMEM	Dulbecco's Modified Eagle's Medium

LIST OF ABBREVIATIONS (CONTINUED)

Ecad	E-cadherin
EMT	Epithelial to Mesenchymal Transition
Ena-VASP	Vasodilator-stimulated Phosphoprotein involved in actin polymerization
ERK	Extracellular-signal-regulated Kinase
Ess	ESX Secretion Pathway
FGF	Fibroblast Growth Factor
GltA	Glucosyl-transferase A
GW	Glycine-Tryptophan Repeat Region
HACEK	Haemophilus, Aggregatibacter, Cardiobacterium, Eikenella, and Kingella
H&E	Hemotoxin and Eosin Stain
HGF	Hepatocyte Growth Factor
IL	Interleukin
IFN-g	Interferon Gamma
InlA	Internalin A
InlB	Internalin B
InlJ	Internalin J
InlK	Internalin K
IPTG	Isopropyl Beta-D-Thiogalactopyranoside
JAK/STAT	Janus Kinase/Signal Transducer and Activator of Transcription
kDA	Kilodalton
LB	Luria Broth

LIST OF ABBREVIATIONS (CONTINUED)

LLO/hly	Listeriolysin O
LPxTG	Leucine-Proline-any amino acid-Threonine-Glycine motif
LRR	Leucine-Rich Repeats
LTA	Lipoteichoic Acid
MAPK	Mitogen Activated Protein Kinase
Mcp1	Monocyte Chemotactic Protein 1
MOI	Multiplicity of Infection
Mpl	Metalloprotease
MRI	Magnetic Resonance Imaging
MVP	Major Vault Protein
MyD88	Myeloid differentiation primary response gene (88)
NADPH	Reduced form of Nicotinamide Adenine Dinucleotide Phoshate
NALP3	Cryopyrin, intracellular inflammasome component
NBD-Phalloidan	7-nitrobenz-2-oxa-1,3-diazole phalloidan
NFkB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural Killer Cells
NOD	Nucleotide binding oligomerization domain receptor
P60	Invasion associated protein
PBS	Phosphate Buffered Saline
PC-PLC/ PlcB	Broad specificity Phospholipase C
PCR	Polymerase Chain Reaction
PI3K	Phosphatidylinositide 3-Kinase

LIST OF ABBREVIATIONS (CONTINUED)

PIP2/3	Phosphatidylinositidyl
PI-PLC/ PlcA	Phosphotidylinositol-Specific Phospholipase C
PrfA	Positive-Regulatory Factor A
PVDF	Polyvinylidine Difluoride
PVMus	Paravertebral Muscle
RGD	Arginine-Glycine-Aspartate Fibronectin binding motif
RNA	Ribonucleic Acid
RT	Room Temperature
SAM	s-Adenosylmethionine
SCID	Severe Combined Immunodeficiency Disorder
SUMO	Small Ubiquitin-Like Modifier
TLR	Toll-Like Receptor
TNFa	Tumor Necrosis Factor alpha
TORCH	Toxoplasma, Others, Rubella, Cytomegalovirus, Herpes
UTR	Untranslated Region
WASP	Wiskott-Aldrich Syndrome Protein
WT	Wild Type

SUMMARY

The goal of this study was to investigate determinants of cardiac tropism exhibited by *L. monocytogenes*. In chapter one, the relevant physiology of *Listeria* as both an environmental saprophyte and mammalian pathogen were discussed. In particular, it was noted that selective pressures in the environment create diversity in disease presentation when infection occurs. In chapter two, we determined that the ability of one cardiotropic isolate of *L. monocytogenes* likely acquired the trait through genetic drift in its common virulence arsenal. A genetic correlation between the invasins *inlA* and *inlB* was found to exist in the cardiotropic strains tested in a previous study. In chapter three, alleles of *inlA* and *inlB* were tested in multiple genetic backgrounds and demonstrated that *inlB* of 07PF0776, a cardiotropic strain of *L. monocytogenes*, supported hyperinvasivity in cardiac myocytes and promoted cardiac colonization in animals. The mutations in the variant *inlB* responsible for this increase appear to reside in both the beta and GW repeats, and confer enhanced stability and increased migration in response to heparan. The ability of the variant *inlB* to promote vertical transmission was tested in chapter four, where it was demonstrated that *inlB* of either source acts as a potentiating factor for vertical transmission. Both *inlA* and *inlB* appeared to mediate uptake into human syncytiotrophoblasts, with *inlA* playing the predominant role. In chapter five, the significance of interactions between allelic variants of the internalins was highlighted. The ratio of one internalin to another dictates invasion efficiency in myocytes and fibroblasts, and the ratio can be altered by a multitude of forces. In this chapter, we will discuss the significance of these findings in the context of the evolution of tissue tropism among isolates of *L. monocytogenes*. Given its environmental source, variants of the species are produced by selective pressures outside of human hosts. No human to human transmission is known to occur, thus making the human a dead end for the bacterium as it

is either cleared or will result in lethality. Success for the bacterium is essentially what we label as pathogenicity, the more pathogenic being more capable of extracting nutrients from its human environment. Such success in terms of bacterial fitness can be derived from a multitude of different mutations in core virulence genes, each of which can alter the physiology of the bacterium, and thus its infection, substantially.

Chapter One: Introduction

Adapted from: McMullen and Freitag, "Listeria monocytogenes." Molecular Medical Microbiology, 2nd Edition. Elsevier, 2015, 2:1345-1361, and is reprinted under the retained right to publish this work in a thesis or dissertation granted by Elsevier Publishing (See author's supplemental thesis documentation for Author's rights for reuse)

1.1 – History and Perspectives of *Listeria monocytogenes* as a Human Pathogen

The gram-positive bacterium *Listeria monocytogenes* is capable of inhabiting the soil as well as the cytosol of mammalian cells, and as a result is an environmental pathogen that poses a continuous threat to food safety. This saprophyte is a facultative intracellular pathogen that is capable of causing severe invasive disease in susceptible human populations. *L. monocytogenes* infections are most commonly associated with food-borne outbreaks involving soft cheeses, deli meats, and produce, but sporadic cases of the disease can also occur. The first human cases of listeriosis were reported in Denmark by Nyfeldt in 1929, and in 1935, Burn showed that *L. monocytogenes* caused human neonatal infections [2, 3]. The first human outbreak of listeriosis directly linked to *Listeria*-contaminated foodstuffs was reported by Schlech *et al.* in 1983 and was traced to contaminated cabbage used in the making of coleslaw [4]. Since then, there have been numerous outbreaks of *L. monocytogenes* in association with a wide variety of food items, resulting in extensive and expensive food recalls and even traveler safety advisories. A recent deadly outbreak traced to contaminated cantaloupes occurred in the latter half of 2011 and was responsible for over 30 deaths out of 147 cases reported across multiple states [5]. In approximately 65% of listeriosis cases, the offending food product has been identified and confirmed by culturing *Listeria* from refrigerated foods in the patient's possession [6].

The annual number of cases generally remains low as a result of extensive surveillance and the relatively high level of resistance to infection exhibited by immunocompetent persons. Despite this, *L. monocytogenes* has one of the highest mortality rates among food-borne pathogens, making infection with *Listeria* clinically a rare, but serious condition [7]. The bacterium is best recognized for its ability to colonize and infect the placenta of pregnant women, subsequently causing fetal infections or abortion. However, *L. monocytogenes* is capable of causing invasive disease in a variety of immunocompromised individuals, including the elderly and persons on immunosuppressive regimens such as chemotherapeutics or long-term corticosteroids. While prompt treatment is generally associated with reasonable prognoses, the mortality rate of listeriosis remains approximately 25-50%, and 30-50% of patients who are cured of listeriosis will ultimately display permanent sequelae as a result of the disease [6].

Since recognition of its outbreak potential in humans, the physiology and growth characteristics of the species inside and outside of host cells have received considerable attention. *L. monocytogenes* has become a model system of intracellular pathogenesis, and studies of this organism within tissue culture and animal models of infection have revealed a variety of processes associated with host signaling cascades, cell physiology, and immune response to intracellular pathogens [7]. From a molecular perspective, studies with *L. monocytogenes* have also clarified how an environmental pathogen mediates the switch from the external environment to life inside a mammalian host [8]. An additional benefit of research focused on *L. monocytogenes* has been the development of attenuated strains that have proven useful as vaccine vectors for the delivery of a variety of antigens. *L. monocytogenes*-based vaccines deliver robust cell-mediated immune responses, and vaccines consisting of antigens from a variety of origins, including those associated with pancreatic and cervical cancers, are

currently undergoing clinical trials to ascertain their safety and efficacy in humans [7]. This chapter will review interesting aspects of *L. monocytogenes* physiology, pathogenesis, and disease to provide background for the work described in the subsequent chapters, and will highlight the diversity of cellular processes that can be learned by studying the interactions of a single bacterium within its mammalian host.

1.2 - *L. monocytogenes* physical characteristics and distribution.

L. monocytogenes is a gram positive, facultative anaerobe that is incapable of forming spores. The species is subdivided into 16 serovars, and the vast majority of human cases are associated with serovars 4b, 1/2a, and 1/2b [9]. *L. monocytogenes* strains have also been grouped into distinct lineages based on southern hybridization using serovar and subspecies-specific probes [10]. *Listeria* possesses one to five peritrichous flagella which confer swimming and tumbling motility at 28°C; most strains have reduced or no evident motility at higher temperatures, including human body temperature (37°C). The optimum growth temperature ranges from 30°C to 37°C, however growth has been reported between temperature ranges as wide as 2°C to 42°C [8]. When grown on blood agar, colonies appear weakly beta-hemolytic.

L. monocytogenes is remarkably resilient, and this resiliency has no doubt contributed to its widespread distribution. The bacterium can survive broad ranges of pH (4.5-10) and salinity (up to 10%) [6, 8]. It has been isolated from a diverse array of animal species, including more than fifty species of mammals as well as birds, rodents, and fish [11]. In addition, *L. monocytogenes* has been found in biofilms associated with machinery surfaces and pipes, and recurrent outbreaks of single strains have been reported in association with food processing plants [12].

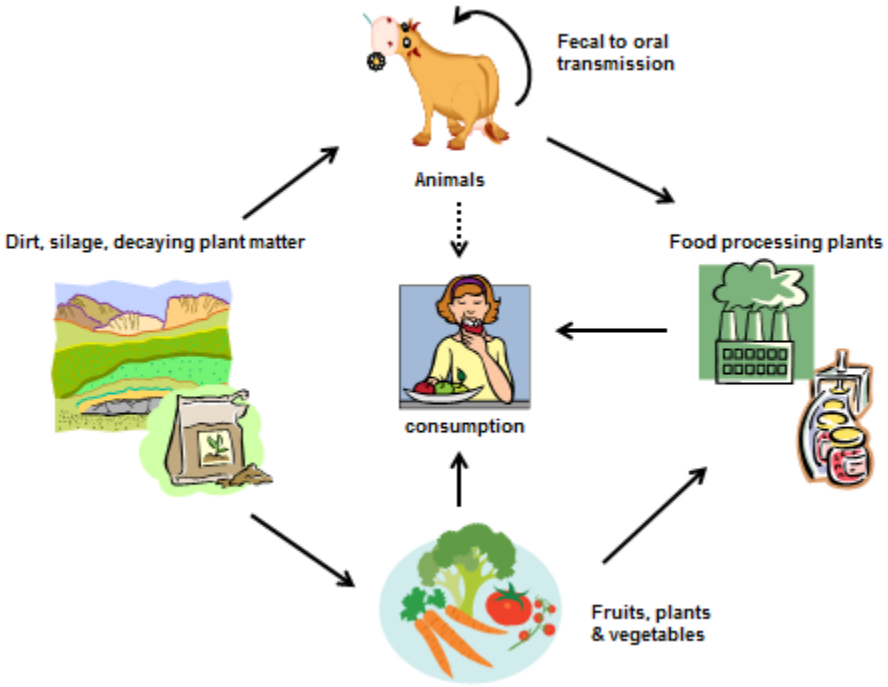


Figure 1: The multiple environments of *Listeria monocytogenes*. The ability of the bacterium to survive ubiquitously in environmental sources allows for dispersion into food supplies at various points in food production. Consumption of contaminated food products is thought to be responsible for the overwhelming majority of Listeriosis cases. *Figure in press: PD McMullen and Nancy E. Freitag, Listeria monocytogenes, Molecular Medical Microbiology, Second Edition, Elsevier publishing.*

L. monocytogenes thus can infiltrate food supplies either prior to food processing or during the course of food processing and packaging (Fig. 1). The rate of invasive disease in the United States is estimated to be approximately 2-9 cases per 1,000,000 persons, however the true incidence of disease is thought to be underestimated as well as underreported. Recent reports suggest an increasing incidence of disease in Europe [13].

1.3 - Clinical Manifestations of Infection

The ability of *Listeria* to survive a variety of environmental conditions may contribute to the diversity of clinical manifestations associated with this organism. Presentation may depend on patient predispositions or potentially on strain-specific bacterial factors that influence the progression of disease. Initial inoculation of the host following ingestion was originally thought to be silent, however gastroenteritis may be apparent within the first 48 hours of exposure [14]. Symptoms of *L. monocytogenes* gastroenteritis mimic the disease produced by other enteric pathogens and may include nausea, watery or bloody diarrhea, abdominal pain and fever. Occurrence of these early symptoms may depend on the size of the ingested inoculum, as suggested by studies in a healthy non-human primate model [15]. Whether these gastrointestinal symptoms can be accounted for by a toxic effect of *Listeria* on intestinal tissues or by bacterial migration across the epithelial border of the small intestine – which occurs during the normal course of infection – is not currently known. In healthy individuals, disease is most often self-limiting.

Illness in pregnant women is characteristically flu-like, with mothers presenting with myalgias, fever, and chills [4]. 70-90% of fetuses carried by mothers infected by *Listeria*

subsequently become colonized. Of these, approximately 50% succumb to the infection *in utero,* while infected live-born neonates have a mortality rate of 24% [6]. One manifestation in the neonate is a condition called *granulomatosis infantisepticum*, and is among the most severe forms of the disease[16]. It is characterized by a diffuse, erythematous rash over the body of the neonate, granulomas in the liver and spleen, and is nearly universally fatal for the infected. The highest levels of *Listeria* in the fetus are typically found in the bowel and in the lung, suggesting infection occurs through the ingestion of contaminated amniotic fluid [16]. Colonization of the placenta prior to the fetal infection, however, is thought to be hematogenous, with bacteria spreading from primary organ targets, including the liver and spleen. In animal models, it has been demonstrated that *L. monocytogenes* replicates within the placenta to such a degree that free bacteria are released back into the maternal circulation [17].

When neonatal contamination occurs during parturition, the clinical features are similar to those caused by *Streptococcus agalactiae* (group B *Streptococcus)* infection [18]. This infection is sometimes associated with purulent conjunctivitis and widespread papular rash over the body, and is complicated by late-onset meningitis. *L. monocytogenes* is one of the three major causes of meningitis in neonates [18]. While mothers typically recover from the infection, if there is involvement in other organs the recovery may be slower or incomplete.

Bacteremia caused by *Listeria* is most common in pregnant women and cancer patients who are on immunosuppressive regimens. Approximately 70-90% of cancer patients diagnosed with listeriosis are bacteremic, and nearly all pregnant women with fetal involvement have culturable *L. monocytogenes* in their blood. In addition to the number of immune perturbations caused by viable bacteria in the blood, the bacteremic state additionally allows for dissemination of *Listeria* throughout the host, subsequently allowing for colonization of new sites [19]. Bacteremia

increases the likelihood of focal damage to additional organ systems, thus worsening the prognosis in patients who are not treated promptly. Endocarditis caused by *Listeria* is thought to be dependent on the bacteremic state, and carries with it a mortality rate of 35%-50%. Recent evidence suggests that subpopulations of *L. monocytogenes* have an enhanced capacity for cardiac invasion, thereby increasing the risk of cardiac disease in cases involving particular isolates [20]. For individuals who develop invasive disease, the signs of generalized infection most frequently occur after an incubation period that can range from 10–70 days [21]. Hematogenous seeding of *L. monocytogenes* results in bacteremia and an influenza-like febrile illness, accompanied by muscle pain, joint pain, headache and backache [22].

Bacterial spread to the brain and meninges are common manifestations of *L. monocytogenes* during invasive disease for all susceptible populations. Several mechanisms have been proposed with regards to *L. monocytogenes'* ability to target the CNS [19, 23]. Direct invasion of the epithelial choroid plexus and blood-brain-barrier endothelial cells by free bacteria is thought to be a major component of CNS infections [24]. It has also been reported that phagocytes infected with *L. monocytogenes* can act as a delivery vehicles, providing a mechanism for direct spread of bacteria from the infected macrophages into underlying brain parenchyma [23]. In animals, rhomboencephalitis can result from direct inoculation of the trigeminal nerve by bacterial transiting through open lesions in the oral cavity, which represents an ascending neurological mechanism for CNS disease. Ascending infections in animals produce circling disease, which is caused by direct destruction of brain stem parenchyma by replicating bacteria [25].

The presentation of meningitis resulting from *L. monocytogenes* infection is typically subacute, with fever being the most consistent symptom. Frequently described as aseptic meningitis, the identification of *L. monocytogenes* as the underlying agent may come by

exclusion of other more likely causes [6]. The CSF of patients with listerial meningitis can have either low or normal glucose, however the presence of gram-positive bacteria and low glucose - textbook presentations of bacterial meningitis - only occur in 30%-40% of cases. In addition, *L. monocytogenes* in the CSF has been confused with diphtheroids, *Haemophilus*, and can often appear to be gram variable, further confounding diagnosis and treatment. 75% of patients with neurological involvement have a white blood cell count below 1,000/ul, thus making infection seem an unlikely cause for the patient's condition [18]. All of these factors, if not accounted for during the workup, may slow the delivery of antibiotics necessary to control the infection. Patient history and predispositions may aid in narrowing the diagnosis, however, even with laboratory analysis the results may be difficult to interpret [6]. Diagnosis oftentimes relies on community surveillance, given the food-borne nature of listeriosis. Populations of elderly or immune-suppressed patients presenting with sterile meningitis should immediately raise suspicion for a potential outbreak.

L. monocytogenes can also cause invasive forms of encephalitis in humans, and these manifestations usually result in permanent lesions. Sequelae develop as bacteria replicate and spread further into the brain parenchyma from the meninges, producing cavitation and subsequent destruction of neuronal tissue. Imagining with MRI and contrast often reveals ring-enhancing lesions, which can be mistaken for tumors [6]. The course of listerial encephalitis may be biphasic and/or subacute. Patients may present initially with a fever that resolves spontaneously into a prodromal period, followed by fulminant rhomboencephalitis with neurological consequences. Mortality rates and localized sequelae can vary depending on the virulence of the particular isolate, its manifestation, as well as the medical history of the host, however CNS infections overall have a 15-26% mortality rate [18].

1.4 – How Bacterial Pathogenesis Translates to Clinical Manifestation

Gastroenteritis is generally thought to require the ingestion of a large inoculum of bacteria, however invasive disease is often silent in this regard, suggesting fewer bacteria may be required in some circumstances [14]. Although *L. monocytogenes* demonstrates resistance to low pH, the extreme acidic environment of the stomach serves as a defense against ingested *Listeria*. Once the surviving *L. monocytogenes* reach the small intestine, they gain access to luminal enterocytes that act as the cellular barrier between the lumen of the small intestine and the lymph and blood channels within the intestinal villus tip [26] (Fig. 2). *L. monocytogenes* is able to bind to and mediate uptake into non-phagocytic enterocytes through interactions with tight junction proteins on the host cell surface, but appear to target luminal-accessible E-cadherin of goblet cells [27]. Transcytosis of bacteria allows for direct invasion of the intestinal villus tip, with subsequent migration into the blood and lymph. Blood from the small intestine is carried via the portal circulation to the liver, where the bacteria are taken up by resident phagocytes. Goblet cells, which express luminal accessible E-cadherin, are thought to be the primary target of intestinal *Listeria*, although phagocytic M cells present within the intestinal epithelium have also been reported to be a portal for *L. monocytogenes* entry [28] (Fig. 2).

In animals infected intravenously with *L. monocytogenes*, the bacteria are taken up first by resident macrophages known as Kupfer cells in the liver, and appear shortly after in splenic dendritic cells [17] (Fig. 3). The fenestrated nature of hepatic and splenic capillary beds permits easy movement of solutes and cells from the blood stream into the organ proper, and may also permit easy diffusion of blood-borne *L. monocytogenes*, explaining the enhanced susceptibility

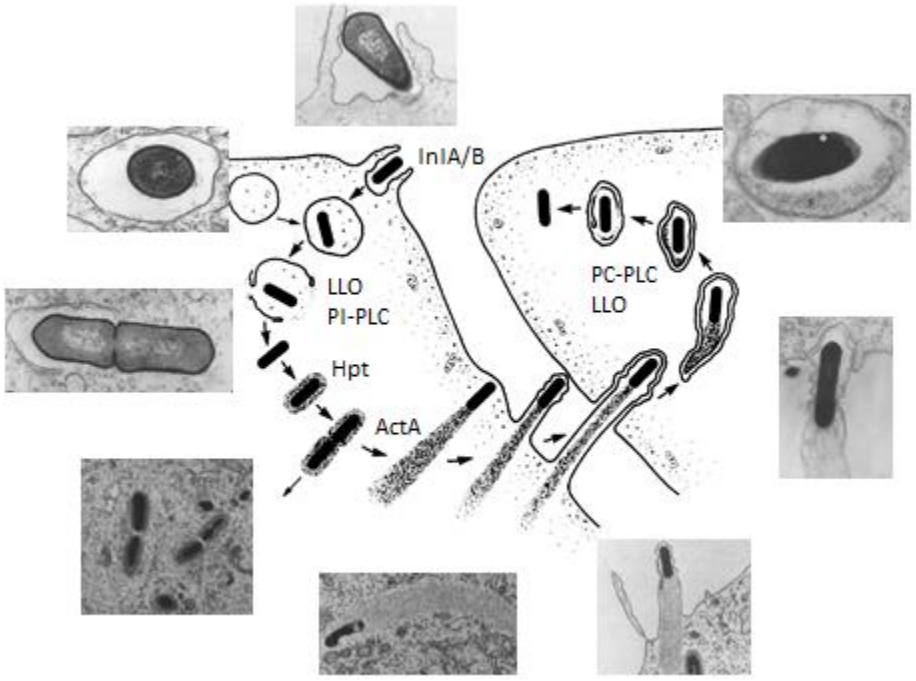


Figure 2: The lifecycle of *Listeria monocytogenes* within mammalian cells. The process begins with cellular invasion, which is most commonly associated with the invasins InlA and InlB. Next, the bacterium is contained within a phagosome or endosome, which it can rupture through the activity of LLO. Once in the cytosol, *Listeria* acquires host nutrients through transporters such as Hpt, initiating cytosolic replication. The production of ActA in the cytosol leads to the nucleation of host-cell actin, which in turn propels the individual bacteria into neighboring cells. Once in adjacent cells, the double-membraned vacuole is lysed through synergistic actions of Pc-Plc and LLO. The process of nutrient acquisition and cytosolic spread are then repeated. *Figure in press: PD McMullen and Nancy E. Freitag, Listeria monocytogenes, Molecular Medical Microbiology, Second Edition, Elsevier publishing. Originally adapted from* [29].

of these organs to early colonization. Cell wall components *of Listeria* activate complement via the alternative pathway, and deposition of C3bi on the bacterium triggers complement-dependent entry into professional phagocytes within the tissues [30].

Within the Kupfer cells and splenic DCs, the bacteria are initially confined to a membrane-bound vacuole. Acidification and toxification of the vacuole are modified through delayed Rab5 colocalization with the endosome and phagosomal membrane disruption [31]. Bacteria lyse the host cell vacuole and replicate within the cytosol of infected macrophages, subsequently spreading into neighboring parenchymal cells. *L. monocytogenes* can also directly invade hepatocytes and splenocytes through the activity of secreted and surface-linked invasins (described later within this chapter). Early in the infection, neutrophils are recruited to the site of infection in order to ingest the bacteria and apoptotic hepatocytes [32, 33]. Interestingly, recent studies have suggested that some classes of neutrophils may be dispensable for clearing *L. monocytogenes* [34]. In the non-immune host, exponential intra-hepatic bacterial growth reaches a maximum 2–3 days after infection [35]. It is thought that as replication continues in the liver, bacteria and phagocytes containing bacteria can migrate to the spleen, where they are taken up by resident dendritic cells there, initiating a splenic replication niche. Replication within these two organs is the most common manifestation of listeriosis in infected animals, thus the liver and spleen are considered as primary target organs of colonization for *Listeria*.

Multiple mechanisms are thought to contribute to bacterial tropism to localized areas of colonization within the host during the septic infection. As many as 70% of blood-borne *Listeria* are extracellular during sepsis [22]. This population is thought to be capable of directly colonizing and invading the endothelial cells of both the blood-brain barrier and the placental trophoblastic barrier [24]. The remaining 30% of bacteria circulating in the blood are carried

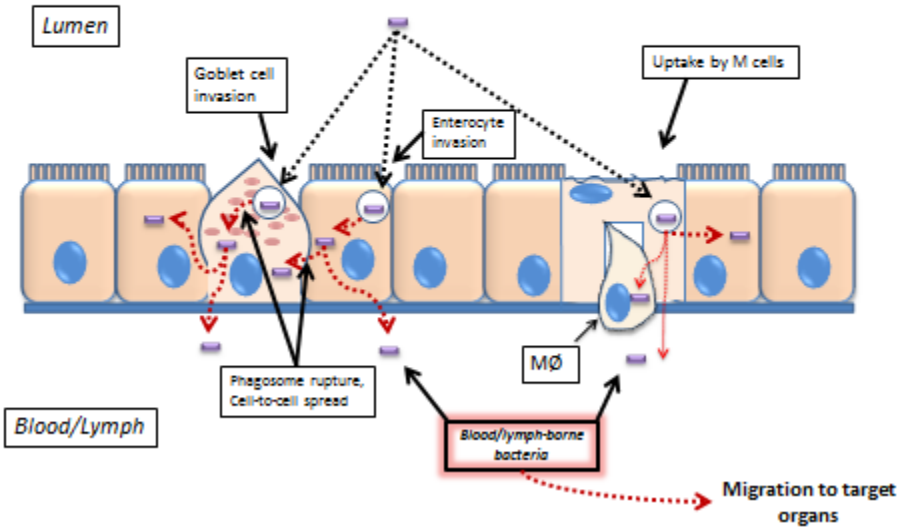


Figure 3: Crossing the intestinal border may rely on the ability of the bacterium to invade multiple cell types. Goblet cells and Enterocytes are invaded through the action of InlA binding to lumen-accessible E-cadherin, while invasion through M-cells may be more passive. *Figure in press: PD McMullen and Nancy E. Freitag, Listeria monocytogenes, Molecular Medical Microbiology, Second Edition, Elsevier publishing*

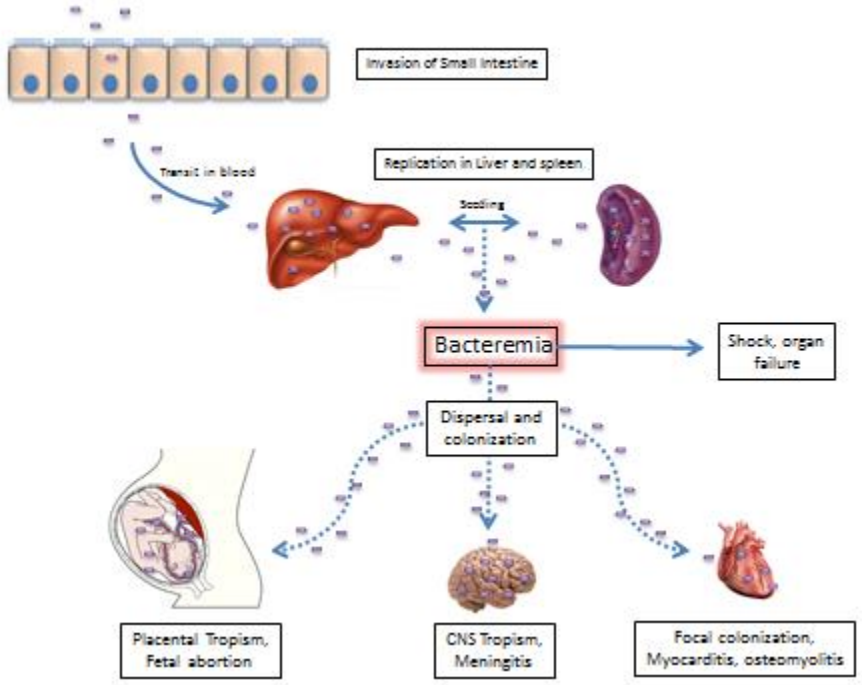


Figure 4: Spread within susceptible hosts following migration across the intestinal border. Blood from the intestines is drained directly into the liver, where the bacteria are capable of replicating within both hepatocytes and Kupfer macrophages. Hematogenous spread then carries the organism to the spleen, where replication can also occur. Sequestration of organisms to the liver and spleen is a natural defense against unregulated spread of blood-borne pathogens, however since *Listeria* is capable of replicating in these organs, bacteremia eventually occurs, allowing for spread to other sites more commonly associated with disease in humans. *Figure in press: PD McMullen and Nancy E. Freitag, Listeria monocytogenes, Molecular Medical Microbiology, Second Edition, Elsevier publishing*

within phagocytes. Rather than limiting bacterial infection, these phagocytes can serve as vehicles for the spread of bacteria from immune cells to tissue parenchyma, a method of bacterial dissemination that has been referred to as the "Trojan Horse" mechanism [22, 23].

Bacterial products generated during *Listeria* infection activate endothelial cells through the NFκB pathway, which contributes endothelial glycocalyx remodeling [19]. This remodeling process is implicated in the recruitment of immune cells to areas of activated endothelium, leading to diapedesis of immune mediators into underlying tissue structures. The endothelial damage-response circuit may play an additional role in increasing bacterial tropism to sites of initial bacterial colonization for organisms capable of transit within phagocytes such as *L. monocytogenes*. Once a replication niche has been established in an organ or tissue, *L. monocytogenes* is capable of reseeding the blood and traveling to additional sites within the host. As an example, *L. monocytogenes* colonizes the placenta in early stages of sepsis, which then serves as a reservoir for bacterial replication and release back into the maternal blood supply [17].

The dissemination of bacteria to organ systems and the establishment of distinct replication niches within the host are largely responsible for acute symptoms of *L. monocytogenes* infection. During the septicemic phase in pregnancy, *Listeria* has access to the highly vascularized uterine decidua, which is in contact with the trophoblast barrier situated on the fetal side of the intervillous chamber. Only a small proportion of circulating *Listeria* are believed to cross this tight barrier, eventually to gain access to fetal circulation and the amniotic fluid [36]. Infection of the CNS is also thought to occur as a result of hematogenous spread. The central nervous system is one of the most vascular organs of the human body, and a very dense capillary network

irrigates the brain parenchyma. The endothelium of these capillaries is in contact with the foot processes of astrocytes, forming the tight junctions that constitute the blood-brain barrier [24, 37]. In the choroid plexus, where cerebrospinal fluid is produced and secreted into the ventricular network, the endothelial capillary network is fenestrated, and the blood–brain barrier in this region consists of a tight monolayer of polarized choroid plexus epithelial cells. *L. monocytogenes* again subverts these tight junction proteins in between the choroid epithelial cells to mediate invasion for the bacterium [37, 38]. Invasion via this route has been suggested to result in transcytosis of bacteria across the choroid plexus, leading to colonization of CNS tissues. Thus, *Listeria* manipulates several different pathways to gain access to host organ systems. Invasion of tissues directly by free bacteria in the blood, 'Trojan-horse' transiting via infected macrophages, and replicative diffusion through tissues all contribute to the migration of *Listeria* within the host.

1.5 - Cellular Invasion, Intracellular Replication, and Spread: The Varied Roles of *L. monocytogenes* Virulence Factors.

Upon entry into the host cell environment, *L. monocytogenes* transitions from an environmental saprophyte to a pathogen that is adapted for survival and replication within host cells. *L. monocytogenes* maintains an impressive repertoire of virulence factors that facilitate the invasion of non-phagocytic cells, the rupture and disruption of the phagosomal compartment, replication within the cytosol, and spread to adjacent cells (Fig. 4). Other products contribute to survival in the host environment, such as bile resistance and resistance to oxidative stresses. Within the host environment, these virulence factors may each serve multiple functions, creating

a myriad pleiotropic, often complicated, effects coordinated around the need for survival within the cytosol.

1.6 – Invasion of Host Cells by Listeria monocytogenes

Cellular invasion of distinct tissue sites by free-living bacteria is thought to be largely mediated by the *inlAB* genetic locus, which encodes two surface invasins, InlA and InlB (reference). These proteins facilitate bacterial invasion of a wide variety of host cell types, and together serve to promote entry of extracellular *Listeria* into intracellular niches, thereby removing free bacteria from the blood and enabling bacterial replication in protected intracellular compartments. InlA, InlB, and other members of the internalin gene family share a common motif structure [39]. Each contains a signal sequence directing protein secretion, two repeat regions of which the first is made up of 7–15 leucine-rich repeats (LRRs) and the second a highly conserved region of about 100 amino acids situated just downstream of the LRR region. All of the internalin family members, with the exception of InIB and InIC, have a putative cell wall anchor sequence LPXTG motif. The best characterized members of the internalin gene family are *inlA* and *inlB*, whose gene products mediate bacterial uptake into non- phagocytic cell types throughout the body [39]. Other members of the internalin family promote a variety of cellular functions, while a number remain uncharacterized.

InlA is a peptidoglycan-linked invasin that binds to the host tight-junction protein E-cadherin, or Ecad [40] (Fig. 5). The presence of functional InlA seems to be most important in the context of the epithelial border of the small intestine, where *L. monocytogenes* transverses the intact cellular barrier in order to reach the blood supply of susceptible hosts. The interaction between

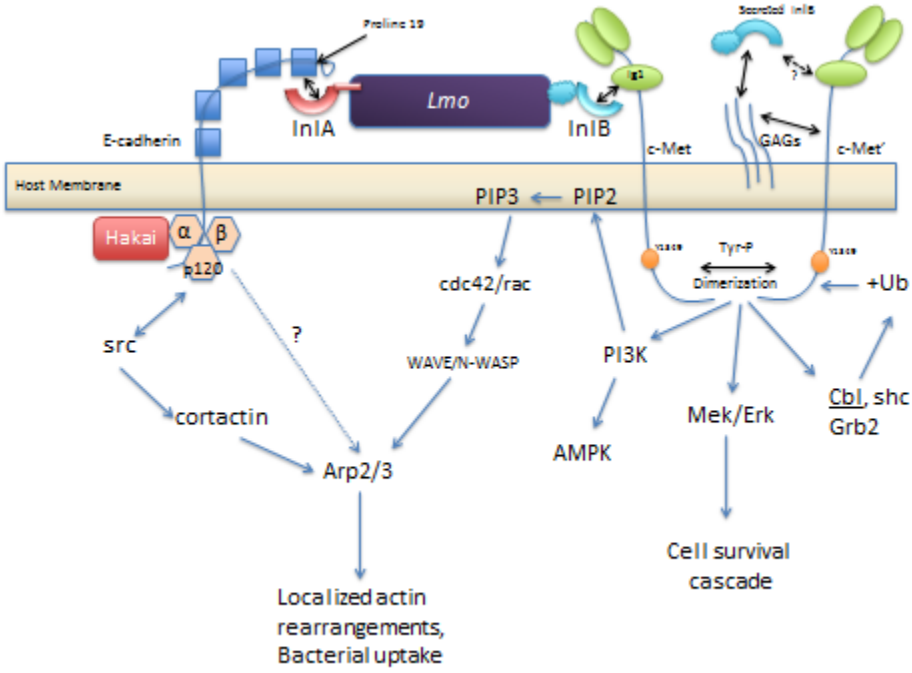


Figure 5: The molecular mechanisms underlying cellular invasion. The invasins InlA and InlB interact with different ligands, and can thus mediate uptake across a number of different cell types. The pathways involved in both mechanisms have been studied in great detail and ultimately induce cytoskeletal rearrangements capable of pulling bacteria into the cell. *Figure in press: PD McMullen and Nancy E. Freitag, Listeria monocytogenes, Molecular Medical Microbiology, Second Edition, Elsevier publishing*

InlA and Ecad has been described in detail and is highly species specific. Human, guinea pig, and gerbil Ecad contain a critical proline at position 19 of the first Ecad repeat domain, which is necessary for the interaction with InlA of *L. monocytogenes* [27, 40, 41]. Although Ecad of mice and rats differs from human Ecad and is not recognized efficiently by *L. monocytogenes* InlA, mutationally altered alleles of *inlA* have been described whose gene products bind Ecad of mice and rats with high affinity, making these alleles 'murinized' for better interaction with the incompatible Ecad of mice [41]. Such mutations, while conferring increased affinity with the natural Ecad ligand present in many strains of mice, may also increase the participatory role of other cadherin pathways in invasion by unintended receptor expansion. To date, however, no natural alleles have been demonstrated to bind to or mediate uptake through other cadherins proteins.

In order to study Ecad and InlA interactions *in vivo*, transgenic mice have been engineered to express humanized cadherin containing a point reversion of the glutamate at position 19 in the first Ecad repeat of the mouse Ecad gene to the critical proline needed for interaction [26]. Using this transgenic model, as well as gerbil models where InlA is reactive, the functional role of InlA in the infective process has been elucidated in greater detail. InlA has been shown to mediate uptake into the CNS by binding to epithelial cells of the choroid plexus, thus transversing the blood-brain-barrier via transcytosis, and has also been shown to work conjunctively with InlB in mediating tropism and uptake into the placenta [36]. InlA exerts its invasive forces through well-described cytosolic pathways that culminate in localized perturbations of the host cytoskeleton. InlA-stimulated signaling through Ecad binding is mediated by recruitment of the α/β-catenins to the targeted Ecad cytoplasmic tail. The cytoplasmic Src protein of the host then phosphorylates the Ecad molecule, which removes the

invasion-restricting cofactor p128 from the cytoplasmic tail of Ecad. The removal of p128 allows for recruitment of the host ubiquitin-ligase Hakai to induce either clathrin-dependent or calveolin-dependent endocytosis. Endocytosis itself is then mediated through actin rearrangements that are dependent on the Arp2/3 complex [40, 42].

InlA is encoded in an operon with a gene encoding another cellular invasion protein, InlB. InlB contains a leucine-rich repeat domain that is structurally and functionally similar to that of InlA. Unlike InlA, however, InlB is not surface-linked but is rather secreted from the cell and remains associated through hydrophobic interactions with lipoteichoic acids (LTA) on the bacterial cell surface [43] (Fig. 5). InlB thus can have both retained and secreted functionalities, although the significance of the secreted portion *in vivo* remains to be demonstrated. When associated with the bacterial surface, InlB mediates uptake through a signaling cascade that culminates in actin rearrangements similar to those of InlA. In contrast to InlA, which seems most critical to bacterial survival at the level of the intestinal enterocyte, InlB is responsible for entry into a variety of cell types, particularly hepatocytes, splenocytes, fibroblasts, and cardiac myocytes. The invasive capacity of InlB has been investigated in a variety of cell lines, such as HepG2 (hepatocyte-like), HeLa (epithelial), and L2 (fibroblast) [39, 43].

InlB exerts its action through the growth factor receptor c-Met, which initiates its cascade through the PI3K pathway. InlB binds c-Met at a site distinct from its natural ligand HGF, and the protein has been crystalized in complex with the Ig-like domain of Met [44]. It has been shown that binding of the Ig-domain by itself is not sufficient for the activity of InlB, and that clustering of c-Met is required for full signaling. Presumably, this clustering allows for more efficient auto-phosphorylation of the cytoplasmic tail of the c-Met proteins, which is essential for activation of PI3K and subsequent recruitment of actin. Production of PIP2/PIP3 from the

activity of PI3K leads to recruitment of small GTPases essential for activation of Arp2/3 and WASP cytoskeletal proteins, which in turn induce actin polymerization in the area local to the activated c-Met receptors [39, 42]. These localized perturbations to the actin cytoskeleton culminate in a zipper-like uptake mechanism that engulfs the bacterium into the host cell.

As mentioned earlier, evidence suggests that the activity of both InlA and InlB is necessary for optimal invasion of some cell types. In a pregnant gerbil model, the coordinated action of InlA and InlB was necessary for full colonization of the placenta [45]. InlA and InlB have been shown to act coordinately in the CNS as well, with InlB mediating uptake into the endothelial cells of the brain capillary beds and InlA mediating uptake into the epithelial cells of the choroid [37]. These interactions lead to the development of meningitis and encephalitis in infected animals and thus demonstrate the tropic influence these two invasins impose on the bacterium through the course of the infection.

Three other bacterial factors, LLO, p60, and ActA, may also contribute to bacterial entry into cells [46-48]. p60 is a major extracellular protein encoded by the *iap* gene, and was first described as an invasion-associated protein because strains whose p60 production is altered are defective for invasion [48, 49]. p60 is a protein of 484 amino acids that contains a putative signal sequence followed by a repeat region of 19 threonine-asparagine units. The protein has murein hydrolase activity and plays a role in normal septum formation and daughter cell separation after division, which explains why mutants that lack p60 produce long chains of bacteria separated by double septa [49]. This lack of daughter cell separation likely underlies the decreased invasivity in these strains, as invasion is more efficient for individual bacteria [50]. ActA, the protein responsible for the actin-based intra-cytoplasmic movement of *L. monocytogenes* (see below), also contributes to cell entry through the binding of heparan sulfate

proteoglycans [47]. Interestingly, the central region of ActA possesses an RGD motif, which raises the possibility that it may also interact with integrins, although this has yet to be demonstrated. Listeriolysin O (LLO), the listerial pore-forming hemolysin, has also been demonstrated to induce bacterial invasion and can induce the uptake of latex beads into host cells [51, 52]. LLO insertion in to host-cell membranes induces localized calcium fluxes, which in many cell types have been associated with massive endocytosis in the region surrounding the fluxing calcium [39, 46].

1.7 – How Listeria Avoids Death: Disruption of Phagocytosis and Escape into the Cytosol

Following entry, *L. monocytogenes* is contained within host cell vacuoles targeted for phagolysosomal degradation. Listeriolysin O (LLO) is a cholesterol-dependent pore-forming toxin produced by the bacterium to disrupt vacuolar membranes and promote bacterial entry into the cytosol [53]. LLO is secreted and aggregates, assembles and inserts into the vacuolar membrane. The vacuolytic activity is stimulated at low pH, thus specifically targeting the pore-forming activity of LLO to the acidified vacuole [54]. LLO confers beta-hemolysis for bacteria grown on blood agar, and is closely related to other Gram-positive cytolysins, such as streptolysin, pneumolysin, and perfringolysin O. Membrane insertion by LLO in acidified vacuoles prevents phagosome maturation, thereby increasing the chances of *L. monocytogenes* escape. The pH-dependence of LLO activity has been traced to an acidic triad of amino acids located in the transmembrane region, and the cytotoxic effect of LLO is tightly controlled by *L. monocytogenes* through a variety of mechanisms which serve to prevent plasma membrane lysis and host cell death [55]. Perturbations in the synthesis, structure, or half-life of LLO lead

ultimately to altered cytotoxicity, which usually reduces bacterial virulence, even when cytotoxicity is increased substantially. Dissolution of the primary vacuolar membrane, where the activity of LLO has been carefully studied, is also enhanced by the activity of two phospholipases, a phosphatidylinositol-specific phospholipase C (PlcA or PI-PLC) and a broad specificity phospholipase (PlcB or PC-PLC) [56].

In addition to its role in vacuolar lysis, LLO may induce changes in cytosolic calcium and potassium levels, which can result in a myriad of consequences depending on the cell-type [57]. Endothelial cells treated with purified LLO show disregulation of NFkB resulting in an upregulation of cellular adhesion molecules on the host-cell surface, which may subsequently act to increase bacterial adhesion via the upregulation of ligands necessary for bacterial adhesion and invasion [24]. LLO appears to interfere with other broad physiologic processes within host cells, as well. One such perturbation involves altering host-cell SUMOlyation by inducing the degradation Ubc9, one of the enzymes involved in the SUMOlyation process [58]. SUMOlyation is a post-translational modification process in eukaryotic cells, where SUMO (an ubiquitin-like polypeptide) is covalently linked to proteins via the activity of three distinct enzymes. LLO-induced degradation of Ubc9 reduces the overall amount of host SUMOlyated proteins both *in vitro* and *in vivo* in mice. The effects of LLO have also been demonstrated to dephosphorylate host cell histones, resulting in the down-regulation of several host genes involved in innate immune responses [57]. LLO-mediated membrane destabilization also induces mitochondrial fragmentation and mitogen-activated signaling cascades, including AMPK and PI3K [59]. These changes have been also related to regulation of autophagy by the host, indicating the down-stream effects of LLO may play a role in intracellular trafficking of the bacterium by redirecting host organelles to an autophagic state [60]. Thus, while commonly

associated with vacuolar lysis, LLO may induce many effects that alter the course of host physiology.

L. monocytogenes' two distinct phospholipases, PI-PLC and PC-PLC, contribute to the disruption of both primary and secondary vacuoles formed during bacterial cell entry and cell-to-cell spread, respectively [61, 62]. PI-PLC assists in the disruption of the primary vacuole in synergy with LLO, while PC-PLC activity is directed more towards the secondary vacuole formed following bacterial spread into adjacent cells [63, 64]. Mutants lacking both PI-PLC and PC-PLC demonstrate vacuolar escape defects in both the primary and secondary vacuoles. In some human cell types, the combined activity of the phospholipases are sufficient for vacuole escape in the absence of LLO, functionally demonstrating their role in vacuole lysis [53]. It has been recently shown that despite their critical roles in vacuole lysis, the presence of *L. monocytogenes* phospholipase induces NADPH-oxidase-dependent generation of oxidative radicals in host cells, which is deleterious to bacterial survival. To balance the need for phospholipases with the negative effect of the generation of oxidative radicals, LLO acts to inhibit the NADPH-oxidase complex from forming on the vacuole membrane, thus functionally compensating for this presumably negative bacterial outcome of phospholipase production [65].

Similar to LLO, the lytic action and thus cytotoxic effects of the phospholipases are tightly regulated. PC-PLC is first secreted by the bacterium as a proenzyme, which is then enzymatically cleaved to an active form in response to environmental cues by an extracellular metalloprotease, Mp l[56, 66]. Secretion in most circumstances outpaces proteolytic activation by Mpl, thus a pool of inactive yet intact PC-PLC is synthesized prior to cytosolic entry by *L. monocytogenes* to remain in close association with the bacteria, becoming sequestered at the membrane-cell wall interface [56]. The drop in pH encountered in vacuoles triggers Mpl-

dependent enzymatic cleavage of the PC-PLC propeptide, releasing a pool of activated PC-PLC for membrane disruption. Mutants of PC-PLC that have been genetically modified so as to lack the propeptide lose compartmentalization of phospholipase activity and compromise host-cell membrane integrity [64]. *L. monocytogenes* has thus evolved multiple check-points to regulate the activity of enzymes whose activities are required for vacuole lysis while avoiding plasma membrane damage.

1.8 - Cytosolic Replication and Cell-to-Cell Spread.

Having gained access to the cytosol, *L. monocytogenes* induces the expression of a number of bacterial factors required for cytosolic replication and spread to adjacent cells. A number of peptide and phosphorylated sugar transport systems facilitate bacterial acquisition of host-derived nutrients. In particular, glycerol and other three carbon sugars serve as carbon sources for intracellular replicating bacteria and *L. monocytogenes* mutants that lack glycerol transport exhibit severe cytoplasmic growth defects [67]. Additionally, host lipid sources such as lipoic acid and branched-chain fatty acids can be scavenged by *L. monocytogenes* via lipid transport systems upregulated specifically in the host cytosol [68]. Iron is also actively scavenged by the invading bacterium through the action of a number of transport channels, including *fur*-regulated systems [69]. The evolution of such complex and targeted mechanisms for growth within intracellular environments further exemplifies the specific capacity *Listeria* to exploit mammalian hosts as replication niches. Indeed, *L.* monocytogenes is completely suited for life within the cytosol, as bacterial replication rates inside of host cells are comparable to those observed in rich broth media.

Shortly after arrival within the cytosol, *L. monocytogenes* expresses a surface protein known as ActA that provides a scaffold for host actin assembly and thereby enables movement of the bacterium through the cytosol and into adjacent cells [70] (Fig. 6). *L. monocytogenes* mutants lacking *actA* reach the cytosol and replicate but are completely incapable of movement into neighboring cells and are severely attenuated for virulence, demonstrating the importance of bacterial cell-to-cell spread for pathogenesis. The ActA protein has three distinct regions, two of which are involved in recruiting components of host actin polymerization processes [71] (Fig. 7). The N region spans from amino acid 21 to 231 and serves to nucleate host-cell actin, as well as recruit the host Arp2/3 actin polymerization complex through homology to cofilin. The P region spans from residue 232 to 393, and contains four proline rich repeats that bind to the Ena/VASP family of vasodilating proteins, enabling the binding of free actin filaments and the recruitment the actin monomer-binding protein profilin [70]. Microscopically, the bacteria begin to colocalize with clouds of actin upon reaching the cytosol. As bacterial replication progresses, ActA becomes localized nearer the poles of dividing bacteria, leading to unipolar localization of actin polymerization, and thus unidirectional travel [50, 72]. The overall rate of motility for a given bacterium correlates well with the length of the actin tail formed while during the cytosolic phase of growth.

Why does the critical need exist for *L. monocytogenes* actin-based motility within cells? Interestingly, *L. monocytogenes* is not unique in its requirement for intracellular movement. Other pathogens such as *Shigella flexneri*, *Rickettsia conorii*, *Burkholderia ssp.*, *Mycobacterium marinum*, and even viruses such as vaccinia virus have evolved distinct forms of actin-based motility within the cytosol of infected cells [42, 73]. One apparent reason underlying the need for microbial movement within the cytosol appears to be the need for pathogens to avoid host

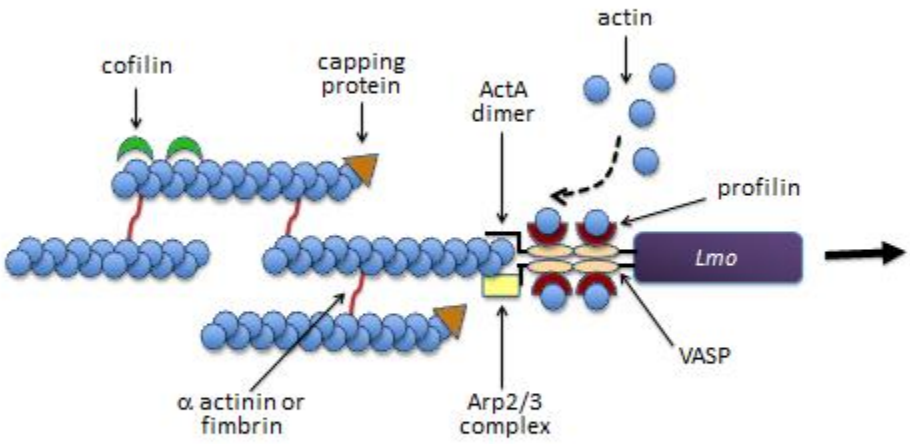


Figure 6: Nucleation of host-cell actin through ActA allows for cell-to-cell spread, as well as dissemination within the three-dimensional organ. ActA itself does not polymerize actin, but instead acts to recruit the host Arp2/3 complex to the bacterial surface. Torsion forces and migration of ActA to the lateral poles of the bacterium allow for unidirectional travel. *Figure in press: PD McMullen and Nancy E. Freitag, Listeria monocytogenes, Molecular Medical Microbiology, Second Edition, Elsevier publishing*

autophagy. Autophagy is a tightly controlled cellular process of cell organelle recycling and foreign body removal initiated under certain stresses, such as infection or cellular starvation. For pathogens such as Group A *Streptococcus* and *Mycobacterium bovis*, the process of cell autophagy contributes to bacterial killing and resolution of intracellular infection [72]. *L. monocytogenes* appears to avoid the process of autophagy through movement, thereby preventing entrapment of cytosolic bacteria in autophagosomal compartments and preventing their uptake and destruction. In addition to ActA-dependent motility as means of directly out-running autophagy, a member of the internalin family of virulence factors, InlK, has also been shown to bind to major vault protein (MVP) to prevent autophagy in an ActA-independent manner [74].

1.9 - Regulation of Virulence Effectors

Expression of the majority of the gene products associated with *L. monocytogenes* pathogenesis is regulated by a transcriptional regulator known as PrfA. PrfA binds to a fourteen base-pair palindromic region located approximately 40 base-pairs upstream of target genes [75, 76]. Approximately 10 genes are directly regulated by PrfA, however an additional 145 accessory genes may exhibit some form dependence on PrfA activity. The core genes upregulated by PrfA include *hly*, *hpt*, *plcA/B*, *actA*, and *inlA/B/C* [8, 75]. Other gene products directly regulated by PrfA include a bile salt hydrolase *bilE* and the *prsA2*-encoded post-translational chaperone [1]. Additional gene products that appear to be indirectly regulated by PrfA include proteins associated with cell wall synthesis and modulation (NamA) and a bacterial chitinase known as ChiA [50, 77]. Mutants of *Listeria* lacking PrfA demonstrate a five-log

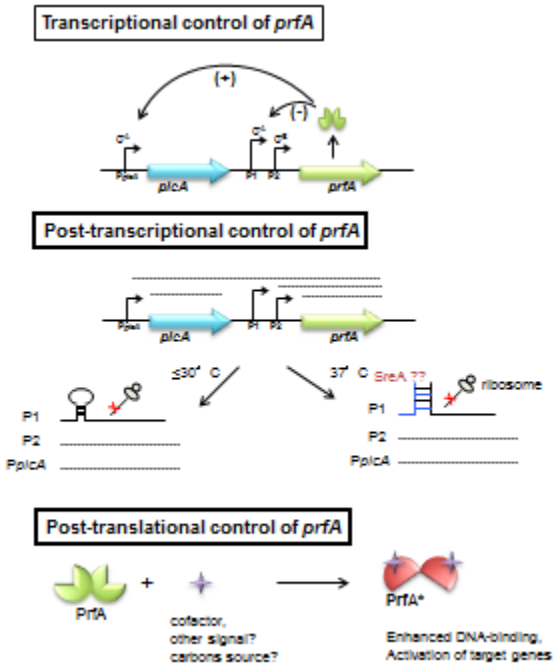


Figure 7: Regulation of the Master Virulence Regulator, PrfA. The regulation of major virulence effectors is controlled by PrfA, the activity of which is tightly regulated by transcription, post-transcriptional, and post-translational forces. *Figure in press: PD McMullen and Nancy E. Freitag, Listeria monocytogenes, Molecular Medical Microbiology, Second Edition, Elsevier publishing*

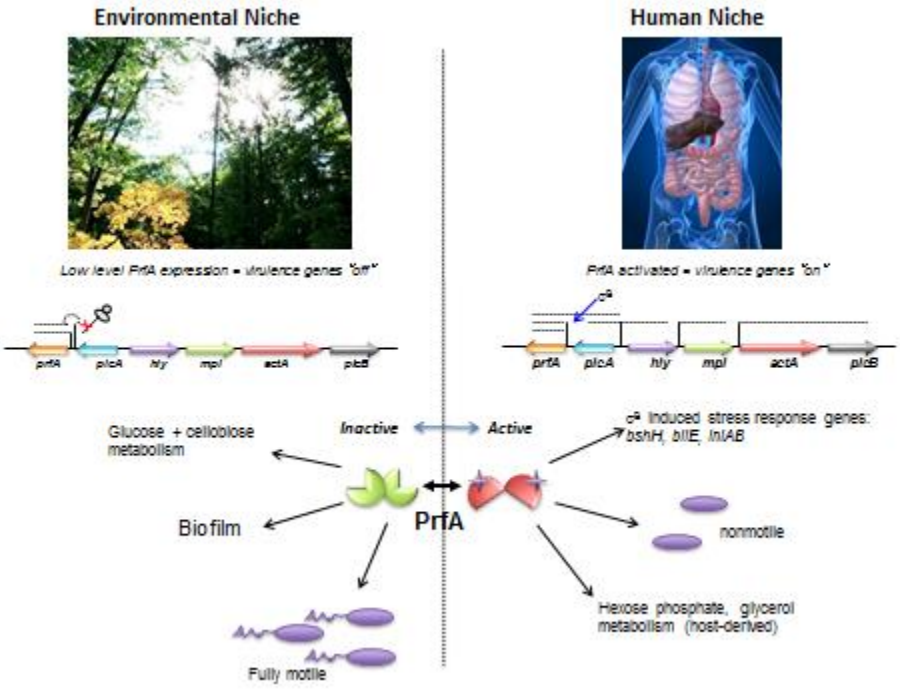


Figure 8: The action of PrfA in environmental sensing The multiple layers of control allow for precise regulation of multiple factors involved in changing from the environmental niche to the human niche. Activation of PrfA leads to physiologic alterations associated with motility, metabolism, and virulence factor production. *Figure in press: PD McMullen and Nancy E. Freitag, Listeria monocytogenes, Molecular Medical Microbiology, Second Edition, Elsevier publishing*

reduction in virulence compared to WT parent strains, indicating a central role for this regulator in the pathogenesis of *L. monocytogenes*.

Regulation of PrfA expression is tightly controlled and involves multiple mechanisms, including transcriptional, post-transcriptional, and post-translational regulation [75] (Fig. 8). The p*rfA* gene product has been demonstrated to both positively and negatively regulate its own expression at the transcriptional level [8, 76, 78]. An RNA-thermosensor in the 5'-UTR of the *prfA* transcript is responsible for limiting complete PrfA expression to environments where the ambient temperature is near 37° [78]. Indeed, mutations in this region which reduce the amount of secondary structure in the 5'-UTR at 30° lead to increased expression of PrfA-dependent effectors at lower temperatures. With regards to sRNA regulation of *prfA* mRNA translation, Loh *et al.* identified of a region of complementarity between a defined location of *sreA*, one of seven putative S-adenosylmethionine (SAM)-responsive riboswitches (SAM riboswitches) in the *L. monocytogenes* transcriptome, and the distal end of the *prfA* 5'UTR [79, 80]. SreA directly interacts with the *prfA* 5'UTR to reduce mRNA translation at 37°C.

The third and possibly most important mechanism for regulating PrfA activity occurs through post-translational modification. PrfA protein belongs to the cyclic AMP (cAMP) receptor protein (Crp)-Fnr family of transcriptional regulators of which there are approximately 400 members [81]. Proteins in this family usually function as dimers and generally require the binding of small molecule co-factors (for example, cAMP for Crp) or other forms of post-translational modification for full activity. There are several lines of evidence that suggests that PrfA is also likely to require the binding of a small molecule cofactor for full activity [8, 75]. PrfA shares significant structural homology with Crp and other family members, and conditions

have been described under which PrfA protein is synthesized but appears inactive (for example, when *L. monocytogenes* is grown in the presence of readily metabolized carbon sources such as glucose and cellobiose) [82]. In addition, Ripio et al described the identification of a *L. monocytogenes* strain that contained a single mutation within *prfA* coding sequences that resulted in the constitutive expression of PrfA-dependent virulence genes in broth culture [83]. The substitution of a serine for a glycine at position 145 within PrfA was suggested to be analogous to an A144T mutation identified within Crp that resulted in the constitutive expression of Crp-dependent gene products (Crp* mutants) [84]. Similar to Crp*, the PrfA G145S mutation alters PrfA protein confirmation and increases the DNA binding affinity of PrfA for its target promoters via to a repositioning of helix-turn-helix DNA binding motif [85]. PrfA G145S and other mutations that appear to constitutively activate PrfA are referred to as PrfA* mutations. Mutants with constitutive PrfA activity display subtle growth defects in rich media, and display defects in competitive fitness when grown in co-culture with wild type strains [86]. However, in the presence of glycerol, the hyperactive *prfA** strains are enhanced for growth, suggesting a *prfA** strain is capable of utilizing nutrients present in rich media, but is locked-in to a physiologic state of glycerol scavenging [87].

Recent studies have focused on the characterization of the putative cofactor binding pocket of PrfA [76]. An examination of PrfA surface charge distribution suggests that the cofactor may be a small, negatively charged molecule. The introduction of positive charge within the pocket interferes with PrfA activation, however activation can be restored by the introduction of mutations that map outside of the pocket that modify PrfA conformation in a manner similar to that which is thought to occur following cofactor binding [76]. Although a specific cofactor for PrfA has yet to be identified, it has been possible to study the consequence of PrfA activation on

L. monocytogenes gene expression, protein secretion, and physiology through the *prfA** strains mentioned previously, thus bypassing the *in vitro* demand for a cofactor [75, 85]. Strains containing such mutations are hypervirulent in animal models of infection and are enhanced for invasion, vacuole escape, and actin polymerization in tissue culture infection models. Thus, constitutive activation of PrfA appears to enhance *L. monocytogenes* fitness within mammalian hosts. The need for PrfA regulation becomes evident when examining the effects of PrfA activation on *L. monocytogenes* fitness outside of host cells. As mentioned previously, strains containing *prfA** alleles are defective for flagella-mediated swimming motility as well as long term starvation adaptation, and exhibit fitness defects when grown in mixed cultures with wild type bacteria [76]. Regulation of PrfA activity is thus required for *L. monocytogenes* to optimize bacterial fitness for its disparate lifestyles inside and outside of host cells (Fig. 9).

1.10 – Host Responses to Infection

Studies in mice have elaborated the immune response to *Listeria* in fascinating detail. In intravenous models of infection, bacteria are quickly taken up in the liver by resident macrophages, or Kupfer cells, while *Listeria* localizes to marginal zone macrophages in the spleen [6]. Once internalized, *Listeria* is present in a membrane- bound vacuole. In phagocytic listericidal cells, endocytosed bacteria are exposed to the non-specific killing mechanisms of these cells. The roles of reactive oxygen radicals, reactive nitrogen intermediates and microbicidal cationic peptides, such as defensins, in the listericidal processes of infected macrophages remain to be defined in detail. Approximately 15% of internalized bacteria are

able to lyse their vacuole and multiply in the cytosol [88]. The remaining bacteria fail to escape from the vacuole before fusion with the lysosomal compartment and are killed.

The predominant early response against *Listeria* infection is largely mediated by innate phagocytes. Pioneering research into innate resistance to *Listeria* was done in SCID mice and demonstrated a T-cell independent mechanism of resistance. It was subsequently elucidated that infected macrophages were capable of producing IL-12 and TNFα, thus activating NK cells to produce IFNγ {Τριππ, 1993 #72}. Mice in which IL-12 is neutralized, or the IFNγ receptor is deleted, demonstrate an increased susceptibility to infection, supporting the notion that macrophage activation and response is critical for controlling *Listeria* infections. Other studies demonstrated roles for neutrophils in early killing of *Listeria* in the liver but not the spleen, with a later influx of macrophages after approximately 24 hours of infection [32]. Regulatory γδ-T cells play additional roles in the innate reaction by limiting collateral damage to host tissues during infection, and providing additional IFNγ for macrophage activation in the early phases of infection [89].

Infection by *Listeria* induces the upregulation of a number of pro-and-anti-inflammatory cytokines early in the infection [90]. Initial release of IL-6 by infected cells recruits neutrophils to sites of early infection [91]. *Listeria* capable of surviving the initial barrage by these phagocytes spread quickly to infect neighboring cells via cell-to-cell spread in both the liver and spleen. Intracellular spread of bacteria largely prevents phagocytosis and processing of free bacteria. Although the role of neutrophils seems minimal, neutrophils recruited to the liver have been shown to secrete CSF-1 and MCP-1, which serve to recruit monocytes to the site of infection [92]. Macrophages derived from these recruited monocytes, when activated, are

largely responsible for clearing the infection, thus implicating an indirect role for neutrophils in the clearing process [91]. Once macrophages arrive at the site of infection, they become activated to produce IL-12 and TNFα, both of which in turn activate NK cells in proximity to the infection [93]. Through the action of granzymes and perforin, NK cells lyse target cells infected by *Listeria*, thus releasing bacteria contained within for phagocytosis by nearby activated macrophages. The cycle of lysing infected cells followed by killing of the released bacteria largely represents innate strategy for resolving *Listeria* infections.

Upon introduction of *Listeria* to the cytosol, cells induce two waves of signaling cascades. The first wave of signaling appears to be dependent on TLR-2, and through NFkB induces a proinflammatory response [94]. Macrophages deficient for MyD88 or TLR2 signaling demonstrate significantly less secretion of the proinflammatory cytokines TNFα, IFNγ, IL-1β, and IL-12; however, it was also demonstrated that the lack of TLR2 in mice had a minimal effect on resistance to infection [95]. Lack of MyD88 in mice demonstrated a much greater increase in susceptibility than did TLR2, implicating other intracellular surveillance pathways in resistance to *Listeria* infection [93]. Indeed, the cytosolic surveillance machinery NOD2 and NALP3 have been proposed to be involved in responses to intracellular *Listeria* by sensing of peptidoglycan fragments and caspase-1 activation, respectively [96].

Following this inflammatory cascade initiated by TLR/MyD88 signaling, a second cascade initiates and culminates in the activation of JAK/STAT through a TLR-independent mechanism [94]. This second cascade results in the expression and release of IFNα/β, which have documented roles in anti-viral responses. Investigation into this cascade reveals induction of these products is beneficial for *Listeria* and indeed has been demonstrated to increase rates of T-cell apoptosis and lead to increased IL-10 secretion by immune mediators [97]. The

IFNα/β response requires the presence of cytosolic bacteria, thus *Listeria* must escape the vacuole in order to initiate this response.

Listeria also induces a robust cell-mediated adaptive immune response following the innate responses mentioned previously. The mediators of adaptive immunity, including T-cell populations, become evident 4-5 days post-infection and peak roughly 7-9 days following inoculation. This occurs via the initiation of either CD4 or CD8-type responses, depending upon the context of antigen presentation [90]. Antigen released by cytosolic bacteria is cycled and presented using the MHC-I pathway, which induces CD8+ cytotoxic T-cell responses, while bacteria phagocytosed and killed within vacuoles are typically presented on MHC-II and induce CD4+ responses [90, 91]. While the CD8+ response is primarily directed at killing infected host cells in a perforin-dependent manner, the role of CD4+ cells is less well defined. Mice lacking CD4+ T cells that are infected with *Listeria* demonstrate reduced granuloma formation in areas colonized by bacteria [98]. CD4+ cells also secrete IFNγ, which induces macrophage activation, the critical component for controlling *Listeria* infection. Thus, a stable population of T-cells appears to be required for final clearance and sterile immunity generated against *Listeria*, and CD8+ T-cells are the most effective mediators of anti-*Listeria* immunity [90]. Additionally, an expansion of γδ-regulatory T-cells becomes apparent upon challenge with *Listeria* and is required to control expansion of inflammation within areas colonized by the bacterium [89].

Although generated during listeriosis, B cells and antibody have a minimal role in controlling infection, presumably due to the intracellular niche occupied by *Listeria*. Circulating antibodies within naïve animals, however, can prevent early dissemination and can also neutralize intracellular bacterial products, such as LLO [99]. Populations of B-cells may also play a role in maintaining CD8+ T-cells during the infection, thus contributing to the primary defense strategy,

albeit in an indirect manner [100]. Following resolution and successful adaptive responses to *Listeria* challenge, animals develop sterile immunity and are protected from subsequent reinfection [91]. This consequence of immunity has been subverted in recent vaccine trials in order to induce strong cell-mediated responses against recombinant antigens expressed by mutant strains of *Listeria* [7].

1.11 - Tissue Tropism in Listerial Infections: Securing Replication Niches within Hosts

Just as environmental forces create selective pressures on the replication of *Listeria* at large, selective pressures within susceptible hosts are thought to contain bacterial replication such that patterns of infection may be observed consistently between multiple isolates. For *Listeria*, the primary targets of bacterial replication are the liver and spleen in mammals, with a propensity to spread beyond these organs in later stages of infection – commonly, the brain and fetus/placenta (where present)[20]. Restriction of bacterial replication can be imagined as a unique interaction between the individual host and the invading microbe. Restriction of replication within hosts can be driven by many different facets, including but not limited to: localized immune responses, presence of cellular receptors for invasion, or nutritional immunity through sequestration of nutrients required for bacterial growth. For *Listeria* it is thought that the intrinsic life-cycle (i.e. rupture of the phagosome, cell-to-cell spread, etc.) plays very little role in niche acquisition within hosts. Rather, the limiting factor appears to be the direct ability of the bacterium to promote its own invasion of host cells. The plethora of susceptible cells indicates that strains of *Listeria* are well-suited to induce their uptake and survive in many different systems. Yet this strikingly flexible ability to invade multiple cell types, including endothelial cells and fibroblasts,

is often met with a very rigid infection phenotype in mice – where bacterial replication is contained largely to the liver and spleen.

It has been shown previously that alterations to the surface-expressed receptors for *Listeria* invasion can influence tissue distribution in infected animals. Such experiments include the replacement of native host-receptors with versions capable of mediating *Listeria* uptake (e.g. insertion of E-cadherin into the hearts of mice in place of N-cadherin promotes *Listeria* invasion of the heart *in vivo*) [101], as well as modifications of the bacterial invasin to modulate receptor recognition [102]. Thus it stands to reason that altered bacterial or host components involved with the process of bacterial invasion may alter tissue tropism at large. Given the large amount of diversity present in the species, isolates of *Listeria* may possess strain-specific alterations that can influence the migration and colonization of host tissues based solely on the large amount of genetic diversity present across the invasion loci of isolated strains. Indeed, when assessed for their ability to colonize particular tissues in infected animals outside of the liver and spleen, several isolates have been shown to be enhanced for replication within tissues such as the heart, placenta, and fetus [20]. The advancement of virulence in these sites is likely derived from stochastic differences between individual strains of *Listeria*, as each strain has evolved a complex and unique chemistry which ultimately drives its interaction with the host.

1.12 – Summary

Listeria's distribution in the environment as a saprophytic organism exposes it to consumption by mammals. Just as the species has evolved features which allow it to survive a variety of external environments, it has also acquired multiple effectors that permit replication and spread within mammalian organs. Spread within hosts is akin to spread within any

environment in that it is shaped ultimately by forces of selection. Ultimately, the infectious process represents the bacterium's attempts to extract nutrients from an external source (i.e. the host), and the host's resistance to that parasitism. The final outcome of its interaction with mammalian hosts (specifically humans) depends on multiple factors: The competence of the individual strain for pathogenesis, the immune status of the affected host, and/or host predisoposition to localized disease (e.g. heart valve lesions, disrupted intestinal barriers, poor hygiene, etc.). Host predispositions aside, there is evidence that subpopulations of *Listeria* may have evolved (or may be evolving) features which allow them to spread and replicate within a greater number of host niches, potentially allowing for more nutrient acquisition from the host.

1.13 – Goals of this Study

Although *Listeria* demonstrates great genotypic and phenotypic diversity, study of the disease in laboratory animals has largely focused on a small number of well-characterized strains. In these strains, manifestations include infections of the liver, spleen, brain, and fetal/placental unit. Infection in other sites, such as the heart, lungs, or kidneys (which are also able to be colonized by other pathogenic organisms), has not been extensively reported in animals. In humans, however, blood-borne *Listeria* have been shown to colonize the heart in some cases. Manifestations of cardiac involvement include endocarditis [103], myocarditis [104], pericarditis [105], vasculitis with aneurysm formation [106], and even fistula formation (fistulas are areas of necrotic tissue that create a communicating channel between two compartments, such as the left ventricle and left atrium [107]). A previous study of a small sample of strains demonstrated that among eleven unique strains, two demonstrated increased cardiac myocyte invasion and cardiac tropism in infected animals [20]. Given the great diversity in genetics present across the species,

it is therefore likely that some strains have evolved the capacity to replicate outside of their characterized niches. The offset between the clinical data and the laboratory observations in animals may, therefore, be the result of bacterial genetics. The overarching goal of this study is to assess features of cardiotropic strains of *Listeria* in the hopes that the factors leading to this alteration in tropism can be identified.

Early investigations into cardiotropism began by sequencing the genome of an isolate taken from a fatal myocardial abscess. Chapter Two details some of the major findings of this sequence information. The strain in question has an enhanced ability to invade cardiac myocytes *in vitro* and colonize the hearts of mice *in vivo*. Sequencing of the genome allowed for comparison of the isolate with two of the better characterized laboratory isolates, each from different serovars of the species. The findings demonstrated that the isolate shared a large degree of homology with both laboratory strains, and suggested that horizontal gene transfer did not play a role in the ability of the isolate to colonize the heart. The sequence also provided crucial sequences of all characterized virulence factors, including LLO, ActA, PlcB, InlA, InlB, and PrsA. The results suggested that alterations to the core set of virulence factors may be sufficient to alter tropism within animals.

Chapter Three begins the investigations into two of these virulence factors, InlA and InlB, and their potential roles in cardiac myocyte invasion. InlA/B have been shown to mediate uptake into a variety of cell types (as mentioned in previous sections of this chapter), and polymorphisms within the genes encoding InlA/B were conserved among isolates more likely to colonize the heart. Using genetic swaps, the polymorphic genes were moved between backgrounds of *Listeria* and these strains were assessed for myocyte invasion and cardiotropism. The gene encoding *inlB* isolated from the clinical isolate was found to be capable of increasing myocyte invasion, as well

as cardiac colonization in mice. Polymorphisms in this gene appear to alter aspects of InlB's function, notably sensing of heparan sulfate, as well as lead to decreases in protein degradation. Mutagensis and studies of another *inlB* allele indicated that synergistic changes to two distinct regions were required for cardiac cell invasion and cardiotropism.

Chapter Four follows up on these findings by investigating the physiology of the cardiac isolate in the context of pregnancy. The clinical isolate which was capable of colonizing the hearts of infected mice to a greater extent also colonized the placenta and fetuses of pregnant mice more so than the laboratory strain commonly used. Not only was colonization of the placenta found to be significantly increased for the cardiac isolate, but the migration from placenta to fetus was also significantly increased, indicating a propensity for vertical transmission. By using a deletion strain of the clinical isolate, InlB was demonstrated to play a critical role in mediating colonization of both the fetus and placenta. The complementation of the InlB-defect was found to be sufficient to increase colonization of the fetus and placenta above the level of the deletion strain. Additionally, over-expression of InlB (regardless of allele) increased placental and fetal colonization above WT levels. These results indicate that InlB is a positive factor for placental and fetal infections from this strain, and that the overall amount of InlB is correlative to the amount of colonization and infection.

Chapter Five returns the focus to both InlA and InlB, and specifically investigates the contributions of each protein to invasion in two other cell types: Caco-2 cells (representative of the intestinal barrier) and L2 cells (representative of lung epithelial cells, colonized during vertical transmission). Using allelic studies identical to those used in Chapter Three, allele-specific effects were noted in both cell types, indicating that alleles of *inlAB* have the potential to alter invasion independently and interdependently in enterocytes, myocytes, and lung epithelial

cells. Additionally, the physical characteristics of internalin expression are described in Chapter Five. Investigations centered around the effect of gene dosage of one member of the internalin operon on the surface abundance of the other, as well as documenting differences in the stability of protein variants from different isolates.

Chapter Six broadly addresses the role of genetic diversity within the internalin operon on tissue tropism by parsing apart molecular features discussed in earlier chapters. The roles of the relative expression of InlA to InlB, stability of protein variants, and interactions with host ligands are discussed in detail in the context of invasion. Additionally, this chapter discusses the advantages of using clinical isolates to study molecular phenomena associated with pathogenesis, and the correlation between replication within isolated host environments with the evolution of individual sub-species. The goal of the thesis overall is provide a better perspective on the complex nature of bacterial and host interactions, and to address the role of bacterial genetic diversity in disease manifestation.

Chapter Two: Genomic Sequence of a Clinical Isolate Recovered from a Myocardial Abscess

Adapted from: McMullen and Freitag, "Genome sequence of Listeria monocytogenes 07PF0776, a cardiotropic serovar 4b strain." J. Bacteriol. 2012 Jul;194(13):3552. Doi: 10.1128/JB.00616-12, *and is reprinted under the retained right to publish this work in a thesis or dissertation granted by the American Society of Microbiology (See author's supplemental thesis documentation for Author's rights for reuse)*

2.1 - Summary

Listeria is known for its propensity to colonize the liver and spleen in infected animals. The bacterium is also capable of colonizing the CNS, as well as the fetal-placental unit of pregnant women. While these are the classical manifestations, *Listeria* is capable of colonizing and replicating within many distinct tissue sites [108, 109]. Previous studies have demonstrated the presence of subpopulations of *Listeria* capable of consistently colonizing the hearts of infected animals [20]. One of the strains tested, 07PF0776, demonstrated not only consistent heart colonization, but also displayed alterations in invasion and was capable of invading cardiac myocytes at a greater rate than the other strains examined. As cardiotropism could be the result of either host predisposition to disease or bacterial virulence products, 07PF0776's specific enhancement for myocyte invasion and colonization suggested the presence of a bacterial specific factor. To further investigate this possibility, a genome sequence of 07PF0776 was performed and analyzed in the context of two other strains of *Listeria*, 10403S and F2379. Analysis revealed some major differences in genomic structure, but overall hinted that the ability

of 07PF0776 to colonize the heart was due to polymorphic differences in its core virulence factors.

2.2 - Introduction

A recent strain labeled 07PF0776 was isolated originally from an HIV-positive man with at T-cell count of 200cells/cc. The patient presented to the emergency with a three week history of chest pains, palpatations, and fever. EKG and physical workup supported a cardiac event, likely a myocardial infarction. As workup progressed, the patient's blood samples began to grow a single strain of *Listeria monocytogenes*. The patient was started on Erythromycin, the standard antibiotic of preference for listeriosis. After three days, the patient's cardiac complaints worsened and EKG supported the presence of a transmural infarct (an area of dead tissue within the myocardium that is capable of disrupting electrical transmission through the heart). Despite intervention, the patient's status progressively declined and he passed at three days post-admission. On autopsy, the patient was found to have a $2cm^3$ abscess in the interventricular septum of the myocardium. This abscess was found to contain a monoculture gram positive organism, which was later determined to be the same strain of *L. monocytogenes* isolated from the patient's blood earlier in the workup. The abscess contained numerous dead phagocytes and bacteria which appeared to be distributed both inside and outside of myocytes. Importantly, given the high level of HIV present in this patient, there was no evidence of underlying HIV-mediated myocarditis. This strongly suggested the isolated strain of L. monocytogenes was the etiologic agent of the fatal myocarditis [20].

As myocarditis caused by *Listeria* is not a well-documented phenomenon, further characterizations of the strain were performed in order to better understand the physiology of the

isolate [20]. These studies demonstrated that 07PF0776 was indeed enhanced for myocyte invasion and cardiac colonization of laboratory animals. While superficial genetic screens were performed on a small selection of virulence factors (InlA, InlB, LLO), a complete genome of the organism in question was lacking.

2.3 - Materials and Methods

Genomic DNA was isolated from 07PF0776 using the Qiagen Blood and Tissue Genomic DNA Extraction Kit. Purity was analyzed by 260/280nm ratios and samples with a ratio greater than 1.80 were considered sufficiently pure for analysis. The isolated DNA was sent to the Genomics Center at the University of Oklahoma, where it was sequenced using the SOLiD platform. This method generates a large number of short reads (40-60bp) by using emulsion PCR to amplify fragmented portions of the genomic DNA. These reads were then mapped to two previously published genomes: 10403S (a serovar 1/2a strain commonly used in laboratories) and F2379 (a serovar 4b clinical isolate also used in laboratories).

2.4 - Results

Alignments indicated the presence of an 11,000bp deletion extending from the latter third of lmo0072 to just upstream of lmo0088. This region contains genes homologous to the Ess secretion pathway of *Staphylococcus aureus* that is required for bacterial virulence and which is an ortholog of mycobacterial T7 secretion systems [110]. This frameshift and deletion was somewhat confirmed by PCR analysis, which showed a difference in running patterns in the region between 10403S and 07PF0776. Several other frameshift mutations were detected, though much smaller in magnitude than the loss of the Ess-like operon. One was detected within the gene encoding InlJ, a surface protein that has been implicated in bacterial adhesion to

hepatocytes [111]. 07PF0776 also contains a frameshift in genes responsible for lipoteichoic acid modifications. Specifically, 07PF0776 is lacking *gltA*, which functions by adding glucose modifications to the lipoteichoic acid [112]. While these changes are interesting to note, the overall genome architecture of 07PF0776 was very similar to both 10403S and F2379. Indeed, examination of unmatched reads from the alignment failed to demonstrate the presence of novel pathogenicity or phage islands which would not align to either 10403S or F2379.

2.5 - Discussion

Even though the genomes of 10403S, F2379, and 07PF0776 were roughly similar, many strain-specific alterations were noted. As mentioned in section 2.3, frameshifts were specifically detected in an Ess-like operon, InlJ, and GltA. The Ess-like operon in *Listeria* has been studied by our lab previously, though the results were not published (Thesis of G. Port, unpublished data). In those studies, deletion of this region appeared to have no effect on virulence in mice or cells in tissue culture. Secretion systems have been shown to also play roles outside of virulence [110, 113], so it is possible the loss of this genetic information has a role in the environmental aspects of 07PF0776's lifestyle. Attempts were made to reamplify this region in 07PF0776, in order to rule out the possibility poor quality reads in the genome sequence, however no products were ever recovered.

Mutants of InlJ have also been characterized, though this protein is known to be strain-specific (F2379 does not possess genomic *inlJ*, whereas 10403S does) [111]. In tissue culture, InlJ appears to function as an invasion for *Listeria*, mediating attachment to the surface of hepatocytes. While this gene does have a known virulence function, its presence varies across strains of *Listeria*, and analysis of 07PF0776 in mice has revealed no defect in liver colonization.

It is possible that 07PF0776 is from a lineage which never possessed InlJ, and thus has evolved other features which compensate for a lack of liver adhesion. One appealing target in this aspect is the LLO of 07PF0776, which has been shown to be more cytotoxic than that of 10403S, as well as having a different migration pattern following SDS-PAGE (Francis Alonzo, Unpublished Data). LLO has been shown to mediate uptake into hepatocytes by perturbing ion flux, thus leading to cytoskeletal perturbations [51], so modifications to the activity of other bacterial factors may compensate for this apparent genetic defect.

Mutants lacking glucosylation machinery have also been studied previously, and have been shown to possess altered lipoteichoic acid structure [112]. Conceivably, alterations to lipoteichoic acid could alter immune responses through TLR4 [95], the retention of LTA-associated virulence factors (such as InlB and Ami) [39, 112], as well as other features such as cell-wall density [114] and adhesion [115]. These changes could conceivably play a role in cardiotropism for this strain, especially given the role of LTA in modulating attachment of other virulence factors, such as InlB and Ami.

By aligning against two distantly related strains of *Listeria*, it was possible to compare the genome content and polymorphic patterns within commonly studied virulence factors. Previous results have catalogued the physiology of 07PF0776, detailing its cytotoxicity, invasiveness, and behavior in cell culture [20]. These results, combined with the genome sequence, suggest that the alterations which allow 07PF0776 to colonize the heart and invade cardiac myocytes lie within the well-characterized virulence factors conserved among all virulent strains of *L. monocytogenes*. Sequencing of specific members of these factors previously revealed genetic correlations between cardiotropism and the sequence of *inlA* and *inlB* [20], however the genetic studies were limited.

Chapter Three: Identification of InlB as a Strain-Specific Factor Capable of Enhancing Invasion in Cardiac Myocytes and Promoting Cardiotropism in Animals

3.1- Summary

Pathogens may face a variety of different environments within an infected host, each with a somewhat unique set of selective pressures limiting pathogen replication. Several isolates of the gram positive pathogen *Listeria monocytogenes* demonstrate enhancements in their ability to replicate within the hearts of infected animals. A subpopulation of clinical isolates from a previous study was found to colonize the hearts of infected animals more efficiently, and some members were also hyperinvasive in cardiac myocytes *in vitro*. Genetic correlations exist within these cardiotropic strains, and indicated related alleles of the invasins *inlA* and *inlB* may be responsible for the increased ability to colonize heart tissue.

Using one of the cardiotropic isolates studied previously (07PF0776) and a non-cardiotropic laboratory strain (10403S), it was found that strains of *Listeria* lacking the invasin *inlB* were significantly impaired in terms of both cardiac myocyte invasion and heart colonization. Complementation of the *inlB* defect revealed an allele-specific feature, such that the *inlB* from 07PF0776 was able to make strains of 10403S hyperinvasive in cardiac myocytes; whereas *inlB* from 10403S was incapable of mediating hyperinvasivity in myocytes, even when placed in a strain of 07PF0776 lacking *inlB*. These results indicate that the *inlB* allele of 07PF0776 can enhance both heart –specific invasion and *in vivo* colonization of mice across different bacterial backgrounds.

The mechanism of InlB's effect on bacterial invasion and tissue tropism relies on bacterial mimicry of a host ligand. InlB binds to the host receptor c-Met, which is present across a variety of host tissues and mediates effects such as wound healing and tissue regeneration during times of injury. In hepatocytes, InlB's interaction with c-Met has been shown to drive invasion of liver cells and thus promote replication within the livers of infected animals. The molecular events in the mechanism of InlB include dissocation from the bacterial surface and local migration on the host surface under the influence of heparan sulfate. Following this migration process, InlB binds to c-Met, induces receptor clustering and bacterial invasion occurs.

To understand the features of InlB from 07PF0776 which contribute to its ability to promote cardiac infections and heart cell invasion, the role of host heparan sulfate and stability of the bacterial gene product were investigated. InlB of 07PF0776 possesses an aspartic acid polymorphism in its beta-repeat (linker) region that is also shared with other previously identified cardiotropic isolates. Site-directed mutagenesis was used in order to convert this aspartic acid to an asparagine, a residue more commonly present in non-cardiotropic strains. This reversion decreased the apparent stability of the InlB from 07PF0776, and made the protein degrade in a pattern similar to what is seen for 10403S InlB. Polymorphisms present in the GW repeat (cell-association) domains of 07PF0776 InlB indicated a substantial amount of surface positive charge is lost in the 07PF0776 variant. As a result, the WT 07PF0776 variant of InlB demonstrates increased responses to heparan sulfate, especially at low concentrations and poor sulfation. Both the enhanced stability and increased response to poorly-sulfated heparan sulfate appear to be crucial for enhancing myocye invasion, and thus are synergizing features that enhance heart colonization in infected animals.

3.2 - Introduction

Listeria monocytogenes is a gram-positive, saprophytic bacterium most commonly isolated from environmental sources such as detritus, silage, and pond water [8]. The species is capable of causing disease in a wide variety of hosts, and is most commonly associated with human disease through the consumption of contaminated food products [29]. The invasive disease listeriosis is almost exclusively limited to immunocompromised populations, including the elderly, persons on chemotherapy and pregnant women [17]. Exposure to *L. monocytogenes* is a silent process for the majority of hosts, though it has been suggested that gastroenteritis may be an initial, unrecognized, manifestation [14].

In susceptible populations the bacteria are capable of causing very severe invasive infections, with the most common manifestations involving the central nervous system or fetal-placental unit, leading to diseases such as meningitis, or causing septic abortion [13]. In less common cases, *L. monocytogenes* can colonize other organs or tissues, leading to localized infections that produce organ-specific pathologies [18]. Bloodborne bacteria such as *L. monocytogenes* largely rely on the circulation of blood for distribution to distinct organ sites. Once within a capillary bed, *L. monocytogenes* is capable of breeching continuous endothelial barriers either via direct invasion or by the 'Trojan horse' mechanism of pathogen delivery by infected phagocytes [38]. With the ability to breech host barriers, subsequent replication of *L. monocytogenes* within tissues relies on the bacterium gaining access to the intracellular compartment, which is largely facilitated by the invasins InlA and InlB [35, 45].

For a small percentage of *L. monocytogenes* infections, the primary manifestation is cardiac in nature, suggesting the heart as a potential site of replication for some strains of *L. monocytogenes*

[20]. Cardiac infections are not commonly reported, so it is unknown whether host or bacterial factors play the predominant role in directing bacteria to the heart. Access of *L. monocytogenes* to the heart during infection is an almost certainty, however, as the two principle manifestations of disease – meningitis and placental/fetal infection – rely on the presence of blood-borne bacteria [38, 45]. These observations implicate the heart's central location and circulatory functions in the spread of bacteria to these organ systems. In addition to generating blood flow throughout the body, the heart also supplies itself with approximately 5% of the total output through the coronary arteries and perfusing capillary beds [116]. As a result, there is exposure of cardiac cells, including myocytes, fibroblasts, and endothelial cells, to circulating *L. monocytogenes* during bacteremia. These cell types represent major constituents of cardiac tissue and each type is capable of sustaining intracellular *L. monocytogenes* [19, 20, 39]. Thus the low frequency of clinical cases may indicate that cardiac tissue is more resistant to *L. monocytogenes* colonization and infection when compared to other target organs, such as the liver, spleen, CNS or placenta.

Previously, we have found that a subpopulation of *L. monocytogenes* clinical isolates demonstrate enhanced cardiac myocyte invasion *in vitro* and enhanced heart colonization in outbred mice [20]. *L. monocytogenes* isolates capable of heart colonization included representatives from different lineages and serovars, and sequencing of several genetic loci indicated that cardiotropic isolates shared highly related sequences of the bacterial invasins InlA and InlB, suggesting that these gene products may contribute to heart cell invasion. InlA and InlB have been previously reported to contribute to *L. monocytogenes* colonization of the placenta [26], as well as the choroid plexus [37], and mediate uptake of *L. monocytogenes* into a wide variety of cell types *in vitro.* In this report, we describe the roles of InlA and InlB in

cardiac cell invasion and demonstrate that an allelic variant of the *inlB* locus enhances cardiac myocyte invasion directly, resulting in increased colonization of the heart during an infection.

3.3 - Results

L. monocytogenes 07PF0776 InlB contributes to cardiotropism

L. monocytogenes strain 07PF0776, originally isolated from the heart of a patient with an invasive cardiac infection, has been shown to more readily colonized the hearts of infected mice in comparison to the widely studied strains 10403S and EDG-e [20]. Female Swiss-Webster mice, aged 6-8 weeks, infected with 10,000 organisms via tail-vein injection exhibited similar bacterial burden in the liver and spleen at 72 hours post-infection, however mice infected with 07PF0776 exhibited both higher bacterial burdens in the heart as well as a higher frequency of cardiac colonization (Fig. 9). Six out of eight infected mice demonstrated significant cardiac colonization for 07PF0776, whereas only two of the eight infected with 10403S had detectable levels of bacteria. Average heart burdens for mice infected with 07PF0776 were on the order of 1,000 CFU at 72 hours post-infection, whereas mice infected with the 10403S strain had less than 100 CFU per organ at the same time point.

L. monocytogenes 07PF0776 also exhibits increased invasion of H9c2 rat cardiac myoblasts *in vitro*, as assessed by gentamicin protection assay (Fig. 10). 07PF0776 exhibited a 3-4 fold increase in H9c2 cell invasion in comparison to 10403S. 07PF0776 was also elevated in comparison to hyper-virulent 10403S strains containing the constitutively activated *prfA* L140F (*prfA**) allele, which has been shown to enhance bacterial colonization of mouse liver and spleen [87], indicating that a general hyper-virulent state was not sufficient to enhance myocyte

invasion. Interestingly, 10403S mutants lacking *inlB* were significantly reduced for invasion compared to the parent strain, while the deletion of *inlA* did not reduce H9c2 cell invasion (Fig. 10). Deletion of *inlB* in the 07PF0776 background similarly produced a striking defect in cardiac myocyte invasion, indicating that InlB plays a major role in mediating *L. monocytogenes* cardiomyocyte invasion.

InlB derived from cardioinvasive 07PF0776 confers enhanced cardiac cell invasion.

To further analyze the roles of InlA and InlB with respect to cardiac myocyte invasion, we compared the ability of 10403S and 07PF0776 alleles of *inlA* and *inlB* to promote cardiac cell invasion in a 10403S mutant strain lacking both *inlB* and *inlA* (Fig. 11). The expression of either *inlB* allele increased invasion of the 10403S Δ*inlAB* mutant, as anticipated, however strains expressing *inlB* derived from 07PF0776 were 3-4 fold more invasive than those expressing *inlB* derived from 10403S (Fig 11). This fold difference was similar in magnitude to the difference observed between 10403S and 07PF0776 strains for cardiac cell invasion using the same assay. When 07PF0776 *inlB*-deletion strains were complemented with either 10403S or 07PF0776 *inlB* alleles, *inlB* derived from 07PF0776 enhanced cardiac cell invasion over strains expressing the 10403S allele, similar to what was observed in the 10403S background (Fig. 12). *inlB* derived from 07PF0776 is thus enhanced for its ability to promote bacterial invasion of

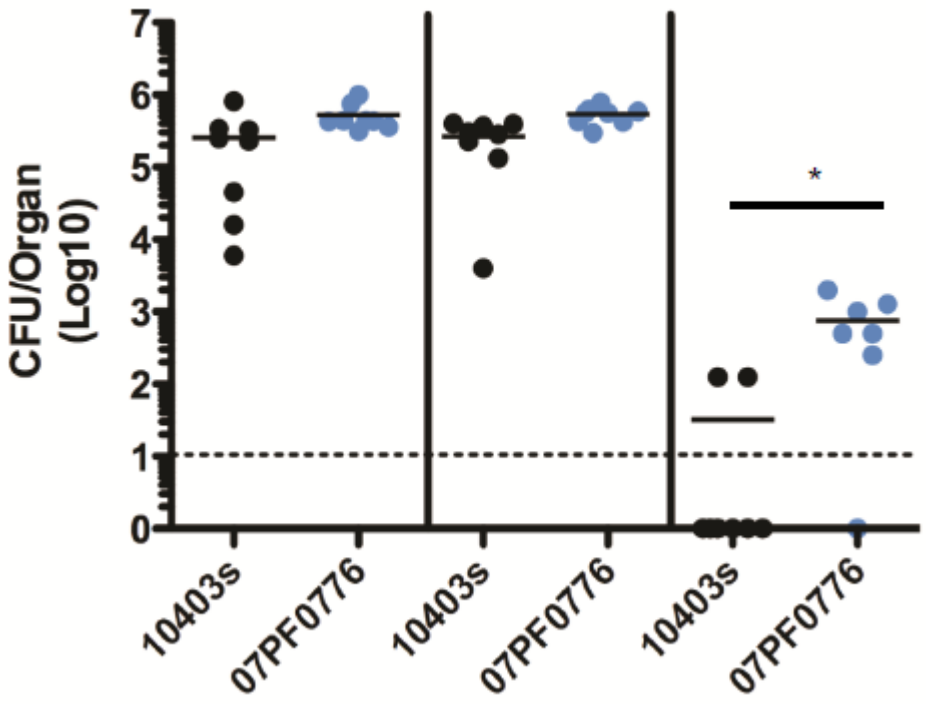


Figure 9: 07PF0776 is enhanced for cardiac colonization in a mouse model of infection. Female Swiss-Webster mice, aged 6-8 weeks, were infected via the tail vein with 10,000 CFU of *L. monocytogenes* 10403S or 07PF0776. Infections were allowed to progress for 3 days, at which point mice were sacrificed and bacterial CFU in the liver, spleen, and heart were enumerated. 07PF0776 and 10403S behave similarly in both the liver and spleen, however more organisms were recovered from the hearts for those mice infected with 07PF0776. * Indicates statistical significance ($p<0.05$ Mann-Whitney Test).

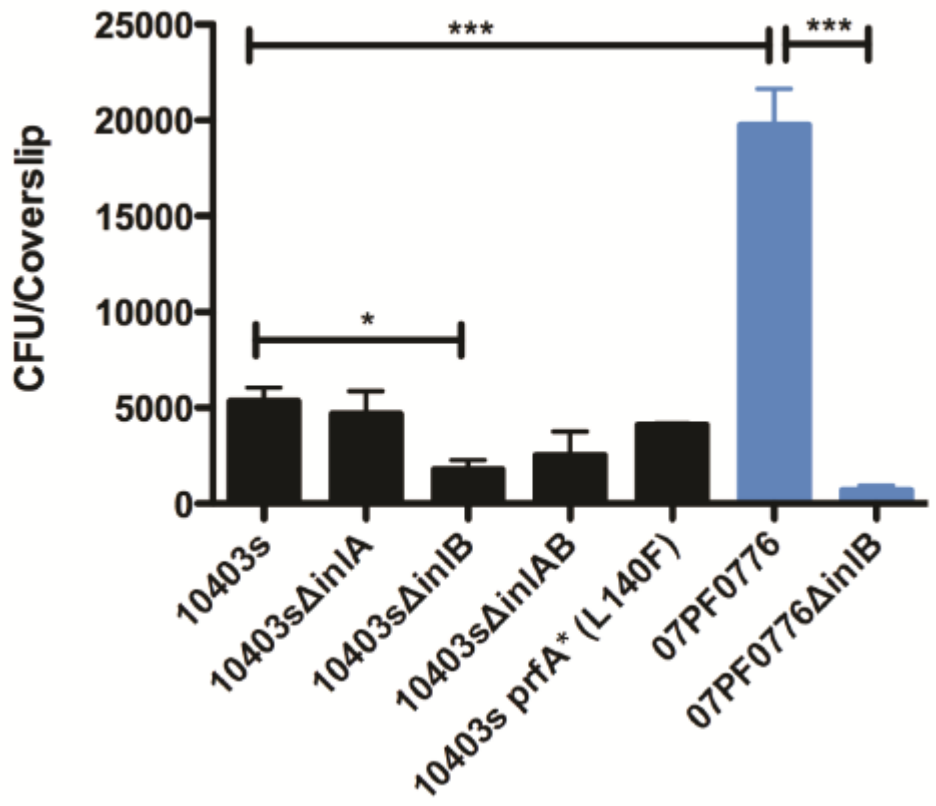
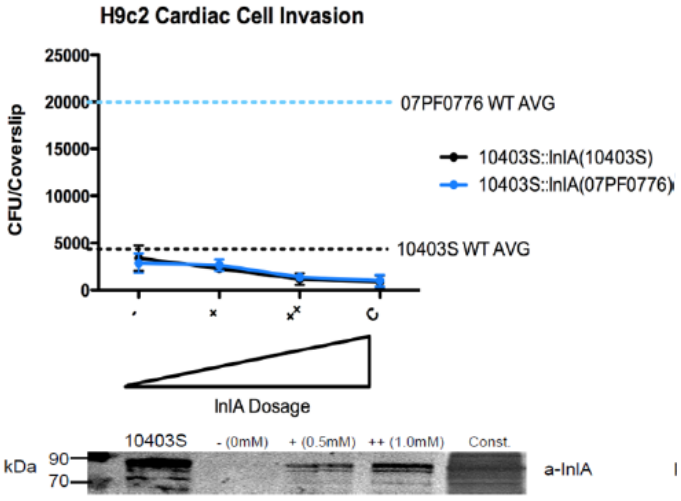


Figure 10: 07PF0776 is enhanced for cardiac myocyte invasion, and InlB is required for full invasion in both 10403S and 07PF0776. Bacterial invasion of cardiac myocytes was assessed using gentamicin protection assays. 300,000 H9c2 cardiac myoblasts were seeded over glass coverslips and allowed to adhere and double overnight. Cells were infected the following morning at an MOI=100, with infections lasting one hour before gentamicin was added to kill extracellular bacteria. Coverslips were removed after one hour in gentamicin and the cells were lysed with ddH20. Bacterial CFU per coverslip was then enumerated by spot plating on LB agar. 07PF0776 is enhanced for invasion compared to 10403s by 3-4 fold. Mutants of either strain lacking InlB are defective for invasion, however a hypervirulent strain of *Listeria* (L140F prfA*) – which overexpresses InlB – is not enhanced for invasion in this cell line. Statistics were calculated using unpaired student's t-test. * represents a p-value less than 0.05, *** represents a p-value less than 0.001.

a



b

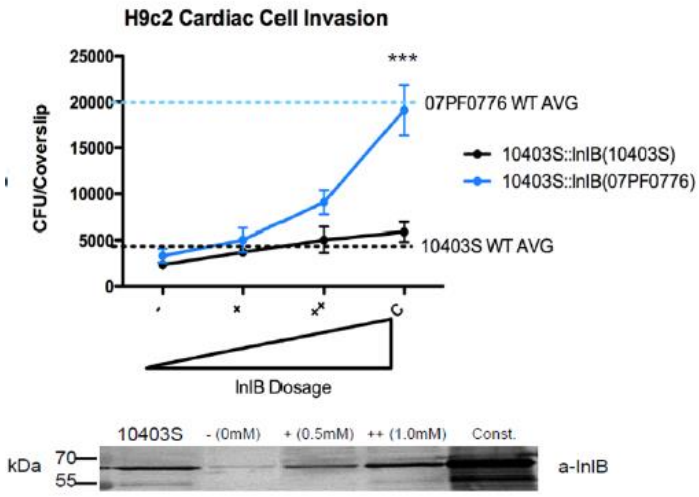


Figure 11 a and b: InlB promotes cardiac myocyte invasion in an allele-specific manner. Strains of 10403S lacking *inlAB* were complemented with alleles of each gene from either 10403S or 07PF0776. The genes were placed in either IPTG-inducible vectors induced at 0.0mM, 0.5mM, and 1.0mM IPTG, or in constitutively over-expressing vectors (C). Western blots indicate the representative expression level of the vectors compared to 10403S WT levels. InlA was found to have no effect, and actually reduced invasion regardless of allele used. Expression of InlB, however, increased bacterial invasion for both alleles tested. InlB derived from 07PF0776 enhanced invasion to a greater extent than that of 10403S, and actually complemented the invasion of 10403S to the level of the 07PF0776 WT strain.

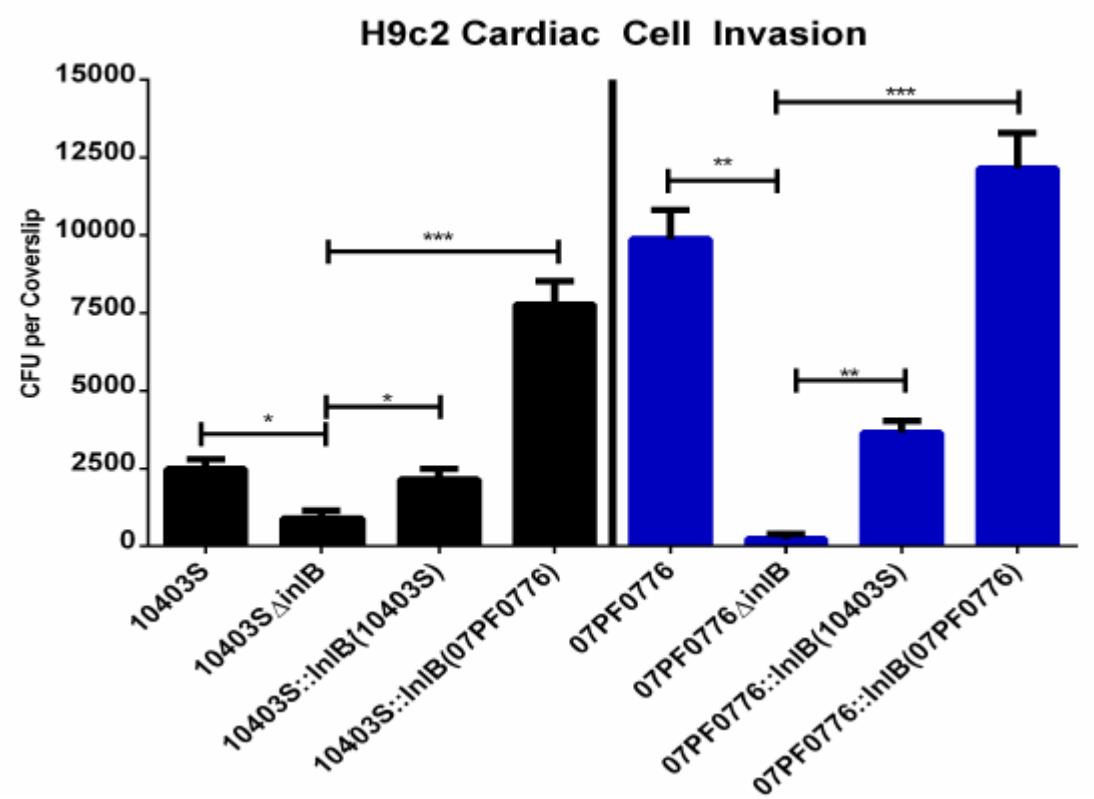


Figure 12: Allele-specific patterns of InlB-mediated invasion are consistent across both bacterial backgrounds. A comparison of the ability of each *inlB* allele to mediate invasion in H9c2 cardiac myocytes in both the 10403S and 07PF0776 backgrounds. Strains of 10403S lacking *inlAB* were complemented with either its native *inlB*, or with the *inlB* of 07PF0776 as previously described. Complementation was also done in a strain of 07PF0776 lacking *inlB*, and also demonstrated an allele-specific enhancement. Data shown in this graph was obtained using constitutively expressing vectors, and demonstrates that the *inlB* of 07PF0776 is enhanced for cardiac cell invasion regardless of genetic background.

cardiomyocytes in cell culture regardless of genetic background tested.

InlB from 07PF0776 Enhances Phosphorylation of c-Met in Myocytes in comparison to InlB from 10403S

InlB is known to signal through the PI3K and Akt pathways through binding to the host surface receptor c-Met [39], and thus behaves as a growth factor for mammalian cells [43]. Binding of c-Met by InlB is followed by clustering of the c-Met receptors and subsequent autophosphorylation of cytoplasmic tyrosines [117]. C-Met itself is present in cardiac myocytes, and has demonstrated roles in mediating responses to injury [118], avoidance of apoptosis [119] and protection against physiologic oxidative stress [120].

To test whether the 07PF0776 InlB protein was enhanced for its ability to stimulate phosphorylation of the c-Met receptor in cardiac myocytes, we examined the levels of phosphorylated c-Met (specifically, phosphorylation of tyrosine 1349 on the C-terminus of c-Met) using a commercially available antibody (Santa Cruz). Initially, we compared the amount of phosphorylated Y1349 (p-Y1349) present at 45 minutes, since the initial gentamicin protection assays were performed with a 45-minute incubation before adding gentamicin. When compared to mock infected cells, the amount of p-Y1349 was increased for all strains expressing *inlB*, however the 07PF0776 strain and strains containing the 07PF0776 allele of *inlB* demonstrated enhanced staining for p-Y1349 at this initial time point (Fig. 13-14). The investigation of earlier time points post-infection demonstrated a consistent elevation at 20, 40, and 60 minutes following bacterial exposure to 07PF0776 and its allele of *inlB*. Additionally, after 60 minutes of exposure to bacteria, myocytes treated with either 07PF0776 or its allele of *inlB* expressed in 10403S began to demonstrate morphologies indicative of cell scattering, a

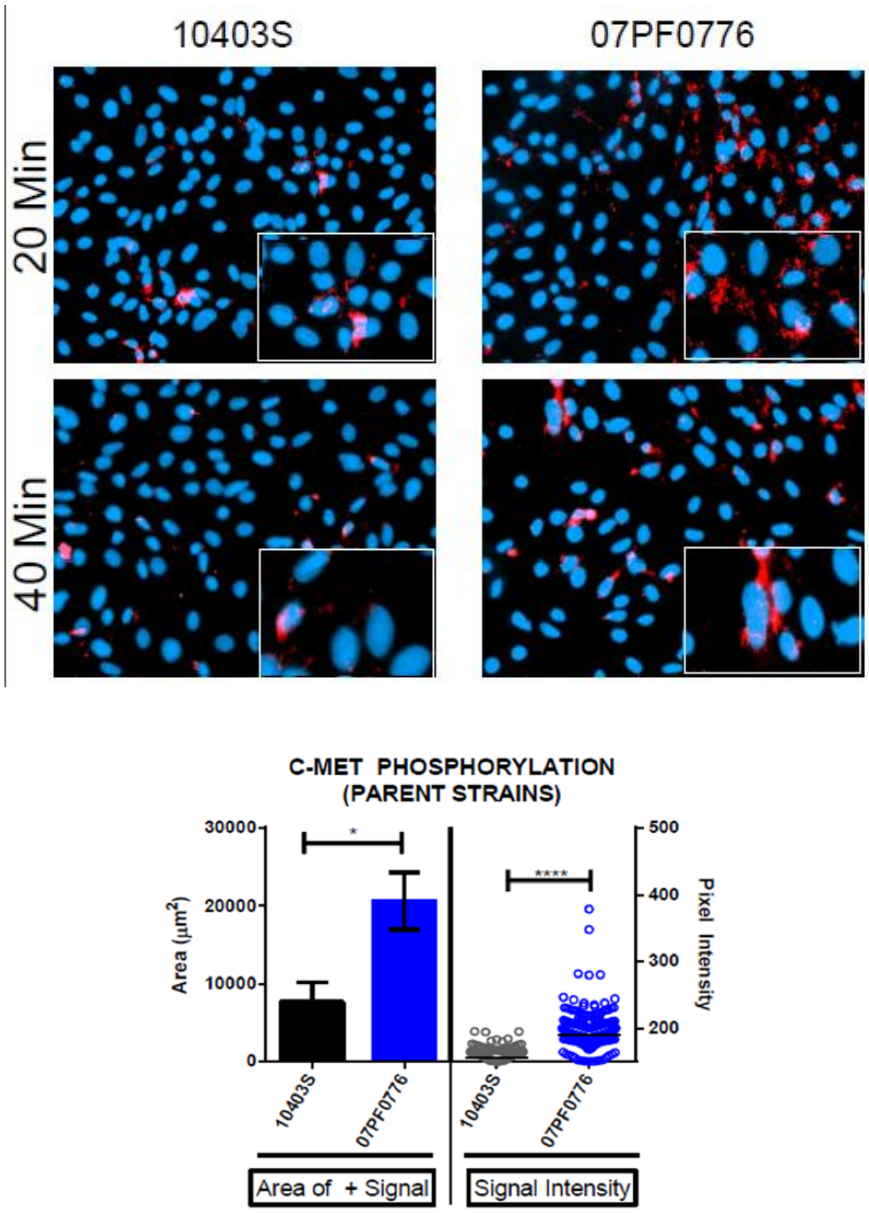


Figure 13: 07PF0776 stimulates Met phosphorylation to a higher degree than 10403S in myocytes. WT strains of 10403S and 07PF0776 were assessed by IF for their ability to phosphorylate c-Met at tyrosine 1349, a demonstration of InlB activity [52]. H9c2 cells were seeded into 24-well plates containing a glass coverslip in each well and allowed to adhere overnight. Infections were allowed to progress as described previously, with coverslips taken at 10, 20, 40, 60, and 120 minutes post-inoculation. Shown in panel (a) are representative images from 20 and 40 minutes post-inoculation. In panel (b), the total area of positive fluorescence and individual pixel intensities for each area of positive staining are graphed for the 60 minute time-point. Even though representative data is used in this figure, levels of phosphorylated c-Met were found to be elevated for 07PF0776 regardless of the time-point tested. Experiments were performed in quadruplicate and repeated three times.

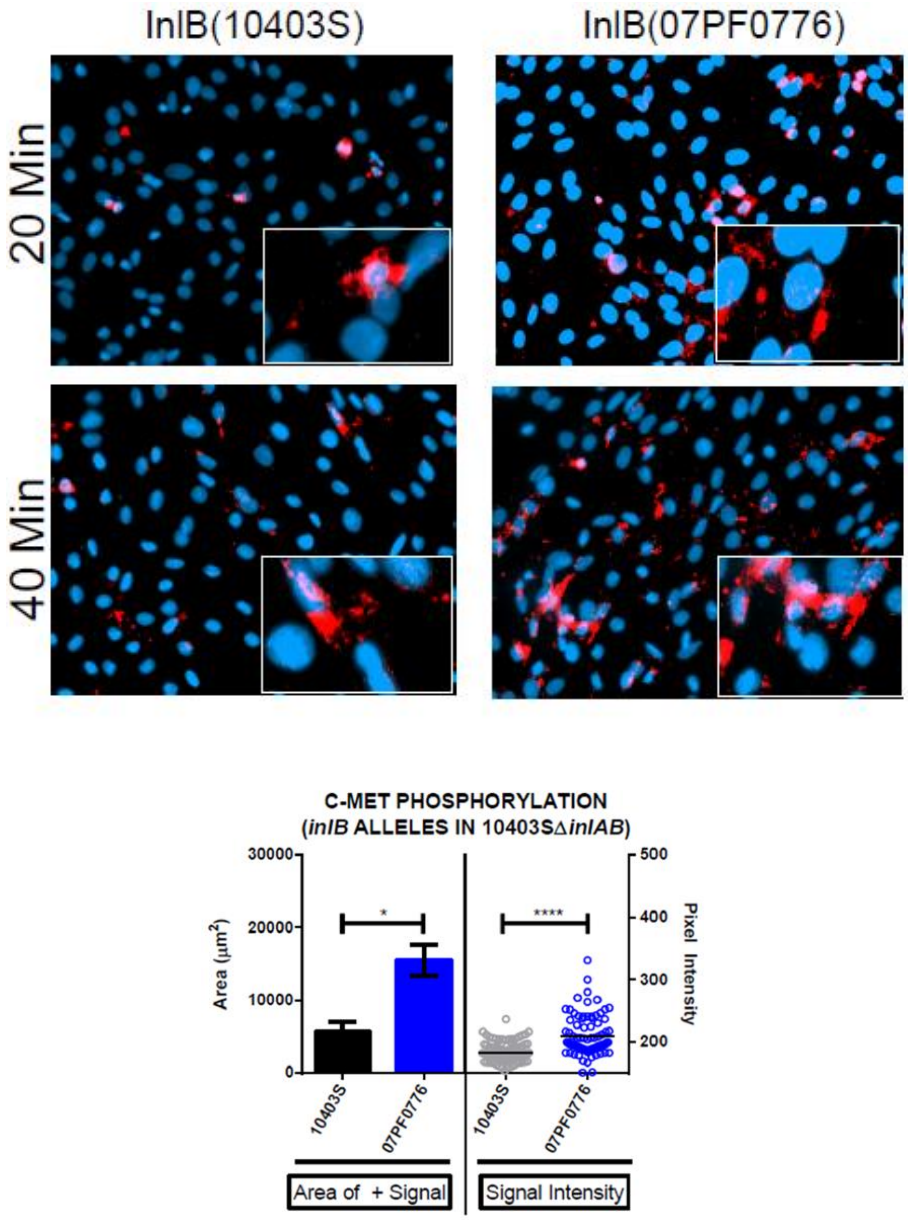


Figure 14: InlB from 07PF0776 more efficiently stimulates Met phosphorylation in myocytes. Strains of 10403S lacking *inlAB* were complemented with either *inlB* of 10403S or 07PF0776 and were assessed by IF for their ability to phosphorylate c-Met at tyrosine 1349, a demonstration of InlB activity [52]. H9c2 cells were seeded into 24-well plates containing a glass coverslip in each well and allowed to adhere overnight. Infections were allowed to progress as described previously, with coverslips taken at 10, 20, 40, 60, and 120 minutes post-inoculation. Shown in panel (a) are representative images from 20 and 40 minutes post-inoculation. In panel (b), the total area of positive fluorescence and individual pixel intensities for each area of positive staining are graphed for the 60 minute time-point. Levels of phosphorylated c-Met were found to be elevated for the *inlB* of 07PF0776 regardless of the time-point tested. Experiments were performed in quadruplicate and repeated three times.

known output of InlB stimulation across several cell types. These results confirm that the 07PF0776 allele of *inlB* possesses an enhanced ability to potentiate signaling from c-Met in cardiac myocytes.

The 07PF0776 InlB enhances cardiac colonization in infected animals

Given that *L. monocytogenes* strains expressing 07PF0776 *inlB* exhibited enhanced cardiac cell invasion in tissue culture, we assessed if *inlB* contributed to cardiotropism *in vivo*. Animals were intravenously infected with 10,000 CFU of *L. monocytogenes* 10403S or 07PF0776 expressing either 10403S or 07PF0776 *inlB.* No significant differences were observed between *L. monocytogenes* strains expressing either *inlB* allele in either the 10403S or 07PF0776 backgrounds in terms of bacterial colonization of the liver and spleen (Fig. 15). However, strains expressing the 07PF0776 allele of *inlB* demonstrated increased cardiac colonization in infected animals (Fig. 15). Importantly, the *inlB* of 07PF0776 was capable of enhancing cardiotropism in infected animals in both the 10403S and 07PF0776 backgrounds (Fig. 16).

Strains expressing the 10403S *inlB* allele were less likely to colonize the heart, and thus animals infected with these strains were less likely to exhibit abscess formation or overt cardiac pathology. Conversely, mice infected with strains expressing the *inlB* from 07PF0776 had increased likelihood of cardiac colonization, and often demonstrated overt cardiac pathology (Fig. 17, 18). Pathologic manifestations of *Listeria* colonization in the heart were most often seen as discrete microabscesses in the myocardium (Fig. 17), and demonstrated immune cell recruitment as well as fulminant tissue destruction in some cases (Fig. 18). Five days after initial infection, collagen deposition in the heart could be visualized for most animals infected

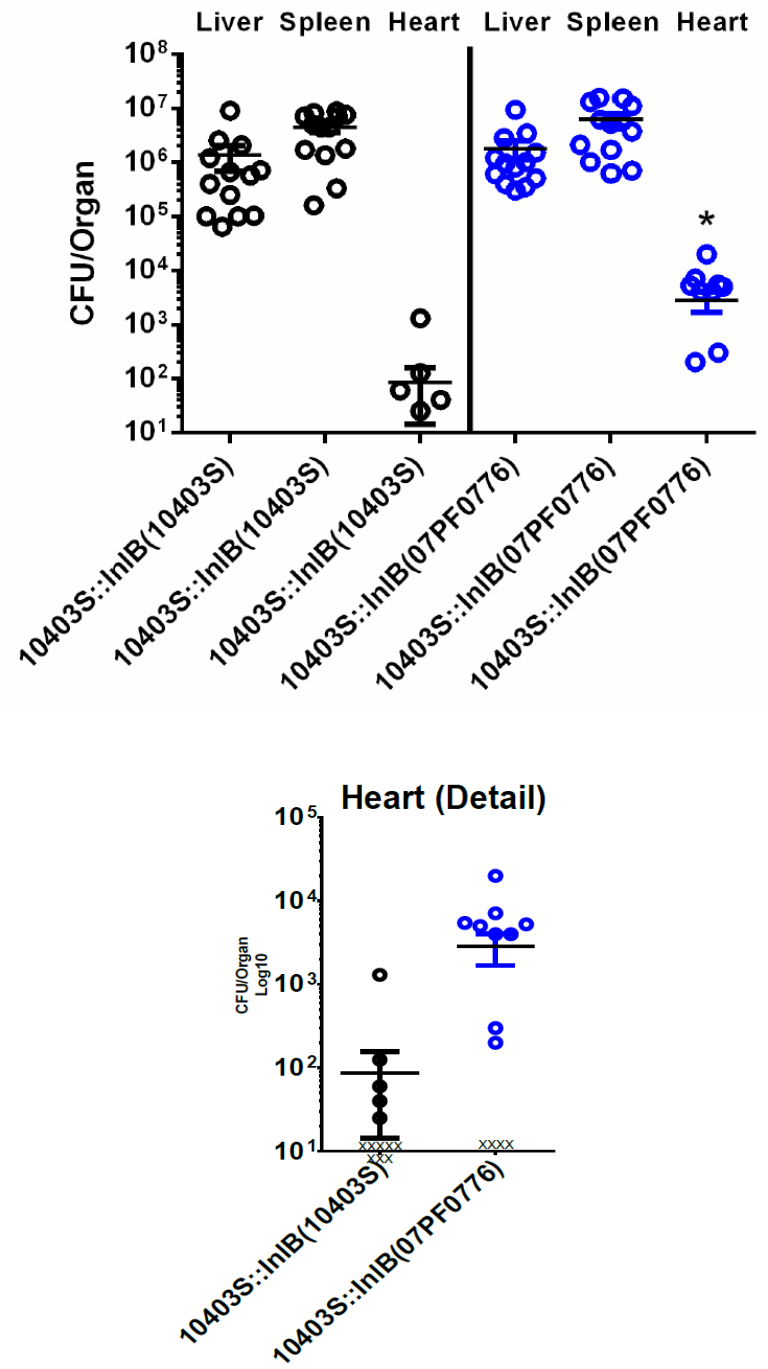


Figure 15: InlB from 07PF0776 enhances cardiac colonization in the 10403S background. Strains of 10403S lacking *inlAB* were complemented with either allele of *inlB* and subsequently used to infect mice via the tail vein at a dose of 10,000 CFU. Infections progressed for 72 hours before the liver, spleen, and hearts of the infected animals were collected and homogenized in 5mL ddH20. Colonization of the liver and spleen was comparable in these strains, however those strains expressing *inlB* of 07PF0776 were enhanced for cardiac colonization in this model.

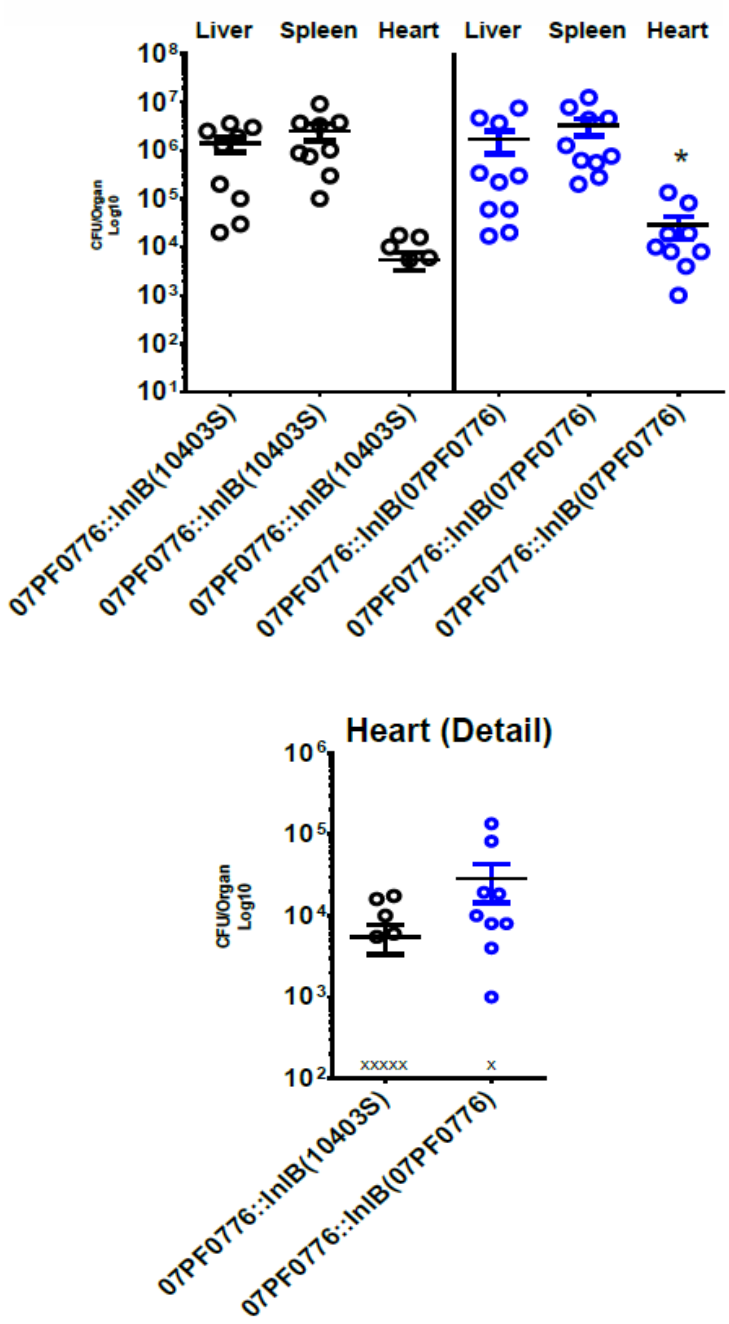


Figure 16: InlB from 07PF0776 promotes cardiotropism in the 07PF0776 genetic background. Strains of 07PF0776 lacking *inlB* were complemented with either allele of *inlB* and subsequently used to infect mice via the tail vein at a dose of 10,000 CFU. Infections progressed for 72 hours before the liver, spleen, and hearts of the infected animals were collected and homogenized in 5mL ddH20. Colonization of the liver and spleen was comparable in these strains, however those strains expressing *inlB* of 07PF0776 were enhanced for cardiac colonization in this model. This enhancement was less so than what was observed in the 10430S background, potentially indicating other strain-specific features in 07PF0776 that are capable of enhancing cardiac colonization

with the 07PF0776 allele, but very few indicators of scarring or fibrosis were observed in the hearts of mice infected with strains expressing the 10403S-derived *inlB* (Fig. 17). Thus, with increased frequency of bacterial colonization of the heart, there was a corresponding increase in the frequency of cardiac toxicity and myocardial disruption.

Mice infected with *L. monocytogenes* strains expressing 07PF0776 *inlB* allele thus experience an increased frequency of cardiac colonization, resulting in morphologic changes in the myocardium. Colonization of the liver and spleen, however, was comparable between the two alleles, and thus pathology in these organs exhibited less discrepancy.

InlB derived from cardiotropic strains contains conserved variations in the beta-repeat region as well as reduced positive surface charge in the GW repeats.

InlB is comprised of multiple domains, each with a functional contribution to signaling events necessary for bacterial invasion (Fig. 19). The N-terminal portion is composed of several leucine-rich repeats (LRR), which bind to the Ig1 domain of the host growth factor receptor c-Met. Following the LRR, there are two other repeat regions, the LRR-adjacent Ig-fold region and the beta-repeat, which contribute to bacterial invasion by so far cryptic mechanisms [121]. The C-terminal domains are three GW repeat motifs, which mediate electrostatic binding to bacterial lipoteichoic acid, as well as heparan-sulfated proteoglycans on the host cell surface [122].

The tissue-specific pattern of enhanced colonization demonstrated by strains expressing the 07PF0776 allele of *inlB* suggested that specific amino acid variations unique to the 07PF0776

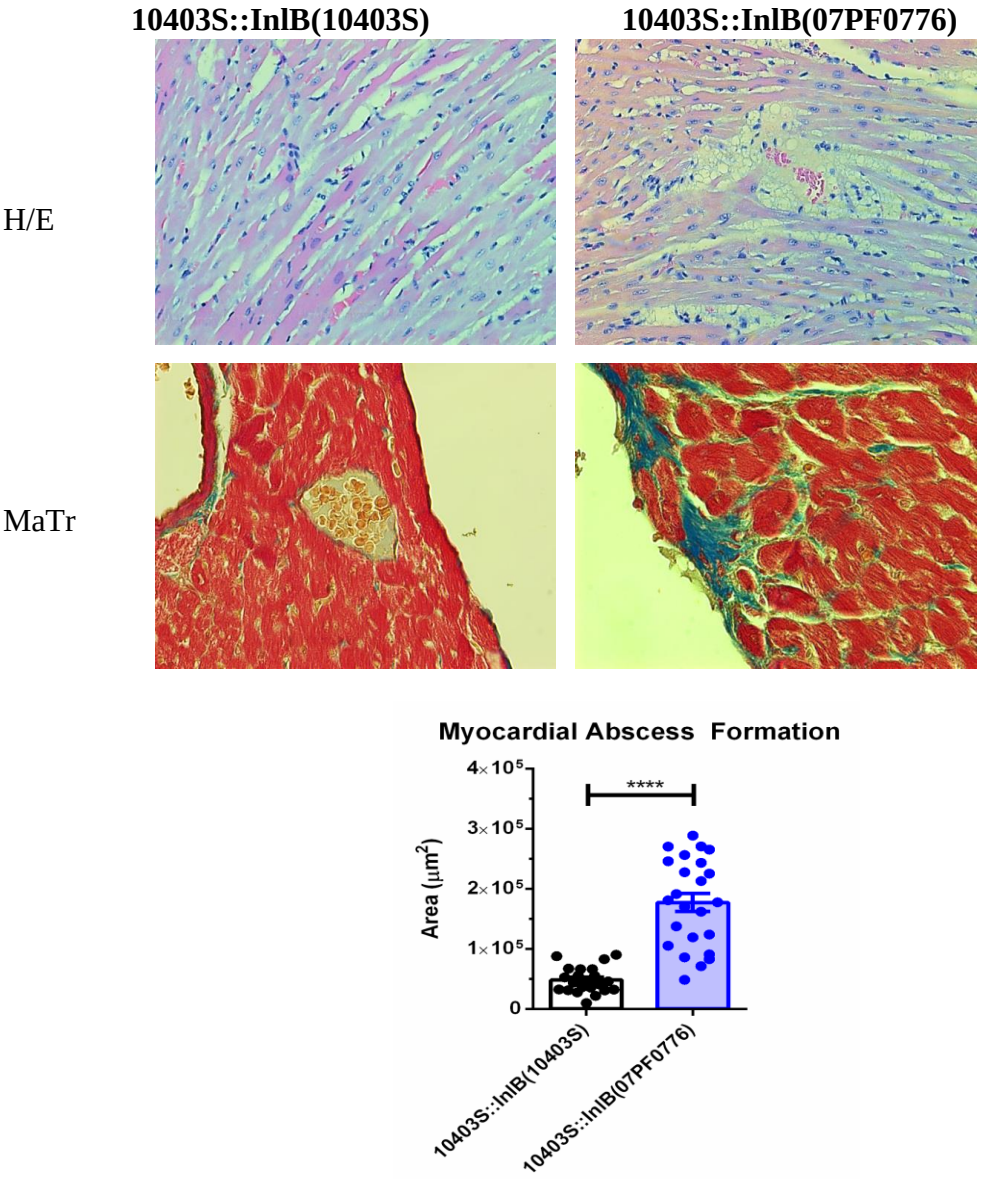


Figure 17: More pathology is noted in hearts infected with strains containing the 07PF0778 variant of InlB. 6-8 week old Swiss-Webster mice were infected with 10,000 CFU and infections were allowed to progress for 3 days (top panels) or 5 days (bottom panels). Hearts were removed and placed in 10 mLs of PBS, then moved to a 4% solution of formalin/PBS for three days. After fixation, hearts were sectioned and stained by the Histology Research Core at the University of Illinois at Chicago for H&E and Masson-Trichrome Staining (MaTr). Hearts of mice infected with strains expressing *inlB* from 07PF0776 were found to demosntrate greater tissue disruption, immune infiltrations, and collagen deposition (blue in MaTr sections). Additionally, abscess formation was measured through Zen and is diagramed in the lower graph. Abscesses rarely progressed to the point where they were grossly visible, thus only microabscess formation was assessed in this figure.

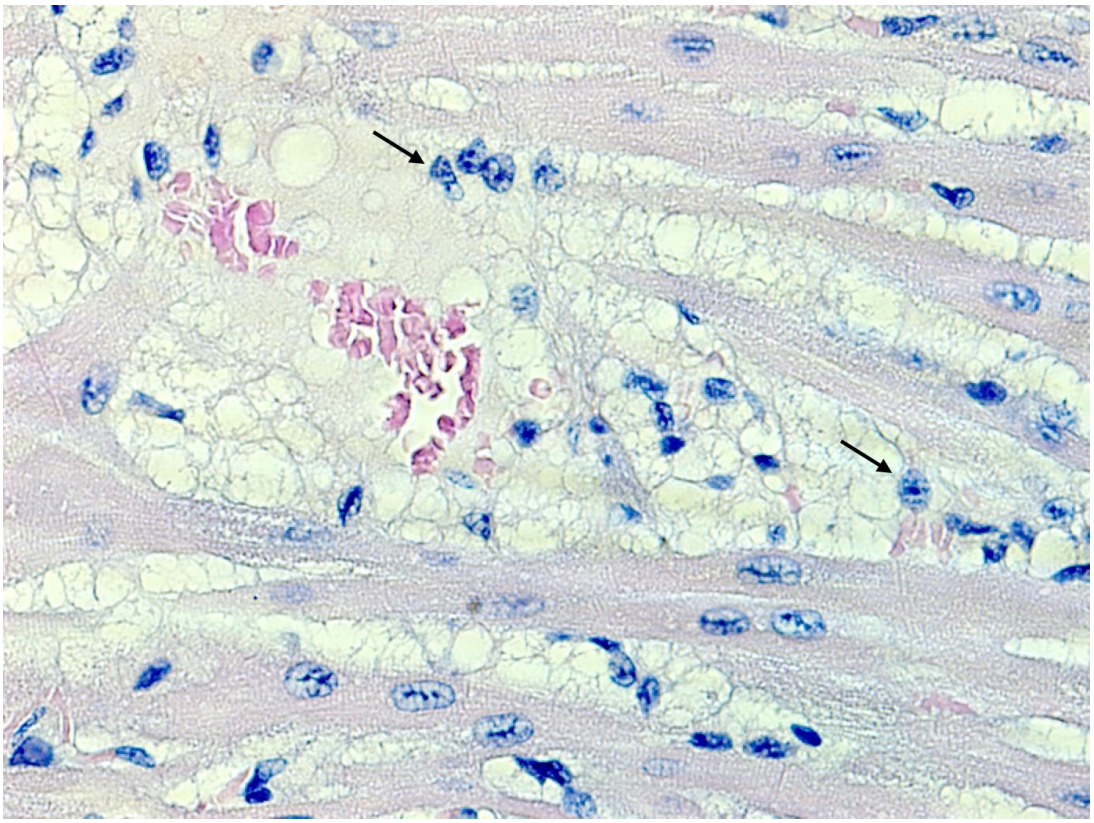


Figure 18: An enhanced image of a myocardial abscess, containing phagocytes. 6-8 week old Swiss-Webster mice were infected with 10,000 CFU and infections were allowed to progress for 3 days. Hearts were removed and placed in 10 mLs of PBS, then moved to a 4% solution of formalin/PBS for three days. After fixation, hearts were sectioned and stained by the Histology Research Core at the University of Illinois at Chicago for H&E staining. 40x Image of a heart taken from an animal infected with 10403S expressing *inlB* from 07PF0776, stained with H&E. In some areas, tissue disruption and myocytolysis (disrupted or dying myocytes) were observed. Additionally, many phagocytes in the area appear to contain intracellular granulations indicative of phagocytosed organism (arrows).

inlB gene product were probably responsible for mediating increased cardiotropism. To elucidate which amino acid variations contributed to enhanced cardiac cell invasion, we aligned sequences of *inlB* isolated from a number of clinical strains assessed for cardiotropism in a previous study [20]. The overall number of polymorphisms across the sequenced alleles was 59, and they were distributed more-or-less evenly across the protein (Fig. 19). Interestingly, the strains previously found to be more cardiotropic in mice uniformly contained an aspartic acid in the beta-repeat at residue 371, whereas the non-cardiotropic isolates contained an asparagine. Previous alignments of this region using in excess of 300 isolates determined that this region is very poorly conserved among strains of *L. monocytogenes* [121], so the conservation of a residue in this region was of particular interest. Other polymorphisms were found to be very close in proximity to D371 among the isolates, including S373, however only D371 was conserved among those strains most capable of colonizing the hearts of infected mice, thus S373 was not pursued further.

In addition to the D371 variation, several polymorphisms were detected among the cardiotropic isolates in the C-terminal region of the InlB protein (Fig. 19). As mentioned previously, the InlB C terminus has been shown previously to mediate interactions with host-surface heparan sulfated proteoglycans [43]. The binding of heparans to InlB has been mapped to the GW repeat regions, extending from amino acid 391 to the end of the protein [117]. The GW repeat regions have been demonstrated to play a crucial role in potentiating the response produced by binding of the LRR of InlB to the IG-1 domain of c-Met [123]. Taken together, these genetic predictions suggest two regions of alterations that could potentially enhance the ability of 07PF0776 InlB to interact with cardiac cells. In order to test the possible contribution of the changes in the beta repeat and the GW repeat region to cardiac myocyte invasion, we

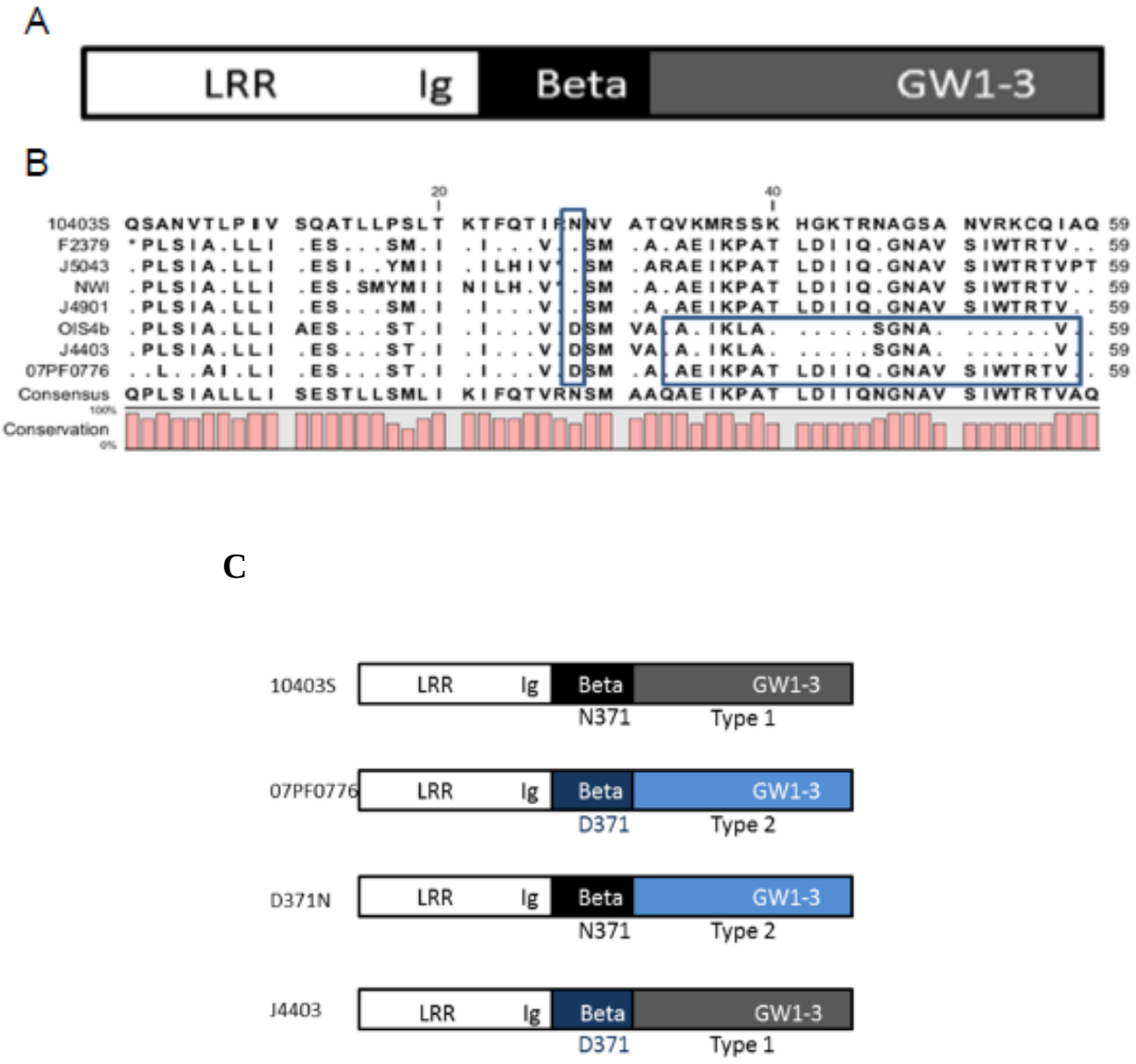


Figure 19: Cardiotropic strains shared homology in the beta-repeat and divergence in the GW repeats. Genetic polymorphisms present within the *inlB* locus of multiple isolates were compared using CLC genomics workbench reader, and revealed the presence of a conserved aspartic acid in the beta-repeat domain (N371D), as well as variance in the GW repeats. In order to test these polymorphisms, mutagenesis of the 07PF0776 allele of *inlB* was performed to revert D371 back to an asparagine. Additionally, to investigate alterations in the GW repeats, a fourth allele (J4403) was cloned and expressed in 10403S lacking *inlAB* as described previously. These four alleles would allow for determining the significance of polymorphisms within the beta and GW repeat regions in terms of cardiac cell invasion and cardiotropism.

began by using site-directed mutagenesis to convert D371 to an asparagine (as was present in non-cardiotropic isolates) within the beta repeat region, thereby generating an allele encoding an InlB protein with the preserved 07PF0776 architecture minus the variant aspartic acid (Fig 19).

For the GW domain, electrostatic modeling of the GW repeat structure (PDB: 1M9S) was used to assess the impact of the changes observed in the genetic alignment with respect to surface charge of the protein (Fig. 20). The polymorphisms were found to alter the surface charge of the GW repeat region, resulting in a less-positively charged molecule (Fig 20). Additionally, several surface polymorphisms appeared to directly alter structural components of the region, leading to a reduction in basic residues protruding from the surface (e.g. K501T). Globally, these changes appeared substantial enough to potentially alter binding interactions between InlB and heparan sulfate. To test the role of these polymorphisms detected in the GW repeats, we cloned and assessed invasion for a fourth allele of *inlB,* designated J4403 (Fig. 19). This allele possesses the D371 residue in the beta-repeat, but lacks a number of the polymorphisms present in the 07PF0776 GW-repeats and thus has GW repeats that more closely resemble those of 10403S.

InlB D371N substitution reduces myocyte invasion and cardiac colonization in mice.

Interestingly, it was observed following the introduction of the D371N mutation that the degradation products of the 07PF0776 InlB, as observed by western blot, were altered to resemble a more 10403S-like pattern (Fig 21). This change in susceptibility to degradation could reflect altered protein stability on the bacterial surface resulting from increased

GW Repeat region of 10403S InlB **GW Repeat region of 07PF0776 InlB**

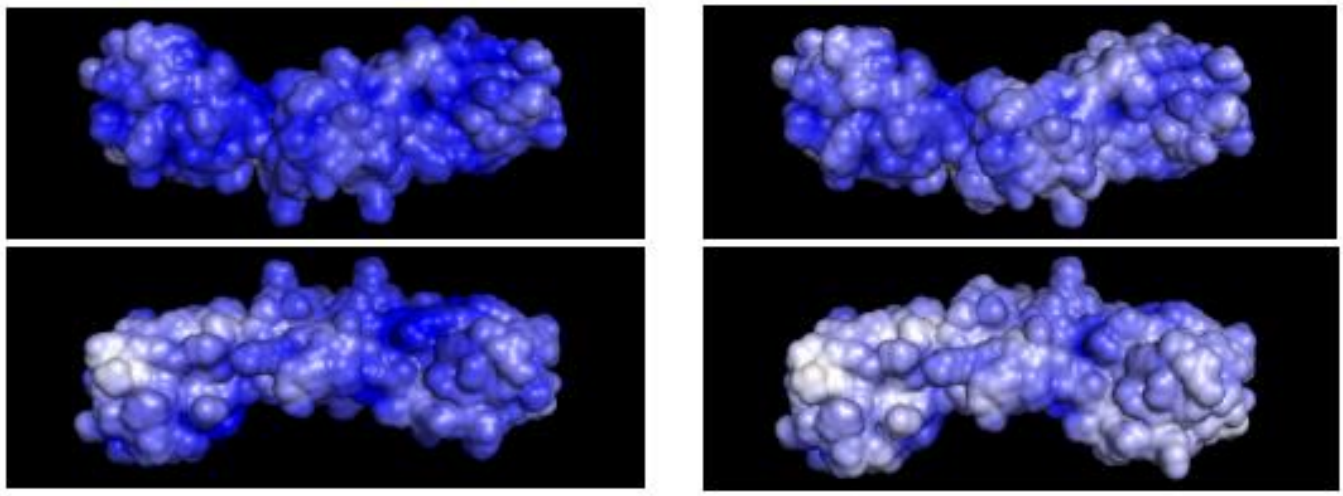


Figure 20: The GW repeat regions are substantially less positively charged in 07PF0776, compared to 10403S. Electrostatic modeling of the GW repeat regions of both 10403S and 07PF0776 were carried out to assess the functional impact of mutations present in the 07PF0776 variant of InlB using PyMol software in conjunction with APBS. Models were retrieved from the protein database (PDB: 1M9S) and mutagenesis of the residues was done in PyMol using the mutagenesis wizard feature. Shown is the solvent-accessible surface charge for both variants of InlB, demonstrating the reduced presence of positive charge and a loss of protruding basic residues from the surface for the 07PF0776 variant. The molecule, however, still retains a great amount of positive charge, indicating it may still function to sense heparan sulfate. (Kev = -5 to +5 was used to generate the surface potential plots shown in this figure.)

susceptibility to local proteases. Indeed, InlB has been shown previously to be susceptible to proteolytic degradation near the beta-repeat [122]. It is possible that a reduction in InlB susceptibility to proteolysis could be a contributory factor to myocyte invasion, as it is anticipated that a more stable form of InlB would better potentiate interactions with host cell receptors.

In tissue culture invasion assays, the InlB D371N mutation in 07PF0776 InlB reduced myocyte invasion to levels observed for 10403S strains (Fig. 21). Additionally, strains expressing InlB D371N were no longer associated with changes in cellular morphology associated with bacterial invasion, including cell-rounding and scattering, as was observed for strains expressing the 07PF0776 InlB (Fig. 21), and InlB D371N strains were additionally reduced for levels of p-Y1349 (Fig. 21). In infected mice, strains expressing InlB D371N behaved similar to 10403S InlB strains with respect to bacterial burdens present in liver and spleen, and were decreased in cardiac colonization when compared to strains expressing 07PF0776 InlB (Fig. 22).

To further assess the role of D371 in the enhancement of InlB-mediated cardiac cell invasion, an allele of *inlB* derived from another cardiotropic *L. monoytogenes* strain, J4403, was investigated. As mentioned previously, the J4403 *inlB* encodes the same beta-repeat mutations found in the 07PF0776 allele, but lacks the additional GW repeat polymorphisms as revealed by the comparative alignment (Fig. 19). Strains expressing J4403 InlB exhibited patterns of invasion in cardiomyocyte cell lines similar to those observed for strains expressing the 10403S InlB, and both strains were substantially less invasive than strains expressing 07PF0776 InlB (Fig. 21). Taken together, these results indicate that efficient cardiomyocyte invasion is dependent on changes present in both the beta and GW repeat regions. These changes appear to

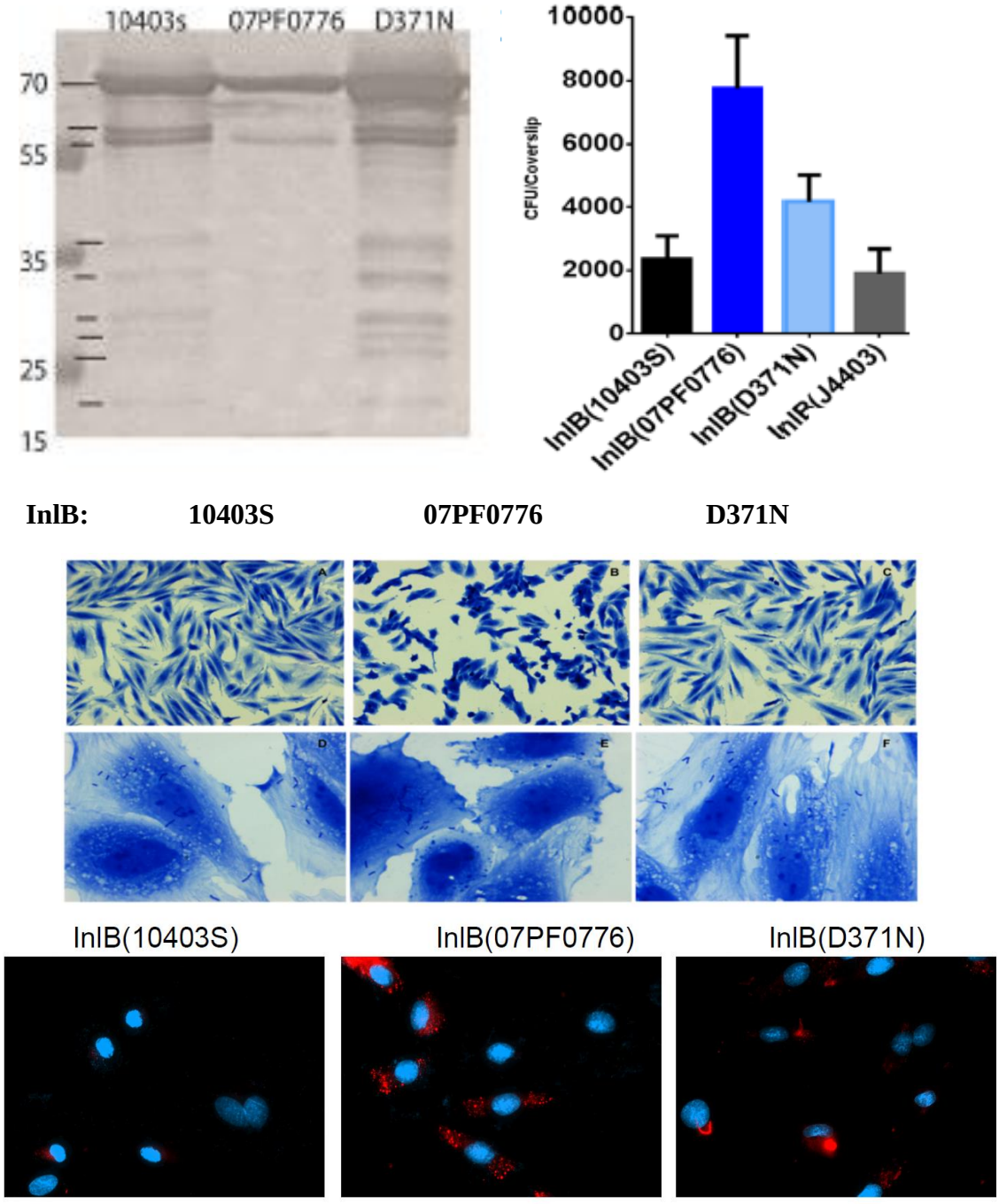


Figure 21: D371 is required for a number of cardiac-specific phenotypes. Mutagenesis of D371 in the 07PF0776 variant of InlB reverts the protein to a degradation phenotype much like 10403S. In cardiac myocytes, gentamicin protection assays demonstrated a decrease in cardiac myocyte invasion, making the D371N variant intermediate to 10430S and 07PF0776. The J4403 variant, which possesses D371 but lacks many of the polymorphisms in the GW repeat, was not enhanced for myocyte invasion, indicating both D371 and GW polymorphisms are responsible for the effects seen in the 07PF0776 variant. D371N also was reduced in both cell-scattering and p-Y1349 formation compared to InlB 07PF0776.

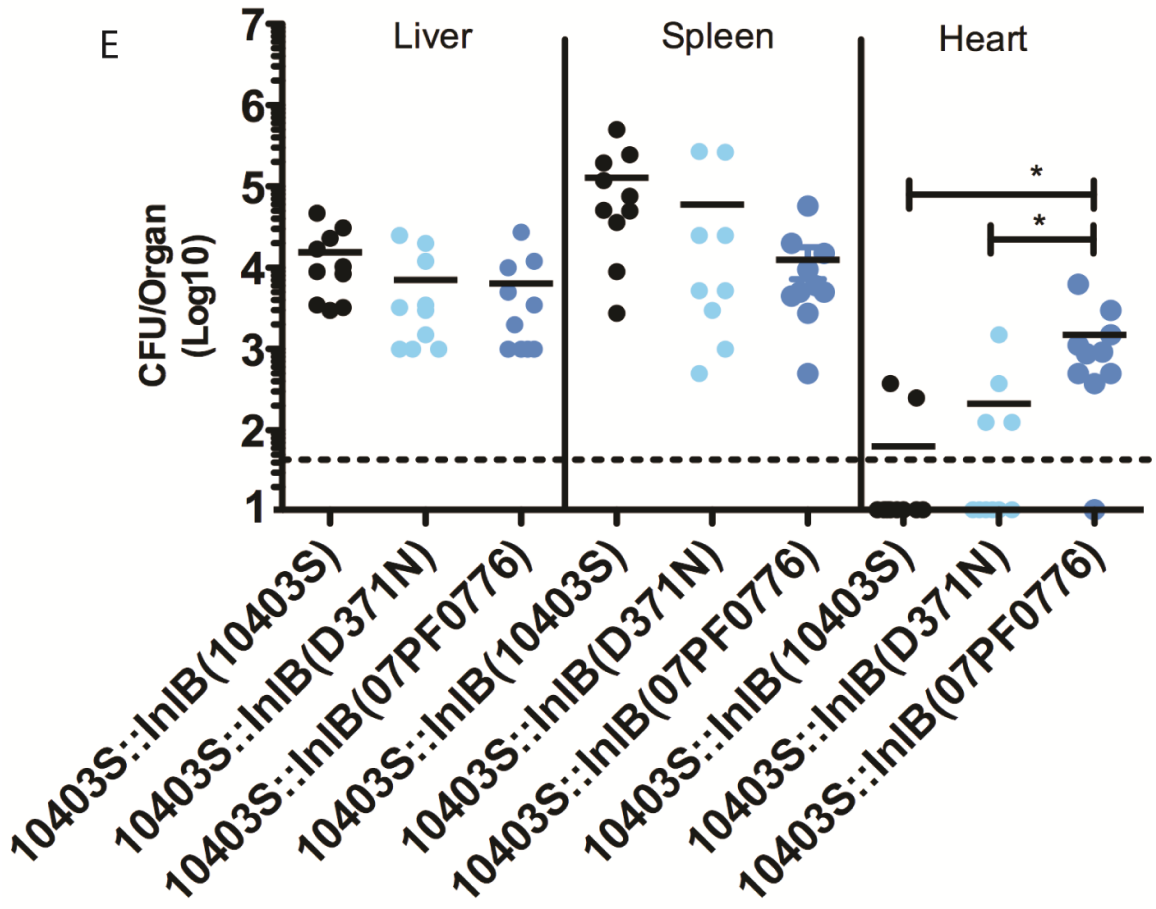


Figure 22: D371 is required for efficient colonization in mice. Female Swiss-Webster mice aged 6-8 weeks were infected with 2,000 CFU via the tail vein, as described previously. Infections were allowed to progress for 72 hours before the animals were sacrificed, and the liver, spleen, and heart homogenized. Bacterial CFU was enumerated by diluting the homogenate and spot plating on LB agar containing kanamycin.

alter both protein stability and surface charge in the heparan-binding regions.

As mentioned previously, InlB has been suggested to interact with a number of potential receptors on the host surface apart from c-Met and HSPGs, including the hyaluronan receptor CD44v6. CD44v6 has also been demonstrated to play a role in the processing of ligand-bound Met [124], and is also highly expressed in cardiac myocytes [125]. Previous reports in the literature are conflicting as to the precise role of CD44v6 in InlB-mediated signaling [124, 126], however it is known that pre-incubating cells with inhibitory peptide specific for blocking the colocalization of CD44v6 with Met can reduce the activity of CD44v6 in the context of Met pre-treated coverslips of cardiac myocytes with inhibitory peptide (KEQWFGNRWHEGYR) [126] at a minimum concentration of 200 ng/mL. Neither the parent strains, nor their respective alleles of *inlB*, responded to inhibition of CD44v6, indicating that participation of this receptor is not responsible for increasing bacterial uptake into cardiac myocytes (Fig. 23).

InlB variants exhibit different binding sensitivities to heparan sulfate.

The altered surface potential present in the GW repeats could alter interactions with cellular HSPGs, and thus have an effect on InlB-dependent Met-phosphorylation efficiency. Binding to heparan can be assessed by inhibition studies, in which bacteria are pre-treated with various concentrations of heparan sulfate and then subsequently allowed to infect cells in tissue culture [43, 123]. When *L.* monocytogenes strains expressing different forms of InlB were pre-incubated with inhibitory concentrations of heparan sulfate, it was observed that strains expressing the cardiotropic 07PF0776 InlB were more sensitive to invasion inhibition, with levels of invasion reduced by approximately 70% after only five minutes of heparan treatment

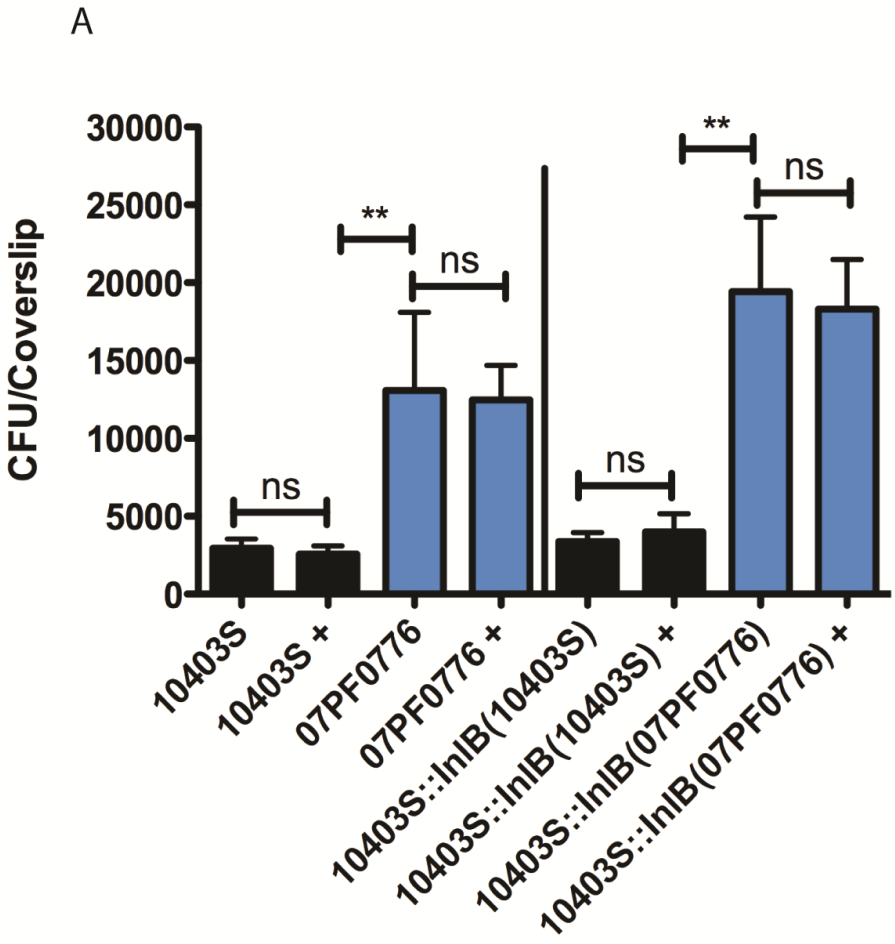


Figure 23: The role of CD44v6 in bacterial invasion in myocytes was assessed by using an inhibitory peptide [124] at a concentration of 200ng/mL. H9c2 cells were seeded into 24-well plates containing glass coverslips. 30 minutes prior to assay, the inhibitory peptide was delivered to treated wells, while non-treated wells received PBS. Cells were then infected at an MOI=100 for one hour before the addition of gentamicin. After one hour of incubation in media containing gentamicin, coverslips were removed and vortexed in 1mL ddH20. Bacterial CFU were enumerated by spot plating on LB agar.

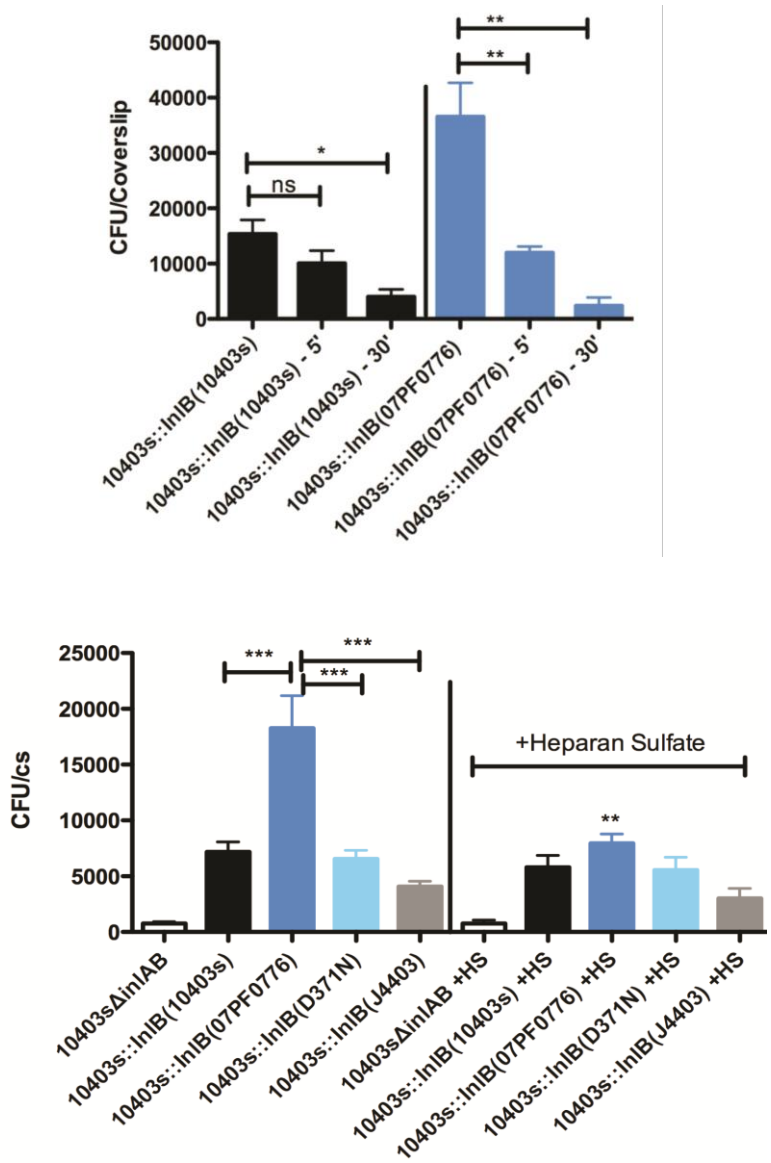


Figure 24: The effect of heparan sulfate pre-treatment on alleles of *inlB*. (a) Strains of 10403S expressing either 10403S or 07PF0776 *inlB* were pretreated with an inhibitory concentration of heparan sulfate (50ug/mL) suspended in PBS for the indicated time points. After 30 minutes, significant reduction was observed for both alleles, however the amount of reduction after 5 minutes was only significantly different for the 07PF0776 variant. (b) A sub-inhibitory concentration of heparan for the 10403S allele was used to assess heparan sensitivity across the other alleles of *inlB* assessed in this study. At a concentration of 25ug/mL for 20 minutes of treatment, heparan sulfate had no effect on any allele apart from the 07PF0776 variant. Assays were performed in H9c2 cells in 24-well plates as previously described. Both experiments are representative of studies done in triplicate.

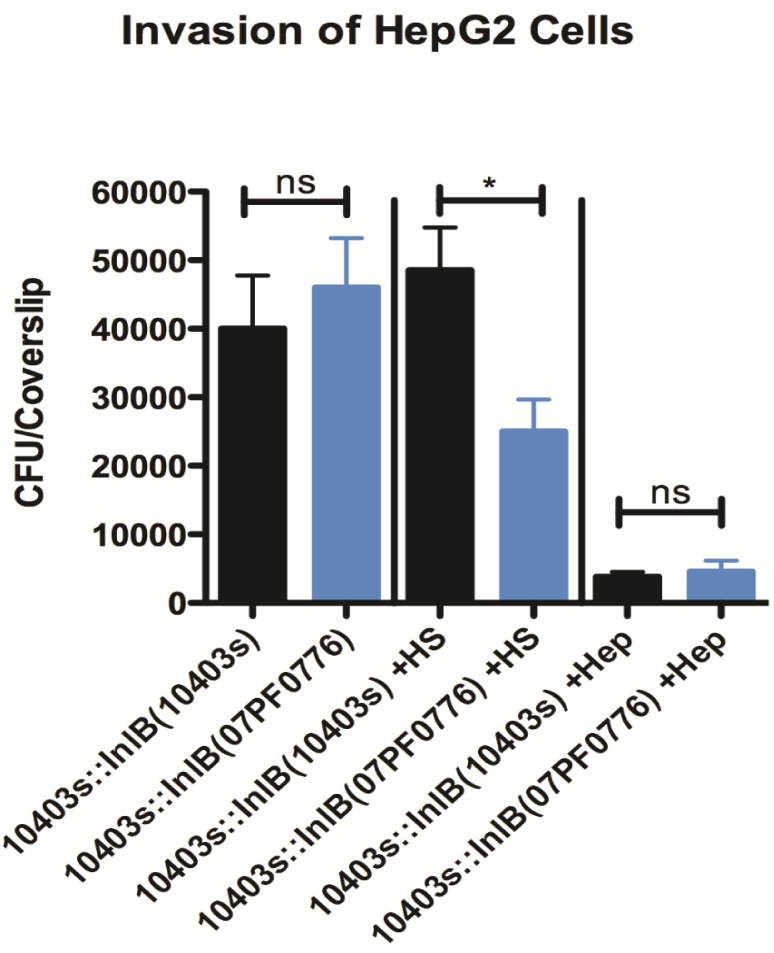


Figure 25: Allele-specific responses to heparan sulfate are observed in HepG2 cells, representative of liver hepatocytes. Cultures of 10403S expressing either *inlB* of 10403S or 07PF0776 were pre-treated for 20 minutes with either PBS, Heparan Sulfate (HS), or Heparin (Hep). Cells were then infected at an MOI=100 and extracellular bacteria were killed by treatment with gentamicin. Both the 10403S and 07PF0776 alleles perform similarly in HepG2 cells without treatment, and both alleles of *inlB* respond to heparin (which has been shown to be more inhibitory than heparan sulfate in previous studies [127]). However, only the 07PF0776 allele of *inlB* is reduced with pre-treatments of heparan sulfate. This figure represents the total average of three independent experiments.

and nearly 100% after thirty minutes (Fig. 24). In comparison, 10403S strains expressing native InlB exhibited modest levels of inhibition with five minutes of heparin sulfate pre-treatment, with 70% inhibition after thirty minutes of treatment.

To further test the sensitivity of both 10403S and 07PF0776 InlB to heparan sulfate, a subinhibitory concentration of heparan sulfate was used. Using this lower concentration, it was found that levels of heparan sulfate well-below the inhibitory level for strains expressing 10403S InlB were still capable of inhibiting invasion for strains expressing 07PF0776 InlB (Fig. 24). In HepG2 liver cells, this sub-inhibitory treatment of heparan sulfate was also observed to reduce invasion only for strains expressing 07PF0776 InlB (Fig. 25), demonstrating enhanced sensitivity in a cell line more commonly associated with InlB-mediated invasion. Irrespective of cell type or allele of *inlB*, the addition of heparin had an overwhelmingly negative effect on InlB-mediated invasion. This result was expected given that InlB has been shown to bind heparin more readily than heparan sulfate [123].

Interestingly, those strains expressing InlB D371N failed to respond to heparan sulfate at the sub-inhibitory concentration, possibly indicating D371 is necessary to stabilize the GW architecture for heparan binding, or that the beta-repeat is functioning as a novel heparan-sensing moiety. The latter option appears less likely, as the J4403 allele of *inlB* was neither hyperinvasive or responsive to heparan sulfate below inhibitory concentrations (Fig. 3.19b). Taken together, these results suggest that the cardiotropic 07PF0776 InlB protein is able to migrate more readily from the bacterial surface to the host cell surface when exposed to concentrations of heparin sulfate that are much lower than those required to trigger the dissociation of 10403S InlB. The differences in 07PF0776 InlB migration and stability appear to contribute to the enhanced stimulation of the c-Met receptor and the subsequent uptake of *L.*

monocytogenes into cardiac cells.

3.4 - Discussion

The outcome of microbial infection can depend upon the ability of a pathogen to establish a replication niche within the host, and a number of microbes are commonly associated with disease affecting specific body sites or tissues. Invasive infections caused by *L. monocytogenes* have primarily been associated with the central nervous system and with placental/fetal infections, although disease pathology in other tissues has been reported. In this study, we have identified amino acid variations within the surface protein InlB that enhance the ability of *L. monocytogenes* to cause cardiac infection. Alterations to both the beta and GW repeat regions of InlB were sufficient for enhancing the uptake of *L. monocytogenes* into cardiac myocytes. These two functional regions appear to be co-dependent as the presence of both the D371 residue in the beta repeat and the GW motif polymorphisms that reduced surface charge were necessary to enhance cardiac myocyte invasion. These subpopulations of *L. monocytogenes* were therefore more likely to colonize and replicate within the hearts of infected mice through amino acid variations in its standing arsenal of virulence factors rather than through the acquisition of a novel virulence factor via, for example, horizontal gene transfer.

What properties of InlB contribute to *L. monocytogenes* uptake into different cell types and tissues? The first function associated with the activity of InlB was bacterial tropism for the liver, which was observed by mutational analyses of *inlB* in animal models and *in vitro* invasion assays of hepatocyte-like cells [43]. Further studies demonstrated that InlB exists in a state of loose-association between binding to lipoteichoic acids or to heparan sulfate, such that it is capable of moving from the bacterial cell surface to associate with host cell surfaces [117, 123]. This

transition appears to be electrostatically mediated; positively-charged InlB is pulled from the weakly-negative lipoteichoic acid by the relatively strong-negative charge of heparan sulfate on the surface of host cells. The length and structure of the heparan chains also play a crucial role in interactions with InlB [127]. The transition of InlB from bacterial-bound to host-bound state subsequently allows for diffusion of the molecule in two-dimensions along the host surface surrounding the bacterium to lead to the binding to c-Met [117]. Upon c-Met binding, InlB forms dimers with a second InlB molecule bound to another c-Met receptor through an interface along the exposed surface of the InlB leucine-rich repeats. The presence of heparan binding regions in the GW repeats directly enhances the amount of c-Met phosphorylation, thus increasing the efficiency of bacterial uptake [122].

Previous studies into the tissue-specific structures of heparan sulfate indicate that heparan sulfate derived from liver is the most highly sulfated (sulfation ratio = 0.95), most heparin-like moiety in animals [128, 129]. Given the affinity of all InlB variants examined in this study for heparin, this suggests that liver heparan – with its highly sulfated, heparin-like moieties – is highly suited to dissociate all of the variant forms of InlB from the bacterial surface [128, 130]. This affinity for heparin, along with the abundance of c-Met on the surface of hepatocytes, likely contributes to the critical role for InlB in mediating *L. monocytogenes* liver colonization. An *inlB* allele encoding a variant protein also capable of responding to poorly sulfated heparan sulfate, however, would more readily dissociate from the bacterial surface in response to heparan isoforms found in other tissues, such as the heart (sulfation ratio =0.65 [129]), in addition to dissociation triggered by the highly sulfated forms found in the liver. Additionally, the binding mechanism proposed for InlB to heparan is complex, with multiple binding and re-binding events that likely serve to migrate the molecule towards its ultimate destination [127].

Alterations to heparan binding, then, have the likelihood of altering both migration on both the bacterial and host surfaces. Myocytes, as mentioned previously, possess the c-Met receptor necessary for InlB-mediated invasion [131, 132], thus the apparent enhancement for *L. monocytogenes* cardiac invasion lies in the increased ability of the variant InlB molecule to dissociate from the bacterial surface and associate with alternate heparan moieties present on cardiac cells to promote invasion.

The pre-treatment of bacterial cultures with heparan sulfate at relatively high concentrations has been previously shown to have a negative effect on InlB-mediated invasion, as was confirmed by our experiments. This inhibition is due to binding of heparan sulfate by InlB, which prevents subsequent InlB association with host-surface-associated heparan sulfate and initiates premature release from the bacterial surface. Pretreatment with a poorly-sulfated form of heparan sulfate derived from bovine kidney, which has a sulfation ratio of ~0.60 [129] was incapable of inhibiting invasion for the 10403S InlB strains in cardiac myocytes and HepG2 hepatocellular carcinoma cells at low concentrations (Fig. 3.19b). This less-sulfated derivative at the same concentration was, however, capable of reducing the invasive potential of strains expressing the 07PF0776 *inlB* allele. With the addition of heparin (sulfation ratio > 2.0) to cultures under the same conditions, a drastic reduction in invasion was observed for strains expressing either *inlB* allele, demonstrating the increased affinity of InlB in general for a more negatively-charged heparan type (Fig. 3.20). Thus, while *L. monocytogenes* strains expressing either the 10403S-derived or the 07PF0776-derived InlB respond to highly sulfated heparin, only the 07PF0776 variant is consistently capable of responding to less sulfated forms of heparan sulfate present on the cell surface.

Our results strongly suggest a critical role for cardiac moieties of heparan sulfate in the

establishment of the heart as a new replication niche for *L. monocytogenes* 07PF0776. The ability of tissue-specific heparan sulfate to act as a modulator of cell signaling has been well documented. For example, the heparan sulfate binding growth factors FGF-2 and FGF-4 exhibit cell-specific functions based on the structure of heparan sulfate present on the cell surface [133]. The cell-specific activities of these two factors appear to be modulated by the binding affinity of each molecule for distinct forms of heparan sulfate, and interestingly these growth factors also show discrepancies in binding affinity in the liver and the heart, where one variant only binds in the liver and the other binds to both the liver and heart heparan moieties. Sulfation of heparan sulfate can also be regulated by extracellular sulfatases, an activity which has been shown to create tissue-specific differences in antithrombin recruitment [130].

In regards to traditional c-Met signaling, the natural ligand hepatic growth factor (HGF) also interacts with heparan sulfate. Mutants of HGF with reduced binding for proteoglycans actually demonstrate increased activity across a number of different tissues due to alterations in ligand trafficking and degradation [134]. InlB mimics the trafficking and interaction patterns of HGF, and our results appear to demonstrate that similar alterations to heparan binding regions of InlB alter specific activities of the molecule.

In addition to sensing and responding to heparan differently through altered proteoglycan affinity, the 07PF0776 allele also possesses a somewhat unique polymorphism in the beta repeat. Mutagenesis of this variant aspartic acid (D371) was accompanied by reductions in myocyte invasion, cardiotropism in animals, and heparan responsiveness *in vitro.* These results were found to coincide with a drastic change in protein stability as observed by western blot (Fig 23). This could indicate that D371 may be playing a structural role, protecting the mature protein from natural proteolytic degradation. Indeed, it has been previously reported that the beta-repeat,

in particular, is susceptible to proteolytic digestion [122], which results in uncoupling of the GW-repeats from the LRR domain. The ability of D371 to reduce degradation was a significant enhancement to cardiac cell invasion only when coupled with alterations in the GW motifs that reduced the total positive charge on the surface of the molecule (Fig 22). Thus, for efficient *L. monocytogenes* cardiac cell invasion, increased InlB stability must be combined with enhanced migration of InlB from the bacterial surface to the host cell surface via heparan sulfate interactions. Overall, our results suggest that the 07PF0776 InlB polymorphisms within the GW repeat are required for enhanced invasive capacity in myocytes, however this altered motif is impotent without the structural stability conferred by substitutions within the beta-repeat, such as D371.

Based on the findings reported here, the poor conservation of the beta and GW repeats found across the InlB proteins encoded by different isolates of *L. monocytogenes* may represent a mode of variation that leads to the exploitation of novel host niches by subpopulations of *L. monocytogenes*. Across the species, these polymorphisms would appear to have the potential to generate diversity in tissue and body sites that support bacterial replication by facilitating cell invasion. Thus through the alteration of a common virulence factor, *L. monocytogenes* gains access to new intracellular habitats within the human host.

3.5 - Materials and Methods

Strains, cells, and culture conditions

Bacterial strains were grown statically overnight at 37˚C in Brain heart infusion media (Difco Laboratories) containing the appropriate antibiotic. For 10403S, BHI was supplemented with

antibiotics where appropriate. For strains complemented with the pIMK-derived expression vectors [135], kanamycin was added to 50 ug/mL. For the IPTG-inducible vector pIMK4, IPTG was added to bacterial cultures at the time of inoculation to concentrations of 0, 0.1, or 1.0mM as indicated.

The rat cardiomyoblast H9c2 cell line was a kind gift of Dr. David Engman (Northwestern University, Chicago, IL, USA). Cells were maintained in DMEM supplemented with penicillin (100ug/mL), streptomycin (100ug/mL), L-glutamine (292ug/mL), and 10% fetal bovine serum (Hyclone), and incubated at 37˚C with 5% CO2. HepG2s were purchased from ATCC and maintained in MEM made according to manufacturer's specifications, incubated at 37˚C with 5% CO2.

Plasmid and mutant strain constructions

The coding regions of *inlA* and *inlB* were amplified by PCR using primers listed in Table 1. Amplified genes or genetic fragments were subsequently ligated into pIMK2, pIMK3, and pIMK4 [135]. Ligations were transformed into XLI-BLU sub-cloning grade competent *E. coli* (Agilent) using the manufacturer's recommended protocol. Cells were plated on LB containing 50 ug/mL kanamycin and allowed to incubate overnight at 37˚C. Constructs were confirmed by restriction digest and by sequencing of plasmid insertions.

An in-frame deletion of *inlB* in the 07PF0776 background was generated using the allelic exchange method described previously [1]. Briefly, the two 500 base pair regions directly upstream and downstream of the coding region were amplified, such that only the start and stop codons of the *inlB* coding region were amplified with the flanking regions. These products were then amplified using SOEing PCR [1], with internal primers (listed in Table 1) overlapping by 20

base pairs to facilitate the SOEing reaction, forming a 1000 bp product. This product was digested using *Bam*HI and *Sal*I restriction sites engineered into the external primers and ligated into the temperature sensitive allelic-exchange vector pKSV7. Allelic exchange was performed as previously described [136], and mutants were selected on the basis of chloramphenicol sensitivity. Screening was performed by isolating genomic DNA using the DNEasy Blood and Tissue Kit (Quiagen), followed by PCR amplification for confirmation of deletion via gel electrophoresis and sequencing.

Bacterial invasion assays in tissue culture cells

H9c2 rat cardiomyoblasts were seeded into 24-well plates containing sterilized coverslips at an initial density of $2.25x10^4$ cells/well ($1.0x10^5$ for HepG2) and allowed to adhere and double in number overnight. Alternatively, some assays were performed in 6 well petri dishes containing up to 10 glass coverslips, seeded with 3.0x10^5 H9c2 cells in 6mLs of DMEM. The cells were then infected with an MOI=100 of *L. monocytogenes* and infections were allowed to progress for 45 minutes at 37˚C in 5% CO2. Following this incubation, media was removed and the coverslips were washed once with PBS warmed to 37˚C. DMEM containing 15ug/mL gentamicin to kill extracellular bacteria was the added to the wells (1mL/well), followed by a one-hour long incubation at 37˚C in the presence of 5% CO2. Following the second incubation, coverslips were removed and vortexed in 1mL ddH20 to lyse the cells and release intracellular bacteria. Serial dilutions were performed and bacterial cfu enumerated by spot-plating on LB agar containing the appropriate antibiotic where necessary. Statistics were calculated using an unpaired two-tailed student's t-test and p-values less than 0.05 were considered significant.

Immunofluorescence staining and quantification

Phosphorylation of c-Met was assessed using a commercial antibody raised against the phosphorylated tyrosine residue 1349 of the Met protein (Santa Cruz Biotechnologies, Inc). Infections were performed as described previously under 'Invasion Assays and Tissue Culture Cells,' with the exception that infections were only allowed to progress for one hour, with coverslips being removed at 20, 40, and 60 minutes post-infection. Coverslips were fixed and stained as described previously [50]. Briefly, after fixing in 5% formaldehyde for 5 minutes, cells were permeabilized using 0.1% Triton in PBS for 10 minutes. Following permeabilization, cells were incubated with NBD-phalloidan (a toxin which binds filamentous actin, dilution = 1:50) for 30 minutes, followed by incubation with the p-Y1349 antibody (dilution= 1:333) for 30 minutes, and incubation with rhodamine-conjugate goat-anti-rabbit antisera (dilution= 1: 400) for 20 minutes. Between each incubation, coverslips were dipped 5 times in PBS to remove excess antibody before proceeding to the next incubation. Analysis of positive regions was done using the Carl Zeiss Zen Lite 2012 Software, and images were taken on a Zeiss Axio Image A2 upright microscope, using the 20x objective. Images of the 40 minute time-point from analysis of the D371N allele were analyzed using both the Zen 2012 software and ImageJ. Analysis of histograms and area fraction were carried out using Prism.

Mouse infections and bacterial burden assessment

Animal procedures were approved by IACUC and performed at the Biologic Resources Laboratory at the University of Illinois at Chicago. Adult 6-8 week old Swiss-Webster mice (Charles River Laboratories) were injected via the tail vein with 200 uL of PBS [0.144g/mL KH2PO4, 9.0g/ml NaCl, 0.795g/mL Na2HPO4] containing either $2.0x10^3$ or $1.0x10^4$ CFU of *L. monocytogenes*. Each strain was injected into 5-10 mice and infections were allowed to proceed for 72 hours. Following the infection, mice were sacrificed using CO2 anesthesia followed by

cervical dislocation. Organ burdens were assessed after homogenizing by plating serial dilutions of the homogenate on LB plates containing the appropriate antibiotic. Animal experiments were repeated twice and results of the two experiments were then averaged. Histology samples were fixed in 5% formaldehyde overnight and subsequently sectioned and stained by the Histology core of the Research Resource Center (RRC) at the University of Illinois at Chicago. Statistics were calculated using a two-tailed Mann-Whitney U-test and p-values less than 0.05 were considered significant.

Heparan sulfate and heparin treatments

Heparan sulfate derived from bovine kidney was purchased from Sigma, and stocked as a 1mg/mL solution kept at 4˚C. For subinhibitory levels, 25uL of the heparan sulfate stock solution was added to 500 uL of bacterial culture for 20 minutes. For inhibitory experiments, 50 uL of the stock solution was added to 500uL bacterial culture and incubated for either 5, 15, or 30 minutes. All timed incubations were carried out at 37˚C in a static water bath. For heparin, heparin sodium salt derived from porcine intestine was purchased from Sigma and stocked as a 10 mg/mL solution kept at -20˚C. 5 uL of the stock heparin solution was added to 500 uL of bacterial culture. Incubation was carried out at 37˚C in a static water bath for 20 minutes. Following the treatment, bacterial suspensions were mixed by vortexing and directly added to cells at the desired MOI.

Chapter Four: InlB Enhances Vertical Transmission of *Listeria monocytogenes* 07PF0776 Independently of Syncytiotrophoblast Invasion and Allelic Variations

4.1 – Summary

Listeria monocytogenes is famous for its ability to cross the placental barrier and infect the developing fetus in humans and other mammals. Features which enhance the bacterium's ability to invade cells within the placenta, as well as those which contribute to cell-to-cell spread, have been implicated in its ability to cause fetal infections. In this chapter, unique isolates of *L. monocytogenes* were assessed for their ability to cause vertical infections in pregnant mice, as well as their ability to invade the cells which comprise the principal barrier to vertical transmission. We found that while some strains were enhanced for vertical transmission in mice, this enhancement may not result from increased invasion of the cells which make up the maternal barrier. Additionally, we found that InlB in 07PF0776 acted as an enhancing factor for vertical transmission, and appeared to do so without substantially increasing invasion in syncytiotrophoblast cells. Complementation of an *inlB-null* strain of 07PF0776 with either 10403S or 07PF0776 *inlB* demonstrated that alleles of *inlB* behaved similarly in pregnant animals, with both variants displaying an ability to enhance colonization of the fetus and placenta. Interestingly in these strains, the higher levels of InlB expression appear to mediate statistically significant increases in vertical transmission compared to WT 07PF0776. Given that both J4403 and 07PF0776 express higher levels of InlB compared to 10403S, it is likely that the amount of enhancing factor present in these two strains plays a significant role in mediating increases in placental and fetal infection during pregnant infections.

4.2 – Introduction

The ability of *Listeria* to cause infections within the developing fetus through vertical transmission has been well-recognized [4, 17, 18, 137]. Advice to avoid soft or unpasteurized cheeses, as well as unprocessed milk, is commonly issued by physicians to pregnant women specifically for the concern of *Listeria* [137]. Because of this, and standards in food surveillance, the yearly number of cases in pregnant women is low [13]. In those women who do get infected, however, the bacterium is capable of colonizing the placenta, where it subsequently seeds into the amniotic fluid and gains access to the fetus [16].

Listeria is not alone in its ability to use the placenta as a replication niche. Other organisms, ranging from viruses to eukaryotic parasites, are capable of honing to the developing fetus for replication [138-142]. The anagram used in the medical field to remember the specific organisms capable of placental/fetal transmission was, until recently, TORCH (which stands for *T*oxoplasma, *O*ther (Group B Strep, *Listeria*), *R*ubella, *C*ytomegalovirus, *H*erpes) [143]. However, recognition of other causes of vertically transmitted fetal infections has led to a modification of the original anagram to a longer form: CHEAP TORCHES, which now stands for *C*hickenpox, *H*epatitis, *E*nteroviruses, *A*IDS (HIV infection), *P*arvovirus B19, *T*oxoplamsa, *O*ther (Listeria, GBS), *R*ubella, *C*ytomegalovirus, *H*erpes Simplex, *E*verything else sexually transmitted (Gonorrhea, Chlamydia, etc.), *S*yphilis [144]. This change in perspective is accompanied by the realization that these organisms likely possess multiple different mechanisms of egress from the maternal circulation in order to colonize the developing fetus.

The ability of *L. monocytogenes* specifically to move from infected mother to child during pregnancy has been associated with a number of virulence factors [36, 45, 145]. Both InlA and

InlB appear to be important for crossing of the placental barrier in some animal models [36, 45]. Others have demonstrated roles for the cell-to-cell propulsion machinery, ActA [145]. These findings implicate both the invasion and spreading capabilities of *L. monocytogenes* as crucial components of vertical transmission. The placenta is not unique in this sense, as the same activities – invasion and spread – are crucial for the colonization of the liver and for *Listeria*'s lifecycle in general [6, 146, 147]. Furthermore, once *Listeria* establishes infections within the placenta, bacteria are capable of reseeding the maternal blood from the placenta just as they do from infected livers [148].

Thus, for *L. monocytogenes*, the forces which dictate its ability to survive within the liver directly contribute to its ability to replicate within the host at large. To summarize the process, it is one of *replicative diffusion*, where the organism spreads by replicating outwards in three-dimensions from a single focus of infection within an organ. This process drives the progression of bacterial migration from the maternal circulation to the fetal compartment. The targeting of specific organs by the bacterium has largely been discussed in the context of the invasins InlA and InlB, as cellular invasion usually initiates the replicative cycle within a tissue [22]. The invasion machinery has been demonstrated to promote uptake, and thus tropism, in cells of the intestine [27], liver [146], brain [37], and placenta [39, 45].

Results presented herein indicate that two clinical isolates, J4403 and 07PF0776, may be enhanced for vertical transmission in outbred Swiss-Webster mice. The *inlB* gene of one of these strains, 07PF0776, was recently found to display a number of features not present in more traditionally studied alleles, such as the variant present in the commonly studied 10403S strain (Chapter Three). These features include an apparent decreased susceptibility to proteolysis, increased sensitivity to heparan sulfate, and an increased ability to mediate invasion in cardiac

myocytes. The variant InlB from 07PF0776 was also capable of enhancing cardiac colonization in infected mice when expressed in 10403S, a strain which did not normally display cardiotropism. Given the roles of the *inlAB* operon in placental invasion, it was hypothesized that the enhanced transmission from mother-to-pup could be due to the alterations present in its InlB. To test the contributions of InlB to vertical transmission, several strains of *L. monocytogenes* were assessed for their ability to colonize the fetus and placenta of infected mice. Infections were done using 10403S, J4403 (a clinical isolate from pericardial fluid), 07PF0776, and a strain of 07PF0776 lacking *inlB* (to assess the role of *inlB* in vertical transmission). InlA was not considered in the mouse model, as mouse E-cadherin does not serve as a high affinity receptor for WT InlA [45]. However, since both proteins have been shown to be involved in tissue tropism to the placenta, the contributions of InlA were investigated using a syncytiotrophoblast-like cell line (BeWo) and gentamicin protection assays.

4.3 - Materials and Methods

Pregnant Infections and Histology of Infected Fetuses and Placentas

Pregnant Swiss-Webster mice were ordered to arrive at day E12 of pregnancy (Charles River Laboratories). On E13, each mouse was injected with 5,000 CFU of organism and placed in a single cage for observation over an 84-hour time course. If during the time course the mother miscarried, abortions were collected as soon as they were noted. At 84 hours post-inoculation, mothers were anesthetized with CO2, exsanguinated by cardiac puncture, and then sacrificed by cervical dislocation. The uterus containing any and all retained pups was collected and placed in 25mL of ddH2O; the heart, liver, and spleen of the mother was also collected and each placed in

5mL of ddH2O. Five mothers were used per strain, and experiments have been replicated twice, giving a total of 120 fetuses and 120 placentas per strain. Some fetuses and placentas were fixed with formalin (4.5% in PBS) for 4-5 days. They were then sectioned, stained with H&E, and mounted by the Histology Research Core at the University of Illinois at Chicago. Data was graphed using Prism, and statistics performed using a Two-tailed T-test with Welch's Correction to correct for unequal variance between the populations due to sporadic colonization of the placenta and fetuses.

Invasion Assays in Syncytiotrophoblast (BeWo) Cells

Invasion Assays were performed using BeWo choriocarcinoma cells, which are syncytiotrophoblast-like in nature. Cells were cultivated in F12K media (ATCC) and seeded into 24-well plates at a density of 1.0x10^5 cells/mL with 1mL into each well. Cells were then allowed to adhere overnight, and were infected the next morning at an MOI=100. Cells were returned to the incubator for 45 minutes to allow the bacteria to initiate invasion. After 45 minutes, cells were washed once in PBS warmed to 37ºC. 1mL of F12K media containing 15ug/mL gentamicin was then added to each well and cells were returned to the incubator for another hour of incubation. Following this incubation in gentamicin (which kills extracellular bacteria), coverslips were removed and vortexed in 1mL of ddH20. Homogenates were diluted in 96-well plates and spot plated onto LB agar. The number of colonies per spot was used in conjunction with the known dilution in order to calculate the number of bacteria per coverslip. Experiments were performed in quadruplicate and repeated a total of three times.

4.4 - Results

Cardiac isolates J4403 and 07PF0776 are Enhanced for Vertical Transmission in Pregnant Mice

Both J4403 and 07PF0776 strains were confirmed to be enhanced for vertical transmission compared to 10403S (Fig. 26). With regards to 07PF0776 specifically, InlB played a significant role in the colonization of multiple organs within the pregnant animal. 07PF0776 InlB significantly contributed to colonization of the liver, spleen, and heart (Fig. 27). The ability of InlB to enhance bacterial migration to the fetus was demonstrated by the significant decrease in fetal colonization exhibited by strains lacking *inlB* (Fig. 27). Nearly 60% of mice infected with WT 07PF0776 exhibited fetal colonization, whereas less than 20% of those lacking *inlB* were found to have detectable levels of bacteria in the fetus.

In the placentas, a breakdown of maternal and fetal compartments was noted to occur in those pups infected with either strain. The amount of dissolution between the two compartments was always greater for those infected with 07PF0776 compared to 10403S (Fig. 28) top panels, star is fetal compartment, cross is maternal). Additionally, histological staining of fetal pups infected with 07PF0776 revealed patterns of tissue degradation and immune infiltration throughout a variety of compartments (Fig. 28). Within the CNS specifically (including the spinal cord), complete degeneration of cerebral layers was observed along with an increased immune infiltrate (Fig. 28 middle panels). Additionally, a loss of neural layers was noted in the cerebral cortex (Fig. 28 middle panels, arrows), indicating irreversible loss of neural tissue. Sporadic inflammation of the meninges was also seen in multiple sections for strains infected with 07PF0776 (Fig. 28 middle panel, star). This pattern of advanced tissue degradation was also

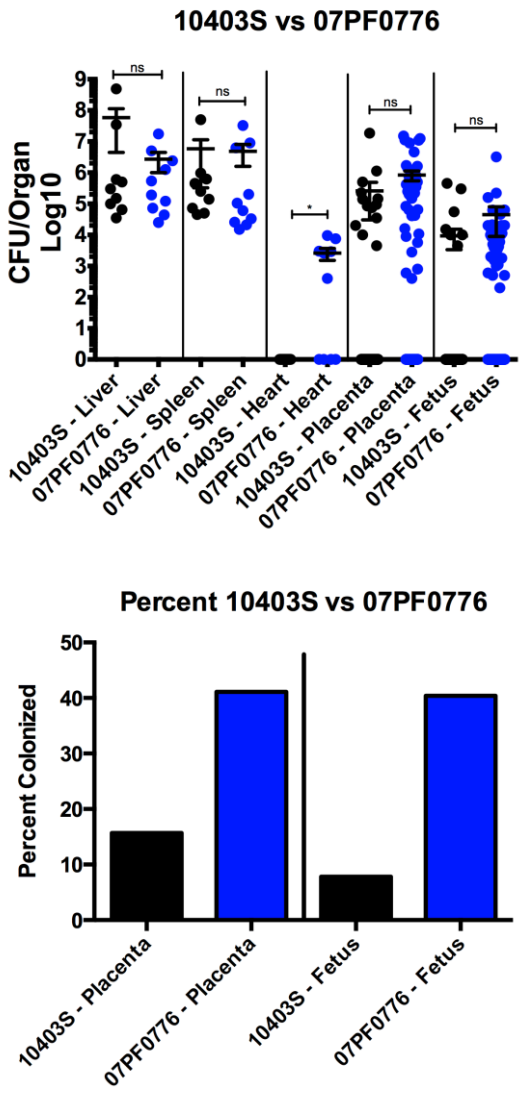


Figure 26: Cardiac Isolate 07PF0776 is Enhanced for Vertical Transmission in Pregnant Mice. Pregnant mice were infected intravenously with 5,000 CFU of either 10403S or 07PF0776 on day E13 of gestation. After 84 hours, mothers were sacrificed and maternal livers, spleens, and hearts were collected. Pups were also isolated and separated from their individual placentas. All organs (including pups and placentas) were suspended in 5mL of ddH20 and homogenized. Bacterial CFU were enumerated by plating serial dilutions of the homogenate on LB agar. While overall burdens between 10403S and 07PF0776 differed only in the heart (top panel), 07PF0776 demonstrated an increased ability to colonize the Fetus and Placenta across all experiments. Roughly 15% of placentas and 10% of fetuses were infected when mice were injected with 10403S, whereas approximately 40% of fetuses and placentas were infected in mice injected with 07PF0776 (bottom panel).

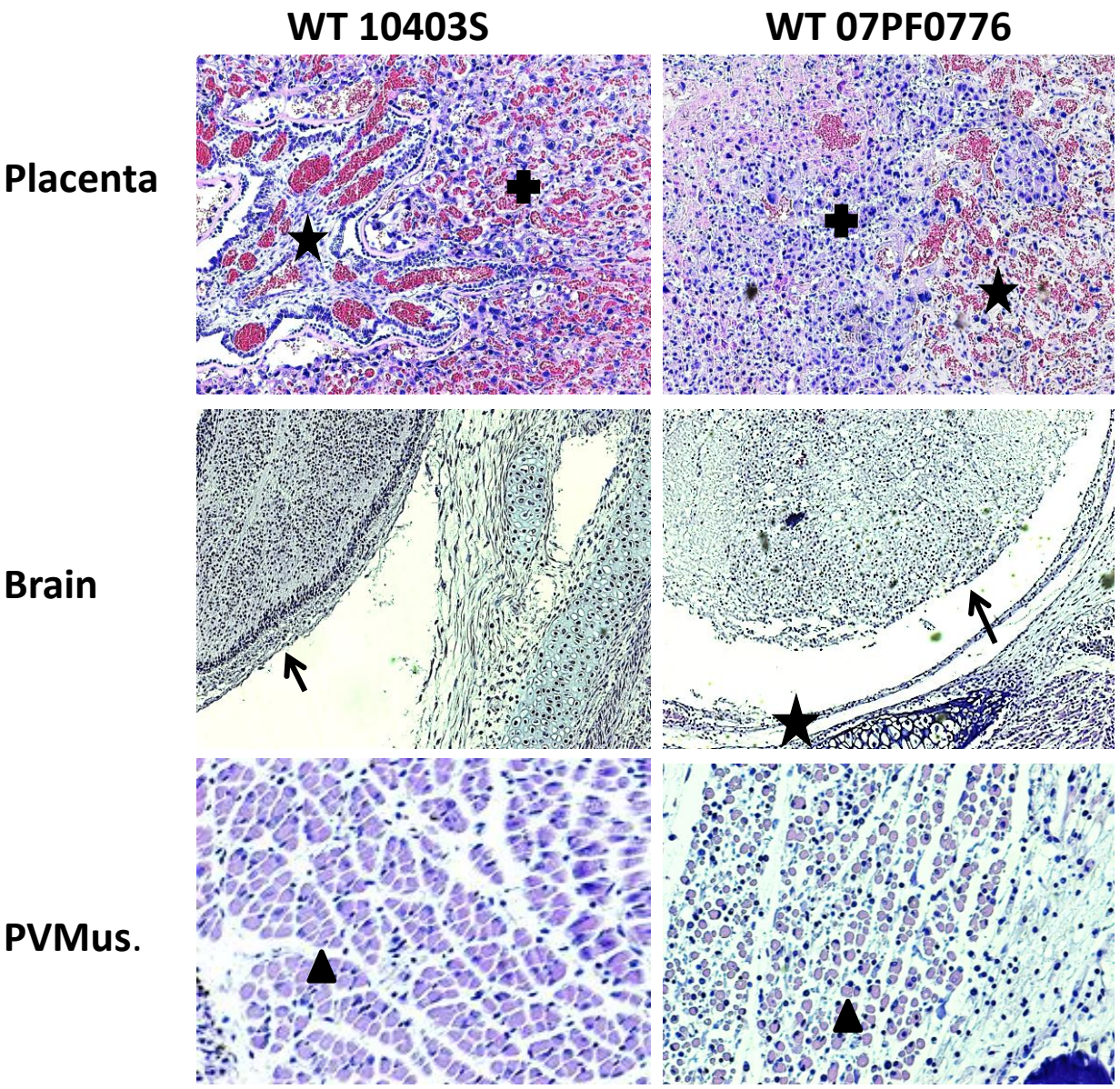


Figure 27: Fetuses infected with 07PF0776 demonstrate increased tissue destruction across a variety of organ compartments. Individual fetuses and placentas were isolated from pregnant mice after five days of infection with either 10403S or 07PF0776. Samples were fixed in 4% formalin, then sectioned and stained by the University of Illinois at Chicago Research Histology Core. Slides were stained with H&E and analyzed using Zen Software on a Carl Zeiss Axio Imager 10 Microscope. (Top panels) Infected placentas demonstrated greater apparent barrier disruption between the maternal (Cross) and fetal (Star) compartments. (Middle panels) The CNS of fetuses infected with 07PF0776 demonstrated loss of cortical layering (arrows), as well as focal meningitis (star). (Bottom panels, PVMus = Paravertebral muscles) Muscle wasting as indicated by X was apparent in the paravertebral muscle columns for those pups infected with 07PF0776 (triangle).

found in paravertebral muscles surrounding the vertebral column (Fig. 28 bottom panels, triangles). Pups infected with 10403S demonstrated normal muscle structure and size, whereas those infected with 07PF0776 displayed destruction of myocytes and loss of muscle mass (muscle wasting).

InlB is an Enhancing Factor for Vertical Transmission in 07PF0776, but the Enhancement is not Allele-specific

InlB in 07PF0776 was found to play a positive role in colonizing the placenta and fetuses (Figure 28, bottom panels), in addition to promoting increased consistency of liver colonization, and increased levels of bacteria in both the spleen and heart (Fig. 28 top panels). Interestingly, there was no allele-specific enhancement observed for the 07PF0776 variant of InlB in mediating vertical transmission, as the expression of either InlB from 10403S or 07PF0776 restored the capacity to colonize the placenta and fetus (Figure 28, bottom panels), however it was evident that the increased expression of either *inlB* allele from the complementing insertion plasmid enhanced vertical transmission in comparison to the wild type 07PF0776 strain (Fig. 29). These data implicate a significant role for InlB in placental and fetal infection, however the 07PF0776 InlB amino acid variations that have been shown to enhance cardiac invasion are not required for enhanced vertical transmission. Consistent with this observation, there was an apparent enhancement of colonization in the hearts of pregnant mice for the InlB from 07PF0776 compared to the 10403S allele, while the bacterial burdens in the liver and spleen were similar (Fig. 28, middle panels).

InlA Mediates Uptake into Syncytiotrophoblast Cells in conjunction with InlB

The effect of the internalins was assessed in tissue culture models of infection by performing

invasion assays on human BeWo choriocarcinoma cells. These cells are representative of the syncytiotrophoblast layer of cells separating the maternal and fetal compartments [149, 150], and thus useful as a model to study bacterial invasion in the context of vertical transmission. Despite the clear enhancement of vertical transmission observed for mothers infected with 07PF0776 during pregnancy, 10403S was actually enhanced for invasion of BeWo cells compared to 07PF0776 (Fig. 30, top left panel). Mutants of 10403S lacking *inlAB* were completely deficient for invasion in BeWo cells. Interestingly, it was found that *inlB* in 07PF0776 played no role in mediating uptake into this cell-line (Fig. 30, top left panel). Using strains of 10403S lacking either *inlA* or *inlAB*, it was found that *inlA* from either bacterial source was sufficient to complement invasion (Fig. 30, top right panel). Surprisingly, the presence of chromosomal *inlB* in 10403S had a negative effect on the ability of *inlA* from 07PF0776 to mediate uptake into BeWo cells (Fig. 30, top right panel), whereas *inlB* on the chromosome had no effect on *inlA* from 10403S. When over-expressed in the *inlAB- null* background of 10403S, InlB could increase the amount of bacterial invasion in BeWo cells independently of InlA. There was no observed difference for alleles of *inlB* in the background, as each variant induced invasion to approximately 25% of WT 10403S.

4.5 - Discussion

Our investigations into pregnancy-associated illness by *Listeria* have thus far focused on two models: invasion into the syncytiotrophoblast and colonization of the fetal-placental unit in infected mice. The enhancement in pregnant animals for 07PF0776 was clearly dependent on InlB, however complementation with alternate alleles of the gene failed to demonstrate strain-specific enhancements, such as those seen in the heart. Of note, the complementation studies

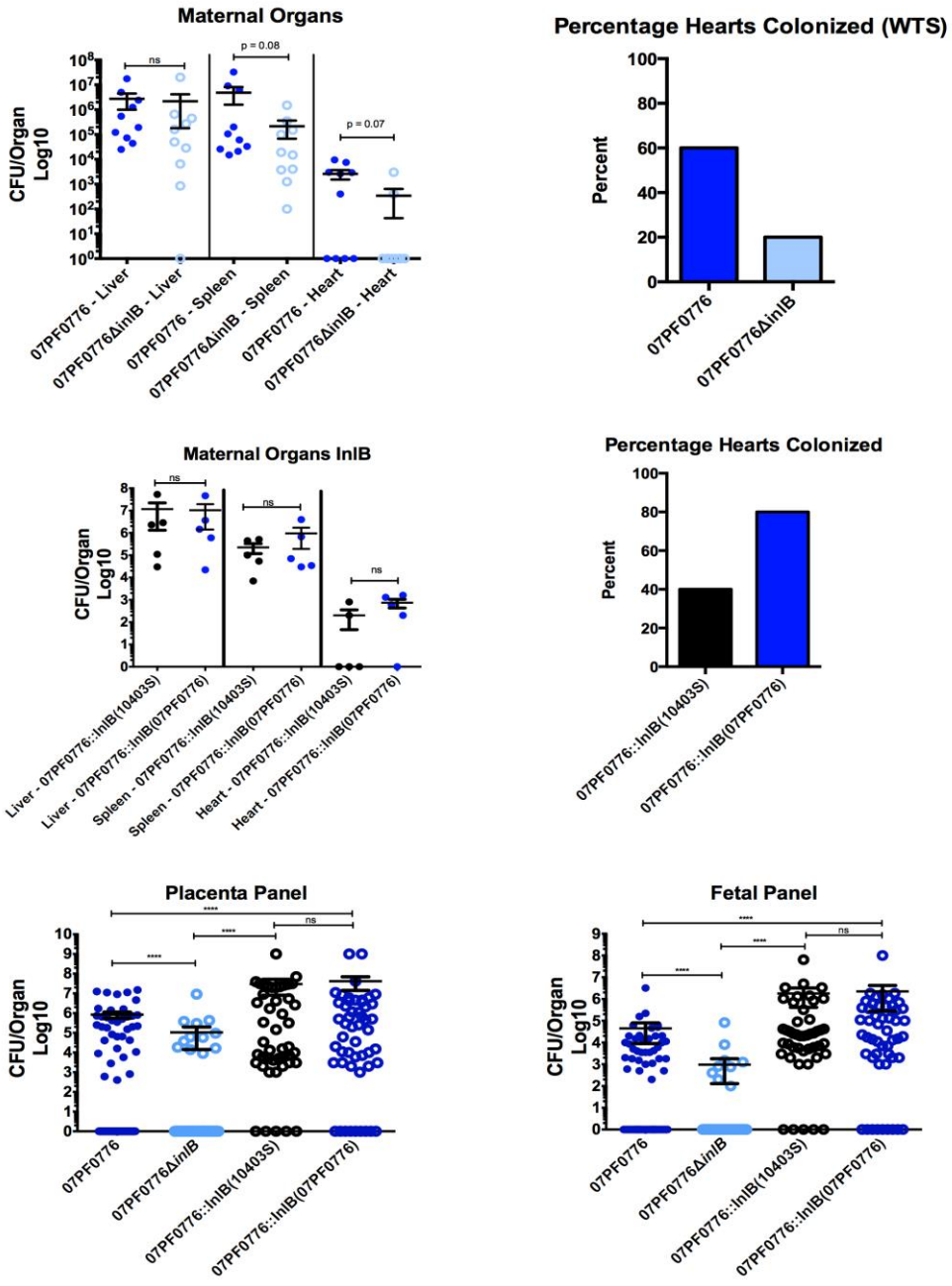


Figure 28: InlB is required for efficient vertical transmission in pregnant animals, independent of allele type. Pregnant mice were infected with 5,000 CFU via the tail vein at day E13 of gestation. After 84 hours, the liver, spleen, heart, and fetuses/placentas were isolated, homogenized, and assessed for bacterial colonization by plating on LB agar. Strains of 07PF0776 lacking InlB were defective for both cardiac and placental tropism (Top, bottom panels). Strains complemented with InlB were restored for their ability to colonize the placenta and fetus regardless of allele used, however only InlB from 07PF0776 enhanced cardiac tropism specifically.

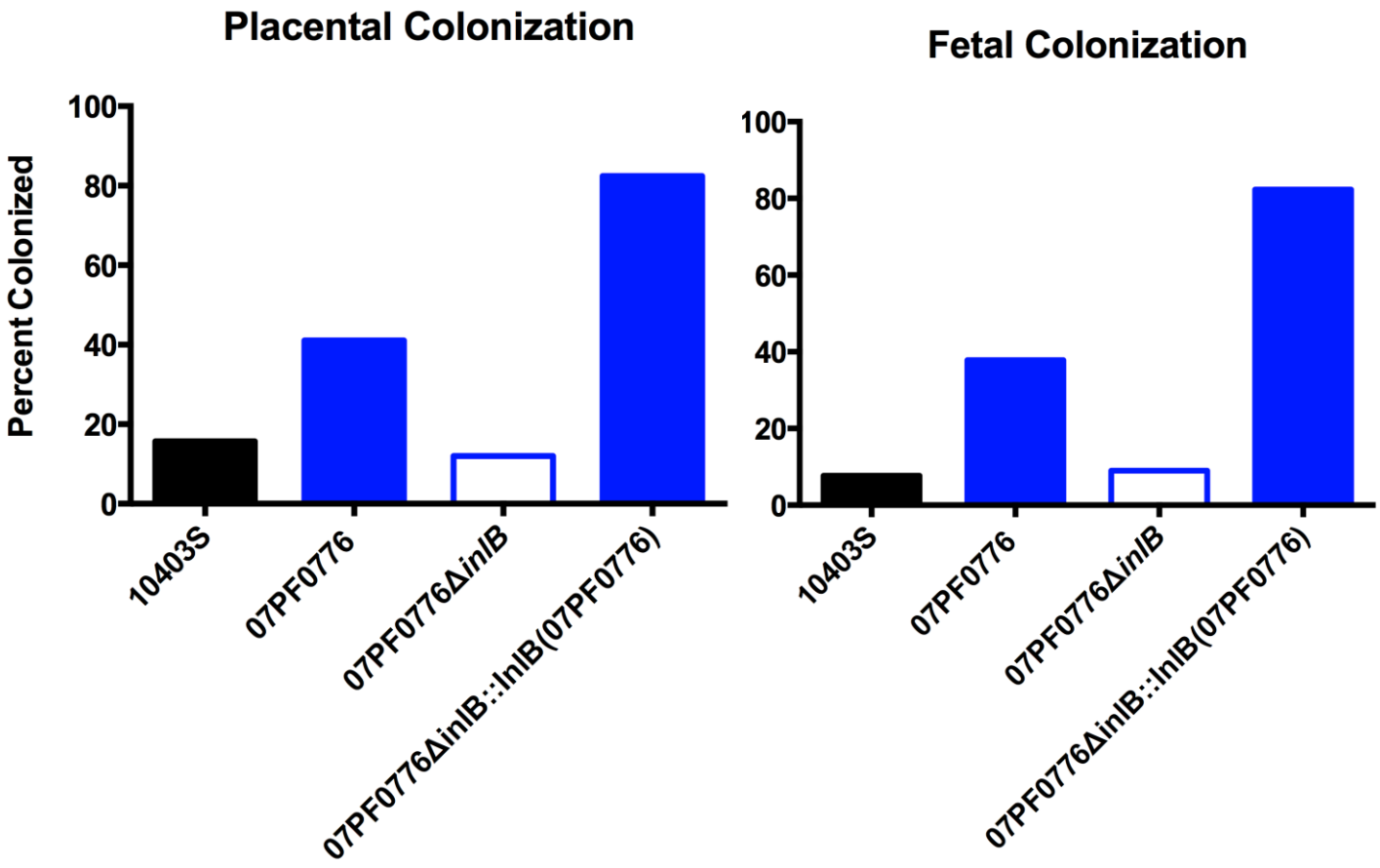


Figure 29: InlB enhances rates of both placental and fetal infections. Pregnant mice were infected with 5,000 CFU via the tail vein at day E13 of gestation. After 84 hours, the liver, spleen, heart, and fetuses/placentas were isolated, homogenized, and assessed for bacterial colonization by plating on LB agar. Numbers represent the percentage of infected fetuses and placentas in each group.

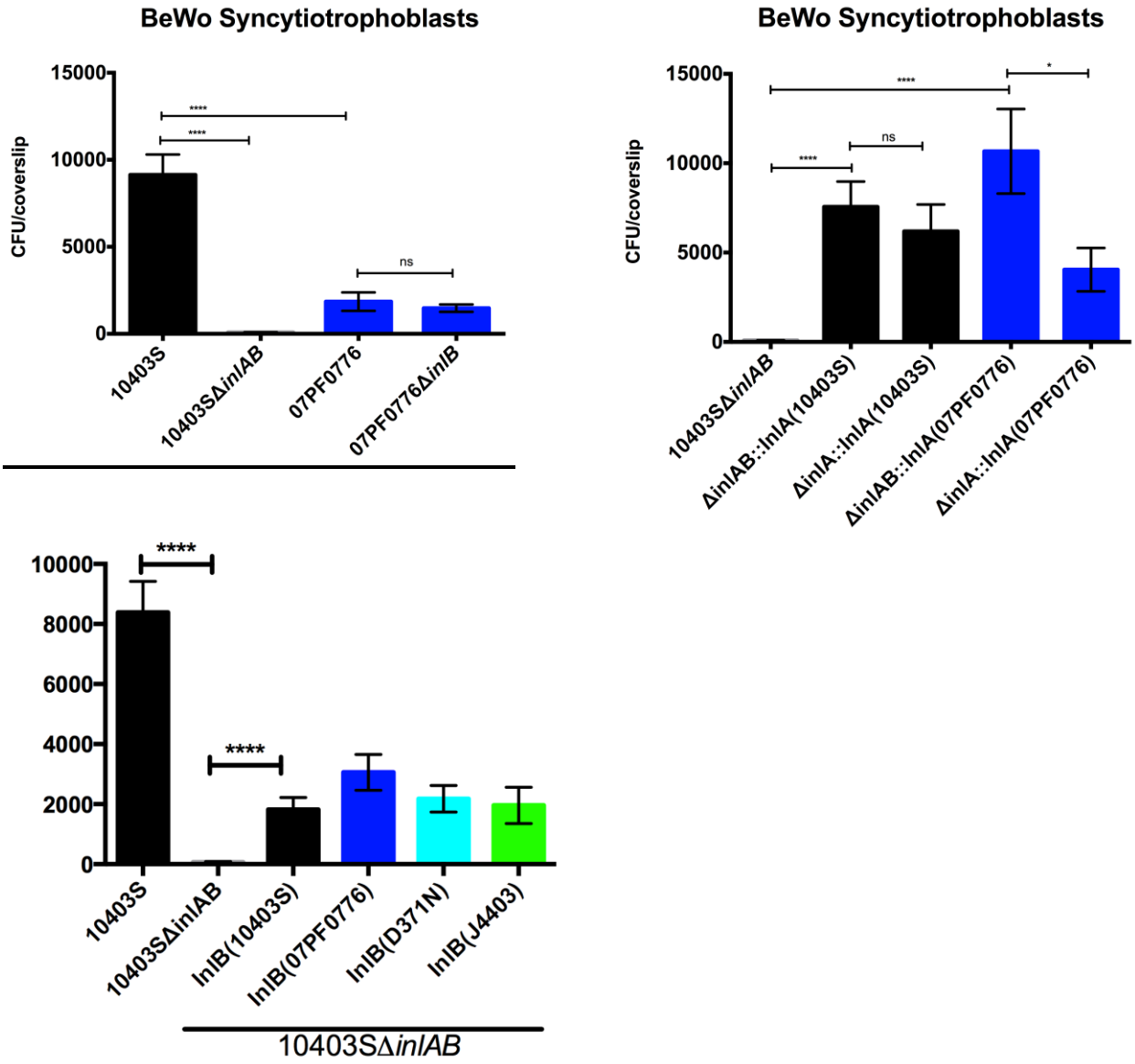


Figure 30: InlA and InlB may act synergistically to mediate invasion into syncytiotrophoblast-like cells. BeWo cells were infected at an MOI=100 and after 45 minutes of incubation, media containing gentamicin was added to kill extracellular bacteria. Coverslips were removed after one hour in gentamicin, and vortexed in 1mL ddH20. Samples were spot plated onto LB agar and enumerated after 24 hours. In this system, 10403S is enhanced over 07PF0776, and *inlB* of 07PF0776 appears to play no role in invasion. Complementing 10403S strains lacking *inlA* resulted in an increase in invasion, but the magnitude of this increase was dampened for strains with a chromosomal *inlB* for the 07PF0776 variant of InlA (panel 2). Variants of InlB did enhance invasion over the strain lacking *inlAB*, however full complementation was not observed for *inlB*, suggesting only complementary role in these cells.

demonstrate that increased expression of either InlB variant increases levels of vertical transmission over WT 07PF0776. Previous experiments (P.D. McMullen, unpublished data) have indicated that the amount of InlB expressed on the surface of WT 07PF0776 is roughly 2-4 fold higher than the amount found on the surface of WT 10403S. The enhancement seen for WT 07PF0776 compared to 10403S in our initial studies may, therefore, be the result of different levels of protein expression between these two strains. In agreement with this hypothesis, the strains of 07PF0776 complemented with *inlB* alleles typically express 2-3 fold more protein than the WT 07PF0776 strain, and likewise are significantly better at colonizing both the placenta and fetus than the WT strain. Thus, while an allelic variant of *inlB* is crucial for the cardiotropic aspects of 07PF0776 (including in pregnant animals), its ability to transmit vertically may simply be due to an increased presence of a factor known to enhance vertical transmission, and not due to strain-specific characteristics of the InlB protein. The amount of each invasin produced is known to vary from isolate to isolate, so raw amounts of their production may well constitute a strain-specific predisposition to pathogenesis.

While 07PF0776 was enhanced for vertical transmission in animals, it failed to demonstrate increased invasivity at the level of the syncytiotrophoblast . This offset could be due to a number of factors. First, the mouse system of infection known to have its limitations in regards to the effects of InlA [45, 102], whereas BeWo cells are human-derived and thus have a compatible ligand for InlA. To test the effects of 07PF0776's InlA in this system, alleles of *inlA* were expressed in two mutant backgrounds of 10403S. In both backgrounds, expression of *inlA* increased bacterial uptake into syncytiotrophoblasts, indicating that 07PF0776 InlA is capable of binding to human E-cadherin and mediating similar levels of uptake. A caveat to this, however, was noted when *inlB* was present in the chromosome of 10403S. In these strains, the ability of

07PF0776 InlA to mediate bacterial invasion was markedly reduced, suggesting an inhibitory effect of InlB on InlA's activity in this model. This reduction by the presence of InlB on this allele of *inlA* may partially explain the reduced invasion of WT 07PF0776 compared to 10403S. More thorough testing needs to be conducted in order to ascertain the exact interplay between InlA and InlB on the bacterial surface, however it is known that 07PF0776 expresses more InlB naturally than 10403S (P.D. McMullen, data not shown). This may skew the ratio of InlA to InlB such that the effect of InlA is masked by the presence of InlB, as it appears to do so in our experiments in the 10403S background. To test the role of these proteins in vertical transmission for these strains, tests in animal models with compatible ligands for InlA must be done. Such models include humanized E-cadherin mice [27], as well as guinea pigs [17].

One other possibility that potentially explains the enhanced levels of migration from mother to pup for 07PF0776 independent of trophoblast invasion involves the immune system. Pregnant hosts display characteristic changes in hormone and cytokine levels which ultimately lead to a minor immunosuppression [151]. Preliminary data from studies into 07PF0776's cardiotropic ability, as well as early studies in the pregnant model, suggest that 07PF0776 induces a more-robust immune response in pregnant animals than 10403S (P.D. McMullen, data not shown). While these results are preliminary, it could suggest that immune dysregulation in animals could play a role in vertical transmission. Many of the features of vertical infections, including abortion and fetal resorbtion, are actually mediated by the maternal immune system [151-153], and *Listeria* is known to traffic to other organs via infected phagocytes [19]. Additionally, immune mediators induced during sepsis have been demonstrated to reduce the efficacy of endothelial barriers, including the blood-brain barrier [154]. Loss of barrier function in compartments could explain the increased propensity of this strain to migrate from the maternal circulation into the

fetal compartment. Along with the studies mentioned in this chapter, we have also collected serum from every infected mother analyzed. We hope to further clarify the potential role of the immune response in pregnant animals by analyzing cytokine levels via Bioplex assay. By studying alternative animal models, alleles of *inlB* in our current model, and the immune response to infection during pregnancy, we hope to identify features of 07PF0776 which enhance its vertical transmission. The identification of such factors would be of great impact to the field, as strains of *Listeria* could potentially be characterized for their abortant potential by simply sequencing genes from the isolated strain. At this point, however, more work needs to be done in order to fully understand the similarities between the strains we find to be enhanced in pregnant systems.

Chapter Five: Further Exploring the Functional Relationships Between InlA and InlB

5.1 - Summary

Listeria is well-versed in its ability to translocate across various host barriers. This ability to migrate across barriers is thought to underlie its ability to colonize organs such as the CNS and placenta of infected individuals [37, 39]. Colonization of these organs relies not only on the ability of the bacterium to cross barriers from the blood, but also on its ability to invade and interact with cells within the effected organ [146]. Two virulence factors in particular, InlA and InlB, have been demonstrated to be crucial to the bacterium's ability to migrate from the blood stream to susceptible organs [20]. In the course of our investigations into tissue tropism mediated by allelic-variants of *inlB*, it was noted that the invasivity of particular strains of *Listeria* appeared to be perturbed by interactions directly between InlA and InlB – a phenomenon not previously reported. In this chapter, the roles of InlA and InlB on invasion were assessed across a variety of cell lines, each representative of cell types *Listeria* may utilize during the course of an infection. Additionally, two features of the internalins (linked expression on the bacterial surface and protein stability to proteases) were also investigated. InlA appeared to be principally responsible for migration across the intestinal barrier, as the protein was sufficient to mediate invasion into Caco-2 enterocytes in a mutant of *Listeria* lacking the entire *inlAB* operon. InlB itself appeared to mediate invasion in cells downstream of intestinal invasion, including fibroblasts (which mediate wound-healing and scar formation in the presence of cytokines) and cardiac myocytes. Additionally, the dynamic between the activity of InlA and InlB appeared to correlate with the amount of each protein present at the time of invasion. The ratio of InlA to InlB appears to be modulated by different forces, including expression-related differences in surface abundance as well as susceptibility to proteases.

5.2 - Introduction

Infection with *Listeria* arises almost universally from the ingestion of contaminated food products [8]. The bacterium is capable of first mediating its own uptake into the enterocytes and goblet cells of the small intestine, wherein it gains access to the blood supply draining from the intestine [26]. Given that the lumen of the intestine is an external environment separated from the internal environment of the host, the epithelial border in the small intestine represents the primary barrier to infection. The migration of the bacterium from the external environment of the lumen to the blood supply is critical for the initiation of later stages of infection [40]. The migration across the epithelial border is mediated by the surface-linked invasin InlA, which binds to E-cadherin present between enterocytes and goblet cells of the small intestine [27]. The importance of this initial step is illustrated by genetic data from epidemic strains of *Listeria*, which all contain intact and functional InlA [9]. Sporadic cases of Listeriosis may occur in which the offending strain is found to lack InlA, however those strains which are most associated with wide-spread outbreaks of Listeriosis are much more likely to contain an intact InlA [9, 102].

After migration across the intestinal barrier, *Listeria* may encounter many other barriers which it must efface and cross in order to gain access to nutrients within target organs [23, 37, 38]. The blood draining from the region of the small intestine where invasion is thought to occur is first carried by the portal circulation to the liver [116]. Indeed, in patients with leaky tight junctions, such as alcoholics, members of the normal flora can be isolated from the liver as a result of this natural flow of blood from the intestinal space to the liver [155]. For *Listeria*, this trafficking results in the introduction of the bacterium to one of its natural replication niches within mammals, as *Listeria* has evolved factors which allow for its survival within Kupfer macrophages as well as hepatocytes themselves [35, 146]. There are other factors which play

into the susceptibility of the liver in the course of infection, including fenestrated capillaries within the liver through which whole bacteria can pass without the need for endothelial cell invasion [156]. Additionally, after several cycles of replication, large amounts of organism may spill into the blood only to be filtered into the liver again as a result of the liver's role in clearing blood-borne organisms from the host [33]. Thus, following penetration into the host by migration across an epithelial border, *Listeria* are introduced to their primary replication niche directly, and can be reintroduced to the liver again in later stages of infection. Success in the liver for *Listeria* may therefore lead to increases in overall bacterial burdens not only in the early stages of infection, but also as the infection progresses. The hepatitis induced by *Listeria* infection is largely subclinical in humans however, and symptoms of Listeriosis are therefore more associated with the colonization of other organs, such as the CNS or placenta (where present) [4, 152]. Both of these commonly targeted regions reside behind additional barriers across which the bacterium must also migrate [38, 45]. In infected animals, the migration across these additional barriers are mediated largely by two mechanisms: Direct invasion of the barrier through the action of bacterial invasins (InlA and InlB), and invasion through infected phagocytes (i.e. The "Trojan Horse" mechanism of tropism) [38, 157].

The internalins InlA and InlB are linked both by genetic organization and by function [36, 39, 43]. In infected animals, they coordinate migration across multiple host barriers by directly mediating cellular invasion, subsequently allowing for replication and/or spread within the targeted organ [22]. Their activities can be synergistic or independent of one another, and strains harboring both genes are substantially more virulent than those strains lacking one or both [38]. In this regard, InlA and InlB may represent successive steps in evolution specifically directed towards gaining access to a protected replication niche and intracellular nutrients in a variety of

mammalian tissues [9]. Both proteins demonstrate species specificity as well, further demonstrating their ability to increase the host range and diversity of *Listeria* infections [41, 45].

As discussed in Chapter Three, genetic drift of *inlAB*, combined with selective forces within mammalian tissues, has the potential to alter tissue tropism by changing how InlA and/or InlB interact with cellular targets. During the course of our studies examining cardiac invasion, it became evident that InlA had the potential to influence InlB function, and vice versa. To further explore this functional crosstalk, we assessed bacterial invasion across multiple cell lines, each representative of the various barriers and cell populations *Listeria* may encounter during the course of infection of susceptible populations.

5.3 - Materials and Methods

Invasion Assays

For myocytes, 3.0x10^5 H9c2 myocytes in 6mLs of DMEM (10% FBS, high glucose, high pyruvate) were loaded into 60mm petri dishes containing 6-10 glass coverslips [20]. Cells were allowed to adhere to the coverslips and dish overnight, and were subsequently infected at an MOI=100 the following morning. Bacteria were allowed to interact with the cells for 1 hour, before the media was removed and the monolayer washed three times with PBS warmed to 37˚C. Media containing 15ug/mL gentamicin was added to kill extracellular bacteria, thus allowing enumeration of only those organisms which invaded the monolayer. After one hour of incubation in gentamicin, the coverslips were removed and vortexed in 5mLs of ddH20. Samples of the homogenate were then plated at various amounts on media containing antibiotics where appropriate. The same procedure was used for L2 cells, but 1.0x10^6 cells were used initially

rather than 3.0x10^5. In the assays shown in Figure 31, where InlA repression was being compared between two alleles of *inlA*, coverslips were vortexed in 1mL of ddH20 and the homogenate was spot-plated at 5 dilutions. This modified recovery method was deployed to better resolve the CFU numbers, since invasion rates were very low for these strains.

For Caco-2 cells, an alternative assay was employed with 24-well plates as previously described [135]. Briefly, 1.0x10^5 cells were inoculated into individual wells of a 24-well plate, each containing a glass coverslip. As with myocytes and L2 cells, Caco-2s were allowed to adhere overnight for infections the following morning. After one hour of incubation without antibiotic, media was removed from each well by vacuum aspiration, and replaced with DMEM containing 15ug/mL gentamicin for one hour. Coverslips were then removed individually and placed in 5mLs of ddH20 and vortexed. Samples of the homogenate were then plated at various amounts on media containing antibiotics where appropriate.

Western Blots and Densitometry

Analysis of protein expression was carried out using western blots as previously described [1]. 10mL cultures of each strain were grown overnight statically at 37˚C. Surface proteins were isolated by first normalizing the amount of bacteria per strain by optical density, then placing 250uL of SDS loading buffer containing 5% BME on the bacterial pellets. Pellets were then resuspended by vortexing, and boiled for 5 minutes at 100˚C. The boiled reaction was then centrifuged for 30 minutes at room temperature, in order to separate the pellet from the soluble protein fraction. Normalized amounts of sample were then loaded onto 10% SDS polyacrylamide gels and separated by 1D PAGE. Proteins were transferred to PVDF membranes using a commercial technique from Invitrogen, and then the membranes were blocked in 2.5% milk

overnight at 4˚C. The next morning, membranes were treated with their respective primary antibodies (for InlA and InlB, these were both polyclonal rabbit antibodies, but the InlB antibody had been purified by M-protein) for one hour at RT. After one hour, membranes were washed three times in PBS-T (PBS containing 1% tween). Following the wash step, membranes were treated with a secondary antibody conjugated to a colorimetric enzyme for one hour (Southern Biotech), and then immediately developed for five minutes at room temperature with the addition of colorimetric substrate. Densitometry was performed using ImageJ.

5.4 - Results

The Contributions of InlA to Cardiac Myocyte Invasion

In Chapter Three, we briefly discussed how InlA influences cardiac cell invasion in a strain of *Listeria* lacking the entire *inlAB* operon. In those studies, the expression of InlA alone had a negative impact on bacterial invasion (Fig. 11). As the ligand for InlA (E-cadherin) is not highly expressed in the myocyte cell line [158], InlA-dependent inhibition of entry was not likely to be the result of altered InlA-E cadherin interactions, nor was it likely that InlA was interacting with N-cadherin (the dominant cadherin present in myocyte cells), as such interactions have been ruled out in previous studies [102]. The presence of InlB in strains lacking *inlAB* was found to increase invasion into myocytes, however it was noticed that the presence of InlA on the bacterial surface modulated this effect, especially for the allele of *inlB* associated with increased myocyte invasion (Fig. 31). While strains expressing InlB from 07PF0776 were still cardiotropic in mice regardless of the presence or absence of *inlA*, in tissue culture the ability of InlB from 07PF0776 to mediate cardiac cell invasion for 10403S was reduced when the 10403S

inlA was present (Fig. 31). The inhibition of 07PF0776 InlB-mediated invasion by 10403S-derived InlA strongly suggests a level of interaction between variants of InlA and InlB in mediating cardiac cell invasion. Interestingly, the presence of InlA in the 07PF0776 background had no negative effect on invasion (Fig. 31). When the different InlA variants were expressed in 10403S strains containing chromosomal *inlB*, InlA from 07PF0776 supported increased levels of invasion in comparison to the 10403S InlA variant (Fig. 31), again suggesting allele-specific alterations to invasion in this cell line.

The Role of InlA in the Invasion of Enterocytes

InlA has been reported to be the principal invasin used for crossing the small intestine. Luminal accessible E-cadherin is present in the small intestine near goblet cells and between extruding enterocytes and acts as the ligand for InlA [27]. It is through these two cellular portals as well as phagocytic M cells [28], that *Listeria* is thought to make the initial migration from the external environment to that of the host. The expression of InlA in 10403S strains lacking *inlAB* resulted in increased amounts of invasion compared to the deletion strain, confirming previous results (Figure 32). It is important to note that in these experiments the pIMK4 vector (which is IPTG-inducible) was used at a level of 1.0mM IPTG. This level of expression corresponds to approximately 80% of 10403S levels (Fig. 11). Still, despite the lower level of expression, the positive effect of InlA is still apparent.

Additionally, the ability of WT 07PF0776 to invade Caco-2 cells was assessed as well, and a slight increase over 10403S was noted (Fig. 32). This slight increase was also seen for the 07PF0776 allele of *inlA* when compared to the 10403S allele. This possibly indicates that

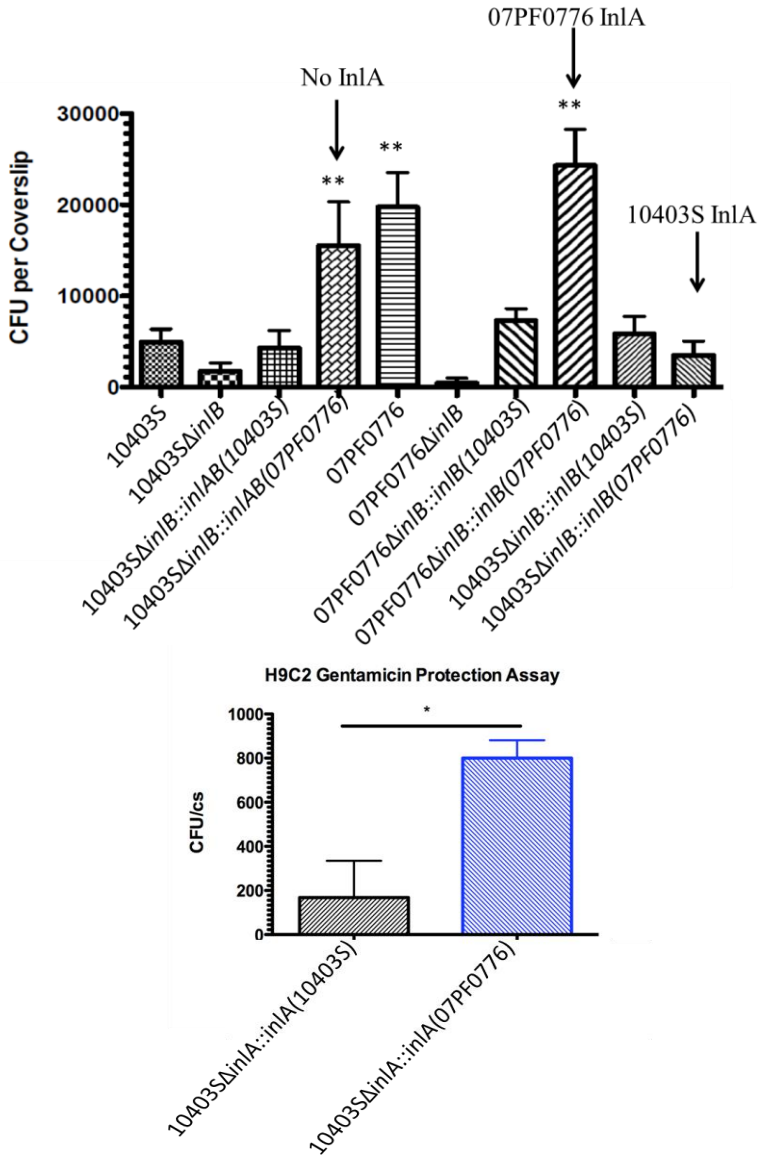


Figure 31: InlA modulates cardiac cell invasion *in vitro* in an allele-specific manner. H9c2 cardiac myocytes were infected with strains of *Listeria* expressing InlB with or without chromosomal *inlA*. (top panel) Strains of 10403S lacking *inlA* clearly demonstrate the allele-specific enhancement for the InlB of 07PF0776, however this effect was significantly reduced in a strain of 10403S which possessed chromosomal *inlA*. This repression was not observed in the 07PF0776 background with its intact *inlA* variant. (bottom panel) Strains of 10403S lacking only *inlA* (thus possessing the functional invasin InlB) were complemented with either 10403S or 07PF0776 alleles of *inlA*. The ability of InlA to inhibit cardiac myocyte invasion was reduced in this background for strains expressing the 07PF0776 variant as compared with the 10403S allele.

07PF0776, in addition to being enhanced for cardiac and vertical infections, may have an enhancement in oral infections as well. The unfortunate caveat to this supposition is the experiment required to confirm it: The E-cadherin of mice used by our laboratory is not compatible with InlA [41]. Thus, another model of infection must be used to move these hypotheses forward. InlB has been shown previously to have little-to-no role in mediating invasion of enterocytes [39], and indeed our results recapitulated this data (Fig. 33).

Listeria Invades L2 Mouse Fibroblast Cells using InlB

Another cell line commonly used to assess virulence among strains of *Listeria* is the L2 cell line derived from rats. These cells are functionally similar to fibroblasts, and are most commonly used in plaque assays (which assess cell-to-cell spread) [75]. Though fibroblasts are not considered to be parenchymal cells of any particular organ, their widespread distribution and roles in modulating wound-healing and inflammation potentially expose them to bacteria during the course of infection [159-161]. Fibroblasts are dispersed throughout tissues underneath of the endothelium as well as through the functional portions of the organ itself [161]. When damage occurs within an organ, inflammatory and chemical mediators, such as cytokines or reactive oxygen species, are capable of modulating protein expression within fibroblasts, altering their physiology substantially [154]. These alterations may be specific to the particular insult, but generally they result in migration and proliferation of fibroblasts within the damaged area [154, 161]. Activated fibroblasts are capable of depositing collagen in order to produce a scar within the tissue [154, 159], but are also capable of mediating scar-less wound healing within an organ through the production of growth factors, such as FGFs [133, 154]. Interestingly, bacterial

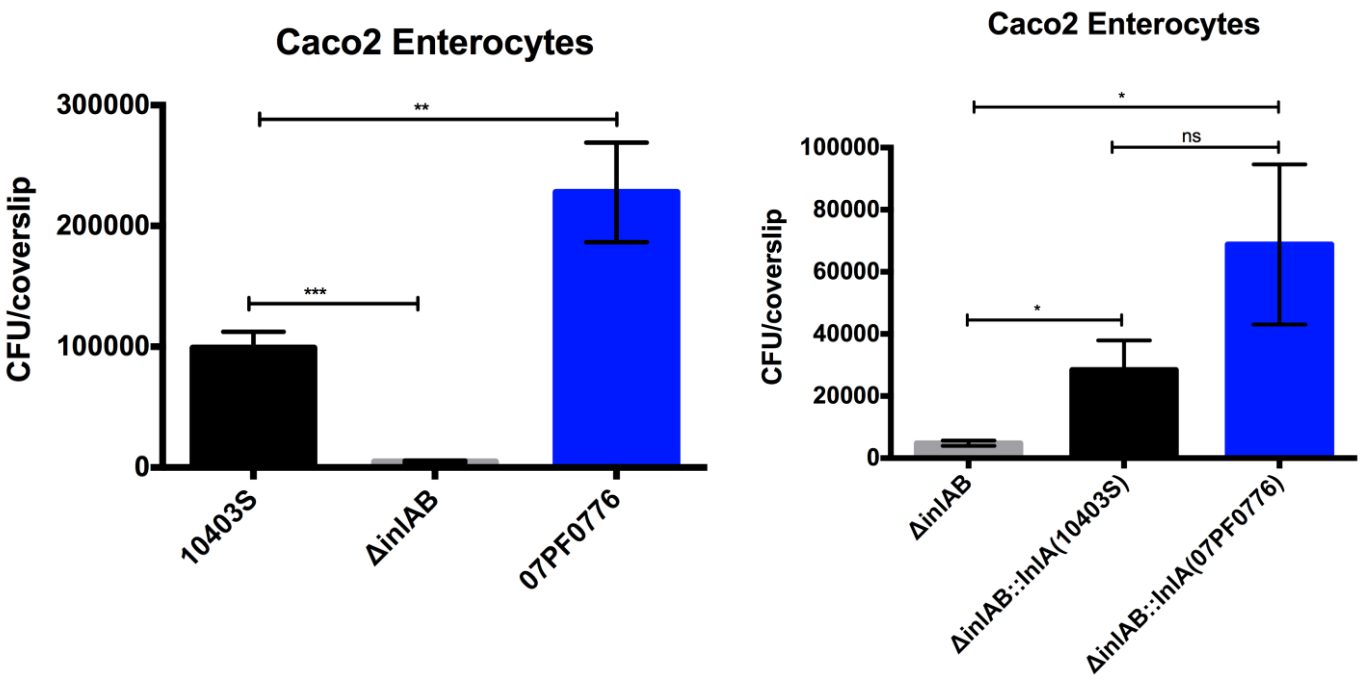


Figure 32: InlA mediates invasion into enterocyte-like cells. CaCo-2 cells were seeded into 24-well plates at a density of 1.0x10^5 cells per well and infected with an MOI=50. Bacterial invasion was assessed by gentamicin protection assays. (a) WT 07PF0776 demonstrated a small but consistent increase over 10403S in this cell line, which was statistically significant. (b) Comparison of InlA from 10403S and 07PF0776 in 10403S Δ*inlAB* also demonstrates a small difference in invasion potential, such that the 07PF0776 InlA is more capable of mediating invasion at this level of expression when compared to the 10403S version. Alleles of *inlA* were induced with IPTG, which results in approximately 80% of the expression level in 10403S WT. The difference between 10403S and 07PF0776 WT strains was significant with a p-value less than 0.05 in an unpaired t-test. The difference between *inlA* alleles was nearly significant with a p-value of 0.0692. These experiments were performed in triplicate.

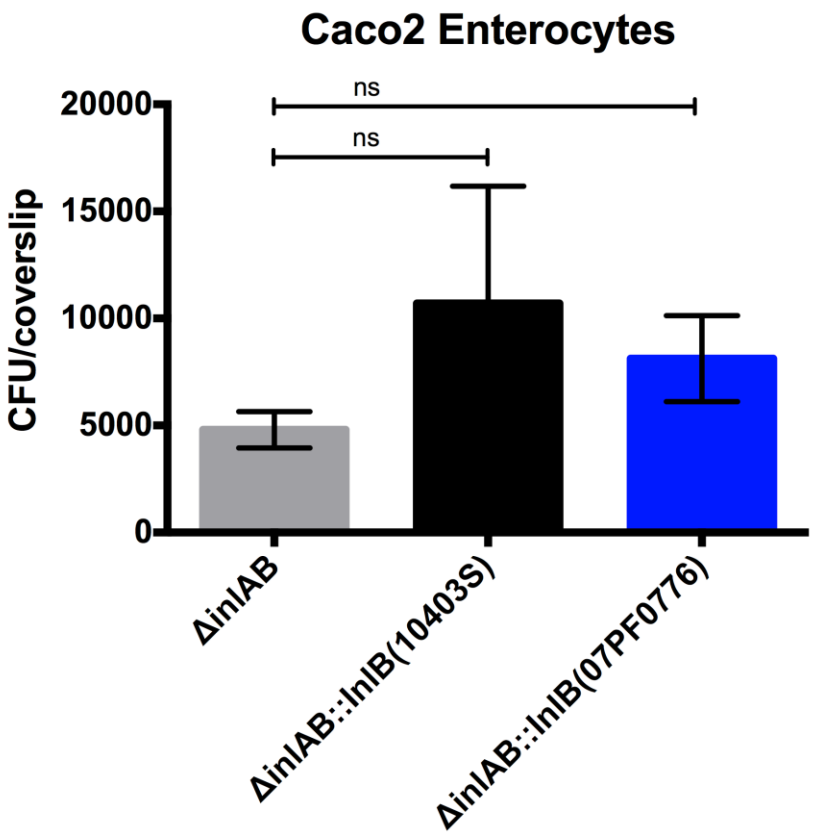


Figure 33: InlB plays no role in mediating invasion into enterocytes. The effect of InlB on CaCo-2 invasion was assessed by gentamicin protection assay, and demonstrated no significant effects on bacterial uptake. CaCo-2 cells were seeded into 24-well plates at a density of 1.0x10^5 cells per well and were infected at an MOI=50. Extracellular bacteria were killed by gentamicin treatment after one hour of incubation. No significant difference was observed between alleles of *inlB* when compared to the background strain lacking *inlAB.*

products – specifically InlB – have been demonstrated to induce wound-healing phenotypes, including cell scattering and wound closure in animals [44, 162].

Fibroblasts are abundant in multiple organs susceptible to colonization by *Listeria*, including the liver [159] and heart [160]. Given the role of InlB specifically in both of these organs, and the role of fibroblasts in mediating wound closure and/or healing following infection, bacterial invasion into L2 fibroblasts derived from murine embryos was assessed by gentamicin protection assays. As with myocytes, bacterial invasion of mouse L2 fibroblast cells was negatively influenced by InlA (Fig. 34). Additionally, there was a statistically significant difference between alleles of *inlA*, with the 07PF0776 allele demonstrating less repression (thus higher levels of invasion), similar to what was observed in myocytes. This difference was 3-4 fold, which is roughly equivalent to the order of enhancement observed for the 07PF0776 allele of InlA in Caco-2 and H9c2 cells (Fig. 31, 32).

Similar to H9c2 myocyte cells, InlB acted as an enhancing factor for bacterial invasion of fibroblasts (Fig. 34). Over-expression of both InlB alleles increased invasion well beyond the WT 10403S strain, a phenomenon not observed in myocytes, suggesting that overall levels of InlB present on the surface were critical to mediating this enhancement. In terms of the allele-specific effects of InlB in this cell line, the 10403S allele of *inlB* now appeared to be enhanced by approximately 5-fold over the 07PF0776 allele. An interesting point to note, apart from the enhancement observed for strains expressing InlB, is that mutants of 10403S lacking either InlA or InlB are not defective for invasion (Fig. 34). This may indicate that *Listeria* can effectively invade and survive within fibroblasts using other proteins that have been associated with cellular invasion.

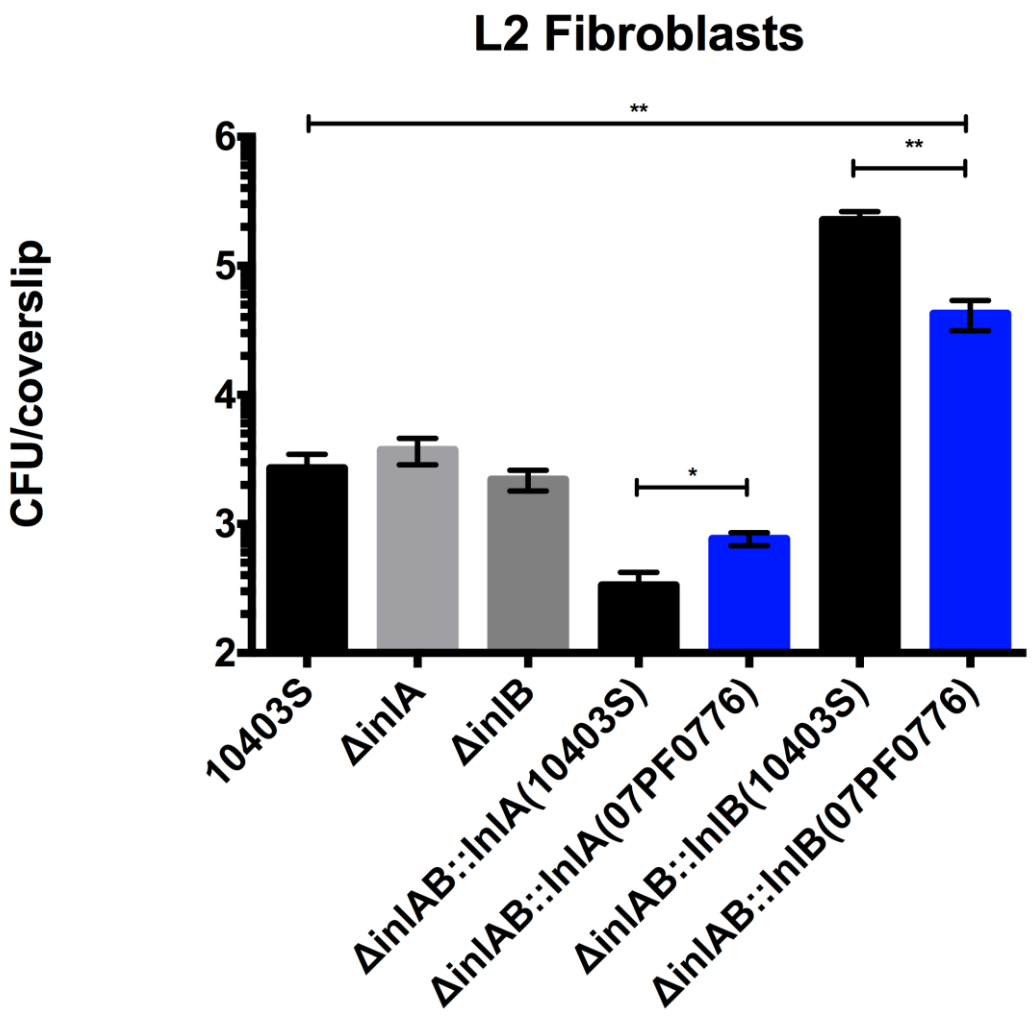


Figure 34: InlB promotes invasion into L2 fibroblasts, with 10403S InlB displaying an enhanced level of invasion over 07PF0776 InlB. Invasion in L2 fibroblasts was assessed by gentamicin protection assay in strains of 10403S lacking *inlAB*, complemented with alleles of either *inlA* or *inlB* from 10403S (WT) or 07PF0776 (car). In this cell line, InlB from 10403S was significantly better than InlB of 07PF0776 in mediating uptake into L2 cells, despite robust levels of invasion for both complemented strains compared to the *inlB* deletion strain. InlA in this cell line had a negative effect on invasion, with 07PF0776 InlA being less inhibitory than the InlA of 10403S. Deletion of either InlA or InlB (either individually or as an operon) had no significant effect on invasion of the 10403S-derived strain, suggesting that over-expression of one internalin relative to the other may modulate invasion for L2 cells.

The Relative Ratio of InlA to InlB is Functionally Significant and Can Be Influenced in Multiple Different Ways

Although encoded in an operon, InlA and InlB share little in terms of their homology and activity. InlA, as mentioned previously in Chapter Three, is covalently linked to the bacterial surface, while InlB exists in a state of loose association with lipoteichoic acid [39, 42]. Both genes can be transcribed bicistronically or individually by different transcription factors [163]. Differences in the activity of one or more of these factors across isolates may feasibly skew the surface profile towards one internalin or the other, thus "resetting" a strain's invasive phenotype. In addition, both InlA and InlB undergo extensive degradation on the surface of the bacterium (Fig. 35) [122]. Differences in these degradation patterns could foreseeably alter the amount of active protein on the surface as well, potentially creating strain-specific differences by altering susceptibility to surface proteases. Interestingly, variants of InlB were found to demonstrate different levels of degradation. The degradation phenotype of InlB was specifically discussed in Chapter Three in the context of a mutation within the beta-repeat region of InlB (N371D), which when reverted to an asparagine resulted in an apparent loss of protein stability (Fig. 23). The quantitative differences in degradation between two alleles of *inlB*, 10403S and 07PF0776 were calculated using densitometry (Fig. 23). Densitometry more clearly demonstrates the increased amount in the primary band for the 07PF0776 variant when compared to the 10403S protein.

Also an interesting point uncovered by these studies is that the expression of the individual components of the *inlAB* operon itself can influence the expression of the adjacent gene. For instance, when InlA was over-expressed in strains of 10403S possessing a chromosomal copy of *inlB*, the amount of surface-associated InlB is reduced (Fig. 36). Conversely, when InlB was

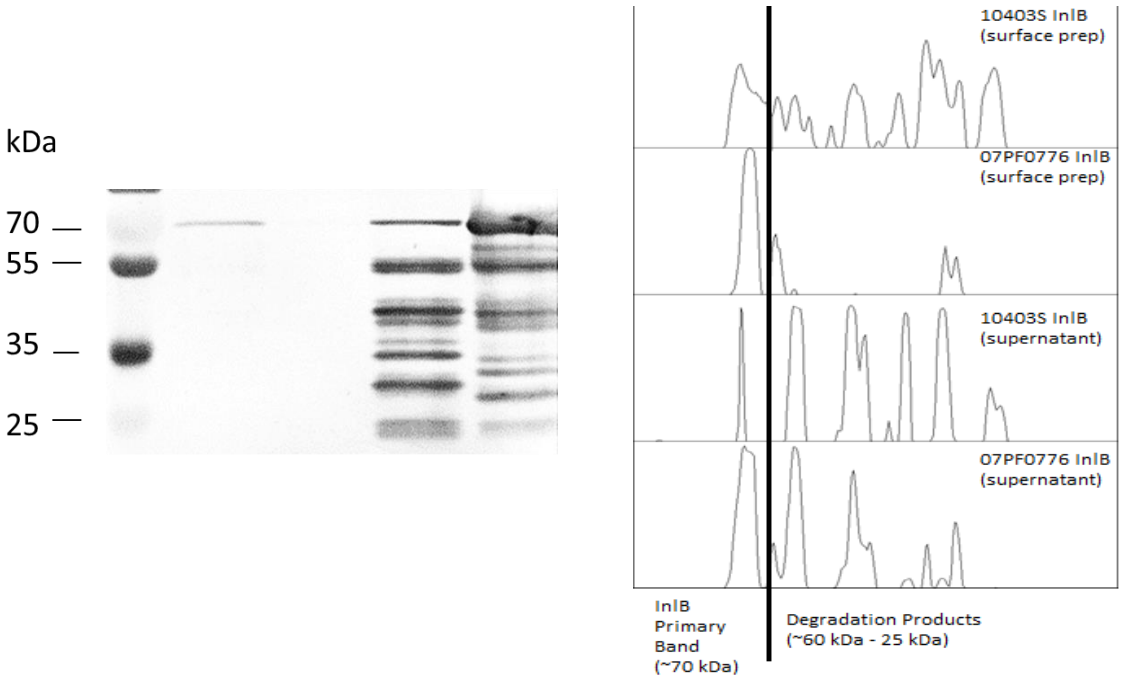


Figure 35: Alleles of InlB display different amounts of degradation in both surface-associated and supernatant fractions. Proteins were isolated from the bacterial surface by boiling in SDS buffer for five minutes. Supernatant fractions were collected after isolation of the bacterial pellet and proteins were precipitated by treatment with trichloroacetic acid as described previously [1]. Samples were normalized and separated by 1D SDS PAGE. (a) Representative western blot from supernatant fractions of 10403S WT (lane 2), 10403S d*inlAB* (lane 3), 10403S::InlB(10403S) (lane 4), and 10403S::InlB(07PF0776) (lane 5). (b) Plots of InlB band intensity were generated using ImageJ (left side of plot is the top of the gel, right side of plot is the bottom of gel, with peaks indicating band intensity). The total amount of detected products for each variant (primary and degradation) was 56,763.45 for 10403S InlB and 46,682.92 for 07PF0776 InlB, indicating that the 10403S allele actually made more product overall. However the amount of product in the primary band for the 07PF0776 variant was more greater in both on the surface and in the supernatant.

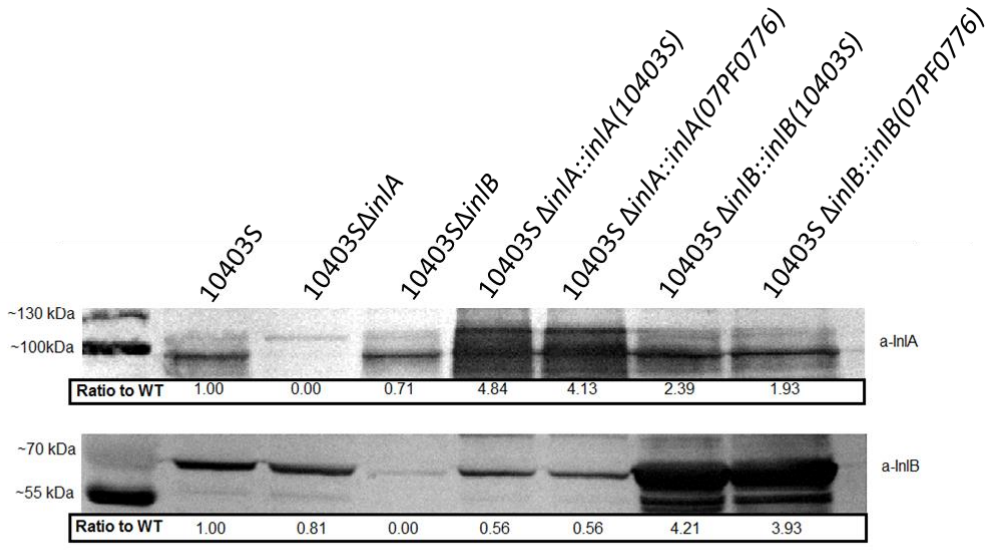


Figure 36: Over-expression of either InlA or InlB perturbs the surface level of the other internalin. Surface-associated proteins were isolated from various strains of *Listeria* by boiling in SDS buffer. Samples were normalized by OD600 and analyzed by Western Blot. The effects of over-expressing one internalin on the surface levels of the other internalin were calculated by densitometry in ImageJ. Increasing expression of InlA decreases surface InlB by approximately 50%, while increasing InlB levels increases the amount of InlA on the surface.

over-expressed, the amount of InlA present increased on the surface (Fig. 36). These results, combined with the cellular invasion assays for these strains, indicate the ability of the internalins to mediate bacterial uptake may not hinge solely on their presence or absence, but rather suggest that multiple forces may coalesce to create unique patterns of activity across different strains of *Listeria*.

5.5 - Discussion

The effects of the internalins on cellular entry have been well characterized for particular cell lines, such as enterocytes [41] and hepatocytes [43]. It is clear, however, that their presence can not only have positive effects on bacterial invasion, but in some cases can actually have negative consequences. The selection against such negative consequences, particularly those seen for InlA in fibroblasts and myocytes, could conceivably reshape the pathogenesis of certain isolates by skewing their ability to invade a variety of cell types. No negative consequences have been observed for the presence of InlB, apart from its inhibition of 07PF0776 InlA in BeWo cells; it appears to either have a positive effect on growth or no effect at all in most cell types. Thus mutations in the *inlAB* locus can arise from both positive and negative selection events and appear to direct strains of *Listeria* to discrete tissue sites during an infection.

The genetic diversity among sequenced isolates in this region is immense. InlA, for instance, has been genetically typed into a constellation of different "allele-types." [9] Those isolates most commonly associated with outbreaks, for instance, tend to share a "type 25" allele of *inlA*, 10403S is in possession of a "type 13," while EGDE (another laboratory isolate commonly used) has a "type 8." [9] While this organization scheme is able to clarify the evolution dynamics of

the *inlA* region, sequence data obtained from our previous studies into cardiotropic strains indicated a number of distinct polymorphisms across isolates which do not fall into "type-able" categories [20]. Some of these mutations were in conserved regions involved with receptor binding and covalent linkage to the peptidoglycan wall. Such non-typeble, strain-specific mutations may underlie the different virulence potentials across isolates of *Listeria*. Further genetic studies into *inlA* and other virulence factors have consistently demonstrated segregation between strains of *Listeria*, their virulence potential, and the sequences of *inlA* and *hly* (LLO) independent of other virulence factors, such as ActA [164, 165].

This point of diversity within *inlAB* is recapitulated by a genetic study of the beta-repeat region of *inlB.* Over 300 alleles of *inlB* were sequenced and aligned in order to identify residues conserved among pathogenic strains [121]. In this alignment, the residues extending from 370 to 380 were found to be incredibly polymorphic, with no consensus sequence being reached in this region [121]. Yet, among our cardiotropic isolates, D371 was found to be conserved in the beta repeat and was also crucial in mediating cardiotropism *in vivo* and cardiac cell invasion *in vitro* (Fig. 24). These results, along with the results presented in this chapter, demonstrate that the diversity present between alleles of *inlAB* has the potential to modulate disease independent of conserved genetic motifs within the locus. Moreover, alleles of *inlA* and *inlB* may hold the potential to open up new portals of entry [102], altering invasive capacity and thus the end-pathogenesis of an isolate. The identification of such functional mutations among the great diversity of alleles present may provide greater insight into the pathogenesis (and thus manifestations) of listerial disease.

Chapter Six: Discussion

6.1 - Summary

The goal of this study was to investigate determinants of cardiac tropism exhibited by *L. monocytogenes*. In chapter one, the relevant physiology of *Listeria* as both an environmental saprophyte and mammalian pathogen were discussed. In particular, it was noted that selective pressures in the environment create diversity in disease presentation when infection occurs. In chapter two, we determined that the ability of one cardiotropic isolate of *L. monocytogenes* likely acquired the trait through genetic drift in its common virulence arsenal. A genetic correlation between the invasins *inlA* and *inlB* was found to exist in the cardiotropic strains tested in a previous study. In chapter three, alleles of *inlA* and *inlB* were tested in multiple genetic backgrounds and demonstrated that *inlB* of 07PF0776, a cardiotropic strain of *L. monocytogenes*, supported hyperinvasivity in cardiac myocytes and promoted cardiac colonization in animals. The mutations in the variant *inlB* responsible for this increase appear to reside in both the beta and GW repeats, and confer enhanced stability and increased migration in response to heparan. The ability of the variant *inlB* to promote vertical transmission was tested in chapter four, where it was demonstrated that *inlB* of either source acts as a potentiating factor for vertical transmission. Both *inlA* and *inlB* appeared to mediate uptake into human syncytiotrophoblasts, with *inlA* playing the predominant role. In chapter five, the significance of interactions between allelic variants of the internalins was highlighted. The ratio of one internalin to another dictates invasion efficiency in myocytes and fibroblasts, and the ratio can be altered by a multitude of forces. In this chapter, we will discuss the significance of these findings in the context of the evolution of tissue tropism among isolates of *L. monocytogenes*. Given its environmental source, variants of the species are produced by selective pressures outside of human hosts. No human to

human transmission is known to occur, thus making the human a dead end for the bacterium as it is either cleared or will result in lethality. Success for the bacterium is essentially what we label as pathogenicity, the more pathogenic being more capable of extracting nutrients from its human environment. Such success in terms of bacterial fitness can be derived from a multitude of different mutations in core virulence genes, each of which can alter the physiology of the bacterium, and thus its infection, substantially.

6.2 - The Study of Clinical Isolates

The results of this project were not possible without the initial characterizations and demonstrations of cardiotropism by Alonzo et al. in 2011 [20]. In this study, more than ten individual isolates from both clinical and laboratory sources were characterized for their virulence in tissue culture and animals, with a specific focus on infections of the heart. From this study, one isolate with high invasivity for cardiac cells was selected as a potential candidate for further study with respect to cardiac infections.

Our in depth characterization of this strain has demonstrated many phenotypic differences compared to other more common strains of *L. monocytogenes*. This thesis has focused only on a few of these phenotypes, but many others remain uncharacterized. In addition to the changes in invasivity, which may be partially or fully explained by the findings presented throughout this thesis, there are differences in cytotoxicity, growth dynamics in broth (reduced lag phase), surface properties (aggregation in broth), and antibiotic resistance (slight kanamycin resistance,). Any of these features in themselves may help contribute to the virulence potential of this isolate (including potentially the differences in antibiotic resistance and sensitivity, as streptomycin

resistance in *Listeria* is associated with a small loss of virulence). These phenotypic readouts could be paired with the genome sequence for later study, in the hopes of understanding more about the basic biology of this very interesting bacterial isolate, as well as creating genetic classification schemes linking bacterial genetics to disease manifestation.

The isolate, described throughout the text as 07PF0776, is essentially a unique moment in the evolution of *Listeria monocytogenes*. While localized in the myocardium of the unfortunate patient in Boston, 07PF0776 was actively competing for resources, avoiding selection, and potentially evolving traits adapted for its immediate environment. For three weeks it grew from a primary lesion into a necrotic abscess. The calculated generation time of 07PF0776 in cardiac myocytes was estimated by Alonzo et al. to be on the order of 50 minutes per division [20] . If we assume colonization occurred at the onset of the patient's chest pain (three weeks prior to his demise) [20], the strain could have undergone roughly 300-600 generations before being isolated by the pathologist. It is unclear whether the strain was predisposed to colonizing cardiac tissue upon initial infection, or whether it evolved traits suited to its environment during the course – though, it is likely a little of each. What is clear is that following its infectious course, the isolate was much more efficient at cardiac infections specifically, both *in vivo* and *in vitro*, than any of the other strains tested.

6.3 - Cardiotropism as a Broad Phenomenon of Infectious Disease

As described briefly in the introduction to Chapter Three, the ability of pathogens to target the heart can be due to a number of different characteristics. Cardiotropic organisms run the gamut from viruses, such as Coxsackie Virus all the way to eukaryotic pathogens such as

Trypanosoma cruzi, the causative agent of Chagas Disease [166, 167]. While many pathogens are capable of targeting the heart, some have evolved specific factors that take advantage of the physiology and/or cell structure of the heart to specifically favor colonization. For *T. cruzi*, the most common cause of myocarditis in the developing world, the ability to invade myocytes through interactions with heparan sulfate is critical to its ability to cause disease [168, 169]. Without its invasion mechanism, the parasite is confined to the extracellular compartment where it is unable to cause myocarditis. For *Enterococcus faecalis*, the sporadic colonization of heart valves is largely dependent on aggregation – a virulence feature mediated by a single bacterial surface protein factor [170, 171]. This aggregation mechanism essentially creates micro-emboli of organisms. These infectious particles are then thought to become trapped within the eddies created by turbulent blood flow within the heart. Thus, the ability of an organism to colonize the hearts of susceptible hosts can be dictated by very different mechanisms specific to each pathogen.

For some pathogens, cardiotropism is not an evolved feature but rather a consequence of their manner of introduction into the host. Members of the HACEK group of organisms (*Haemophilus, Aggregatibacter, Cardiobacterium, Eikenella, and Kingella*) collectively cause approximately 10-15% of endocarditis cases, but there is no collective evolved trait among these organisms that predisposes them to cardiotropism [154, 172]. Instead, their predisposition has to do with the manner in which they enter humans. The HACEK organisms are gram-negative oral symbionts that enter the blood stream upon disruption of the oral mucosa. Each of these organisms individually rarely causes endocarditis, including the deceptively named *Cardiobacterium hominis*. Instead, they cause a plethora of conditions, the course and severity of which depend on interactions between the individual bacterium and the host [154]. Indeed,

the most common cause of bacterial endocarditis in healthy individuals is *Streptococcus viridans*, which itself is also an oral symbiont and is capable of causing a multitude of infectious manifestations apart from the heart [154, 173].

For *Listeria*, cardiotropism is a phenomenon confined to particular subpopulations of the species [20]. Each strain we have tested previously has the ability to invade myocytes and initiate the replicative cycle. Moreover, for each strain there are individual mice that exhibit cardiac-specific colonizations [20]. It is the overall frequency and magnitude of colonization that varies among isolates of *Listeria*. This variance in laboratory animals is supportive of the presence of enhancing factors capable of increasing transit into, or enhancing replication within, heart tissue. Such enhancements include modified invasins, such as the InlB of 07PF0776 mentioned throughout this thesis, but could also be the result of other factors as well. These may include: increased bacterial egress from the liver into the blood (thus increasing flow of bacteria from liver to heart), increased resistance to toxic molecules associated with the myocardium (such as high levels of ROS or extracellular proteases), or the random presence of a known enhancing factor which performs a specific cardiotropic function (such as a plasmid-derived aggregation substance in *Enterococcus* [171]).

It is likely, given the diversity in genetics and physiology among *Listeria*, that there are additional and perhaps overlapping mechanisms of cardiotropism. The discovery of a novel *inlB* allele type capable of mediating enhanced cardiac cell invasion and cardiotropism, while indicative of a potential path to the heart, does not necessarily dictate that InlB is involved in all cardiotropic mechanisms. Indeed, the *L. monocytogenes* strain J4403 is naturally cardiotropic in mice, yet the InlB of J4403 does not enhance cardiac myocyte invasion, nor does it confer cardiotropism to strains of 10403S (Chapter Three). This result demonstrates that cardiotropism

for *Listeria* does not hinge solely on InlB, but likely involves other mechanisms. By researching these mechanisms, we not only learn more about microbial strategies that favor cardiac infection, but also become better informed regarding the evolutionary pressures of pathogens across different tissue environments within susceptible hosts. Such factors that should be investigated further in these clinical isolates include (but are not limited to): variants of LLO which demonstrate differences in red blood cell lysis [20], two variants of ActA present across the species (since ActA behaves as a heparan sulfate binding protein in some cell lines [174]), and changes in the absolute levels of known virulence contributors (which may indicate global changes in virulence potential).

6.4 – An Overview of Factors Influencing Tissue Distribution within Infected Hosts

A convenient way to summarize the progression of disease to other organs after the bacteria have reached the blood is to think of it in terms of two equally important factors: The ability to leave the bloodstream, and the ability to then survive within the invaded organ. Resistance to a pathogen exiting the bloodstream is primarily dictated by the integrity of capillaries within the organ targeted [154]. Once infection is established, disruption of barrier systems within the host can allow the pathogen to colonize sites away from its initial hold. For *Listeria,* the progression begins in the liver and spleen, and can eventually lead the organism to many different sites [101, 164]. A representation of this process is shown in Figures 37 and 38, with special attention given to the state of barriers present in the host throughout the infection.

Many pathogens are capable of targeting the liver and spleen initially, simply because both organs have evolved characteristics supportive of their roles in filtering the blood stream

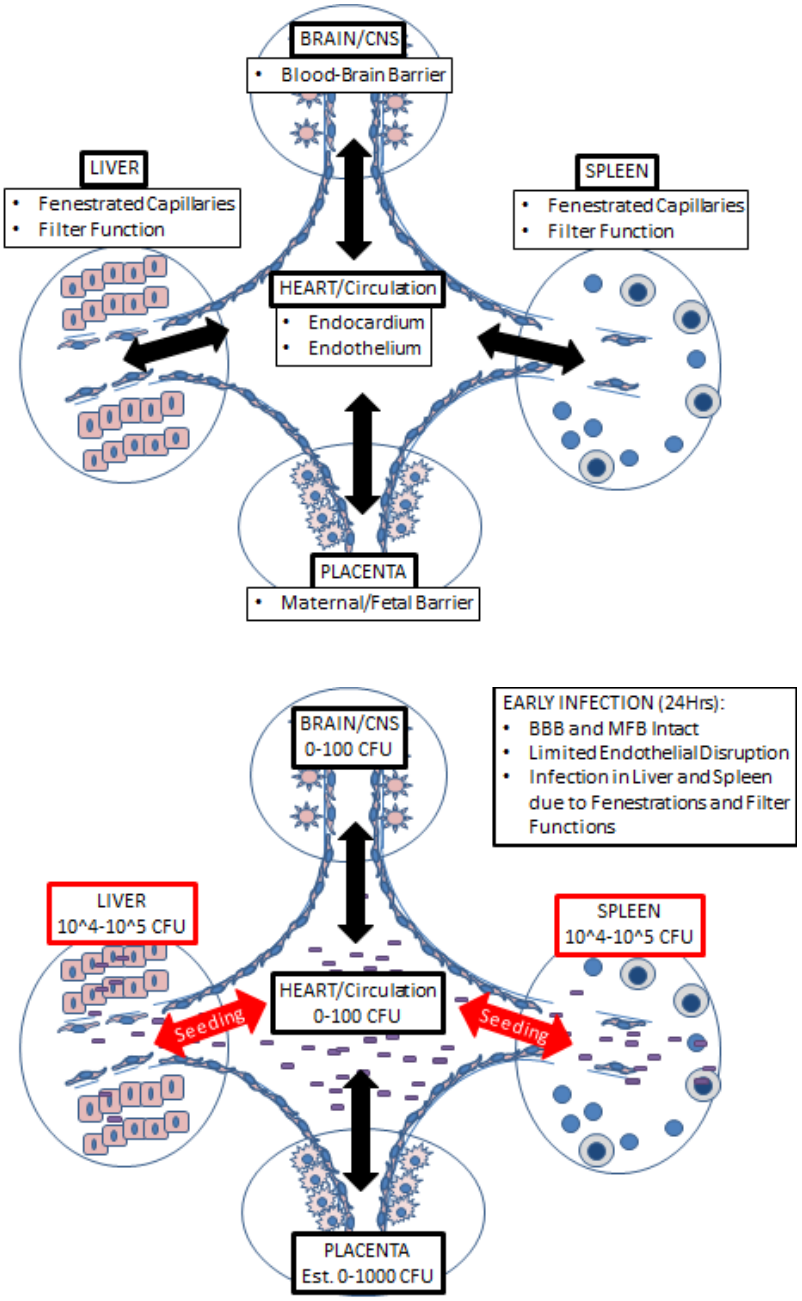


Figure 37: Relationships between initial organs of colonization and the progression of infections to include additional organ systems. The capillary structures of the liver, spleen, brain, and placenta are highlighted. Additionally, the flow of sterile (black) or infected (red) blood to and from each organ is depicted. Colonization begins in the liver and spleen, and progresses to include the CNS, heart, and placenta (where present). Typical burdens within these organs are shown for laboratory experiments involving animals.

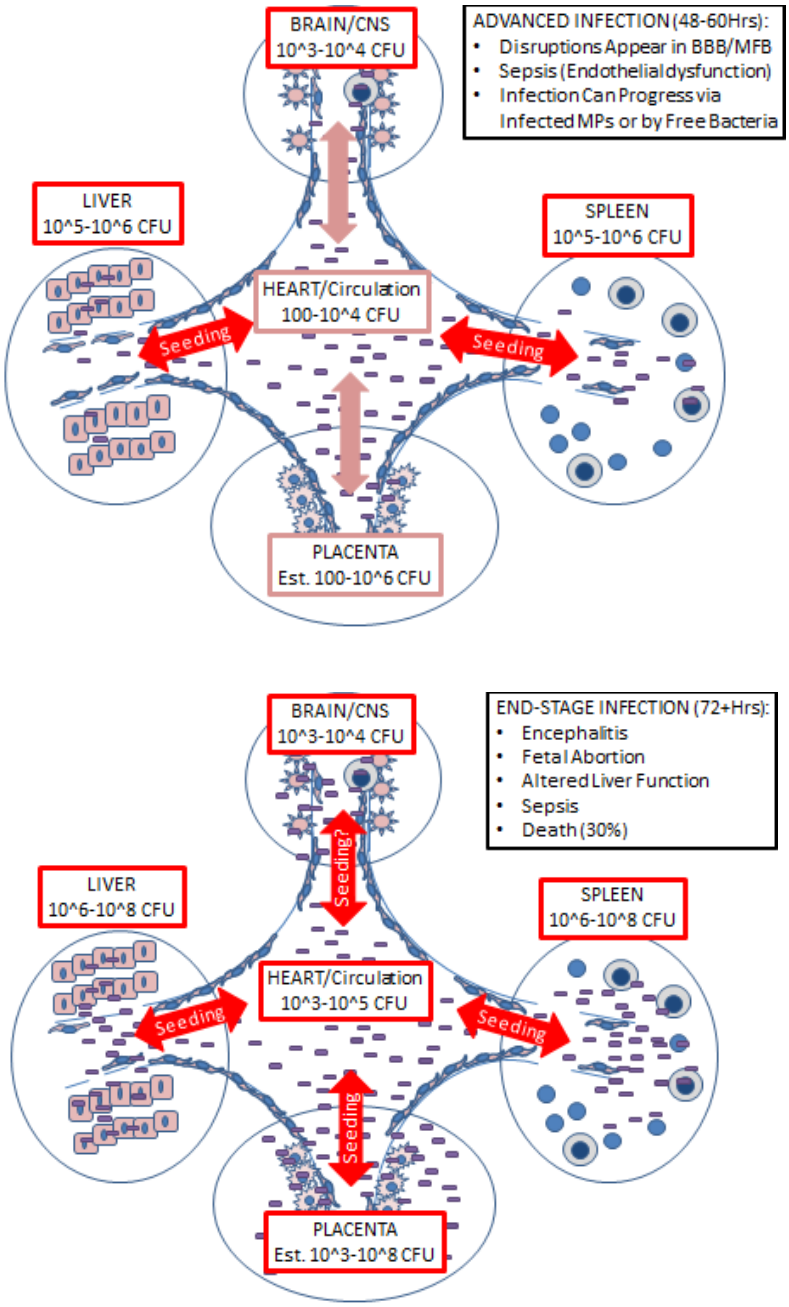


Figure 38: Relationships between initial organs of colonization and the progression of infections to include additional organ systems, continued. The capillary structures of the liver, spleen, brain, and placenta are highlighted. Additionally, the flow of sterile (black) or infected (red) blood to and from each organ is depicted. Colonization begins in the liver and spleen, and progresses to include the CNS, heart, and placenta (where present). Typical burdens within these organs are shown for laboratory experiments involving animals.

[33, 154]. Both the liver and spleen contain fenestrated capillaries, with the fenestrations in the spleen being large enough for entire red blood cells to egress into the organ [175]. The liver is a frequent target of cancer metastases from a variety of different tumor lineages [175], simply due to its anatomic ability to filter and trap offending agents, whether they be cancer cells or microbes. As such, the susceptibility of these organs in particular within laboratory animals is likely derived from their inherent ability to be breached and colonized by particulates in the blood stream. The second most common sites of *Listeria* infection are the CNS and placenta, which both possess barriers known for their integrity: The blood-brain barrier and the maternal-fetal barrier, respectively[149, 176]. The blood-brain barrier is composed of non-fenestrated endothelial cells, which are intimately attached to a layer of pericytes, creating a double layer of cells between the blood stream and the parenchymal cells of the brain [154, 175]. This barrier is further reinforced by astrocytes, which adhere tightly to the pericytes via specialized foot-processes. The maternal-fetal barrier is composed of two cell types: Syncytiotrophoblasts and cytotrophoblasts [149]. Syncytiotrophoblasts were studied in Chapter Four of this thesis, and represent the primary cellular barrier between the maternal and fetal blood supplies [150]. Syncytiotrophoblasts lie beneath a non-fenestrated endothelial layer complete with basement membrane, which itself is still a more integrated barrier than that which is found in the liver or spleen [154].

In any organ in the body, elevated levels of cytokines produced by the host in response to the bacterium cause isolated patches of endothelial dysfunction [177]. As the levels of inflammatory mediators rise during the infection, endothelial dysfunction becomes more widespread, and begins to effect the integrity of both barrier systems in two ways: 1.) Through the loss of endothelial cells directly through a shedding mechanism, and 2.) Increased leukocyte trafficking

into the affected area [178, 179]. The loss of endothelial integrity exposes the underlying organs to free bacteria within the blood. Additionally, the leukocytes targeting these regions may themselves be carrying *Listeria*, thus directly facilitating the bacterium's spread into novel sites of replication [23]. If the strain is competent at replicating within the tissue it has invaded, levels of bacteria within the organs can reach such high levels that the bacteria begin to seed from these regions back into the blood stream [148].

With this in mind, it is clear that a variety of forces can coalesce to create the manifestation of "tissue tropism." Not only is it heavily influenced by bacterial derived factors, as discussed throughout this text, but may also be altered substantially by aspects of the particular host involved. Many, if not most, of the people exposed to *Listeria* are completely capable of resisting colonization altogether, thus limiting the bacterium to its environmental niche. But through a number of selection events, likely in mammals outside of humans, the bacterium has evolved capacities that allow it access to numerous sites within susceptible targets. The imperfect selection of virulence factors in the external environment for human virulence likely translates to variable amounts of pathogenesis across different strains, possibly creating some strain-specific manifestations of disease. Cellular invasion is but one aspect of *Listeria*'s lifecycle that has been shaped by these pressures, and tissue-specific pressures for the rest of its lifecycle may exist as well.

6.5 – How the Changes to 07PF0776's InlB Promote Cardiac Infections

In the pursuit of identifying the mechanism behind 07PF0776's cardiotropic InlB, we sought to characterize how the molecule behaved in the context of its known activities. The mechanism

of action for InlB was discussed in both Chapter One and Chapter Three, but briefly recall that it has primarily three steps: dissociation from the bacterial surface, association with the host surface, and direct binding to c-Met (Fig 39). Our investigations revealed that the first step appears to be altered, such that the 07PF0776 allele responds to lower concentrations of less sulfated forms of heparan sulfate (Fig. 24, 25), while retaining sensitivity to heparin (Fig. 25). This *in vitro* result implies changes in the first and second mechanistic aspects of InlB's influence on bacterial invasion. We thus reasoned that the increase of invasion in myocytes (where heparan is less sulfated) with retained invasion in hepatocytes (where heparan is highly sulfated and more similar to heparin) was likely responsible for the ability of 07PF0776 InlB to enhance cardiac invasion while retaining high invasivity for hepatic cells (Fig. 39). But how do we explain the increased sensitivity to heparan sulfate when the electrostatic plots of the variant InlB clearly demonstrate a *loss* of positive surface charge (Fig. 20)? Recall from Chapter Three that many heparan-binding factors, such as growth factors and coagulation factors, have alternative variants capable of binding heparan with different affinities [133, 180, 181]. This feature of eukaryotic heparan-binding factors does in fact play a substantial role in directing variants to particular tissues [182]. While we did not directly demonstrate altered interactions with the surface of intact host cells, the altered interactions with proteoglycans seen in the 07PF0776 InlB likely belie its ability to act across tissues.

Our conservative mechanism of action has so far focused on the migration of InlB from the bacterial surface – which was demonstrated to be enhanced for the 07PF0776 variant under the influence of a less-negative heparan sulfate moiety. Because of InlB's interaction with both lipoteichoic acid and heparan sulfate, the loss of positive charges on the surface likely reduces interactions with lipoteichoic acid on the bacterial surface, while clearly retaining affinity for

more negative molecules, such as heparan and heparin. This basic mechanism imagines the process as a "tug of war" between the negative changes on the bacterial and host surfaces, with the amount of positive charge on InlB contributing to aspects of both retention to and migration from the bacterial cell. C-met itself is conserved across tissues as a dependence receptor for HGF-mediated tissue responses to injury [183]. While it is known that transcripts of c-Met can be alternatively spliced (and thus produce variant isoforms of the receptor), expression profiles suggest that the canonical isoform is expressed universally across a variety of cell types [184, 185]. The biological significance of c-Met variants, outside of their capacity to promote tumor formation, remains unclear [186]. Additionally, no studies have been undertaken to assess if any tissue-specific distribution exists for splice variants of c-Met. Since all InlB variants were capable of high levels of invasion in hepatocytes, it seemed unlikely that binding to c-Met itself was altered. Additionally, cell-specific enhancements were seen in fibroblasts for the 10403S InlB compared to 07PF0776's InlB (Fig. 34), again supporting a role for potentiating factors apart from InlB's interaction with c-Met.

Proteoglycans are typically described as "bottle-brush" structures, and are composed of a core protein which is heavily decorated with glycosaminoglycans such as heparan sulfate, dermatan sulfate, or chondroitin sulfate [154]. Their role in promoting signaling events through c-Met have been demonstrated for both the natural ligand, HGF, and for InlB [127, 187]. While HGF can interact with a variety of glycosaminoglycans to stimulate signaling through c-Met [188], InlB has been specifically shown to require heparan sulfate, and does not respond to other glycosaminoglycans including dermatan sulfate or chondroitin sulfate [122, 127]. While this narrows our characterizations considerably, heparan sulfate remains one of the most diverse moieties in the mammalian host [129]. It is not only altered by tissue-specific synthesis

machinery, but can also be modified extensively under a variety of stimuli [133, 182, 189]. The specific differences in molecular structure from organ to organ have been studied in great detail, and demonstrate that even though "heparan sulfate" is present at comparable levels in both the heart and liver, the molecular patterns of disaccharide composition between the two organs are quite different [129]. Additionally, the dominant proteoglycan core proteins expressed within the heart are structurally different than those expressed within the liver [190]. In myocytes, the dominant proteoglycan core proteins tend to be glypicans [191], which have their glycosaminoglycan chains proximal to the surface of the host membrane [192], and thus are more distant from the incoming bacterium. In the liver and placenta, the predominant heparan sulfate proteoglycans are typically the syndecans [193, 194], which possess glycosaminoglycan chains further from the host surface [192], thus closer to the incoming organism. The synthesis of these studies suggests that a bacterium contacting hepatocytes would be more likely to encounter highly-sulfated, heparin-like sidechains than one would on myocytes (where the heparan moieties are less sulfated and closer to the host membrane). Figure 40 illustrates how proteoglycan structure and disaccharide charge may intertwine to create tissue-specific effects.

This hypothesis begs the question, why is this InlB heart specific? If recognizing forms of heparan sulfate which contain less heparin moieties is an aspect of the mechanism, and most cell types have less heparin moieties than the liver, why does this strain not invade all cell types at a greater capacity, thus colonizing every organ in an infected animal? The answer likely lies within the roles of the organs themselves. Recall that both the liver and spleen (primary targets

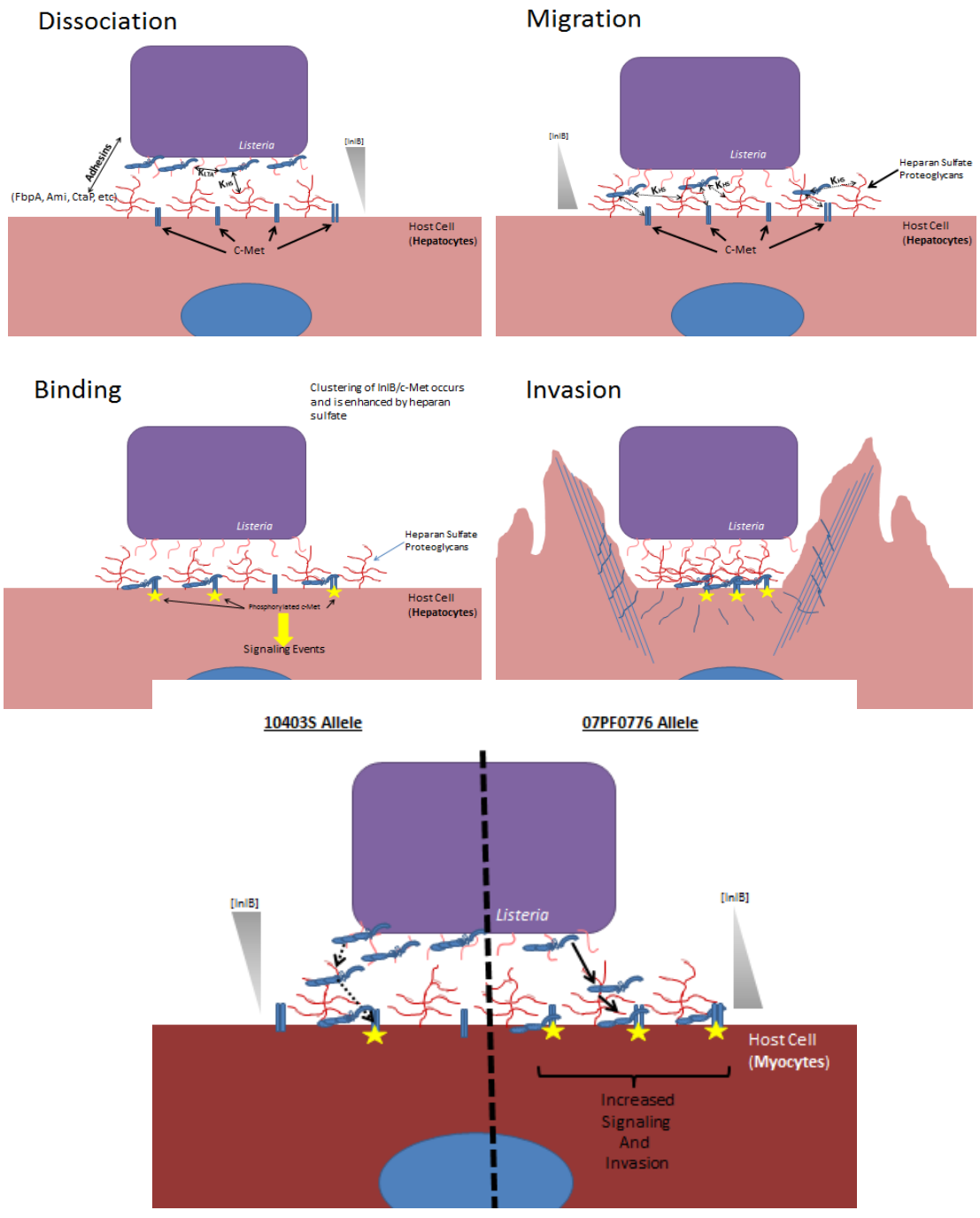


Figure 39: An overview of the invasion mechanism associated with InlB and how it is altered in myocytes for the 07PF0776 variant. InlB is retained on the bacterial surface through electrostatic interactions with lipoteichoic acid until it encounters a susceptible host cell. At this point, highly charged heparan sulfate moieties remove InlB from the bacterial surface and promote its migration to the host ligand, c-Met.

of *L. monocytogenes* in laboratory animals) have been characterized as "blood filters," which collectively serve to sequester blood-borne organisms to sites where neutrophils and other mediators of immunity can better target offending organisms [33, 154]. The structure of capillary beds within these organs also promotes colonization by microbes, as the endothelial layer within both the liver and spleen is highly discontinuous and provides very little resistance to microbial egress from the blood into the organ [154]. The heart serves primarily as the moving force of the blood which is filtered by both the liver and spleen, and thus is naturally exposed to 100% of the cardiac output it generates. As a result of its anatomy, the heart in particular is extremely susceptible to colonization by microbes from every domain of life, as mentioned in the first portion of this chapter. In short, while heparan sulfate moieties likely explain the enhanced function of 07PF0776's InlB in the heart, the anatomic structure of mammals predisposes the heart to microbial exposure during infection, and is likely responsible for the cardiac specific nature of the variant InlB in regards to tissue tropism.

The specific selection mechanisms for or against InlB activity in heart remain to be elucidated, and are likely numerous. Given the findings presented in Chapter Three, two key aspects of future investigation should include the specific interactions with these diverse heparan moieties, as well as the contribution of protein stability in the presence of tissue-specific proteases. Heparan environments can be modified *in vitro* using commercially available enzymes, including sulfatases, epimerases, and acetylases [182]. The artificial modification of these moieties in tissue culture could be used in future studies to determine the forces underlying these effects we observed. The local protease environment can also be modulated by external forces, such as siRNA treatments and specific inhibitors of proteases [195, 196]. In support of

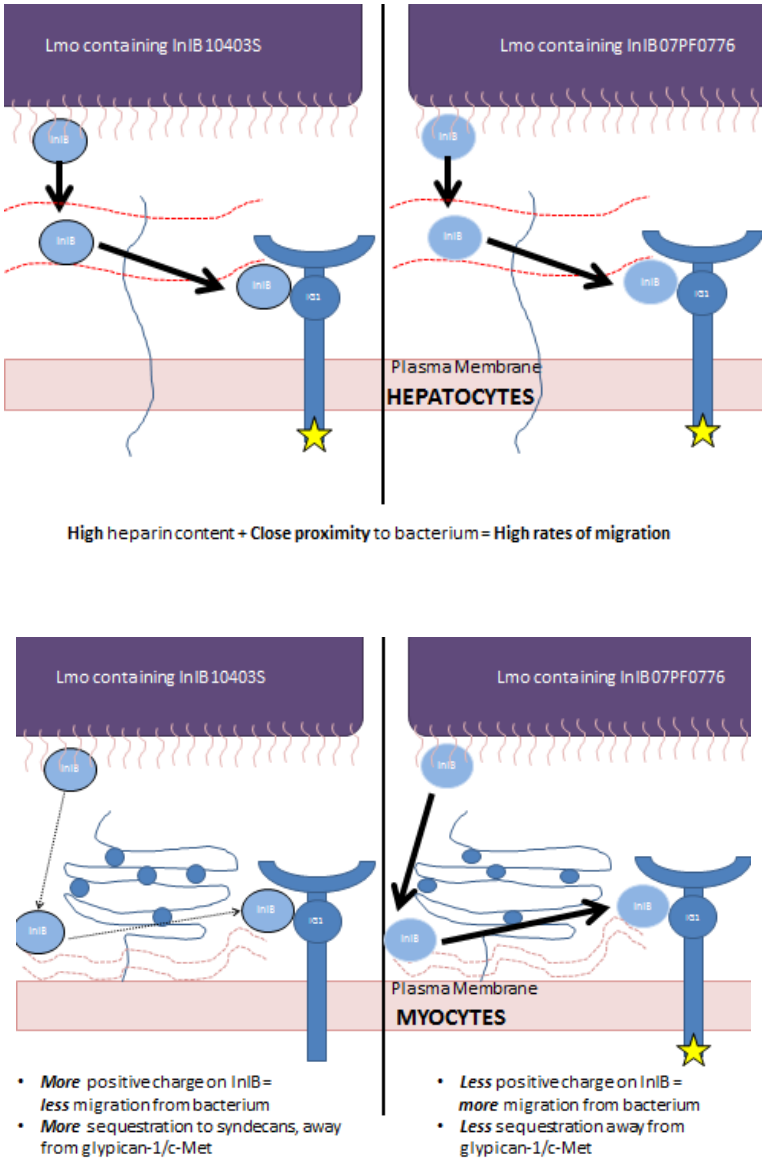


Figure 40: The potential of role of proteoglycan structure in mediating InlB dispersal from the bacterial surface for hepatocytes and myocytes. In the liver (and placenta), syndecans may exert a greater "tug" on InlB, thus mediating greater release and signaling in those organs. However, the dominance of glypican in myocytes, combined with reduced sulfation overall in the heart, may reduce the amount of dispersive force. This effect would potentially permit greater levels of migration and invasion for a variant of InlB capable of migrating in the presence of relatively lower concentrations of heparan sulfate. Additionally, what syndecans are present in myocytes appear to be sequestered away from the c-Met receptor, indicating that a molecule capable of greater levels of migration between different proteoglycan cores may inherently stimulate more c-Met.

this particular avenue of research, a preliminary assay demonstrated an allele-specific enhancement for the 10403S allele in myocytes pre-treated with protease inhibitor (data not shown). The addition of inhibitor in this experiment increased invasion to a level somewhat comparable to the 07PF0776 allele, but not fully enough to explain the entire enhancement observed in myocytes. While not complete, these studies can be expanded on to investigate the selective pressure induced by tissue proteases.

The drastic differences in cellular microenvironments, combined with the diversity of the *inlB* alleles across *L. monocytogenes* isolates, likely means that many enhancements may be found across the species. Whether these advancements in one cell type or another translate to alterations in tissue tropism depends on how these enhancements intertwine with the lifecycle evolved by *Listeria* thus far. In the case of cardiotropism, the heart itself may be at a special risk of colonization solely because of its role in circulating the blood during an infection. Evidence for this supposition was presented at the outset of this chapter, as a myriad of pathogens exploit the heart as a replication niche during their replicative cycle, and many of the organisms that have been isolated from the hearts of patients lack any apparent cardiotropic factors that specifically function to promote cardiac invasion. Thus while the individual mutations present across the alleles of *inlB* may have altered activities across a number of cell types, the ability of this activity to translate into replicative success in the host depends upon exposure to that organ and the resistance of that particular organ to colonization.

The heart, with its central location and critical role in host viability, may simply be the next most accessible target outside of the liver and spleen for *Listeria* in non-pregnant animals. Our studies into pregnant animals have so far demonstrated that InlB variants do not enhance placental (and thus) fetal colonization. This result may be somewhat expected, as the relative

level of heparanated syndecans in the placenta is very high [194]. The abundance of this particular proteoglycan in both the liver and placenta, with its heparan side chains relatively close to an incoming bacterium, may negate any allele-specific enhancements for variants of InlB, as the closeness of heparan moieties to the bacterium precludes a need to migrate from the surface more efficiently.

6.6 - The Dynamic Between InlA and InlB as Tropic Factors for *Listeria*

Throughout our studies, we have observed sometimes conflicting roles for InlA and InlB in the process of bacterial invasion *in vitro*. Such examples of conflicting activity include the negative effect of InlA on the invasion of H9c2 cardiac cells and L2 fibroblast cells, while InlB demonstrates a positive role in these cells (Chapter Five). While expression of either the 10403S or 07PF0776 InlA allele was not associated with any detectable perturbations of tissue tropism in mice, this may be due to the species-specificity of the molecule. Our data in Caco-2 cells (Fig. 32), H9c2 cells (Fig. 31) and BeWo cells (Fig. 27) indicate that allelic variants of InlA may demonstrate altered invasion in some cell lines, and that this altered invasion may be due to differences in the repressive effect of InlA in some cell lines. But, what are some explanations for the interactions between these two molecules – such that sometimes they behave synergistically, independently, or competitively? One of the first observations noted in our studies was the ability of InlA over-expression to reduce the amount of surface-associated InlB (Fig. 36). Conversely, in strains where InlA was present, increasing levels of InlB enhanced the amount of surface InlA somewhat (Fig. 36). Thus there is a clear relationship between the levels of either InlA or InlB and its cognate internalin. This relationship may account for differences in

invasivity between isolates of *Listeria* that have different levels of either InlA or InlB compared to more well-studied variants of the species.

Suppose, for instance, that high expression of InlA on the surface results in an altered surface phenotype as a consequence of InlA's covalent attachment to peptidoglycan. An overwhelming amount of covalently attached InlA may act to disperse InlB into the supernatant (as it appears to do in our western blots), potentially as a result of steric interference preventing the association between InlB and lipoteichoic acid. This reduction in InlB surface association would result in less InlB being present at the time of bacterial effacement of the host cell – meaning there would be *less* of an effect for InlB in mediating invasion. In this way, a strain of *Listeria* with an InlA-dominant surface would be reduced for InlB-mediated invasion, and thus potentially be less likely to colonize sites where InlB is required. In the studies detailed in Chapter Five, primarily those involving H9c2 cardiac myocyte cells, there is a clear enhancement of the 07PF0776 allele of *inlA* to permit invasion over the level of the 10403S allele, indicating potential differences in this repressive effect (Fig. 31). These intriguing results seem to indicate that a potential difference between the InlA variants lies in how they interact with InlB on the bacterial surface.

While we have not yet undertaken studies that would clarify this relationship further, there are some features of both internalins from 07PF0776 that have the potential to alter this relationship. As mentioned briefly in the previous section, one of the hypotheses involves changes in susceptibility of the internalins to either bacterial or host proteases. Proteases play a tremendous role in mediating the transitions between host environments for *Listeria*, and many bacterial factors can be targeted by several classes of proteases and chaperones [1, 197, 198]. If the internalins of the 07PF0776 variant were more, or less, susceptible to proteases – either bacterial or host – it may potentially reduce the amount of active InlA/B on the surface at the time of

interaction between the bacteria and host, independent of either protein's transcriptional levels of expression. In particular, the 07PF0776 variant (along with the J4403 and other cardiotropic variants of InlA) has a point-mutation in the Ig-fold region of InlA that introduces a predicted cleavage site for metalloproteinases in a region of the protein with little intrinsic structure. Metalloproteases can not only be found in a variety of different host sites, but are also produced by the bacterium in order to activate its phospholipase [56, 63]. Thus, proteases may impact protein survivability both near and far from host surfaces. This may directly contribute to the retention and thus activity of other virulence determinants, including InlB, for individual strains of *Listeria*, thus impacting survival in particular cell types.

While the susceptibility of the proteins to proteases is one avenue of future study, there are other potential explanations for the interaction between InlA and InlB. Another possibility is that the 07PF0776 variant of InlA is capable of weakly binding InlB on the bacterial surface, such that InlA now acts as a weak reservoir for InlB, rather than a dispersant. If this InlA variant was capable of binding to InlB directly, the presence of InlB may mask the effects of InlA somewhat. This hypothesis is somewhat supported by the experiments performed in BeWo cells mentioned previously in Chapter Four, where chromosomal InlB in 10403S seems to interfere with the ability of the 07PF0776 variant of InlA to mediate uptake into the BeWo cell line (Fig. 30 top right panel). Additionally, the genes for these proteins are encoded in an operon, and have multiple different regulatory mechanisms. Post-transcriptional regulation of InlA has been demonstrated previously [40, 199], thus it is possible that alterations to transcription factors outside of the *inlAB* operon may influence the relative levels of InlA to InlB. Further studies into the relationship between the mature proteins encoded by the *inlAB* operon are needed to fully understand the apparent cell specific cooperativity and competing activities between them. These

interactions, combined with the allele-specific differences gained through genetic drift or horizontal DNA exchange, have the potential to alter invasivity at particular cell types, which may influence virulence and tropism for *Listeria* at large.

6.7 - InlB as a Mediator of Nutritional Immunity

The greatest portion of this thesis has focused on the activity of InlB as a cellular invasin. A perhaps greater portion of our future thought, however, should be directed to InlB as a mediator of physiologic change within cells – perhaps within tissues as well. This consideration comes from the realization many of the readouts of InlB activity in tissue culture (i.e. cell scattering, wound healing) are, in fact, the result of orchestrated signaling events within the cell [121]. These phenotypes have been replicated in various cancers through manipulation of the same pathways involved in InlB signaling [200]. Indeed, principle pathways of InlB's effects include the PI3K, MAPK, AMPK, and ERK pathways [52, 201], which have all been extensively researched in the cancer field as pro-survival mediators in tumor cells. Each of these mediators, in turn, initiates cascades that promote a variety of cellular functions, including resistance to apoptosis [202, 203] , aspects of ROS control [120], and resistance to DNA fragmentation [200]. Of interest specifically to host-pathogen interactions, the perturbations can also include altered innate immune signaling and function of immune cells [204-207]. In support of this, InlB has been shown to alter aspects of host immunity specifically through c-Met, including the production of IL-6 by mast cells in the blood [208].

Of equal interest to bacterial physiologists is the potential that these pathways ultimately provide the bacterium benefit through the process of *nutritional immunity*, in which the

bacterium directly manipulates the physiology of infected cells such that selective pressures within the cell are reduced. Simply put, InlB may flip a switch that promotes reorganization of cellular processes so both local innate immunity and intracellular nutrient processes are both profoundly altered in such a way that bacterial survival is improved, independent of InlB's functions as a bacterial invasin. In many cancer cell lines, for instance, a mutation which generates hyperactivity along the c-Met axis is capable of mediating the process of *Epithelial-to-Mesenchymal Transition (EMT)* [209]. This process is marked by the de-differentiation of epithelial structure to one more closely resembling fibroblasts and other mesenchymal cell types commonly found in primordial developing embryos [210]. In agreement with this, those cells under the influence of c-Met may demonstrate invasivity in tissues – a feature shared with primordial migratory cells in the developing embryo [211]. Features of EMT also included perturbations to tight junctions between cells, including alterations to the levels of E-cadherin (recall, E-cadherin is a ligand for InlA) [212, 213]. In epithelial cells, there is a discrepant loss of E-cadherin and a conversion to N-cadherin [212], and such changes are thought to partially explain the altered migration exhibited by EMT. Additionally, changes to nutritional states within cancer cells have been documented to occur under the influence of EMT-associated pathways, such as the AMPK pathway [214]. In short, when the pathways downstream of c-Met become activated to the extent that cells begin to demonstrate phenotypic effects *in vitro* (such as they do with InlB stimulation of c-Met), profound changes in cellular physiology are likely to have already occurred.

Since the initial pathways stimulated by InlB match those involved in EMT in other cell lines, and the physiologic outputs of InlB match those seen in EMT, the role of InlB in inducing EMT should be considered a priority for future studies. The literature to date provides the beginning

events (c-Met stimulation) and the end result (changes to cellular physiology), but what remains to be seen is whether InlB is capable of activating specific mediators of these transition processes. Transcriptional regulators play a crucial role in the dedifferentiation process and their role resides directly between the signaling events initiated by InlB and the phenotypic outputs of InlB stimulation. Chief among these factors are the nuclear factors Snail and Slug, which have been implicated specifically in the wound-healing type of EMT [215]. (Recall that purified InlB is capable of mediating wound healing across a variety of cell types and has been studied as a potential wound-healing adjuvant [162]). The fact that outputs specific to EMT are observed for cells under the influence of InlB is extremely suggestive that the bacterium could manipulate the cellular reversion process for its purposes. Examples of both bacterial and viral-induced EMT have been reported previously, suggesting that some pathogens may utilize these mechanisms to "rewire" cellular physiology to their ultimate benefit [212, 216]. It remains to be seen whether or not *Listeria* is among those pathogens capable of mediating this change, however the evidence is extremely suggestive that this is the case (Fig. 41). If a causal link is found for InlB in the induction of EMT, it is also possible that variants of InlB may induce varying degrees of the process, in much the same way variants of InlB inducing varying degrees of invasion across multiple cells lines.

6.8 - The Link between Bacterial Genetics and Tissue Tropism

The acquisition of novel virulence characteristics can be associated with great alterations in disease. For enteric pathogens, the classification of disease itself can be correlated directly with the presence of known aggravating factors in the bacterial genome, such as virulence plasmids in

Escherichia coli or pathogenicity islands in *Salmonella typhi* [154]. As a result, the genotyping of these organisms can be used to directly predict disease outcome and severity, especially in the context of large-scale outbreaks, where multiple similar cases arise almost simultaneously. Unfortunately, our ability to classify microbial disease in this manner is limited. It is apparent for many pathogens, including some strains of *Streptococcus* and *Staphylococcus* that enhancements to disease or pathophysiology occur as a result of random alterations to commonly-shared virulence factors [154].

Our data are not the first studies into the allele-specific activities of virulence factors in strains of *Listeria* isolated from diverse sources [164, 165], but they are the first to investigate the contribution of these factors to activities outside of the typical *Listeria* lifecycle. For this environmental pathogen capable of surviving diverse stresses, transitioning to a human host is effectively a dead end, as no human to human transmission occurs. Thus, the genomic differences present across isolates of *Listeria* may arise specifically from selective pressures outside of humans, possibly in animals where transmission has been shown to occur via fecal-oral transmission. In this regard, when an infection does occur, the ability of the bacterium to proliferate and survive in as many environments as possible before host demise likely represents the evolutionary priority for *Listeria*. 07PF0776 has a demonstrated ability to proliferate in the hearts, placentas, and fetuses of infected animals to a greater amount than 10403S and other strains, indicating its potential to spread quickly within the model host. Our data suggests that the alterations present within conserved virulence factors underlie the ability of this strain to spread so quickly and efficiently.

While inconvenient for classifying disease based solely on bacterial genetics, our studies indicate that variants of known virulence factors can be categorized in a broader way apart from

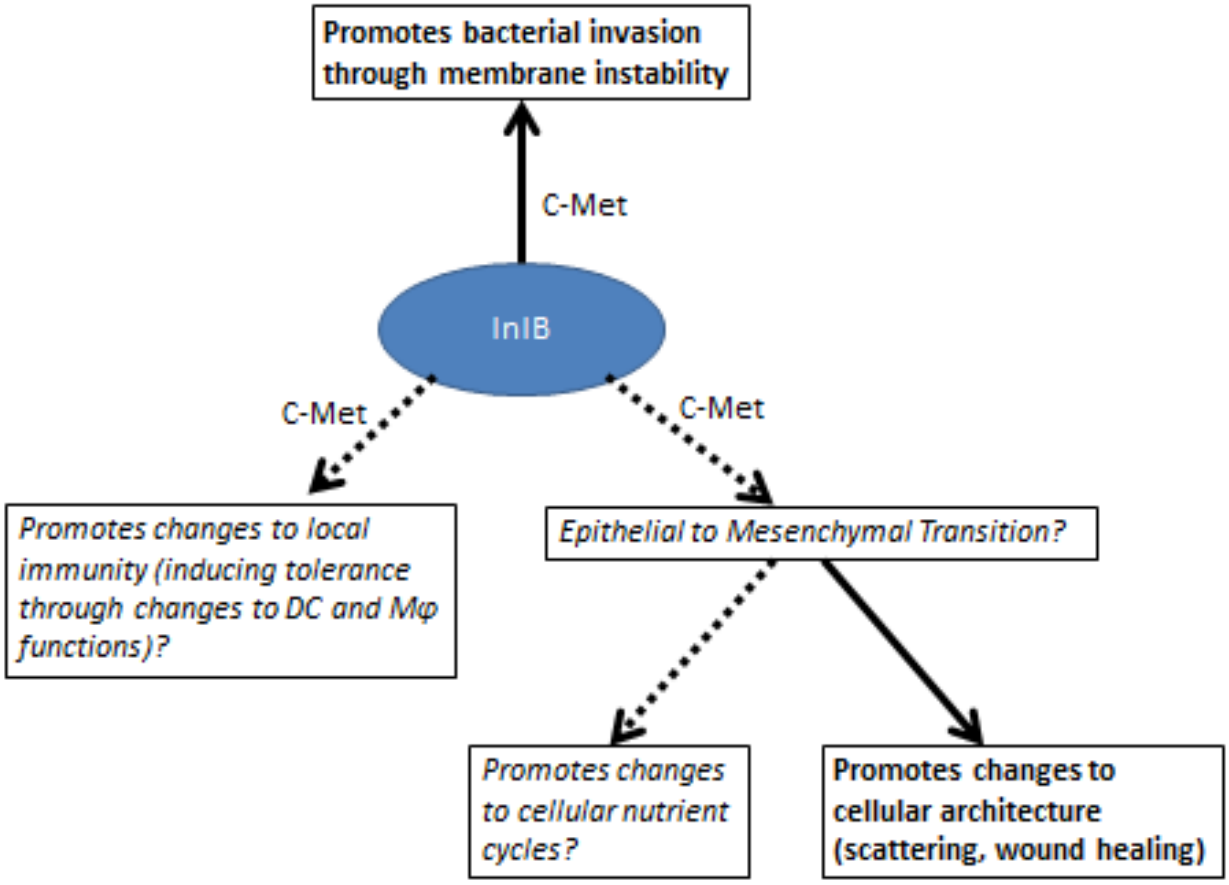


Figure 41: InlB signaling through c-Met may produce multifaceted responses, each of which is capable of modulating host physiology for the benefit of the bacterium. InlB has been conclusively linked to bacterial invasion and changes in cellular architecture (including cell-scattering and wound healing phenotypes). However, multiple effects of c-Met stimulation are known outside of these phenotypes, and include changes to local immunity, nutrient states, and dedifferentiation of cellular morphology (epithelial to mesenchymal transition). Those pathways that have been demonstrated to be involved in InlB's mechanism of action are represented by solid arrows and bold text, whereas those which remain to be investigated are drawn with dashed arrows and italicized text.

genetics (including protein stability and electrostatic features). The characterization of these factors, particularly from interesting or unique isolates such as 07PF0776, may ultimately lead to the identification of enhancing residues, such as D371 or the altered GW motifs, within known virulence factors which alter disease outcome. Our understanding of virulence for *Listeria* in the future will likely expand as we continue to investigate both the genetic and physiologic diversity present within the species. Once we begin to associate phenotypes with specific genetic patterns, we can begin to classify disease outcome based on bacterial genetics – the initial goal of our inquiries. Such classification schemes could potentially be used to identify particularly virulent isolates during the early course of an outbreak, and potentially predict patient populations susceptible to a particular clone. While studying the specific contributions of allelic variance to virulence within hosts is daunting, such studies also inform us of the greater nature of the species as a whole and demonstrate that uniqueness among microbes and hosts creates unique interactions between living, competing forces.

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8.) APPENDIX

8.1) Table of Strains used in these Studies

STRAIN	DESCRIPTION
10403S	Laboratory strain, originally derived from a skin abscess
10403S Δ*inlA*	10403S with an in-frame deletion of *inlA*, the coding sequence of Internalin A
10403S Δ*inlB*	10403S with an in-frame deletion of *inlB*, the coding sequence of Internalin B
10403S Δ*inlAB*	10403S with an in-frame deletion of *inlAB*, the operon sequence for both Internalin A and B
10403S Δ*inlA:: pIMK4(InlA-10403S)*	10403S with an in-frame deletion of *inlA*, complemented with an IPTG-inducible, chromosomally integrated vector. Chromosomal InlB is present, InlA level maxes at 80% 10403S expression. InlA Allele expressed is 10403S.
10403S Δ*inlAB:: pIMK4(InlA-10403S)*	10403S with an in-frame deletion of *inlAB*, complemented with an IPTG-inducible, chromosomally integrated vector. Chromosomal InlB is absent, InlA level maxes at 80% 10403S expression. InlA Allele expressed is 10403S.
10403S Δ*inlA:: pIMK4(InlA-07PF0776)*	10403S with an in-frame deletion of *inlA*, complemented with an IPTG-inducible, chromosomally integrated vector. Chromosomal InlB is present, InlA level maxes at 80% 10403S expression. InlA Allele expressed is 07PF0776.
10403S Δ*inlAB:: pIMK4(InlA-07PF0776)*	10403S with an in-frame deletion of *inlAB*, complemented with an IPTG-inducible, chromosomally integrated vector. Chromosomal InlB is absent, InlA level maxes at 80% 10403S expression. InlA Allele expressed is 07PF0776.
10403S Δ*inlB:: pIMK4(InlB-10403S)*	10403S with an in-frame deletion of *inlB*, complemented with an IPTG-inducible, chromosomally integrated vector. Chromosomal InlA is present, InlB level maxes at 80% 10403S expression. InlB Allele expressed is 10403S.
10403S Δ*inlAB:: pIMK4(InlB-10403S)*	10403S with an in-frame deletion of *inlAB*, complemented with an IPTG-inducible, chromosomally integrated vector. Chromosomal InlA is absent, InlB level maxes at 80% 10403S expression. InlB Allele expressed is 10403S.
10403S Δ*inlB:: pIMK4(InlB-07PF0776)*	10403S with an in-frame deletion of *inlB*, complemented with an IPTG-inducible, chromosomally integrated vector. Chromosomal InlA is present, InlB level maxes at 80% 10403S expression. InlB Allele expressed is 07PF0776.

STRAIN	DESCRIPTION
10403S Δ*inlAB:: pIMK4(InlB-07PF0776)*	10403S with an in-frame deletion of *inlAB*, complemented with an IPTG-inducible, chromosomally integrated vector. Chromosomal InlA is absent, InlB level maxes at 80% 10403S expression. InlB Allele expressed is 07PF0776.
10403S Δ*inlA:: pIMK2(InlA-10403S)*	10403S with an in-frame deletion of *inlA*, complemented with a constitutively over-expressing, chromosomally integrated vector. Chromosomal InlB is present, InlA level maxes at ~3-5x 10403S expression. InlA Allele expressed is 10403S.
10403S Δ*inlAB:: pIMK2(InlA-10403S)*	10403S with an in-frame deletion of *inlAB*, complemented with a constitutively over-expressing, chromosomally integrated vector. Chromosomal InlB is absent, InlA level maxes at ~3-5x 10403S expression. InlA Allele expressed is 10403S.
10403S Δ*inlA:: pIMK2(InlA-07PF0776)*	10403S with an in-frame deletion of *inlA*, complemented with a constitutively over-expressing, chromosomally integrated vector. Chromosomal InlB is present, InlA level maxes at ~3-5x 10403S expression. InlA Allele expressed is 07PF0776.
10403S Δ*inlAB:: pIMK2(InlA-07PF0776)*	10403S with an in-frame deletion of *inlAB*, complemented with a constitutively over-expressing, chromosomally integrated vector. Chromosomal InlB is absent, InlA level maxes at ~3-5x 10403S expression. InlA Allele expressed is 07PF0776.
10403S Δ*inlB:: pIMK2(InlB-10403S)*	10403S with an in-frame deletion of *inlB*, complemented with a constitutively over-expressing, chromosomally integrated vector. Chromosomal InlA is present, InlB level maxes at ~3-5x 10403S expression. InlB Allele expressed is 10403S.
10403S Δ*inlAB:: pIMK2(InlB-10403S)*	10403S with an in-frame deletion of *inlAB*, complemented with a constitutively over-expressing, chromosomally integrated vector. Chromosomal InlA is absent, InlB level maxes at ~3-5x 10403S expression. InlB Allele expressed is 10403S.
10403S Δ*inlB:: pIMK2(InlB-07PF0776)*	10403S with an in-frame deletion of *inlB*, complemented with a constitutively over-expressing, chromosomally integrated vector. Chromosomal InlA is present, InlB level maxes at ~3-5x 10403S expression. InlB Allele expressed is 07PF0776.
10403S Δ*inlAB:: pIMK2(InlB-07PF0776)*	10403S with an in-frame deletion of *inlAB*, complemented with a constitutively over-expressing, chromosomally integrated vector. Chromosomal InlA is absent, InlB level maxes at ~3-5x 10403S expression. InlB Allele expressed is 07PF0776.
07PF0776	Clinical isolate derived from a myocardial abscess
07PF0776 Δ*inlB*	07PF0776 with an in-frame deletion of *inlB*, the coding region which encodes the invasin Internalin B
J4403	Clinical Isolate derived from pericardial fluid

STRAIN	DESCRIPTION
07PF0776 Δ*inlB::* *pIMK2(InlB-10403S)*	07PF0776 with an in-frame deletion of *inlB*, complemented with a constitutively over-expressing, chromosomally integrated vector. Chromosomal InlA is present, InlB level maxes at ~2-3x 07PF0776 expression. InlB Allele expressed is 10403S.
07PF0776 Δ*inlB::* *pIMK2(InlB-07PF0776)*	07PF0776 with an in-frame deletion of *inlB*, complemented with a constitutively over-expressing, chromosomally integrated vector. Chromosomal InlA is present, InlB level maxes at ~2-3x 07PF0776 expression. InlB Allele expressed is 07PF0776.
10403S Δ*inlB::* *pIMK2(InlB-D371N)*	10403S with an in-frame deletion of *inlB*, complemented with a constitutively over-expressing, chromosomally integrated vector. Chromosomal InlA is present, InlB level maxes at ~3-5x 10403S expression. InlB Allele expressed is 07PF0776 with aspartic acid 371 mutagenized to an asparagine.
10403S Δ*inlAB::* *pIMK2(InlB-D371N)*	10403S with an in-frame deletion of *inlAB*, complemented with a constitutively over-expressing, chromosomally integrated vector. Chromosomal InlA is absent, InlB level maxes at ~3-5x 10403S expression. InlB Allele expressed is 07PF0776 with aspartic acid 371 mutagenized to an asparagine.
07PF0776 Δ*inlB::* *pIMK2(InlB-D371N)*	07PF0776 with an in-frame deletion of *inlB*, complemented with a constitutively over-expressing, chromosomally integrated vector. Chromosomal InlA is present, InlB level maxes at ~2-3x 07PF0776 expression. InlB Allele expressed is 07PF0776 with aspartic acid 371 mutagenized to an asparagine.
10403S Δ*inlB::* *pIMK2(InlB-J4403)*	10403S with an in-frame deletion of *inlB*, complemented with a constitutively over-expressing, chromosomally integrated vector. Chromosomal InlA is present, InlB level maxes at ~3-5x 10403S expression. InlB Allele expressed is J4403.
10403S Δ*inlAB::* *pIMK2(InlB-J4403)*	10403S with an in-frame deletion of *inlAB*, complemented with a constitutively over-expressing, chromosomally integrated vector. Chromosomal InlA is absent, InlB level maxes at ~3-5x 10403S expression. InlB Allele expressed is J4403.
07PF0776 Δ*inlB::* *pIMK2(InlB-J4403)*	07PF0776 with an in-frame deletion of *inlB*, complemented with a constitutively over-expressing, chromosomally integrated vector. Chromosomal InlA is present, InlB level maxes at ~2-3x 07PF0776 expression. InlB Allele expressed is J4403.

8.2) Table of nucleotides used in these studies

Name	Sequence and description
inlAf	5'-ATAT[CCATGG]ggAGAAAAAAAACGATATGTATGTATG-3' Forward primer for cloning the *inlA* region of 10403S and 07PF0776. NcoI site with integrated ATG start site is show in brackets. The two guanines following the NcoI site inherently make the first amino acid of the protein a glycine, and are necessary when using the NcoI site in order to prevent frameshift.
inlAr	5'-TTTT[CTGCAG]CTATTTACTAGCACGTGCTTTTTTAG -3' Reverse primer for cloning the *inlA* region of 10403S and 07PF0776. PstI site is shown in brackets.
inlBf	5'-ATAT[CCATGG]ggGTGAAAGAAAAGCACAACC-3' Forward primer for cloning the *inlB* region of 10403S, 07PF0776, and J4403. NcoI site with integrated ATG start site is show in brackets. The two guanines following the NcoI site inherently make the first amino acid of the protein a glycine, and are necessary when using the NcoI site in order to prevent frameshift.
inlBr	5'-ATAT[GTCGAC]TTATTTCTGTGCCCTTAAATTAGC -3' Reverse primer for cloning the *inlB* region of 10403S and J4403. PstI site is shown in brackets.
inlBr-1403	5'-ATAT[GTCGAC]TTATTTCTGTGCCCTTAAATTAGC -3' Reverse primer for cloning the *inlB* region of 07PF0776. SalI site site is shown in brackets.
1403dinlBf1	5' -ATATGGATCCAAACGCCAAAAGCCGGATA -3' Forward primer used for leading 500bp fragment of InlB knockout vector
1403dinlBf2	5' -CAAGGAGAGGATAGTGTGGGTACCTGACCTACGAAAAGCTATTTC -3' Forward primer used for trailing 500bp fragment of InlB knockout vector
1403dinlBr1	5' -GAAATAGCTTTTCGTAGGTCAGGTACCCACACTATCCTCTCCTTG -3' Reverse primer used for leading 500bp fragment of InlB knockout vector
1403dinlBr2	5' -ATATGTCGACGTGAAATTATTGCTGGTAGC -3' Reverse primer used for trailing 500bp fragment of InlB knockout vector

VITA

05/2008 - 05/2016 M.D.-Ph.D. University of Illinois College of Medicine

08/2004 - 05/2008 B.A Miami University, Microbiology and Molecular Biology

Membership and Honorary/Professional Societies: American Society of Microbiology, American College of Physicians, American Physician Scientist Association

Medical and Graduate School Awards: Richard and Mary Finkelstein Award (American Society of Microbiology General Meeting, Denver 2013); NIH F31Research Grant (UIC, 2012); AHA Student Research Award (UIC, 2012 - Deferred for F31); Midwest MicrobialPathogenesis Conference Travel Grant (MMPC, St. Louis 2011)

Work Experience:

08/2006 - 05/2008 Average Hours/Week: 10 Miami University , OH, United States Student Tutor, Provided one-on-one tutoring for the following courses: Botany/Microbiology/Zoology 115-116, General Chemistry 141-142, Organic Chemistry 241-242, General Microbiology 201-202, General Physics 171172, Biochemistry 331

08/2005 - 05/2008 Average Hours/Week: 10 Miami University , OH, United States Supplemental Instructor, Provided supplemental lectures and study materials for the following courses: Botany/Microbiology/Zoology 115-116, General Chemistry 141-142, Organic Chemistry 241-242, General Microbiology 201-202, General Physics 171-172, Biochemistry 331

08/2005 - 05/2008 Average Hours/Week: 15 Miami University , OH, United States Student Manager, Oversaw the hiring, duties, and discipline of student employees at an on-campus dining hall

Research Experience

05/2010 - 06/2014 Average Hours/Week: 40 University of Illinois at Chicago, IL, United States Graduate Research Assistant, PhD Candidate, Dr. Nancy Freitag Studied the role of bacterial invasins in mediating tissue tropism, specifically cardiotropism, in mice infected with Listeria monocytogenes. The project grew to include facets of cardiac, hepatic, splenic, and neurological pathology, as well as genetic variationand cloning of virulence alleles from multiple strains of Listeria. Additionally, the project involved studying the role of these allelicvariants in mediating maternal-fetal transmission in novel mouse and tissue culture models.

07/2009 - 08/2009 Average Hours/Week: 40 University of Illinois at Chicago, IL, United States Graduate Research Assistant, Dr. Nancy Freitag Constructed genetic mutants of Listeria monocytogenes that were used to study virulence factor regulation by the master regulator PrfA

05/2009 - 07/2009 Average Hours/Week: 40 University of Illinois at Chicago, IL, United States Graduate Research Assistant, Dr. Mary Jo LaDu Studied serum fractions from mice containing Alzheimer's-associated apoE alleles for potential biomarkers of disease progression using dot-blots, western blots, and fractionation

05/2008 - 08/2008 Average Hours/Week: 40 University of Illinois at Chicago, IL, United States Graduate Research Assistant, Dr. Nancy Freitag Studied the effects of genetic deletions which caused bacterial chain formation on virulence, motility, and viability in multiple settings

01/2004 - 05/2008 Average Hours/Week: 15 Miami University, OH, United States Undergraduate Research Assistant, Dr. Kelly Abshire Studied the effects of sub-lethal silver toxicity in Escherichia coli and Pseudomonas aeruginosa using 2DPAGE analysis Publications

Peer Reviewed Journal Articles/Abstracts

McMullen PD, Freitag NE.. Assessing bacterial invasion of cardiac cells in culture and heart colonization in infected mice using Listeria monocytogenes. Journal of Visual Experiments. 2015, May; 99(e52497): 10.3791/52497. Cited in PubMed; PMID: 26065439. Pub Status: Published.

Salazar JK, Wu Z, McMullen PD, Luo Q, Freitag NE, Tortorello ML, Hu S, & Zhang W. A prfA-like transcription factor gene lmo0753 contributes to L-rhamnose utilization in Listeria monocytogenes associated with human foodbore infections. Applied Environmental Microbiology. 2013, Sep; 79(18): 5584-5592. Cited in PubMed; PMID: 23835178. Pub Status: Published.

Frencher JT, Ryan-Pasyeur BK, Huang D, Wang RC, McMullen PD, Letvin NL, Collins WE, Freitag NE, Malkovsky M, Chen CY, Shen L, Chen ZW.. SHIV antigen immunization alters patterns of immune responses to SHIV/malaria coinfection and protects against life-threatening SHIV-related malaria. Journal of Infectious Disease. 2013, Apr; 208(2): 260-270. Cited in PubMed; PMID: 23568175. Pub Status: Published.

McMullen PD, Gillaspy AF, Gipson J, Bobo LD, Skiest DJ, and Freitag NE. Genome Sequence of Listeria monocytognes 07PF0776, a Cardiotropic Serovar 4b Strain. Journal of Bacteriology. 2012, Jul; 194(13): 3552.Cited in PubMed; PMID: 22689239. Pub Status: Published.

Alonzo, F III, McMullen, PD, Freitag, NE. Actin polymerization drives septation in namA hydrolase mutants, demonstrating host correction of a bacterial defect. Infection and Immunity. 2011, Jan; 79(4): 1458-1470. Cited inPubMed; PMID: 21263016. Pub Status: Published.

Peer Reviewed Book Chapters

McMullen PD, Freitag NE. (2014). Listeria monocytogenes. In Tang & Sussman & Liu & Poxton & Schwartzman (Ed.), Molecular Medical Microbiology, 2nd Edition (pp. 1345-1361). London: Elsevier.

Poster Presentation

Alonzo F III, McMullen PD*, Freitag NE. (2010). Actin Polymerization Drives Septation in NamA Hydrolase Mutants Poster presented at: Midwest Microbial Pathogenesis Conference; St. Louis, MO.

McMullen PD, Freitag NE. (2011). Probing the role of surface proteins InlA and InlB in mediating cardiotropism for Listeria monocytogenes Poster presented at: Midwest Microbial Pathogenesis Conference; Ann Arbor, MI.

McMullen PD, Freitag NE. (2012). Investigating the Tropic Effects of the InlAB locus of Listeria monocytogenes Reveals Allele-Specific Contributions to Cardiotropism Poster presented at: Midwest Microbial Pathogenesis Conference; Milwaukee, WI.

McMullen PD, Freitag NE. (2013). Investigating the Tropic Effects of the InlAB locus of Listeria monocytogenes Reveals Allele-Specific Contributions to Cardiotropism Poster presented at: American Physician Scientist Association General Meeting; Chicago, IL.

McMullen PD, Freitag NE. (2013). The inlAB invasion locus confers cardiotropism to subpopulations of Listeria monocytogenes Poster presented at: American Society of Microbiology General Meeting; Denver, CO.

McMullen PD, Freitag NE. (2013). The InlAB invasion locus confers cardiotropic features to subpopulations of Listeria monocytogenes Poster presented at: University of Illinois College of Medicine Research Forum; Chicago, IL.

Oral Presentation

McMullen PD, Freitag NE. (2013). The inlAB invasion locus confers cardiotropism to subpopulations of Listeria monocytogenes Oral Presentation presented at: American Society of Microbiology, Richard and Mary Finkelstein Lecture Series; Denver, CO.

Other Awards/Accomplishments Orton K Stark Award (Highest award for senior microbiology students at Miami University, 2008), Howard Hughes Summer Research Fellow (Miami University, 2007), Founding Father Gamma Upsilon Chapter of Delta Tau Delta Fraternity (Miami University, 2007), Summer Undergraduate Research Fellow (Miami University, 2006)

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JOURNAL OF BACTERIOLOGY:

Title: Genome Sequence of Listeria monocytogenes 07PF0776, a Cardiotropic Serovar 4b Strain

Author: P. David McMullen, Allison F. Gillaspy, Jenny Gipson et al.

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