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Forward genetics to identify *Listeria monocytogenes* components that activate mammalian cytosolic surveillance pathways

by

Chelsea Elaine Witte

A dissertation submitted in partial satisfaction of the requirements for the degree of

Doctor of Philosophy

in

Microbiology

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, BERKELEY

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Abstract

Forward genetics to identify *Listeria monocytogenes* components that activate mammalian cytosolic surveillance pathways

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Doctor of Philosophy in Microbiology

University of California, Berkeley

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Listeria monocytogenes is Gram-positive facultative intracellular pathogen that is ubiquitously present in the environment. During infection, *L. monocytogenes* is phagocytosed by host cells and then rapidly escapes from the phagosome into the host cytosol. In the cytosol the bacterium is able to replicate and spread to neighboring cells without leaving the intracellular compartment. Maintenance of the intracellular niche is critical for *L. monocytogenes* pathogenesis, but there have been several reports indicating cytosolic bacteria can induce inflammasome-mediated host cell death. Additionally, our laboratory previously identified a host transcriptional profile induced by *L. monocytogenes* upon entry into the cytosol termed the cytosolic surveillance pathway, characterized by the robust upregulation of IFN β and co-regulated genes. Although the host components of the signaling pathways leading to cell death and IFN- β production are becoming better defined, the bacterial ligands were largely unknown. To gain insight into the bacterial components and ligands required for stimulation of these two pathways, we performed a forward genetic screen and isolated mutants that affected induction of host cell death and/or IFN- β .

We identified a transposon insertion in *lmo2473* that caused hyperstimulation of inflammasome-mediated cell death, pyroptosis. Further characterization revealed these mutants, and to a lesser extent wild-type *L. monocytogenes*, lysed in the macrophage cytosol. Intracellular bacteriolysis released bacterial DNA that was detected by the cytosolic DNA sensor AIM2 to trigger pyroptosis. Tight regulation of other bacterial factors capable of stimulating inflammasomes, such as flagellin, likely contribute to the low levels of pyroptosis induced by *L. monocytogenes*.

We also isolated a mutant that harbored a transposon insertion in the gene *lmo0052*, renamed *pdeA*. Biochemical characterization of the gene product determined PdeA was a cyclic di-AMP phosphodiesterase. Inactivation of this protein resulted in accumulation of the nucleotide and increased stimulation of IFN- β . As cyclic di-AMP was only recently identified as a bacterial second messenger, we used mutants that modulate enzymatic processing of c-di-AMP to interrogate the role of its signaling in *L*.

monocytogenes physiology and pathogenesis. This work implicates c-di-AMP, not only as the IFN- β -stimulatory ligand, but also as a critical molecule involved in bacterial growth, response to stress, and cell wall stability.

In summary, using a forward genetic approach, we have identified mechanisms by which host cells recognize and respond to *L. monocytogenes* invasion of the cytosol. Other mutants isolated in this screen merit further characterization to potentially unveil additional bacterial components involved in these responses.

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In memory of those who always supported and encouraged me but were unable to see the end of this endeavor: Bernard and Adeline Witte, Dale VonBehren Sr., and Dale VonBehren Jr.

"I am among those who believe science has great beauty. A scientist in his laboratory is not a mere technician: he is also a child confronting natural phenomena that impress him as though they were fairy tales." -Marie Curie

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General Introduction to *Listeria monocytogenes* Pathogenesis

Listeria monocytogenes: Food-borne pathogen

Listeria monocytogenes is a Gram-positive, non-spore forming bacterium belonging to the phylum Firmicutes and is closely related to *Bacillus* and *Staphylococcus* species. *L. monocytogenes* has a facultative intracellular life cycle and is the causative agent of the food-borne illness listeriosis. First described in 1926, it was initially isolated from laboratory rabbits and guinea pigs presenting with mononucleosis [1].

L. monocytogenes is ubiquitous in the environment and has been isolated from soil, water, sewage, silage and decomposing plant material. Consumption of contaminated feed is thought to be the primary mechanism of infection for a variety of animals including cattle, sheep and swine. Farm animals may represent a source for amplification of the pathogen and subsequent distribution through fecal contamination of produce. Although there are reports of zoonotic transmission of L. monocytogenes, it is estimated that 99% of listeriosis cases are due to ingestion of contaminated items [2,3]. Food-borne transmission was not fully appreciated until 1983 when a direct link was established between listeriosis and consumption of contaminated food following an outbreak in Canada [4]. The incidence of L. monocytogenes is very high in many raw foods including fruits, vegetables, seafood and non-pasteurized milk, likely due to its ubiquitous environmental presence, but industrial processing also introduces the pathogen to many food products. L. monocytogenes is highly adaptable to normally inhospitable environmental conditions and can survive in a wide range of pH (4.5-9), high salt concentrations (up to 10%) and temperatures between 1-45°C [5] [6]. Additionally, the bacterium is able to colonize and form biofilms on surfaces, further contributing to its persistence in the food processing industry [7]. These unique attributes make L. monocytogenes a substantial problem in many refrigerated ready-to-eat products, salads, and delicatessen items.

Due to the ubiquitous presence of L. monocytogenes in the environment as well as the food processing industry, consumption of the bacterium is likely very common. In contrast, the incidence of listeriosis disease is very low with approximately 0.3 cases per 100,000 people [8]. Although listeriosis only represented 0.65% of laboratory confirmed cases of food-borne illness in the United States in 2010, it accounted for more than 23% of food-borne illness-associated mortalities [8]. The most common clinical manifestations of human listeriosis in non-pregnant adults are gastroenteritis and fever. For individuals with underlying health conditions or immunosuppression, exposure to L. monocytogenes can be much more problematic and frequently lead to invasive disease characterized by meningoencephalitis and/or septicemia and can be fatal. L. monocytogenes is the fourth leading cause of bacterial meningitis following Streptococcus pneumoniae, Neisseria meningitides, and group B Streptococci [9]. Populations most susceptible to invasive listeriosis include HIV patients who are estimated to be 300-1000X more likely to contract listeriosis than the general population, pregnant women who have a 17X higher rate of infection, and people over age 70 with a 3X increase in the incidence of listeriosis [10]. It is estimated that up to 20% of the healthy population may be asymptomatic carriers of L. monocytogenes [9,11].

Pregnant women, especially those in their third trimester, are particularly susceptible to *L. monocytogenes* infection. In these women, listeriosis can be asymptomatic or present as flu-like symptoms, but in some cases *L. monocytogenes* invades the placenta and infects the fetus, resulting in feto-listeriosis. This causes

abortion in approximately 20% of cases [12]. Alternatively, it can result in birth of a stillborn baby or newborn with neonatal listeriosis, which may include bacteremia, pneumonia, and/or meningitis [9,12].

In vivo infection with L. monocytogenes

The oral infectious dose of *L. monocytogenes* sufficient to cause human listeriosis is unknown, but in an outbreak of gastroenteritis traced to contaminated chocolate milk it was estimated that individuals consumed as many as 2.9×10^{11} bacteria [13]. Following ingestion, *L. monocytogenes* must transit through the stomach to the small intestine to cause productive infection. There is a correlation between antacid or cimetidine treatment and listeriosis, indicating in healthy individuals the acidity of the stomach is a mechanism of resistance to infection [14]. Additionally, the intrinsic and adaptive acid resistance systems of *L. monocytogenes* allow survival in the low pH of the gastric fluid and may increase the virulence potential of the organism [15,16,17].

Once in the small intestine, *L. monocytogenes* invades the intestinal epithelium using a mechanism that requires interaction between the bacterial surface protein Internalin A (InIA) and the host receptor E-cadherin. The site and mechanism of *L. monocytogenes* invasion are controversial, but may occur at the intestinal vili tip, the junctions of goblet cells, and/or through M cells associated with Peyer's Patches, depending on the infection model [18,19,20,21]. Following invasion, *L. monocytogenes* rapidly disseminates to the mesenteric lymph nodes, liver, and spleen [20,22,23]. Gastric infection of guinea pigs revealed *L. monocytogenes* dissemination occurs in two waves: first, directly from the intestine, followed by a secondary route through the mesenteric lymph nodes [24]. In cases of severe invasive listeriosis the bacterium also traffics to the central nervous system and meninges. The gall bladder has been appreciated as an additional site of *L. monocytogenes* colonization [25,26] and may represent an asymptomatic carrier reservoir similar to that of the *Salmonella enterica* serovar Typhi carriers of typhoid fever [27].

Although most listeriosis cases are acquired by the oral route of infection, the majority of pathogenesis and immunological studies are performed by intravenous infection of mice, due to the cost, reproducibility, and genetic tools available with this model. Based on these intravenous murine infection models, it is estimated that nearly 90% of the *L. monocytogenes* inoculum traffics to the liver and the remaining 10% to the spleen. In the liver, bacteria are cleared from the blood by Kupffer cells, resident hepatic macrophages [28]. The subsequent rapid infiltration of neutrophils results in initial killing of extracellular and cell-associated bacteria [28,29]. Mice that are deficient for this early innate immune response by neutrophil-depletion are highly susceptible to infection [30,31,32]. *L. monocytogenes* that escape neutrophil-killing enter and replicate within hepatocytes. After approximately three days of exponential bacterial growth, the bacterial load begins to decrease due to specific CD8+ T cells that lyse *L. monocytogenes*-infected host cells to control infection [33,34,35,36]. These T cell responses are responsible for sterilizing immunity [37].

Intracellular life cycle

During infection *L. monocytogenes* is taken up by phagocytic cells or can induce its own uptake into a wide variety of other cell types mediated by proteins of the

internalin family, of which the best characterized are Internalin (Inl) A and B. InlA is largely responsible for internalization in the small intestine, as discussed above [38]. InlB binds to the heptocyte growth factor receptor (Met) that is expressed on hepatocytes to promote uptake in the liver [39].

Initially after cellular invasion, L. monocytogenes is contained within a host phagosome but is able to escape into the cytosol, a mechanism dependent on activity of the cholesterol-dependent cytolysin (CDC) Listeriolysin O (LLO). The CDCs are the largest family within the bacterial pore-forming toxins. LLO is unique in that it is the only CDC produced by an intracellular bacterium. Studies using small fluorescent probes revealed that LLO forms membrane perforations that contribute to delayed phagosomal maturation by disrupting the ion gradient of the phagosome, including an increase in the phagosomal pH and decrease in calcium ions [40]. L. monocytogenes mutants deficient in LLO production (ie. disruption of the LLO-encoding gene hly) remain trapped in the phagosome and are unable to replicate. Phagosomes containing Δhly mutants acidify, have increased calcium ion concentration, and eventually acquire LAMP-1, indicative of lysosomal fusion [40]. The inability of the $\Delta h l y$ mutant to escape into the cytosol leads to more than 5-logs of attenuation in the murine model of listeriosis [41,42,43]. It is appreciated that LLO must be regulated on multiple levels (transcriptional, translational, activity pH optimum, degradation by host cell) to compartmentalize its activity to the phagosome [44]. L. monocytogenes mutants that fail to properly regulate LLO activity lyse their host cells, effectively eliminating the intracellular niche, and are cleared by neutrophils leading to several logs of attenuation [45]. Secreted phospholipases C (PLCs) also contribute to bacterial escape from this primary vacuole as mutants deficient for PLCs have a decrease in escape efficiency [46] [47].

Following escape from the phagosome, L. monocytogenes actively replicates in the host cell cytosol and can reach numbers of more than 100 bacteria per cell. The doubling time of cytosolic L. monocytogenes is approximately equivalent to that of bacteria in rich broth, suggesting the nutrients required for growth are readily available in this compartment [41]. Interestingly, extracellular bacteria or intracellular pathogens that remain within a membrane-bound compartment are unable to replicate when directly inoculated into the host cytosol by microinjection; whereas bacteria, like L. monocytogenes, that naturally replicate in the cytosol were able to grow [48]. These observations suggest that there may be specific metabolic requirements for cytosolic replication based on the nutrients available. L. monocytogenes mutants deficient for the hexose phosphate transporter, Hpt, have an increased doubling time in tissue culture cells, suggesting that uptake of hexose phosphates during intracellular infection is an important carbon and energy source for the bacterium [49]. Additional studies using auxotrophic mutants, screens for genes involved in intracellular replication, and metabolic profiling are beginning to elucidate how L. monocytogenes is able to specifically replicate in this environment [50,51,52,53,54,55].

L. monocytogenes propels itself through the host cytosol and outward within long filopodia-like projections that are taken up by neighboring cells. A single bacterial protein, ActA, is sufficient for this actin-based motility as ActA-coated beads are also propelled through host cells [56]. The cell wall-anchored ActA acts as an actin nucleation-promoting factor and is a scaffold for the actin polymerization complex Arp2/3. *L. monocytogenes* $\Delta actA$ mutants are able to replicate in the primary infected

cells but are defective for intra- and intercellular spread [57]. Hijacking the host actin machinery allows *L. monocytogenes* to spread cell-to-cell while maintaining its intracellular niche and avoiding the extracellular environment.

The primary virulence factors of L. monocytogenes are under control of a master regulator transcription factor, positive regulatory factor A (PrfA). Ten genes have been defined as the core PrfA regulon and include inlA, inlB, inlC, hpt, plcA, hly, mpl, actA, plcB, and prfA [58]. The PrfA-regulated genes are highly upregulated during intracellular infection suggesting there is a host-specific cue(s) sensed by the bacterium signaling transition to a pathogenic state. One of the proposed regulators of virulence gene expression is sugar availability. Cellobiose, from decaying plant material, and glucose, carbon sources available to L. monocytogenes in the environment, lead to downregulation of *hly*, whereas phosphorylated hexoses, like glucose-1-phosphate that could be derived from host glycogen, do not negatively affect hly expression [49,59,60]. In addition to host- or environmental-specific nutrients, temperature also affects PrfA expression. Activity of an RNA thermosensor promotes translation of PrfA-regulated genes at 37°C, the approximate physiologic temperature of a mammalian host [61]. As the master regulator of virulence gene expression, regulation of PrfA itself is a complex, multifactorial process and central to the transition of L. monocytogenes to its intracellular life cycle.

Innate immune responses to *L. monocytogenes* infection

The mammalian innate immune system is important for immediate response to microbial invasion, but also shapes the development of acquired immunity [62]. Evolution of a molecular recognition system that relies on a limited number of proteins and that will maintain its function throughout microbial evolution requires the targeting of motifs that remain constant across microbial genre. As such, innate immune receptors called pattern-recognition receptors (PRRs) generally recognize conserved microbial components that are often essential for survival or growth, those to which microbes are evolutionarily constrained. These conserved microbial molecules are referred to as pathogen-associated molecular patterns (PAMPs) [63].

A large family of PRRs consists of the membrane-bound Toll-like Receptors (TLRs), of which there are at least 13 members in mammals. A subset of TLRs are localized to the cell surface, such as TLR2, 4, and 5 which are triggered by lipoproteins, lipopolysacchrides, and flagellin, respectively. Another group of TLRs including TLR3, 7, and 9 signal from the phagosome and respond to nucleic acids. Stimulation of most TLRs activates a signaling cascade dependent on the adaptor molecule MyD88, whereas TLR3 requires an alternative adaptor, Trif. TLR4 is the only known receptor that can use either adaptor. TLR detection of an appropriate ligand leads to activation of the transcription factor NF- κ B and expression of inflammatory cytokines including TNF α , IL-1 β , and IL-6. Activation of TLR3, 4, 7, 9 can also stimulate production of type 1 interferon, IFN- β .

Transcriptional profiling of primary bone marrow-derived macrophages (BMDMs) infected with *L. monocytogenes* reveals a complex pattern of host gene expression that involves hundreds of genes [64,65]. This is a result of stimulation of multiple, often overlapping, innate immune signaling pathways. Host genes induced during *L. monocytogenes* infection can be separated into two categories- an

"early/persistent" gene cluster and a "late response" group [65]. Only genes within the early/persistent cluster were induced during infection with both wild-type *L*. *monocytogenes* and Δhly mutants that remained trapped within the phagsome. Furthermore, infection of BMDMs deficient in MyD88 eliminated induction of this group of genes indicating stimulation of the early/persistent gene cluster was dependent on TLRs and emanated from the cell surface or phagosomal compartments (Figure 1-1)[64]. MyD88-dependent TLR signaling is clearly important for host defense to *L*. *monocytogenes* infection as MyD88-deficient mice are highly susceptible to infection [66,67]. Eliminating a single TLR does not significantly increase susceptibility to *L*. *monocytogenes* suggesting that host detection of multiple bacterial ligands contributes to the MyD88-dependent response. This approach, using bacterial mutants as well as BMDMs or mice deficient in components required for host signaling pathways, is referred to as "genetics-squared" [68].

The host expression pattern of late response genes is referred to as the cytosolic surveillance pathway (CSP) [69]. There are nearly two-dozen primary genes that are induced by this pathway, of which IFN- β is the most highly upregulated. As its name suggests, bacterial detection by the CSP requires cytosolic access and *L. monocytogenes* Δhly mutants do not stimulate IFN- β (Figure 1-1). Similarly, the CSP is independent of the TLRs but requires the cytosolic PRR STING and transcription factor IRF3 [70,71]. STING has recently been shown to directly bind cyclic di-nucleotides to trigger the downstream signaling cascade [72]. Previous studies from our laboratory revealed expression of bacterial multidrug resistance transporters (MDRs) was critical for detection by the CSP during *L. monocytogenes* infection [73]. Subsequent biochemical analysis of mutants with altered MDR expression led to the identification of the IFN- β -stimulatory ligand, cyclic di-AMP [74].

In addition to the CSP, another host response to cytosolic invasion is induction of programmed cell death that restricts microbial replication by eliminating the intracellular niche. One cell death pathway is dependent on formation of large multi-protein complexes referred to as inflammasomes. Each inflammasome is composed of a receptor, often a Nod-like receptor (NLR), coupled to caspase-1 either directly through compatible protein domains or indirectly through the adaptor molecule ASC. Recent studies have shown direct ligand detection by the leucine rich repeat domains of NLRs [75,76]. This interaction stimulates complex formation and subsequent autocatalysis of the zymogen pro-caspase-1 (Figure 1-1)[77]. Active caspase-1 leads to host cell death and cleavage of pro-IL-1 β and pro-IL-18 into their active, secreted forms. Together, caspase-1-mediated cell death and processing and secretion of proinflammatory cytokines is referred to as pyroptosis [78]. Although the magnitude of pyroptosis, NLRs involved, and bacterial ligands recognized during *L. monocytogenes* infection have been controversial [79,80,81], it is clear that mutants which induce excessive host cell death are highly attenuated [45].

Interferon and bacterial infection

Type 1 interferons were originally defined as part of the antiviral response critical to restrict viral replication. As such, many viruses have evolved mechanisms to prevent stimulating IFNs or to block IFN signaling. It is now appreciated that many intracellular bacteria also stimulate production of Type 1 interferons including the pathogens

Mycobacterium tuberculsosis, Legionella pneumophila, and *Franciscella tularensis* [82,83,84]. For each of these pathogens, including *L. monocytogenes*, access to the host cytosol, by escape into the compartment or through a bacterial secretion system, is required for IFN- β induction.

Although Type 1 interferons are induced by a number of bacterial pathogens, the role of interferons during infection remains unclear. As in viral infections, Type 1 IFNs prevent productive infection with *Chlamydia trachomatis* [85,86] and contribute to restricting *L. pneumophila* replication [87,88]. Contrary to its antibacterial role in some infections, Type 1 IFNs can also suppress innate immunity. Mice deficient in IRF3 or the Type 1 IFN receptor (IFNAR) are more resistant to *L. monocytogenes* infection [89,90]. Similarly, IFNAR^{-/-} mice are more resistant to infection with *M. tuberculosis* and *F. tularensis* [82,91]. This suggests that in some cases Type 1 IFNs are detrimental to the host during infection and may benefit the pathogen. In summary, the role of Type 1 IFNs induced during bacterial infection is complex and is still incompletely characterized.

Inflammasomes and bacterial infection

There are multiple mechanisms that all result in death of a host cell including apoptosis, pyroptosis, oncosis, and autophagic cell death. Similar to induction of IFNs, stimulation of host cell death can have different roles in pathogenesis depending on the bacterial infection. For example, some pathogens induce host cell death as a mechanism to dampen the host response or to invade deeper tissues of the host. In the case of *L. monocytogenes* and many other intracellular pathogens, activation of cell death pathways is a host defense to eliminate the infected cell as well as the intracellular replicative niche of the pathogen. Both *Rickettsia* and *Chlamydia* have evolved mechanisms to prevent activation of host cell death pathways to promote productive bacterial replication [92,93].

In contrast to apoptosis that is considered an immunologically "silent" cell death, pyroptosis couples host cell death with secretion of proinflammatory cytokines. Murine infection models using mice deficient in caspase-1 revealed a crucial role for inflammasomes in the host defense, as these mice were more susceptible to infection with *Salmonella typhiurium*, *Shigella flexneri*, and to a lesser extent *L. monocytogenes* [94,95,96].

Although the host pathways of cytosolic bacterial recognition are becoming better defined, until recently, the listerial ligands responsible for these responses had not been identified. The studies described in the subsequent chapters identify bacterial mutants that affect induction of the CSP and/or inflammasome activation. From this, it is evident that bacterial DNA released due to infrequent bacteriolysis stimulates an AIM2-dependent inflammasome. Additionally, isolation of a mutation in a gene encoding a c-di-AMP phosphodiesterase further support c-di-AMP as the IFN- β -stimulatory ligand secreted during intracellular infection. This has also allowed for the characterization of c-di-AMP signaling in pathogenesis and physiology of *L. monocytogenes*.

Figure 1-1



Figure 1-1: Innate immune pathways triggered by *L. monocytogenes*. During infection of macrophages *L. monocytogenes* is detected by at least three distinct innate immune pathways: one that is dependent on MyD88, one that is dependent on STING/IRF3, and an AIM-2 mediated inflammasome pathway. Each pathway results in a distinct host response to infection. Dashed lines represent infrequent events.

Chapter 2

Forward genetic screens to identify *Listeria monocytogenes* mutants that induce diminished or enhanced induction of the cytosolic surveillance pathway and/or host cell death

A portion of this chapter was submitted in:

Chelsea E. Witte, Kristy A. Archer, Chris S. Rae, John-Demian Sauer, Joshua J. Woodward, Daniel A. Portnoy. Innate immune pathways triggered by *Listeria monocytogenes* and their role in the induction of cell-mediated immunity. 2012 Advances in Immunology

Summary

Infection with *Listeria monocytogenes* induces long-term, protective immunity. The ability of *L. monocytogenes* to stimulate a robust CD8+ T cell response has led to the development of *L. monocytogenes*-based vaccine platforms, including anti-tumor therapy and infectious disease therapeutics. As the innate immune response influences, and may be required for the adaptive response, we aimed to understand mechanistically how *L. monocytogenes* is detected by the host cell. Once the bacterium enters the host cytosol, it induces at least two signaling pathways: i) a STING/IRF3-dependent pathway that leads to expression of IFN- β and co-regulated genes, and ii) low levels of caspase-1-dependent inflammasome activation, resulting in IL-1 β and IL-18 secretion and pyroptotic cell death. To identify bacterial components involved in the stimulation of either of these pathways, we performed a forward genetic screen and isolated bacterial mutants that influenced IFN- β production and/or host cell death. Using bacterial mutants isolated from this screen we identified the listerial ligands that activate each of these host signaling pathways as well as additional bacterial factors that may be involved.

Introduction

Intracellular pathogens represent a unique threat to the host immune system. Their intracellular life cycles allow these microbes to minimize detection by extracellular innate immune mechanisms. As such, the host has evolved a series of intracellular pattern recognition receptors responsible for detecting microbial invasion and triggering a signaling cascade to mount the appropriate cellular response.

One of the host signaling pathways stimulated by cytosolic *L. monocytogenes* is dependent on Interferon Regulatory Factor-3 (IRF-3) and leads to transcriptional upregulation of dozens of genes including IFN- β . This pathway is referred to as the cytosolic surveillance pathway (CSP) and IFN- β expression is quantified as a proxy for the entire transcriptional profile. DNA transfected into macrophages recapitulated the response leading to expression of IFN- β , but there was no evidence that this represented the endogenous ligand [64,84]. A previous forward genetic screen in our lab identified bacterial multidrug resistance transporters (MDRs) as required for *L. monocytogenes* detection by the CSP. The magnitude of IFN- β stimulation directly correlated with level of expression of MDRs, though other bacterial factors involved or the listerial ligand detected by the host remained unknown [73].

There have been many recent reports that *L. monocytogenes* robustly activates the inflammasome via three different cytosolic sensors: Nlrp3, Nlrc4, and/or AIM2 [79,80,97,98,99,100,101,102]. However, in our hands, the amount of inflammasomemediated pyroptosis by wild-type *L. monocytogenes* is less than 5% of the infected cells in non-stimulated bone marrow-derived macrophages (BMDMs) and approximately 15% in TLR2-prestimulated BMDMs. The release of IL-1 β is equally low [98]. The increase observed in TLR2-stimulated cells is likely due to TLR-dependent upregulation of inflammasome components (ie. pro-IL-1 β) and increased phagocytosis. The discrepancy in the literature is likely due to differences in infection protocols such as multiplicities of infection and the duration of experiments. Compared to infection with other intracellular pathogens such as *Legionella pneumophila*, the magnitude of inflammasome activation in response to *L. monocytogenes* infection is very low [103]. Based on these observations and the negligible *in vivo* role for caspase-1 in *L. monocytogenes* infection [103], we hypothesized that *L. monocytogenes* may actively inhibit inflammasome activation and sought to identify these mechanisms through bacterial forward genetics.

Although the host pathways of cytosolic bacterial recognition are becoming better defined, until recently, none of the listerial ligands responsible for these responses had been identified. To elucidate the bacterial ligands involved, we developed a comprehensive forward genetic screen to identify bacterial mutants that induced enhanced or diminished levels of IFN- β expression and/or host cell death (Figure 2-1). Three groups of transposon insertion mutants were identified in this screen: those that affected both IFN- β and host cell death, those that only affected IFN- β , and those that only affected host cell death. Analysis of mutants in the first group revealed intracellular bacterial DNA release, by bacteriolysis or other mechanisms, as a common means of stimulation of both IFN- β and pyroptosis. One mutant that induced more IFN- β compared to wild-type harbored a transposon insertion within the gene *lmo0052*, encoding a c-di-AMP phosphodiesterase. This supported the recent identification of c-di-AMP as the IFN- β -stimulatory ligand. Further analysis of additional mutants from this screen will likely enhance our understanding of the bacterial components involved in induction of the CSP and activation of host cell death pathways.

Results and Discussion

Genetic screen to identify bacterial mutants

Prior to the screen, libraries of *L. monocytogenes* transposon mutants were constructed using a *Himar1 mariner* transposon [104]. In contrast to the previously used *Tn917*-derivative, the design of this *Himar1* transposon minimizes polar effects, has low site-specificity that eliminates insertional "hot spots," and has full genomic coverage.

Isolated mutants were grown to stationary phase overnight at 30°C and used to infect primary BMDMs. Following 6 hours of infection, supernatants from the infected macrophages were harvested and used to assess the ability of each mutant to stimulate (i) the STING/IRF3-dependent pathway as measured by IFN-β production and (ii) host cell death as measured by lactate dehydrogenase (LDH) release. In vitro secondary screening of individual transposon mutants on sheep's blood agar plates eliminated transposon mutants that displayed decreased hemolysis, as these mutants likely had phagosomal escape defects. Additionally, mutants were also plated on swarm plates (BHI plates containing 0.35% [wt/vol] agar) to discern mutants with motility defects that could account for lower rates of infectively and consequently low levels of IFN-β and LDH release. Furthermore, mutants with severe intracellular growth defects were also eliminated as the diminished IFN- β and cell death induced by these mutants was likely a result of fewer cytosolic bacteria per macrophage. The remaining mutants identified in the screen can be classified into three groups: those that induced altered levels of both cell death and IFN- β pathways, those that affected IFN- β , or those that only affected cell death. Of the nearly 5,000 transposon mutants screened, 108 were initially confirmed and sequenced. Of those, 28 met a 2-fold cutoff and were chosen for further analysis (Tables 2-1, 2-2, 2-3). Examination of Tables 2-1, 2-2, and 2-3 reveals a number of phenotypes that although reproducible, are currently unexplainable.

Analysis of transposon mutants that affected LDH release and IFN-β production

We identified several transposon insertion mutants that affected both host cell death and IFN- β production (Table 2-1). Characterization of one of these mutants that harbored a transposon insertion in *lmo2473*, revealed it was lysing in the host cytosol. This resulted in release of bacterial DNA that was detected by the host receptor AIM2, leading to pyroptosis ([98], see Chapter 3 for more details). As DNA transfected into the macrophage cytosol could also stimulate IFN- β , intracellular bacterial DNA release may be a general mechanism that could affect both signaling pathways. To test this hypothesis, we performed a secondary screen with each transposon insertion mutant transformed with a luciferase reporter plasmid encoding firefly luciferase under control of the cytomegalovirus promoter [98]. Bacteria that release DNA in the host cytosol will deliver the reporter plasmid and the infected host cell expresses luciferase. Indeed, for several of the mutants, increased stimulation of cell death and IFN- β correlated with increased DNA released as measured by host luciferase production (Table 2-1). In addition to *lmo2473*, these mutants harbored transposon insertions in *lmo1746* (similar to

ABC transport permease, uncharacterized in *L. monocytogenes*), *lmo2554* (*lafB*), and *lmo0707* (*fliD*).

Lmo2554, renamed LTA anchor formation protein B (LafB), is a galactosyltransferase that catalyzes the addition of a galactose (Gal) residue from UDP-Gal onto the membrane-bound glucose-diacylglycerol (Glu-DAG) [105]. The resulting glycolipid Gal-Glu-DAG is a critical intermediate for lipoteichoic acid synthesis and deletion of *lafB* reduces total wall-associated LTA [105]. Although decreased production of LTA did not significantly affect bacterial replication in BMDMs (data not shown), *lafB::Himar1* and $\Delta lafB$ mutants induced 2-fold more cell death and 1.3-fold more IFN- β than infection with wild-type *L. monocytogenes* (Table 2-1, Figure 2-2A). Consistent with increased intracellular DNA release, induction of pyroptosis and IL-1 β stimulation was abolished in macrophages deficient in the DNA sensor, AIM2 (Figure 2-2A,B). We hypothesized that *L. monocytogenes* mutants with reduced LTA may have weakened cell walls and therefore, be more susceptible to environmental stresses. For example, transit through the host phagosome may result in increased lysis in the cytosol and release of DNA and perhaps other stimulatory ligands.

The gene *lmo0707* encodes a flagellar capping protein FliD. Mutants defective in FliD were predicted to secrete flagellin, similar to what has been shown for *L*. *monocytogenes* $\Delta flgK$ (flagellar hook-associated component) mutants [101]. Indeed, *fliD::Himar1* mutants had more flagellin monomers present in the supernatant of broth culture compared to wild-type *L. monocytogenes* (Figure 2-3A). Interestingly, the transposon insertion in *fliD* did not completely eliminate bacterial motility (data not shown). To test whether increased release of flagellin monomers resulted in increased inflammasome-mediated cell death, we infected Nlrc4-deficient macrophages and found LDH release induced by *fliD::Himar1* mutants was partially reduced (Figure 2-3B), but was not rescued to wild-type levels. Based on the intracellular bacterial DNA delivery assay that demonstrated *fliD::Himar1*-infected cells expressed nearly 3-fold more luciferase than those infected with wild-type *L. monocytogenes*. We hypothesize that increased DNA release through the uncapped flagellar apparatus may account for the remaining Nlrc4-independent cell death.

Using a bacterial forward genetic approach we identified AIM2 inflammasome activation resulting from release of bacterial DNA into the host cytosol, through bacteriolysis like wild-type *L. monocytogenes* and $\Delta lmo2473$ mutants, or potentially through the flagellar apparatus like *fliD::Himar1* mutants. We did not identify a novel mechanism used by *L. monocytogenes* to inhibit inflammasome activation, though mutants identified in this screen support that tight regulation of bacterial virulence factors, like flagellin, aid in avoidance of this pathway that is detrimental to pathogenesis. Mutants isolated in this study support that misregulation of flagella production or monomer secretion also increases Nlrc4 inflammasome activation [101,103]. It is appreciated that *L. monocytogenes* downregulates flagellin production at 37°C, the approximate temperature of a host or tissue culture infection. This is an intrinsic mechanism to evade inflammasome activation.

LDH release is a general phenotype of death or dying cells but is not indicative of the mechanism of death. We pursued mutants that affected caspase-1-mediated cell death to elucidate *L. monocytogenes*-induced inflammasome activation. Further analysis of

mutants that affect cell death may reveal additional cell death pathways activated by *L*. *monocytogenes*.

Analysis of transposon mutants that affected IFN-ß production

There are at least two ligands capable of stimulating the CSP: DNA and c-di-AMP. The secondary screen for intracellular DNA release allowed us to identify several mutants that only affected IFN- β and were likely independent of DNA release. We isolated a mutant harboring a transposon insertion in *lmo0052*, which was predicted to encode a hypothetical protein. This mutant induced nearly five-fold more IFN- β compared to infection with wild-type *L. monocytogenes*. Based on homology to the *Bacillus subtilis* protein YybT [106] and biochemical characterization, we determined that *lmo0052* encoded a c-di-AMP phosphodiesterase (PdeA). Transposon inactivation of *lmo0052* (renamed *pdeA*) was predicted to prevent degradation of c-di-AMP, thereby leading to the accumulation of high levels of c-di-AMP and consequently higher levels of IFN- β induction. Further characterization of *L. monocytogenes* $\Delta pdeA$ mutants can be found in Chapter 4.

We isolated two independent transposon insertions in *lmo1348*, a gene predicted to encode a component of the glycine cleavage system, GcvT. The glycine cleavage system is critical for generation of single carbon units used to synthesize methionine, nucleotides and methylated molecules [107]. GcvT is an aminomethyltransferase that catalyzes the transfer of the methylene group of glycine from the carrier protein GcvH onto tetrahydrafolate. Disruption of gcvT resulted in a reduction in IFN- β production compared to infection with wild-type L. monocytogenes (Table 2-1). Surprisingly, infection with $\Delta gcvT$ mutants carrying the luciferase reporter plasmid resulted in approximately 10X more expression of luciferase than infection with wild-type bacteria. Although the results of the delivery assay indicated bacterial DNA did enter the host cytosol, it did not result in increased pyroptosis. Additionally, $\Delta gcvT$ mutants were able to replicate like wild-type L. monocytogenes in BMDMs (Figure 2-4A), further supporting that the DNA delivery is not due to frequent bacteriolysis. To assess if gcvTdeficiency or decreased host detection affected bacterial growth in vivo, we infected mice intravenously with either wild-type or $\Delta gcvT L$. monocytogenes. Following 48 hours of infection, 1.5 logs fewer $\Delta gcvT$ mutants were recovered from both spleens and livers of infected mice (Figure 2-4B). The paradox that $\Delta gcvT$ mutants deliver the luciferase reporter plasmid but do not induce more IFN-ß compared to wild-type bacteria may suggest that the reporter plasmid, from $\Delta gcvT$ mutants, is not recognized by the host cell, perhaps because it is modified differently than DNA in wild-type L. monocytogenes. An alternative hypothesis is that the $\Delta g c v T$ mutation affects production or modification of cdi-AMP. Based on the observation that $\Delta m dr M$ mutants induce approximately a third the IFN- β levels compared to wild-type bacteria, we hypothesize that c-di-AMP is the predominant IFN-β-stimulatory ligand. Whether mutations in the glycine cleavage system affect the ability of c-di-AMP to be detected by STING remains to be tested.

Materials and Methods

Transposon library construction

The *Himar1 mariner* transposon library was generated in *L. monocytogenes* 10403S using the delivery system pJZ037, as previously described [104]. Individual isolated colonies were selected, used to inoculate wells of Brain Heart Infusion (BHI) media in a 96-well plate, and grown up overnight at 37°C. Each well was supplemented with BHI containing 5% glycerol and stored at -80°C prior to use.

Bacterial strains and cell culture

Wild-type *L. monocytogenes*, Δhly mutants and mutants from the transposon library were inoculated into 96-well plates containing BHI medium and grown overnight at 30°C prior to infection. BMDMs were prepared and frozen from femurs of 6-8 week old C57BL/6 female mice (The Jackson Lab, Bar Harbor, ME), as previously described [108]. For *in vitro* secondary screening, broth growth curves, and genomic DNA isolation, cultures were grown overnight in BHI media at 37°C with shaking. Nlrc4^{-/-} macrophages were from Dr. Vishva Dixit (Genentech, San Francisco CA).

Transposon Mutant Screen

 $5x10^4$ BMDM were plated in 100 µL media per well of a 96-well plate and incubated overnight. BMDMs were infected with 4 µL overnight bacterial cultures for one hour. The media was aspirated and replaced with media containing 50 µg/mL gentamicin. After 30 minutes the gentamicin-containing media was replaced with fresh media. After 6 hours total infection, supernatants were harvested for analysis.

To measure LDH release, 60 μ L infection supernatant was added to 60 μ L of LDH detection reagent, as previously described [109] in triplicate in 96-well plates. Absorbance was measured on a SpectraMax 340 spectrophotometer (Molecular Devices) at wavelength 490nm and lysis values were calculated as a percentage of cells lysed with 1% TritonX-100. To assess IFN- β production, supernatants were analyzed by ISRE-L929 luciferase reporter cells, as previously described [73].

The transposon insertion site was identified in the *L. monocytogenes* genome as previously described [104].

Blood plate screen for ahemolytic mutants

Blood agar plates were prepared containing 1% glucose 1-phosphate supplemented with 5% defibrinated sheep's blood (HemoStat Laboratories, Dixon, CA). Wild-type *L. monocytogenes*, Δhly mutants, and transposon insertion mutants were plated and incubated for 24-48 hours at 37°C, then evaluated for zones of hemolysis.

Swarm plate screen for amotile mutants

Wild-type *L. monocytogenes*, $\Delta flaA$ mutants, and transposon insertion mutants were grown overnight at 30°C. One microliter of each culture was stab inoculated into semisolid (0.35% [wt/vol] agar) BHI plates and incubated for 24-48 hours at 30°C.

Intracellular growth curve

 $2x10^6$ macrophages were plated overnight and then infected with bacteria at a multiplicity of infection of one bacterium per ten macrophages. Bacterial replication was quantified as previously described [41].

Lactate dehydrogenase release and IL-1ß ELISA

Macrophages were pretreated for 12-16 hours with 100 ng/mL Pam3CSK4 (Invivogen, San Diego, CA) prior to infection in 24-well plates. $5x10^5$ macrophages were infected with *L. monocytogenes* strains at a multiplicity of infection of 5 bacteria per cell for 30 minutes. At 30 minutes post infection, media was replaced with media containing 100 ng/mL Pam3CSK4 and 50 µg/mL gentamicin. Six hours post infection, supernatants were harvested and analyzed for lactate dehydrogenase release, as described above, and IL-1 β secretion. IL-1 β secretion was determined using mouse IL-1 β ELISA Ready-SET-Go according to the manufacturer's instructions (eBioscience, San Diego, CA).

AIM2 knockdown

AIM2 shRNA knockdown vectors were a gift from Dr. Katherine Fitzgerald and immortalized C57BL/6 macrophages were a gift from Dr. Russell Vance. Lentiviralmediated knockdowns were performed using the pLKO.1 system as previously described [110] in immortalized C57BL/6 BMDMs [111].

Intracellular bacteriolysis

L. monocytogenes strains were engineered to carry the luciferase reporter plasmid, pBHE573 [98]. $5x10^5$ IFNAR^{-/-} macrophages were infected at a multiplicity of infection of 5 bacteria per cell. At 1-hour post infection media was replaced with media containing 50 µg/mL gentamicin. Six hours post infection supernatants were removed, and cells were lysed with TNT lysis buffer (20 mM Tris, 200 mM NaCl, 1% triton [pH 8.0]) and transferred to 96-well plates. Luciferase reagent was added to each well [112] and luminescence was measured by luminometer (VICTOR3, PerkinElmer; Waltham, MA).

Western blot detection of flagellin

To assess flagellin secretion in broth, cultures were centrifuged and proteins in the supernatants were precipitated with 10% trichloroacetic acid (Sigma). Samples were separated on a 10% SDS-PAGE gel (Invtirogen) either stained with Coomasie stain or transferred to polyvinylidene membrane (Millipore, Billerica, MA). Membranes were probed for flagellin using anti-*Listeria* sera (Denka Seiken, Coventry, UK) followed by HRP-conjugated secondary antibody (GE Healthcare, Buckinghamshire, UK.

In vivo mouse infections

Prior to infection, bacterial strains are grown to stationary phase ($OD_{600}=1.2$) at 30°C, then back diluted and grown at 37°C with shaking until $OD_{600}=0.3-0.5$. Cultures were diluted in 1XPBS and used to intravenously infect female C57BL/6 mice between 6-8 weeks of age with a final inoculum of approximately 10⁵ bacteria. At 48 hours post infection, mice are sacrificed and organs collected. Bacterial burdens were enumerated by plating organ homogenates on LB plates and incubated overnight.

Figure 2-1



Figure 2-1: Schematic of genetic screen. *L. monocytogenes* transposon insertion mutants were grown overnight and used to infect primary BMDMs. Following 6 hours of infection, supernatants from the infected cells were analyzed for IFN- β stimulation by L929-ISRE reporter cells, which produce luciferase in response to IFN- β , as well as for host cell death measured by lactate dehydrogenase release.

Table 2-1: *L. monocytogenes* transposon mutants identified in forward genetic screens that affected both IFN- β and host cell death.

<i>L. monocytogenes</i> gene harboring transposon insertion	Strain number	Description*	Fold IFN-β induction ^a	Fold Host cell death ^b	Relative bacteriolysis ^c
Mutants that affected	d both IFN-β and	cell death			
lmo0707	DP-L5915	FliD, flagellar capping protein	1.9	2.3	2.9
lmo1746	DP-L5916	Similar to ABC transporter, permease	1.5	1.9	1.4
lmo2045	DP-L5917	Hypothetical protein	2.0	2.5	0.7
lmo2167	DP-L5918	Hypothetical protein	1.5	0.4	0.3
lmo2473	DP-L5919	Hypothetical protein, similar to <i>B. subtilis</i> YvcK	2.7	4.0	19.7
lmo2503	DP-L5920	Similar to cardiolipin synthase	1.9	2.0	0.8
lmo2554	DP-L5921	Similar to galactosyltransferase	1.3	2.0	3.4

Values are represented as fold induction compared to infection with wild-type *L*. *monocytogenes*. ^aIFN- β induction was quantified either by qRT-PCR on RNA isolated from infected macrophages or by bioassay on supernatants from infected macrophages. ^bHost cell death was measured by lactate dehydrogenase release into the supernatant of infected macrophages. ^cRelative bacteriolysis was measured by luminescence as indirect readout of host expression of the reporter plasmid, pBH573, as previously described (Sauer et al., 2010). *description based on NCBI annotation of the *L. monocytogenes* EGD-e genome. **Table 2-2:** *L. monocytogenes* transposon mutants identified in forward genetic screens that affected IFN- β production.

L. monocytogenes gene harboring transposon insertion	Strain number	Description*	Fold IFN-β induction ^a	Fold Host cell death ^b	Relative bacteriolysis ^c
Mutants that affected	d IFN-β product	ion			
lmo0052	DP-L5896	Cyclic di-AMP phosphodiesterase	5.0	NS	0.5
lmo0165	DP-L5897	Predicted O-methyltransferase	0.4	NS	1.0
lmo0559	DP-L5507	Pgl, 6-phosphogluconolactonase	3.0	NS	ND
lmo0583	DP-L5898	Similar to preprotein translocase SecA subunit	1.6	NS	0.5
lmo0597	DP-L5899	Similar to transcriptional regulator, CRF/FNR family	2.3	NS	1.2
lmo0781	DP-L5900	Similar to mannose-specific phosphotransferase component IID	2.4	NS	1.4
lmo0847	DP-L5901	Similar to glutamine ABC transporter	0.4	NS	0.7
lmo1348	DP-L5902	GcvT, glycine cleavage system aminomethyltransferase	0.4	NS	10.2
lmo1408 [#]	DP-L5396	LadR, negative regulator of MDR transporter, MdrL	3.0	NS	ND
$\Delta lmo1617^{\#}$	DP-L5444	MdrM, MDR transporter	0.3	NS	ND
lmo1618 [#]	DP-L5418	MarR, negative regulator of MDR transporter, MdrM	0.3	NS	ND
lmo1745 [#]	DP-L5398	VirR, putative two-component system response regulator	3.0	NS	ND
lmo2114	DP-L5903	Similar to ABC transporter (ATP-binding protein)	1.7	NS	1.1
lmo2477	DP-L5904	GalE, UDP-glucose 4-epimerase	1.8	NS	1.9
lmo2589 [#]	DP-L5397	TetR, negative regulator of MDR transporter, MdrT	20.0	NS	ND
lmo2691	DP-L5905	Autolysin, N-acetylmuramidase	1.8	NS	1.9
lmo2783	DP-L5906	Similar to cellobiose phosphotransferase system, component IIC	1.5	NS	4.6

Values are represented as fold induction compared to infection with wild-type *L*. *monocytogenes*. ^aIFN- β induction was quantified either by qRT-PCR on RNA isolated from infected macrophages or by bioassay on supernatants from infected macrophages. ^bHost cell death was measured by lactate dehydrogenase release into the supernatant of infected macrophages. ^cRelative bacteriolysis was measured by luminescence as indirect readout of host expression of the reporter plasmid, pBH573, as previously described (Sauer et al., 2010). ND, no data. NS, not significantly different compared to wild-type *L. monocytogenes*. *description based on NCBI annotation of the *L. monocytogenes* EGD-e genome. [#]denotes mutant was identified and published in previous screen [73]. **Table 2-3:** L. monocytogenes transposon mutants identified in forward genetic screens that affected host cell death.

<i>L. monocytogenes</i> gene harboring transposon insertion	Strain number	Description*	Fold IFN-β induction ^a	Fold Host cell death ^b	Relative bacteriolysis ^c
Mutants that affected	d host cell death				
lmo0540	DP-L5907	Similar to penicillin-binding protein	NS	18	1.8
lmo0687	DP-L5908	Hypothetical protein	NS	1.8	2.8
lmo0692	DP-L5909	CheA, two-component sensor histidine kinase, involved in chemotaxis	NS	0.5	0.6
lmo0785	DP-L5910	Similar to transcriptional regulator, NifA/NtrC family	NS	1.6	0.2
lmo0893	DP-L5911	RsbV, anti-anti-sigma factor	NS	0.5	1.5
lmo2474	DP-L5912	Hypothetical protein	NS	4.4	7.2
lmo2638	DP-L5913	Similar to NADH dehydrogenase	NS	2.5	0.7
lmo2639	DP-L5914	Hypothetical protein	NS	1.9	0.7

Values are represented as fold induction compared to infection with wild-type *L*. *monocytogenes*. ^aIFN- β induction was quantified either by qRT-PCR on RNA isolated from infected macrophages or by bioassay on supernatants from infected macrophages. ^bHost cell death was measured by lactate dehydrogenase release into the supernatant of infected macrophages. ^cRelative bacteriolysis was measured by luminescence as indirect readout of host expression of the reporter plasmid, pBH573, as previously described (Sauer et al., 2010). ND, no data. NS, not significantly different compared to wild-type *L. monocytogenes*. *description based on NCBI annotation of the *L. monocytogenes* EGD-e genome.

Figure 2-2



Figure 2-2: Induction of pyroptosis induced by $\Delta lafB$ mutants is AIM2-dependent. Cell death (A) and IL-1 β (B) were measured following 6-hour infection at an MOI of 5 of Scramble or AIM2 knockdown immortalized macrophages with the indicated strains.





Figure 2-3: *fliD::Himar1* mutants secrete flagellin monomers and hyperstimulate the Nlrc4 inflammasome. (A) Coomassie stained TCA-precipitated culture supernatants from the indicated bacterial strains. (B)Western blot analysis of TCA-precipitated culture supernatants from the indicated bacterial strains. Blots were probed with rabbit anti-*Listeria* sera followed by anti-rabbit-HRP antibody. (C) Cell death was measured following 6-hour infection at an MOI of 5 of wild-type C57BL/6 or Nlrc4^{-/-} BMDMs.

Figure 2-4



Figure 2-4: Intracellular and *in vivo* growth of *L. monocytogenes* $\Delta gcvT$ mutants. (A) Representative intracellular growth curve of wild-type *L. monocytogenes* (circles) and *gcvT::Himar1* mutants (triangles) in BMDMs. (B) C57BL/6 mice were infected with 1x105 CFU of wild-type or *gcvT::Himar1* mutants. Organs were harvested 48 h.p.i, and bacterial load per liver (closed symbol) and spleen (opened symbol) was enumerated.

Chapter 3

Listeria monocytogenes triggers AIM2-mediated pyroptosis upon infrequent bacteriolysis in the macrophage cytosol

The majority of this chapter was published in

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Summary

A host defense strategy against pathogens is the induction of cell death, thereby eliminating the pathogen's intracellular niche. Pyroptosis, one such form of cell death, is dependent on the activation of the inflammasome. In a genetic screen to identify *Listeria monocytogenes* mutants that induced altered levels of host cell death, we identified a mutation in *lmo2473* that caused hyper-stimulation of IL-1 β secretion and pyroptosis following bacteriolysis in the macrophage cytosol. In addition, strains engineered to lyse in the cytosol by expression of both bacteriophage holin and lysin or induced to lyse by treatment with ampicillin stimulated pyroptosis. Pyroptosis was independent of the Nlrp3 and Nlrc4 inflammasome receptors, but dependent on the inflammasome adaptor ASC and the cytosolic DNA sensor AIM2. Importantly, wild-type *L. monocytogenes* were also found to lyse, albeit at low levels, and trigger AIM2-dependent pyroptosis. These data suggested that pyroptosis is triggered by bacterial DNA released during cytosolic lysis.

Introduction

Intracellular pathogens have evolved to survive and replicate within the protected environment of their host cells, concealed from many innate and adaptive immune responses. One host strategy to limit microbial replication is programmed cell death, thereby eliminating the intracellular niche of the pathogen [77,113]. Many intracellular pathogens have evolved mechanisms to counter these responses by either avoiding detection or by actively inhibiting programmed cell death pathways [113,114]. The mechanisms leading to activation and/or avoidance of programmed cell death, from both the host and pathogen perspective, remain incompletely defined.

One cell death pathway resulting from microbial infection is pyroptosis, a proinflammatory cell death triggered upon activation of an inflammasome complex. Multiple inflammasomes have been described and each responds to unique stimuli [77]. Inflammasomes are composed of a receptor, often a nod-like receptor (NLR), coupled to caspase-1, either directly or through the adaptor molecule ASC. Ligand recognition by a NLR leads to the autoproteolytic cleavage of the zymogen pro-caspase-1 [77]. Active caspase-1 can lead to cell death and cleavage of pro-IL-1 β and pro-IL-18 into their active, secreted forms [115].

Listeria monocytogenes, the causative agent of listeriosis, is a Gram-positive facultative intracellular pathogen which grows rapidly within host cells while largely avoiding induction of host cell death [116]. Upon internalization, *L. monocytogenes* escapes from the primary phagosome through the activity of a cholesterol-dependent cytolysin, Listeriolysin O (*hly*) [117]. Indeed, *L. monocytogenes* mutants that induce host cell death due to misregulation of Listeriolysin O are severely attenuated [45]. Recently, *L. monocytogenes* infection has been found to trigger pyroptosis, although the magnitude of this response and which NLR(s) recognize *L. monocytogenes* have been controversial. The adaptor molecule ASC is a central component required for *L. monocytogenes*-induced pyroptosis [79,80,81]. Nlrp3 and Nlrc4 have been reported to detect *L. monocytogenes* infection to varying degrees [80,101,118], while other groups find no role for Nlrp3 [79]. *L. monocytogenes* flagellin has a minor role in inflammasome activation; however, the dominant ligand(s) remains unknown [101].

We performed a forward genetic screen to identify *L. monocytogenes* mutants that resulted in altered induction of host cell death. The mutant with the most robust phenotype was identified as a transposon insertion in *lmo2473*. Deletion of *lmo2473* resulted in mutant bacteria that hyper-induced pyroptosis. Bacterial cell lysis caused either by the loss of *lmo2473*, expression of bacteriophage holin and lysin or treatment with antibiotics led to inflammasome-mediated cell death and IL-1 β release that was dependent on the adaptor molecule ASC. We found that detection of DNA released during bacteriolysis in the cytosol was a natural mechanism of inflammasome activation by wild-type *L. monocytogenes* and that the receptor AIM2 was essential for this process. We propose that ASC-dependent inflammasome responses to other cytosolic pathogens similarly proceed through activation of an AIM2-dependent inflammasome due to bacteriolysis.
Results

Identification of *L. monocytogenes* mutants that hyper-induce pyroptosis

Upon infection of macrophages, *L. monocytogenes* activates a modest level of host cell death. To gain insight into how *L. monocytogenes* interacts with cell death pathways, we screened a bacterial *Himar1* transposon library for mutants that showed increased activation of host cell death. Following infection, cell death was indirectly measured by the amount of macrophage lactate dehydrogenase released into the supernatant. The mutant that caused the most cytotoxicity harbored a transposon insertion in *lmo2473* (data not shown) and in-frame deletion of *lmo2473* resulted in a similar increase in host cell death (Figure 3-1A). Cell death was dependent on caspase-1 and accompanied by IL-1 β release, indicating that $\Delta lmo2473$ mutants were inducing pyroptosis (Figure 3-1A, B). Both cell death and IL-1 β secretion returned to wild-type levels following complementation of *lmo2473* (data not shown). Access to the cytosol was required for induction of pyroptosis as neither Δhly nor $\Delta hly\Delta lmo2473$ double mutants were cytotoxic (Figure 3-1C, D). Furthermore, caspase-1 was cleaved into its active subunits following infection with either wild-type *L. monocytogenes* or $\Delta lmo2473$ mutants but not Δhly mutants (Figure 3-1E).

To address which host inflammasome components were required to activate caspase-1, we infected Nlrc4-, Nlrp3- or ASC-deficient macrophages and found that cell death and IL-1 β secretion induced by both wild-type and $\Delta lmo2473$ were independent of Nlrp3 (Figure 3-1A, B). In agreement with previous observations, IL-1 β secretion was partially dependent on Nlrc4 (Figure 3-1B). Nlrc4-deficent macrophages responded as expected to *Salmonella typhimurium*, a known Nlrc4-stimulus (Figure 3-2A). The inflammasome activation induced by wild-type *L. monocytogenes* and $\Delta lmo2473$ mutants was independent of Nlrp3, although purified Listerolysin O was able to stimulate this Nlrp3 inflammasome, as previously demonstrated (Figure 3-2B)[119]. Also consistent with previous reports, wild-type- and mutant-induced IL-1 β secretion and cell death were fully dependent on ASC (Figure 3-1A, B) [79,80,81,101].

Cytotoxic strains of *L. monocytogenes* are severely attenuated (Glomski et al, 2003), therefore, we hypothesized that hyper-induction of pyroptosis by $\Delta lmo2473$ would negatively affect its virulence. Although growth in broth culture was unaffected (data not shown), $\Delta lmo2473$ mutants were severely defective for intracellular growth in macrophages compared to wild-type bacteria (Figure 3-3A). The growth defect was largely rescued (~90%) in caspase-1-deficient macrophages that are unable to undergo pyroptosis (Figure 3-3B). These data indicated that $\Delta lmo2473$ mutants hyper-activated caspase-1/ASC-dependent pyroptosis compared to the low level activation by wild-type bacteria, subsequently resulting in a severe intracellular growth defect.

Wild-type and $\Delta lmo2473$ L. monocytogenes lyse in the macrophage cytosol

lmo2473 encodes a protein of unknown function that is conserved in many Gram positive and Gram negative bacteria. *Bacillus subtilis* mutants lacking *yvcK*, a homologue of *lmo2473*, have defects in cell wall biosynthesis leading to aberrant cell morphology and eventual bacteriolysis in broth culture [120]. Although the intracellular growth of $\Delta lmo2473$ mutants was mostly rescued in the absence of caspase-1 (~90%), this mutant still showed a measurable defect. In addition, we observed irregular cell morphology of $\Delta lmo2473$ mutants in the cytosol of infected macrophages (data not shown). Taken

together these observations suggested that $\Delta lmo2473$ mutants may lyse in the host cytosol.

To test whether $\Delta lmo2473$ lyses in the host cytosol, we developed a reporter system to indirectly measure bacteriolysis. A luciferase reporter plasmid was constructed that encoded firefly luciferase under control of a cytomegalovirus (CMV) promoter. Lysis of cytosolic bacteria results in release of the reporter plasmid into the host cell and subsequent expression of luciferase from the CMV promoter. As a control for bacteriolysis we designed a *L. monocytogenes* strain expressing PSA bacteriophage holin and lysin from the *actA* promoter. Luciferase production by macrophages infected with the holin/lysin control strain was ~100-fold higher than that produced in macrophages infected with wild-type L. monocytogenes (Figure 3-4). Macrophages infected with $\Delta lmo2473$ mutants expressed ~5-10-fold more luciferase than those infected with wildtype L. monocytogenes. Furthermore, intracellular wild-type L. monocytogenes treated with ampicillin, a β -lactam antibiotic, but not with chloramphenicol, a protein synthesis inhibitor, resulted in bacteriolysis and increased production of luciferase from infected macrophages. Importantly there was a statistically significant increase in lysis of wildtype bacteria compared to bacteria trapped in the vacuole ($\Delta h l y$). These data indicated that there was a reproducible, but low level of lysis of wild-type bacteria in the host cell cytoplasm, however, $\Delta lmo2473$ mutants, the holin/lysin-expressing strain, and β -lactamtreated bacteria lyse at a higher frequency than wild-type bacteria.

Bacteriolysis triggers inflammasome activation

Since $\Delta lmo2473$ mutants lysed in the cytosol of host macrophages and hyperinduced pyroptosis, we hypothesized that bacteriolysis triggers pyroptosis. Therefore, to test this hypothesis, we measured host cell death and IL-1 β secretion from macrophages infected with the holin/lysin control strain or ampicillin-treated bacteria. Similar to macrophages infected with $\Delta lmo2473$ mutants, cells infected with the holin/lysinexpressing strain or bacteria treated with ampicillin underwent pyroptosis and secreted IL-1 β at a higher level than wild-type infected cells (Figure 3-5A, B). Conversely, chloramphenicol treatment following infection resulted in decreased cell death and IL-1 β secretion. Bacterial access to the cytosol was still required for antibiotic stimulated host cell death as Δhly mutants treated with ampicillin did not stimulate pyroptosis (data not shown). Similar to pyroptosis stimulated by wild-type *L. monocytogenes*, inflammasome activation induced by the holin/lysin-expressing strain or ampicillin-treated bacteria was predominantly dependent on caspase-1 and ASC. This data indicated that bacteriolysis in the cytosol, either by expression of autolysins or treatment with β -lactam antibiotics, results in caspase-1/ASC-dependent pyroptosis.

AIM2 recognition of DNA released by bacteriolysis leads to pyroptosis

Lysis of intracellular bacteria could result in the release of many bacterial components into the host cytosol, including DNA. AIM2 was recently identified as a cytosolic DNA receptor that stimulates ASC-dependent inflammasome formation [121,122,123]. We hypothesized that DNA released due to bacteriolysis in the cytosol of host macrophages would be recognized by AIM2 and induce pyroptosis. To test this hypothesis we developed stable shRNA-mediated AIM2 knockdowns in C57BL/6 immortalized macrophages. Lentiviral-based shRNA knockdown resulted in >75%

reduction of AIM2 mRNA in stably transduced immortalized macrophages compared to scramble shRNA transduced cells (Figure 3-6A). Immortalized macrophages transduced with a scrambled shRNA responded similarly to wild-type bone marrow-derived macrophages with respect to LDH release and IL- β secretion following infection with L. monocytogenes (Figure 3-1A, B and Figure 3-6C, D). Similar to the response to DNA (pdA-dT), knockdown of AIM2 resulted in significant decreases in both cell death and IL-1 β secretion following infection with $\Delta lmo2473$ mutants, the holin/lysin-expressing strain, and ampicillin-treated bacteria (Figure 3-6B, C). Meanwhile, pyroptosis in response to Salmonella typhimurium, a known Nlrc4-activator, was largely independent of AIM2. Importantly, even the low level of pyroptosis induced by wild-type L. monocytogenes was partially AIM2-dependent. Consistent with this observation, cleavage of caspase-1 to the active p10 subunit was diminished in the AIM2-knockdown macrophages (Figure 3-6D). Knockdown of AIM2 also resulted in a partial rescue of the intracellular growth defect of $\Delta lmo2473$ (~50%) (Figure 3-6E). In addition, purified L. monocytogenes genomic DNA was also capable of inducing cell death and IL-1ß when transfected into the cytosol of wild-type macrophages (Figure 3-7A, B). Together this data indicated that bacteriolysis of L. monocytogenes in the cytoplasm released DNA that was sensed by AIM2 and triggered inflammasome activation.

Discussion

The results of this study show that *L. monocytogenes* strains that lyse in the host cytosol induced cell death and IL-1 β secretion. Inflammasome activation was largely independent of Nlrp3 and Nlrc4, but was primarily dependent on the recently described DNA receptor, AIM2. These results suggested that DNA released during bacteriolysis is a ligand for inflammasome activation during infection with *L. monocytogenes*. Our data suggests that AIM2 detects bacterial infection and may represent a conserved mechanism for the detection of intracellular bacterial pathogens.

Intracellular pathogens, by definition, require live host cells to support replication; accordingly, *L. monocytogenes* has evolved multiple mechanisms to minimize host cell death. Maintenance of this protected niche is tightly regulated and is central to virulence. Indeed, *L. monocytogenes* mutants that kill their host cells are severely attenuated in mice. For example, the pore-forming toxin Listeriolysin O (LLO), which facilitates bacterial escape from the phagosome, is under transcriptional, translational, and post-translational control that compartmentalize its activity to an acidic phagosome to minimize damage to the host plasma membrane [124,125]. *L. monocytogenes* mutants that fail to properly compartmentalize LLO activity induce non-inflammasome-mediated host cell death and are avirulent [45].

Additionally, flagellin, a known activator of pyroptotic cell death [126,127,128], is tightly controlled during *L. monocytogenes* infection. During growth at 37°C, transcription of *flaA*, the gene encoding flagellin, is repressed through the activity of the transcriptional repressor MogR [129]. Deletion of *mogR* results in significant attenuation of virulence. Previous reports have demonstrated a role for *L. monocytogenes* flagellin in induction of pyroptosis, particularly when it is misregulated by deletion of the flagellar adaptor molecule FlgK [101]. The wild-type *L. monocytogenes* used in this and in Warren *et al* both contain a mutation in MogR that makes control of flagellin expression less stringent [130], potentially explaining a partial role for Nlrc4 in response to wild-

type bacteria. The intricate regulation of LLO and flagellin demonstrate how *L*. *monocytogenes* has evolved to avoid induction of host cell death and maintain its intracellular niche.

The results of this study are consistent with the premise that *L. monocytogenes* causes very low levels of pyroptosis and that lack of host cell death is an essential aspect of *L. monocytogenes* pathogenesis. Indeed, *L. monocytogenes* strains engineered to hyper-activate the inflammasome by ectopically secreting flagellin are highly attenuated [103]. Other groups, however, have reported much higher levels of inflammasome-activation by wild-type *L. monocytogenes* and some have observed a role for Nlrp3. We do not observe a significant role for Nlrp3, even in *L. monocytogenes* strains that activate high levels of inflammasome activation. However, we and others have found that purified LLO added to the outside of cells induces Nlrp3-mediated IL-1 β secretion (Figure 3-2B, [118]). Therefore it is possible that some of the discrepancies in the literature can be explained by the level of extracellular LLO.

The transposon mutant from our screen that caused the most cytotoxicity had an insertion in *lmo2473*. This gene encodes a protein of unknown function and resides in an operon with a predicted NADH dehydrogenase (*lmo2471*) and two additional genes encoding proteins of unknown function (*lmo2474* and *lmo2472*). The architecture of the operon is unique to *Listeria* species, although homologues of *lmo2474-lmo2472* are frequently found together in other organisms. YvcK in *Bacillus subtilis* is a close homologue of Lmo2473 with 46% sequence identity. *B. subtilis* $\Delta yvcK$ mutants were defective for growth with Kreb's cycle intermediates as a sole carbon source [120]. Growth on Kreb's cycle intermediates resulted in phenotypes indicative of cell wall biosynthesis defects including loss of optical density and abnormal cell morphology. Although *L. monocytogenes* is unable to utilize Kreb's cycle intermediates as primary carbon sources [131], our observation that $\Delta lmo2473$ mutants lyse in the cytosol indicates that similar to the role of YvcK in *B. subtilis*, Lmo2473 mutant will help elucidate the role of this gene during wild-type *L. monocytogenes* growth.

Identification of lmo2473 and the observation that $\Delta lmo2473$ mutants appeared to have cell wall defects led to the hypothesis that bacteriolysis was responsible for hyperinduction of the inflammasome. To directly evaluate the role of bacteriolysis in triggering pyroptosis, we constructed a strain designed to lyse and deliver DNA to the cytosol. The holin/lysin-expressing strain resulted in high levels of inflammasome activation. This observation, coupled with the low levels of pyroptosis stimulated by wild-type bacteria, lead us to the conclusion that cytosolic wild-type *L. monocytogenes* undergo very little bacteriolysis. Increased autolysis, resulting in pyroptosis, can result from genetic mutation, antibiotic stress or a number of other environmental conditions.

Treatment of intracellular bacteria with β -lactam antibiotics resulted in bacteriolysis and induction of AIM2-dependent pyroptosis, while chloramphenicol treatment did not. Ampicillin, a cell wall synthesis inhibitor, promotes autolysis, however, chloramphenicol is a bacteriostatic protein synthesis inhibitor and therefore does not result in bacteriolysis. Clinically, listeriosis is treated with ampicillin, often in combination with an aminoglycoside antibiotic (gentamicin) [132]. It is possible that ampicillin is a powerful antibiotic *in vivo* because in addition to targeting bacterial cell wall synthesis, β -lactam activity may increase bacteriolysis leading to induction of pyroptosis. Rational design of antibiotics that not only target centrally important bacterial processes but also help stimulate host innate immune processes may be an attractive approach for the development of antimicrobials.

Similar to previous reports with *Francisella*, we observed some level of host cell death that was independent of caspase-1 but dependent on the inflammasome adaptor ASC (Figures 3-1A and Figure 3-5A)[83]. It is possible that some inflammasome components intersect with caspase-1-independent cell death pathways in the cell. A recent report demonstrated that bacteriolysis of *Shigella flexneri* triggered caspase-3-dependent apoptosis in epithelial cells; however, in these cells bacteriolysis of *L. monocytogenes* did not stimulate any caspase activation [133]. The function of AIM2 in different cell types remains incompletely understood, but it is clear that a variety of cells can recognize and respond to bacteriolysis in the cytosol. Furthermore, inflammasome components may interact with multiple cell death pathways independent of caspase-1.

In the future it will be important to test other cytosolic pathogens, such as *Francisella tularensis*, to determine if they are also recognized by AIM2 and whether bacteriolysis is a conserved pattern of pathogenesis. Future studies to identify the role of AIM2 during acute infection and the development of acquired immunity will further highlight the role of the inflammasome in innate immunity to intracellular bacterial pathogens.

Materials and Methods

Bacterial strains and cell culture

Wild-type 10403S *L. monocytogenes* and isogenic mutants were grown in Brain Heart Infusion media at 30°C overnight without shaking to stationary phase (OD₆₀₀ =1.3-1.6) for macrophage infections. Deletion of *lmo2473* was performed by splice overlap extension and introduced into *L. monocytogenes* by allelic exchange [134]. Bone marrow-derived macrophages were prepared and frozen from femurs of 6-8 week old female mice as previously described [108]. All knockout mice in this study were in the C57BL/6 genetic background. C57BL/6 mice were from The Jackson Lab (Bar Harbor, ME), IFNAR^{-/-} mice were previously described [135], caspase 1^{-/-} and Nlrc4^{-/-}, Nlrp3^{-/-,} and ASC^{-/-}femurs were from Dr. Vishva Dixit (Genentech Inc., South San Francisco, CA).

Macrophage infections and treatments

Macrophages were pretreated for 12-16 hours with 100 ng/ml Pam3CSK4 (Invivogen, San Diego, CA) prior to infection. 5×10^5 bone marrow-derived macrophages were infected with *L. monocytogenes* at a multiplicity of infection (MOI) of 5 bacteria per cell in 24-well plates for 30 minutes. At 30 minutes post infection media was removed and replaced with media containing 50 µg/ml gentamicin and 100 ng/ml Pam3CSK4. Six hours post infection, supernatants were collected and analyzed for LDH release and IL-1 β secretion. Where indicated, infected macrophages were treated with 1 µg/ml ampicillin or 100 µg/ml chloramphenicol at 2 hours post infection.

For infections with *Salmonella typhimurium* LT2, overnight cultures were grown in Luria Bertani (LB) broth at 37°C with shaking. Prior to infection of macrophages, cultures were diluted 1:100 into LB broth and grown for 3 hours at 37°C with shaking. Infections were then performed similar to *L. monocytogenes* infections described above

resulting in an MOI of \sim 5 bacteria per cell. Visual inspection of the infected macrophages reveals that this MOI results in greater than 90% of the macrophages being infected with one or more bacteria.

Poly (dA-dT) was purchased from Sigma-Aldrich and transfected into cells using Lipofectamine2000 (Invitrogen, Grand Island, NY) at a concentration of 500 ng of Poly (dA-dT) per well. Transfections were carried according to the manufacturers protocol for a total of 6 hours at which point samples were collected for LDH and IL-1βanalysis.

Lactate Dehydrogenase release and Interleukin-1β ELISA

To measure LDH release, 60 μ L infection supernatant was added to 60 μ L of LDH detection reagent, as previously described [109] in triplicate in 96-well plates. Absorbance was read on a SpectraMax 340 spectrophotometer (Molecular Devices) at wavelength 490nm and lysis values were calculated as a percentage of cells lysed with 1% TritonX-100.

IL-1 β secretion was determined using mouse IL-1 beta ELISA Ready-SET-Go according to the manufacturer's instructions (eBioscience, San Diego, CA).

Caspase-1 Western blot analysis

Macrophages were pretreated for 12-16 hours with 100 ng/ml Pam3CSK4 (Invivogen, San Diego, CA) prior to infection. 1x10⁶ bone marrow-derived or immortalized bone marrow-derived macrophages were infected at a multiplicity of infection of 5 bacteria per cell in 6-well plates for 30 minutes. At 30 minutes post infection media was remove and replaced with media containing 50 mg/ml gentamicin and 100 ng/ml Pam3CSK4. Six hours post infection, supernatants were collected and precipitated with 10% Trichloroacetic acid (EMD Biosciences, La Jolla, CA). sSamples were separated by SDS-PAGE (Invitrogen, Grand Island, CA) and transferred to polyvinylidene membrane (Millipore, Billerica, MA). Membranes were probed for caspase-1 (Santa Cruz Biotechnology, Santa Cruz, CA) followed by HRP-conjugated secondary antibody (GE Healthcare, Buckinghamshire, UK).

Listeriolysin O inflammasome activation

Listeriolysin O protein was purified as in Zemansky *et al.* Macrophages were pretreated for 12-16 hours with 100 ng/ml Pam3CSK4 (Invivogen, San Diego, CA) prior to infection. $5x10^5$ bone marrow-derived macrophages per well in 24-well plates were treated with 50 ng/ml purified Listeriolysin O for 6 hours. Supernatants were assayed for IL-1 β secretion by ELISA.

Intracellular growth curves

 $2x10^{6}$ macrophages were pretreated with 100 ng/ml Pam3CSK4 (Invivogen, San Diego, CA) overnight, infected with bacteria at a multiplicity of infection of one bacterium per cell, and replication was quantified as previously described [117].

Holin/lysin and luciferase reporter construction

The *actA* promoter was amplified from 10403S genomic DNA and cloned into the MCS of a derivative of the site-specific integration vector pPL2 [136]. The holin-lysin

gene cassette was then amplified from PSA genomic DNA (a gift from Richard Calendar, University of California, Berkeley) and cloned downstream of the *actA* promoter.

The luciferase reporter plasmid was constructed in a stepwise manner. The RP4 *oriT* was cloned into the plasmid pAM401 [137] resulting in the plasmid pAM401oriT allowing for direct conjugation from *E. coli* into *L. monocytogenes*. The modified firefly luciferase gene (luc+) was digested from pGL3-Control (Promega, Madison, WI) and ligated into pAM401oriT. The CMV enhancer, immediate early promoter and chimeric intron were subcloned from pRL-CMV (Promega) and ligated into the pAM401oriT-luc plasmid, resulting in the luciferase reporter plasmid pBHE573.

Luciferase reporter delivery system

5x10⁵ non-stimulated bone marrow-derived IFNAR^{-/-} macrophages per well of 24well plates were infected with *L. monocytogenes* carrying the luciferase-expressing reporter plasmid, pBHE573, at a multiplicity of infection of 5 bacteria per cell for one hour. At one hour post infection, media was removed and replaced with media containing 50 µg/ml gentamicin. Six hours post infection, supernatants were removed and cells were lysed with TNT Lysis Buffer (20 mM Tris, 200 mM NaCl, 1% triton, pH 8.0). Cell lysates were transferred to opaque 96-well plates and luciferase reagent added as previously described [138]. Luciferase activity was measured by luminometer (VICTOR3, PerkinElmer).

AIM2 knockdown

AIM2 shRNA knockdown vectors were a kind gift from Dr. Katherine Fitzgerald and immortalized C57BL/6 macrophages were a gift from Dr. Russell Vance. Lentiviralmediated knockdowns were performed using the pLKO.1 system as previously described [110] in immortalized C57BL/6 bone marrow-derived macrophages [111].

Macrophage gene expression by qRT-PCR

RNA was purified from 2x10⁶ immortalized macrophages using the RNaqueous kit(Ambion, Austin, TX). RNA was then DNase treated, processed and analyzed as previously described [135].

Preparation of L. monocytogenes genomic DNA

L. monocytogenes genomic DNA was isolated using the MasterPure Gram Positive DNA Purification Kit (Epicentre Biotechnologies, Madison, WI) then digested to completion with EcoRI and BamHI (New England Biolabs, Ipswich, MA) and purified using the PCR cleanup kit (Qiagen, Valencia, CA). 5x10⁵ bone marrow-derived macrophages per well in 24-well plates were transfected with Lipofectamine2000 (Invitrogen) and 500 ng/ml prepared genomic DNA.

Statistical analysis

Statistical Analysis was performed using Analyse-It (Leeds, UK). Students' t-test or one way ANOVA followed by a post-hoc LSD test was performed as indicated. *represent p-values of <.05.





Figure 3-1: Induction of pyroptosis by *L. monocytogenes*. Cell death (A,C) and IL-1 β (B, D) were measured following a 6-hour infection at an MOI of 5 with the indicated strains in wild-type, caspase-1^{-/-}, ASC^{-/-}, Nlrp3^{-/-} or Nlrc4^{-/-} bone marrow-derived macrophages. Data are presented as the average of at least 3 independent experiments and the error bars represent the standard deviation of the mean. *indicates these values are statistically different with a *p* value <0.05 using a one-way ANOVA followed by a post-hoc LSD analysis. (E) Caspase-1 processing in *L. monocytogenes* infection. Wild-type bone marrow-derived macrophages were infected for 6 hours at an MOI of 5 with the indicated strains. Proteins were precipitated from macrophages supernatants and Western blotted then probed for the active p10 fragment of caspase-1.



Figure 3-2: Nlrc4^{-/-} and Nlrp3^{-/-} macrophages respond to known stimuli. (A) Cell death was measured following 6-hour infection of wild-type or Nlrc4^{-/-} bone marrow-derived macrophages with the indicated strains, each at an MOI of 5. (B) IL-1 β was measured from the supernatant of wild-type or Nlrp3^{-/-} bone marrow-derived macrophages following either 6-hour infection with the indicated strains at an MOI of 5 or 6-hour treatment with 50 ng/mL purified Listeriolysin O. * indicates these values are statistically different with a *p* value <0.05 by the Students' t-test.



Figure 3-3: Induction of pyroptosis leads to attenuated intracellular replication. Intracellular growth of wild-type *L. monocytogenes* (solid line), $\Delta lmo2473$ mutant (dotted line) and $\Delta lmo2473$ mutant complement (dashed line) was quantified in wild-type C57BL/6 (C) or caspase-1^{-/-} bone marrow-derived macrophages (D). Growth curves are representative of at least three independent experiments. *indicates these values are statistically different with a *p* value <0.05 using a Students' t-test



Figure 3-4: Delivery of plasmid DNA by *L. monocytogenes* strains. Lysis was measured by delivery and expression of luciferase from the luciferase reporter plasmid, pBHE573, in IFNAR^{-/-} bone marrow-derived macrophages following a 6-hour infection at an MOI of 5. Luciferase expression is represented by relative luminescence units. Data are presented as the average of at least 3 independent experiments and the error bars represent the standard deviation of the mean. *indicates these values are statistically different with a *p* value <0.05 using a Students' t-test



Figure 3-5: Pyroptosis and IL-1 β release induced by *L. monocytogenes* that lyse. Cell death (A) and IL-1 β (B) release were measured following a 6-hour infection of wild-type, caspase-1^{-/-} or ASC^{-/-} bone marrow-derived macrophages at an MOI of 5 with the indicated strains. Data are presented as the average of at least 3 independent experiments and the error bars represent the standard deviation of the mean. *indicates these values are statistically different with a *p* value <0.05 using a one-way ANOVA followed by a post-hoc LSD analysis





Figure 3-6: Knock-down of AIM2 abrogates inflammasome activation in response to *L. monocytogenes*. (A) The percentage of AIM2 mRNA following shRNA knockdown in immortalized macrophages was measured by qRT-PCR. mRNA analysis represents the average of 3 independent experiments and error bars represent the standard deviation of the mean. Cell death (B) and IL-1 β (C) were measured following a 6 hour infection at an MOI of 5 of Scramble or AIM2 knockdown immortalized macrophages with the indicated strains. Data are presented as the average of 3 independent experiments and the error bars represent the standard deviation of the mean. (D) Caspase-1 processing in *L. monocytogenes* infection. Scramble or AIM2 knockdown immortalized macrophages were infected for 6 hours at an MOI of 5 with the indicated strains. Proteins were precipitated from macrophages supernatants and Western blotted then probed for the active p10 fragment of caspase-1. (E) Representative intracellular growth of wild-type *L. monocytogenes* (squares) or $\Delta Imo2473$ (triangles) in Scramble (solid lines) or AIM2 knockdown (dotted lines) immortalized macrophages. *indicates these values are statistically different with a *p* value <0.05 using a Students' t-test

Figure 3-7



Figure 3-7: Purified *L. monocytogenes* DNA induces pyroptosis. Cell death (A) and IL- 1β secretion (B) were measured following 6-hour infection with the indicated strains or 6-hour treatment with 500 ng bacterial genomic DNA. Data are presented as the average of 3 independent experiments and the error bars represent the standard deviation of the mean.

Chapter 4

The second messenger cyclic di-AMP is critical for optimal *L. monocytogenes* growth and establishment of infection

A majority of this chapter will be submitted as:

Chelsea E. Witte, Thomas P. Burke, Aaron T. Whiteley, John-Demian Sauer, Daniel A. Portnoy, Joshua J. Woodward. The second messenger cyclic di-AMP is critical for optimal *L. monocytogenes* growth and establishment of infection.

Summary

Listeria monocytogenes infection leads to robust induction of an innate immune signaling pathway referred to as the cytosolic surveillance pathway (CSP), characterized by expression of IFN- β and co-regulated genes. We previously identified the IFN- β stimulatory ligand, cyclic di-AMP, as well as the bacterial protein responsible for its biosynthesis, DacA, and multi-drug resistance transporters necessary for secretion. To identify additional bacterial factors involved in L. monocytogenes detection by the CSP, we performed a forward genetic screen for mutants that induced altered levels of IFN- β . One mutant that stimulated elevated levels of IFN-β harbored a transposon insertion in the gene lmo0052. Lmo0052 renamed here PdeA, has homology to the cyclic di-AMP phosphodiesterase YybT of Bacillus subtilis and is able to degrade c-di-AMP to the linear dinucleotide pApA. Depletion of c-di-AMP by conditional mutation of the di-adenylate cyclase DacA or over-expression of PdeA demonstrated that optimal bacterial replication is dependent on c-di-AMP production. Bacterial transcriptional responses revealed a role for PdeA and c-di-AMP in acid resistance and regulation of resuscitation-promoting factors. Additionally, mutants with altered levels of c-di-AMP have differential susceptibility to peptidoglycan-targeting antibiotics suggesting the molecule may be involved in regulating cell wall stability. During intracellular infection, increases in c-di-AMP production led to hyper-activation of the CSP. Conditional deletion of dacA also led to increased IFN- β and a concomitant increase in pyroptosis, a result of increased bacteriolysis in host cells and bacterial DNA release. Mutants defective for production of c-di-AMP grew poorly intracellularly and were highly attenuated in vivo. These data suggest c-di-AMP coordinates bacterial responses to stress, growth, and cell wall stability and also plays a crucial role in the establishment of bacterial infection.

Introduction

Listeria monocytogenes is a Gram-positive, facultative intracellular pathogen that is the causative agent of the food-borne illness listeriosis. Infection primarily causes disease in immuno-compromised individuals, the elderly, and pregnant women, presenting a significant mortality (up to 25%) to those who contract the disease [9]. A well-characterized infection cycle and a murine model of infection make *L*. *monocytogenes* attractive for studying basic aspects of infection and immunity. During infection, *L. monocytogenes* is phagocytosed by host cells and then rapidly escapes from the vacuole into the host cytosol. Once in the cytosol, the bacterium is able to replicate and spread to neighboring cells without leaving the confines of the intracellular compartment.

Several innate immune pathways detect L. monocytogenes once it enters the host cell cytosol. Activation of one such pathway is mediated by an inflammasome and results in pyroptosis, an inflammatory host cell death. Recent work has demonstrated that L. monocytogenes-induced pyroptosis is due to detection of bacterial DNA released during infrequent bacteriolysis [98]. L. monocytogenes also triggers a specific host transcriptional response called the cytosolic surveillance pathway (CSP) characterized by the expression of IFN- β and co-regulated genes. Until recently, the nature of the bacterial ligand(s) involved in this process remained unknown. A forward genetic screen in our laboratory previously showed that bacterial multidrug resistance transporters (MDRs) of the Major Facilitator Superfamily (MFS) play a central role in host detection during infection, with levels of CSP activation correlating with MDR expression [73,74]. Biochemical analysis of secreted fractions from strains that over-express MDRs led to the identification of cyclic di-adenosine monophosphate (c-di-AMP) as the secreted molecule recognized by the host during infection [74]. Given the structural similarity to c-di-GMP, a well-characterized bacterial-specific second messenger nucleotide, we hypothesize that c-di-AMP serves an analogous but distinct secondary signaling role in L. monocytogenes.

To date, a single di-adenylate cyclase domain (DAC, DUF147) has been implicated in generation of c-di-AMP [139]. Similarly, the DHH/DHHA1 domain pair contained in the *B*. subtilis protein YybT is the only identified protein architecture able to specifically degrade the nucleotide [106]. By sequence homology we previously identified a single DAC domain-containing protein, DacA, responsible for the synthesis of c-di-AMP in *L. monocytogenes* [74]. However, the identification of a c-di-AMPspecific phosphodiesterase or the effects of c-di-AMP signaling in *L. monocytogenes* have not been explored.

In this study we address the effects of c-di-AMP signaling on *L. monocytogenes* physiology and pathogenesis. We have identified the *L. monocytogenes* phosphodiesterase, PdeA, involved in c-di-AMP degradation. By genetically modifying the expression of DacA and PdeA we were able to interrogate the role of c-di-AMP in *L. monocytogenes*. We found that conditional deletion of *dacA* or over-expression of *pdeA* resulted in diminished bacterial replication. We also identified several processes affected by production of the molecule, including acid stress resistance, cell wall stability, and expression of resuscitation promoting factors, also known as stationary phase survival factors (Rpf/Sps). In addition, we show that diminished levels of c-di-AMP dramatically reduced the ability of *L. monocytogenes* to establish infection *in vitro* and *in vivo*. Finally, infection with these mutants resulted in increased stimulation of host innate immune

signaling pathways. We propose that c-di-AMP is a critical signaling molecule required for optimal bacterial growth, cell wall stability, and the ability to establish infection within the host.

Results

Identification of *L. monocytogenes* PdeA, a c-di-AMP phosphodiesterase.

A transposon insertion in *L. monocytogenes lmo0052* was identified in a forward genetic screen for mutants that affect host cell death and IFN- β production (Figure 4-1A) [98]. Disruption of *lmo0052* resulted in elevated levels of IFN- β compared to infection with wild-type *L. monocytogenes* (Figure 4-1B). Sequence analysis identified Lmo0052 as a homolog of *B. subtilis* YybT, which was recently shown to have c-di-AMP phosphodiesterase activity [106]. Lmo0052, renamed here PdeA, has 50% identity and 74% similarity to YybT and shares the multi-domain structure of YybT with two N-terminal transmembrane domains, a PAS signaling domain, a modified GGDEF domain, and C-terminal DHH and DHH-associated domains. Purified PdeA catalyzed conversion of c-di-AMP to the linear dinucleotide pApA, and purification of truncated constructs showed this activity was localized to the DHH/DHHA1 domains, thereby confirming previous biochemical characterization of homologous proteins (Figure 4-1C)[106]. Together these data suggest that PdeA is a c-di-AMP phosphodiesterase.

PdeA, DacA and MDRs alter secretion of c-di-AMP.

The coordinated activity of PdeA, DacA, and bacterial MDRs each modulates cdi-AMP levels generated in and secreted by L. monocytogenes. To define how each of these proteins affects c-di-AMP, we constructed a clean deletion of pdeA and overexpressed the gene. To achieve high levels of over-expression, pdeA was placed downstream of PactA in the PrfA* G145S background strain, where the promoter is constitutively active at high levels [140]. We also manipulated expression of DacA. Multiple attempts to delete *dacA* failed to isolate a clean deletion of the gene from L. *monocytogenes* suggesting it may be essential. In each instance, double recombination and plasmid curing led to regeneration of wild-type alleles. Allelic exchange to generate genomic deletion relies on serial passage of bacteria to cure the plasmid following chromosomal integration. Because the process of plasmid excision and curing is a rare occurrence, mutants with growth defects have a competitive growth disadvantage. To address this, we introduced of a second copy of the *dacA* gene using an IPTG-inducible integration vector, pLIV2 [141]. In this genetic background, successful deletion of the chromosomal *dacA* was accomplished in the presence of inducer ($c\Delta dacA$). Removal of IPTG from these cultures resulted in a conditional *dacA* mutant [142]

Multiple attempts to measure intracellular levels of c-di-AMP were unsuccessful. To define how expression of these proteins affects c-di-AMP metabolism, c-di-AMP secretion by each strain was measured in chemically defined minimal media. As previously reported, ectopic over-expression of bacterial MDRs or DacA resulted in increased levels of c-di-AMP in the culture supernatant [74]. Both the conditional deletion of *dacA* and over-expression of *pdeA* were predicted to lead to depletion of c-di-AMP. Indeed, lower levels of c-di-AMP were observed in culture supernatants of these strains (Figure 4-2), although it should be noted that c $\Delta dacA$ mutant reached 50% of the levels of growth of other strains in minimal media. The clean deletion of *pdeA* was

predicted to result in high levels of intracellular c-di-AMP, as has been shown in *B. subtilis* and *Staphylococcus aureus* mutants deficient in PdeA homologs [143,144]. Surprisingly, PdeA-deficiency did not significantly affect levels secreted into the supernatant during broth culture (Figure 4-2) even though increased IFN-β was observed during infection.

Deletion of pdeA affects bacterial response to acid stress.

Given the putative secondary signaling role of c-di-AMP we hypothesized that altered levels of the signal will have direct or indirect transcriptional effects. Therefore, we performed whole genome microarray analysis comparing $\Delta pdeA$ mutants to wild-type bacteria (data not shown). We found expression of a glutamate decarboxylase, gadD2(lmo2362) and a glutamate transporter, gadT2 (lmo2363), was up-regulated in the PdeAdeficient mutants. Together GadD2 and GadT2 comprise one of two glutamate decarboxylase systems in *L. monocytogenes* and are associated with bacterial survival in severe acid stress [145,146]. To test whether PdeA-deficiency affected *L. monocytogenes* resistance to acid stress, we subjected cultures to severe acid, pH 2.5. *L. monocytogenes* $\Delta pdeA$ mutants were better able to survive acid stress with 2-logs more viable bacteria recovered after 4 hours at pH 2.5 compared to wild-type bacteria (Figure 4-3A). Similarly, mutants deficient in PdeA-homologs in *B. subtilis* [106] and *L. lactis* [147] are more resistant to acid stress. These data suggest that c-di-AMP regulates stress responses, specifically to acid stress, at the transcriptional level.p

Deletion of PdeA leads to increased expression of resuscitation-promoting factors.

Also based on microarray transcriptional analysis we identified two genes highly upregulated in $\Delta pdeA$ mutants, lmo2522 and lmo0186, that were homologous to the *B*. *subtilis* resuscitation-promoting factors/stationary phase survival (Rpf/Sps) family members, *yocH* and *yabE*, respectively (data not shown). Rpf, the first Rpf/Sps family member, was initially identified in *Micrococcus luteus* and shown to be required for stimulating growth of dormant bacteria [148]. Based on these observations, we hypothesized that the absence of these two proteins may affect *L. monocytogenes* replication *in vitro* or *in vivo*. However, deletion of *lmo2522* and *lmo0816*, either independently or in combination, did not affect *L. monocytogenes* growth or initiation of growth from stationary phase (Figure 4-3B). Similarly, deletion of *lmo2522*, *lmo0186* or both Rpf/Sps family members did not affect the ability of *L. monocytogenes* to replicate *in vivo* as equivalent bacteria were recovered from the spleens and livers of mice 48 hours post infection (Figure 4-3C). Nevertheless, the effects of c-di-AMP levels on Rpf/Sps transcription establish a potential link between c-di-AMP and bacterial growth.

Intracellular c-di-AMP levels affect bacterial growth rate.

Our transcriptional profiling suggested a connection between c-di-AMP and regulation of growth control based on the up-regulation of Rpf/Sps expression. Deletion of *pdeA* did not affect *in vitro* bacterial replication suggesting that high c-di-AMP levels do not affect bacterial replication (Figure 4-4A). To test whether c-di-AMP is required for bacterial growth, we either over-expressed PdeA or altered levels of DacA expression. Increased expression of PdeA led to slower bacterial replication with a doubling time of

56 minutes compared to 41 minutes for wild-type (Figure 4-4B), indicating depletion of cellular c-di-AMP levels may slow bacterial growth.

Conditional *dacA* mutants were highly attenuated with a doubling time of 84 minutes compared to 44 minutes for wild-type *L. monocytogenes* (Figure 4-4C), indicating that DacA is required for optimal bacterial replication. In the presence of IPTG $c\Delta dacA$ grew indistinguishable from wild-type *L. monocytogenes*, establishing complementation of the phenotype (Figure 4-4C). To address whether DacA protein or its enzymatic product c-di-AMP is critical for growth, we generated $c\Delta dacA$ mutants in a background strain containing an inducible copy of the *B. subtilis* checkpoint protein, DisA, of which *L. monocytogenes* does not have a homolog ($c\Delta dacA$ pLIV2:*disA*). In addition to the DAC domain, *B. subtilis* DisA contains a DNA binding domain that leads to differential localization to the bacterial chromosome, in contrast to the membrane-anchored DacA. This orthogonal approach to generate c-di-AMP in *L. monocytogenes* was able to rescue the growth defect of the *dacA* conditional mutants (Figure 4-4D). These data are consistent with the hypothesis that c-di-AMP is a pro-growth molecule required for optimal replication.

Synthesis of c-di-AMP by *L. monocytogenes* is predicted to generate intracellular and extracellular pools of c-di-AMP due to secretion through MDRs. This raises the possibility that c-di-AMP functions as an extracellular signal, perhaps regulating the growth of *L. monocytogenes*. To address this hypothesis, we supplemented c $\Delta dacA$ mutants with extracellular c-di-AMP between 0-10 μ M, concentrations spanning and far exceeding the secreted levels observed in broth culture. No affect on growth was observed at any concentration tested, suggesting that intracellular c-di-AMP is necessary to regulate bacterial replication (data not shown).

DacA deletion affects bacterial cell wall stability.

Cell wall synthesis is closely connected to bacterial growth and replication. We hypothesized that the growth attenuation of the $c\Delta dacA$ strains could be due to defects in its cell wall. To test this hypothesis, we evaluated the susceptibility to peptidoglycantargeting antibiotics by disk diffusion assays. *L. monocytogenes* $\Delta pdeA$ mutants were more resistant to cell wall-targeting antibiotics (Figure 4-5A). Conversely, increased susceptibility to these same antibiotics was observed with both the *dacA* conditional mutants and the *pdeA*-over-expressing strains (Figure 4-5A). Interestingly, deletion of both *lmo2522* and *lmo0186* together also conferred increase sensitivity to cefuroxime (Figure 4-5B). We also measured minimum inhibitory concentrations (MIC) of cefuroxime for each of the *L. monocytogenes* strains (Figure 4-5C). Although the differences in susceptibility by MIC were not as drastic as quantified by disk diffusion assay, the trends confirmed the results of the disk diffusion assays. Together, these data supported a role for c-di-AMP in regulating cell wall structure.

Mutation of the S. aureus PdeA-homolog, GdpP, rescues the growth and morphological defects of lipoteichoic acid-deficient mutants [144]. To test whether high cellular c-di-AMP also rescued defects due to the lipoteichoic acid-deficiency in L. monocytogenes, we transduced the transposon insertion disrupting pdeA into $\Delta lmo0927$ mutants. Lmo0927 has been shown to function as an LTA synthase and mutants deficient in this protein grow very poorly at 37°C [105]. This defect was partially rescued upon inactivation of *pdeA* in this genetic background, extending the findings by Corrigan *et al.* to *L. monocytogenes* as well (Figure 4-5D).

Mutants with depleted levels of c-di-AMP likely have defects in their cell walls that led to increased susceptibility to cell wall-targeting antibiotics. These observations led us to the hypothesis that low c-di-AMP may also lead to increased bacteriolysis during broth culture due to cell wall instability. To test this hypothesis, we transduced a transposon conferring β -galactosidase expression into wild-type *L. monocytogenes* and c $\Delta dacA$ [149]. β -galactosidase protein is expressed and maintained within the cytoplasm of the bacterium. Bacteriolysis during growth in broth leads to β -galactosidase release into the culture medium, which can be quantified by the rate of hydrolysis of orthonitrophenyl- β -galactoside (ONPG). As expected, c $\Delta dacA$ mutants release nearly 10-fold more β -galactosidase than wild-type *L. monocytogenes* (Figure 4-5E).

We hypothesized that $c\Delta dacA$ mutants may experience osmotic stress during growth in broth, leading to the observed bacteriolysis phenotype. Therefore, we grew the $c\Delta dacA$ and WT strains in BHI media containing betaine (100 μ M) or carnitine (100 μ M), two osmoprotectants, or NaCl (2% w/v), an osmostabilizer. Increased broth hypertonicity (2% NaCl) had a significant effect on reducing $c\Delta dacA$ lysis (Figure 4-5E). Wild-type *L. monocytogenes* exhibited a small but significant increase in bacteriolysis as a result of increased NaCl. The presence of compatible solutes betaine or carnitine had no effect on lysis or growth rate (data not shown). Together increased susceptibility to peptidoglycan-targeting antibiotics and increased bacteriolysis in broth culture indicate cdi-AMP is involved in regulating bacterial cell wall stability.

<u>C-di-AMP is required for *L. monocytogenes* to establish infection.</u>

To interrogate the role of c-di-AMP during *L. monocytogenes* infection, we utilized strains with altered levels of the signaling molecule in a tissue culture model for intracellular replication and a murine model of infection. Primary bone marrow-derived macrophages were infected with wild-type *L. monocytogenes*, $\Delta pdeA$, and the *pdeA*-over-expressing strain. The intracellular growth kinetics of the *pdeA*-deficient mutants were identical to infection with wild-type bacteria, whereas *pdeA*-over-expressing strains were unable to replicate to the same levels (Figure 4-6A). Similarly, depletion of *dacA* in the absence of inducer attenuated bacterial replication (Figure 4-6B). The c $\Delta dacA$ mutants grew with comparable kinetics (70 minutes between 5 and 8 hours post infection) to those observed in media without inducer. However, while bacteria in broth were able to reach the same final density as wild-type *L. monocytogenes*, the mutant strain in macrophages reached a plateau at 5 hours post infection, 1-log below wild-type levels.

To address whether c-di-AMP levels affected the ability of *L. monocytogenes* to establish *in vivo* infection, we infected mice intravenously with wild-type *L. monocytogenes* or $\Delta pdeA$ mutants. The bacterial loads recovered from both livers and spleens were indistinguishable (Figure 4-6C). Intravenous infection of mice with $c\Delta dacA$ mutants resulted in the recovery of nearly 4-logs fewer bacteria after 48 hours compared to infection with wild-type *L. monocytogenes* or mice infected with the $c\Delta dacA$ strain maintained on water containing IPTG (Figure 4-6D). These results suggested that c-di-AMP was not only required for *in vitro* replication but also for intracellular growth and *in vivo* establishment of infection.

Mutants with altered c-di-AMP levels have increased detection by host innate immune pathways.

C-di-AMP secreted by *L. monocytogenes* during infection leads to Type 1 interferon production [71,74]. As demonstrated in Figure 4-2, although *pdeA*-deficient mutants are predicted to contain high levels of intra-bacterial c-di-AMP, the amount secreted by these mutants into the culture supernatant was comparable to levels observed with wild-type *L. monocytogenes*. To address this paradox, we infected murine bone marrow-derived macrophages and quantified induction of IFN- β by qRT-PCR. PdeA-deficient mutants stimulated nearly 5-fold more IFN- β than wild-type *L. monocytogenes* (Figure 4-7A), suggesting that more c-di-AMP is secreted in the host cell cytosol. This intracellular-specific release of c-di-AMP suggests that some condition within the host cell may trigger c-di-AMP secretion during infection and is physiologically distinct from the signal that leads to secretion during *in vitro* broth growth.

Transit through the host vacuole may present a number of potential stresses to bacterium. To test this hypothesis, we used chemical inhibitors or deficient macrophages to target several of these potential stresses and assess their affect on c-di-AMP secretion, as measured by IFN- β expression. Preventing phagosomal acidification by macrophage pretreatment with the vacuolar ATPase inhibitor Bafilomycin A reduced the amount of IFN- β induced during infection with both wild-type *L. monocytogenes* and $\Delta pdeA$ mutants, whereas IFN- β stimulation by *tetR*::*Tn917* was only minimally affected (Figure 4-7B). Inhibition of phagosomal oxidases or nitric oxidases, independently or in combination, did not affect IFN- β production (data not shown). These data suggest that environmental acidification, such as in the host phagosome, may stimulate bacterial secretion of c-di-AMP by a mechanism that may be dependent on expression or activity of MDRs.

Because host cells recognize c-di-AMP, resulting in stimulation of the cytosolic surveillance pathway, we hypothesized that mutants with low levels of the molecule (e.g. $c\Delta dacA$ mutant) would induce less IFN- β than wild-type bacteria. Surprisingly, infection of bone marrow-derived macrophages with $c\Delta dacA$ mutants resulted in a significant increase in IFN- β (Figure 4-7A). The increased CSP activation during $c\Delta dacA$ infection is especially striking given the attenuated intracellular growth of the $c\Delta dacA$ mutants (Figure 4-6B).

Bacterial DNA delivered to the host cell cytosol can recapitulate the IFN- β response [64,84]. Since host detection of c Δ *dacA* mutants is likely independent of c-di-AMP, we hypothesized that these mutants may also release more DNA while in the host cell. Unlike c-di-AMP, cytosolic DNA can also stimulate another innate immune pathway mediated by the AIM2 inflammasome. Inflammasomes are multi-protein complexes comprised of a receptor coupled to Caspase-1 that assemble in the cytosol; stimulation of which results in Caspase-1 activation and an inflammatory host cell death called pyroptosis. To address whether c Δ *dacA* mutants induced pyroptosis, cell death was indirectly measured by quantifying the amount of macrophage lactate dehydrogenase released following infection. Indeed, infection with c Δ *dacA* mutants led to more than a 2-fold increase in macrophage cell death relative to levels observed with wild-type *L. monocytogenes* or the conditional mutant in the presence of IPTG (Figure 4-7C). Infection of macrophages deficient for the DNA receptor, AIM2, resulted in a reduction

in cell death following infection with *dacA* conditional mutants, indicating bacterial DNA is released from $c\Delta dacA$ mutants and stimulates cell death (data not shown).

We, and others, have shown that microbial DNA can be released during intracellular infection due to bacteriolysis in the cytosol [98,150]. As noted above, mutants with low levels of c-di-AMP lysed in broth culture. Intracellular lysis and release of DNA is a likely explanation for AIM2-dependent inflammasome activation and IFN- β stimulation. To assess the extent to which mutants lyse in host cells, we utilized a luciferase reporter plasmid encoding firefly luciferase under control of the cytomegalovirus promoter [98]. Lysing bacteria release the reporter plasmid, and the infected host cell expresses luciferase. Indeed we observed 4.5-fold more intracellular bacteriolysis during infection with c $\Delta dacA$ mutants compared to infection with wild-type bacteria (Figure 4-7D). Interestingly, macrophages infected with $\Delta pdeA$ mutants expressed only half as much luciferase as those infected with wild-type *L*. *monocytogenes*. Together these data suggest that modulating bacterial production of c-di-AMP affects *L. monocytogenes* stimulation of the CSP as well as the AIM2inflammsome pathway.

Discussion

The process of signal transduction provides a means by which cells can detect and respond to environmental cues. These molecular cues can signal the presence of favorable or unfavorable conditions for growth, leading to a response commensurate with the environmental change. Secondary signaling is a distinct signaling process specifically mediated by a small molecule message. All secondary signaling systems have three fundamental components: (i) a mechanism by which to generate the signal, (ii) a method to degrade the signal, and (iii) a mechanism to transduce the signal. Both signal production and degradation are regulated through environmental cues that modulate enzymatic processing of the signaling molecule, resulting in the phenotypic outputs communicated during the signaling process. In this study we report the characterization of c-di-AMP in *L. monocytogenes*. Specifically, we identified two proteins involved in generating and degrading the nucleotide, as well as the phenotypic effects of altered levels of c-di-AMP. Our results uncovered a unique role for c-di-AMP as a secondary signaling molecule, which had important consequences for basic bacterial physiology and infection.

To affect signal output we modulated expression levels of two proteins involved in c-di-AMP signaling, the di-adenylate cyclase, DacA, and the phosphodiesterase, PdeA, which catalyzed the synthesis and degradation of the di-nucleotide, respectively (Figure 4-8). Although efforts to directly quantify intracellular c-di-AMP have been unsuccessful, we measured secreted c-di-AMP *in vitro* during stationary phase and *in vivo* using induction of IFN- β as an alternative for intracellular levels. As predicted, both overexpression of PdeA and conditional deletion of DacA led to low levels of secreted nucleotide, consistent with the assigned functions of these two proteins. Surprisingly, *ApdeA* mutants did not secrete more c-di-AMP *in vitro* but induced 5-fold more IFN- β relative to wild-type *L. monocytogenes* during infection, indicating increased c-di-AMP secretion selectively in BMDM (Figure 4-7A). A number of possible explanations could account for this seeming discrepancy. Treatment of BMDM with Bafilomycin A, which prevents vacuolar acidification, prior to infection led to a drastic reduction in IFN- β levels induced by $\Delta pdeA$ mutants as well as wild-type bacteria (Figure 4-7B). IFN- β induction by mutants over-expressing MDRs was only minimally affected. This suggests environmental acidification may be a signal for the bacterium to secrete c-di-AMP during infection and may activate MDR expression or activity. Conversely, growth medium used for *in vitro* c-di-AMP secretion is buffered to approximately pH 7 and does not significantly acidify in stationary phase, which may explain why $\Delta pdeA$ mutants only secrete wild-type levels of c-di-AMP in broth culture. An additional explanation is the possibility that another c-di-AMP phosphodiesterase exists. PdeA may function to regulate c-di-AMP levels primarily in host cells, while another protein may alter levels in stationary phase secreted nucleotide levels. Two distinct phosphodiesterase protein families degrade the related second messenger c-di-GMP. Furthermore, non-sporulating Actinobacteria, including *Mycobacteria*, *Gordonia*, and *Rhodococcus*, do not have homologs of YybT but do have predicted DAC domain-containing proteins, indicating the existence of other c-di-AMP-degrading proteins.

Many second messengers are generated in response to environmental stress. Production and degradation of the signaling molecule is often regulated through environmental cues that modulate enzymatic processing of the signaling molecule. We have shown that coordinated activity of DacA, PdeA, and bacterial MDRs regulate c-di-AMP levels in L. monocytogenes (Figure 4-8). It is not clear what signal inputs modulate DacA activity, leading to increased c-di-AMP production. The domain architecture and previous biochemical characterization of PdeA-homolog YybT supports its role as a central hub for stress sensing with regulated degradation of c-di-AMP [151]. PAS domains, similar to the one present in PdeA, function broadly as signal sensory domains that bind a diverse array of small metabolites [152]. Indeed, Rao et al. showed that the PAS domain of B. subtilis YybT binds heme. Coordination of nitric oxide by this heme modulates activity of the downstream domains [151]. Furthermore, in vitro data showed ppGpp inhibits YybT activity, suggesting the possibility of c-di-AMP involvement in nutritional adaptation [106]. Although many bacteria are predicted to metabolize c-di-AMP in a PdeA- and DacA-dependent manner, MDR-mediated secretion of c-di-AMP is currently unique to L. monocytogenes. MDRs are known to have diverse substrate specificity, primarily involved in efflux of toxic molecules including antibiotics and bile [153]. The ability of MDRs to actively secrete c-di-AMP may be a bi-product of the promiscuous substrate specificity of these transporters. However, because these proteins play a role in alleviating small molecule induced stress, it is intriguing to speculate that decreasing intracellular levels of c-di-AMP induced by MDR-mediated secretion provides a rapid means of communicating stress in response to toxic metabolites.

Clearly much remains to be uncovered regarding the signal inputs that affect c-di-AMP levels in *L. monocytogenes*. Furthermore, we do not yet understand what the molecular targets transduce the signal communicated by the nucleotide. However, the work presented here provides insight into the phenotypic effects of altered nucleotide levels and is again consistent with a role in stress adaptation. Components of the glutatmate decarboxylase system were upregulated in PdeA-deficient mutants, which correlated with an increase in resistance to acid stress (Figure 4-3A). Increased levels of c-di-AMP allow the bacterium to survive acidification that is lethal to wild-type bacteria. It is not clear what signal inputs sensed by DacA or PdeA would lead to this enhanced acid resistance.

Our results also demonstrate a connection between c-di-AMP production and bacterial cell wall stability. The Gram-positive bacterial cell wall provides both a primary surface of interaction with and a structural barrier to the extracellular environment. The strengthening of the cell wall induced by high levels of c-di-AMP may provide increased protection to the bacterium in certain environments. Gram-positive bacteria are under an internal pressure of 15-25 atm [154], and the elastic meshwork of peptidoglycan provides a reversible means of withstanding the turgor exerted by osmotic forces. We propose lysis of $c\Delta dacA$ mutants is due to a weakened cell wall that cannot withstand the high pressure of the cell. In vitro lysis was stabilized by the presence of NaCl in the growth medium (Figure 4-5D), which helps to decrease the intracellular pressure by lowering the relative hypo-osmotic force experienced by the bacterium. Indeed, spheroplasts are susceptible to lysis and must be osmotically stabilized by the addition of sucrose or NaCl [155,156]. Additionally, sensitivity of L. monocytogenes mutants to cell wall-targeting antibiotics had an inverse correlation with c-di-AMP levels (Figure 4-5A,C). There are a number of ways in which c-di-AMP signaling could alter cell wall stability. First, c-di-AMP may regulate peptidoglycan production and overall wall content. Two other genes, *lmo2119*, a gene of unknown function, and *glmM*, glucosamine mutase, are adjacent to dacA on the L. monocytogenes chromosome. GlmM has been shown to serve a crucial function in peptidoglycan synthesis, generating the precursor for all peptidoglycan biosynthesis, glucosamine-1-phosphate [157]. Although speculative, this genetic organization, which is highly conserved among bacteria that have predicted DacA homologs, suggests a role for controlling peptidoglycan production. Second, c-di-AMP may affect peptidoglycan cross-linking through altered peptidoglycan-binding protein (PBP) synthesis. PBPs play a central role in the maturation and stabilization of bacterial peptidoglycan by generating peptide cross-links between adjacent peptidoglycan strands. Given the correlation between the c-di-AMP production and susceptibility to antibiotics that target PBPs, it is possible that altered expression or activity of L. monocytogenes PBPs may contribute to wall stability regulated by this molecule. This possibility is further supported by observations made in S. aureus where increased peptidoglycan cross-linking was shown to correlate with increased c-di-AMP production [144].

The results reported here uncover a distinct characteristic that makes c-di-AMP unique from other bacterial second messengers. Although other second messengers have effects on growth, c-di-AMP is unique in that its presence is required for growth. The stringent response alarmone ppGpp, for example, is produced under conditions of amino acid starvation and prevents chromosomal replication by binding DNA primase replication at the origin [158]. Although speculative, c-di-AMP may also regulate bacterial replication by a similar mechanism, in which its presence is required to induce a critical process of bacterial replication. Additionally, increased nucleotide levels result in up-regulation of pro-growth Rpf/Sps members, which are required to reinitiate replication of some dormant bacteria [159]. Transcriptional upregulation of these factors suggests that the presence of high levels of c-di-AMP promotes a pro-growth state in the bacterium. Although no effect on growth *in vitro* or *in vivo* was observed from deletion of the two Rpf/Sps homologs in *L. monocytogenes*, these proteins may play a more prominent role during environmental conditions not tested here or in other bacteria that

have more characteristic dormancy programs. Furthermore, *pdeA* is predicted to be within an operon with a ribosomal protein and a replicative DNA helicase (Figure 4-1A). This genomic architecture is conserved among many microbes with predicted PdeA-homologs and may suggest a link between PdeA production and transcription and translation for the basic function of bacterial replication. This is consistent with work in *B. subtilis* demonstrating that high levels of c-di-AMP allow the bacterium to bypass signals that halt sporulation (ie. DNA damage, acid stress) and continue replication/sporulation [106]. Together these results support a role for c-di-AMP in regulating bacterial replication as a pro-growth signal.

Given the importance of c-di-AMP in basic bacterial growth it is perhaps not surprising that L. monocytogenes requires c-di-AMP for the establishment of infection. Like all bacteria, the goal of pathogens is to obtain nutrients to support bacterial replication. Unique to pathogens, however, is the ability to access nutrients in discrete host niches, each of which includes a distinct set of stresses. Using a tissue culture model of infection, we observed evidence that c-di-AMP may be necessary for the bacterium to survive in a phagolysosome and adapt to the changing environment upon entry into the host cytosol. Infection with the $c\Delta dacA$ and PdeA-over-expressing strains illustrated important defects resulting from limited c-di-AMP production during infection (Figure 4-7A,B). First, during infection with the $c\Delta dacA$ strain, half of the bacteria present at 30 minutes died by two hours post infection (Figure 4-7B). The $c\Delta dacA$ strain has low c-di-AMP from the start of infection and, as a consequence, during passage through the phagolysosome. We hypothesize the survival defect observed with this strain is due to an inability to withstand the stresses associated with traversing the primary vacuole, including acid, oxidative or nitrosative stresses. In contrast, this observation was not seen during infection with a strain that over-expresses PdeA (Figure 4-7A). Although these two strains, $c\Delta dacA$ and PdeA-over-expressing, both resulted in lower levels of c-di-AMP production during infection, they differed in the timing of c-di-AMP depletion. The actA promoter, which is only active following cytosolic entry of the bacterium, drives pdeA-over-expression and c-di-AMP depletion only once the bacterium enters the host cytosol. This observation indicates a unique adaptation must be accompanied by the ability to grow within the infected host cell and may be triggered by distinct conditions traversing the vacuolar compartment. Therefore, we propose that the inability of low c-di-AMP-producing strains to replicate within host cells reflects an adaptation to this unique environment. Together these results indicate c-di-AMP is necessary both to survive in the phagosome and also to replicate in the cytosol.

To monitor for and appropriately respond to the presence and action of microbes, the host relies on a series of germ-line encoded receptors that comprise the innate immune system. Evolution of a molecular recognition system that relies on a limited number of proteins and that will maintain its function throughout microbial evolution requires the targeting of molecular motifs that remain constant across microbial genre. As such, innate immune receptors generally recognize conserved and essential components of microbial function or growth, those to which microbes are evolutionarily constrained. C-di-AMP is directly detected by the host during intracellular infection [74]. Due to its critical role in fundamental bacterial processes including replication, cell wall stability, and stress responses as well as a widespread distribution in bacteria and archaea, c-di-AMP is an ideal pathogen-associated molecular pattern (PAMP). Our data suggests that *L. monocytogenes* is also evolutionarily constrained to produce c-di-AMP. Mutants that produce decreased levels of the molecule also stimulate Caspase-1 activation and pyroptosis, eliminating the intracellular niche of the bacterium. Maintaining the intracellular niche is crucial to *L. monocytogenes* pathogenesis, and bacteria that exhibit cytotoxicity are severely attenuated [45]. Together, these observations indicate that production, degradation, and secretion of the nucleotide must be tightly controlled to allow bacterial replication, to maintain its intracellular niche, and control detection by host signaling pathways.

We have identified several proteins involved in c-di-AMP regulation in *L. monocytogenes* and implicated the molecule in regulation of bacterial growth, response to stress, and cell wall stability. Future work to uncover the signals that modulate enzymatic processing of c-di-AMP and the molecular targets of the molecule will be critical to further understand how c-di-AMP regulates the bacterial processes described here. Importantly, c-di-AMP is also the IFN- β -stimulatory ligand detected by the host cell during infection with *L. monocytogenes*. Currently, we have a very limited understanding about the role of c-di-AMP within the host niche and the signals that induce secretion of the molecule during infection. Furthermore, many microbes, including pathogens like *Mycobacterium tuberculosis, Chlamydia trachomatis, Staphylococcus aureus*, and *Streptococcus pyogenes*, are predicted to produce c-di-AMP, although we do not yet know if host detection of c-di-AMP occurs during infection by these microbes. Clearly cdi-AMP plays a central role in bacterial replication and establishment of infection, features which may make c-di-AMP metabolism an attractive area for future research focus.

Materials and Methods

Bacterial strains, growth media and cell culture

For *in vitro* growth, acid stress susceptibility, and gene expression experiments, wild-type 10403S *L. monocytogenes* and isogenic strains were grown in Brain Heart Infusion (BHI) media at 37°C overnight to stationary phase ($OD_{600}\approx3.0$). All *Escherichia coli* strains used for in-frame gene construction, complementation, and protein expression were grown on Luria-Berani (LB) medium. Antibiotics were used as indicated at the following final concentrations: streptomycin, 200 µg/mL; chloramphenicol, 7.5-20 µg/mL; kanamycin 30 µg/mL.

Bone marrow-derived macrophages were prepared and frozen from the femurs of 6- to 8-week-old female C57BL/6 mice, from Jackson Laboratory (Bar Harbor, ME), as previously described [160]. IFNAR-/- mice were previously described [64]. Immortalized C57BL/6 macrophages were a gift from Russell Vance. AIM2 shRNA knockdown vectors were a gift from Kate Fitzgerald. Lentiviral-mediated knockdowns were performed using pLKO.1 system as previously described [110].

Deletion of *pdeA* (*lmo0052*), *lmo2522*, and *lmo0186* were performed by splice overlap extension and introduced into *L. monocytogenes* by allelic exchange [47]. Overexpression of *pdeA* was achieved by placing the gene downstream of the P_{actA} . Briefly, P_{actA} was amplified using the forward primer GGT ACC GGG AAG CAG TTG and reverse primer ATA GCC TGA CAT ATC GAT TCC CTC, and *pdeA* was amplified using forward primer GAG GGA ATC GAT ATG TCA GGC TAT and reverse primer ATTA CGG CCG TTA TGT TTC TCC CTT. The two fragments were combined by

SOE PCR, and the product was digested with KpnI and EagI and subsequently ligated into digested pINT vector. The plasmid was transformed into SM10 *E. coli*. Following confirmation by sequencing at the UC Berkeley Sequencing Facility, it was conjugated into wild-type *L. monocytogenes* or PrfA* G145S mutants.

Construction of dacA conditional mutant

The IPTG inducible pLIV2:*dacA* plasmid [74] was modified to contain a tetracycline resistance cassette (Tet^R). Tet^R was amplified from the Tn917 transposon using the forward primer GAG GAG GTA TAC CCA TAT TGT TGT ATA AGT GAT GAA ATA and the reverse primer GAG GAG GAC CCG GTC AAT TCC TGT TAT AAA AAA AGG ATC AAT, which contain BstZ17I and Tth111I restriction sites, respectively. The pLIV2:*dacA* plasmid was digested with these two restriction enzymes to remove the chloramphenicol resistance cassette. The PCR Tet^R was similarly digested and ligated into digested pLIV2:*dacA*. The resulting plasmid, pLIV2-Tc:*dacA*, was subsequently integrated into the *L. monocytogenes* genome as described previously.

Deletion of the chromosomal copy of *dacA* was accomplished using the pKSV7oriT plasmid. Briefly, 1000 base pairs flanking the 5' and 3' ends of the *dacA* gene were amplified and subsequently combined by SOE PCR. A total of 6 amino acids from the original open reading frame were retained to limit disruption of downstream genes. The SOE amplified product was digested with PstI and SalI and ligated into similarly digested pKSV7-oriT. The plasmid was then conjugated into *L. monocytogenes* through the donor strain *E. coli* SM10. Subsequent selection for integration, plasmid excision, and plasmid curing were as previously described [47]. The primers used to generate the pKSV7oriT: *AdacA* construct were, GAG GAG GTC GAC CGC AGC CTG GAC GAT TC, ATT TAA AAT TCG ATC CAT CAT TCG CTG GAA AAA TCC ATC ACT TCA CCT C, GAG GTG AAG TGA TGG ATT TTT CCA GCG AAT GAT GGA TCG AAT TTT AAA T, GAG GAG CTG CAG CTG TGT CTT GGT TAT TAC TAT CTG.

<u>Construction of c∆dacA pLIV2::disA</u>

The c $\Delta dacA$::pLIV2-*disA* strain DP-L5937 was constructed using the c $\Delta dacA$ strain DP-L5932 integrated with pLIV2-*disA*, an IPTG inducible variant of pPL2 (Lauer et al., 2002) expressing *disA* amplified from an asporogenic derivative of *B. subtilis* strain ZB307 (Zuber and Losick, 1987) using primers GAG GAG CGG CCG CAT ATG GAA AAA GAG AAA AAA GGG GCG and CTC CTC GTC GAC TCA CTC GAG CAG TTG TCT GTC TAA ATA ATG CTT C.

Protein expression and purification

Using *L. monocytogenes* genomic DNA as a template, *pdeA* fragments were amplified encoding the PAS-GGDEF-DHH/DHHA1 (Δ 83PdeA) truncation using primers CCA TGG ATG CCG ATG GGA ATA CTG CTG TAT GAT and CTC GAG TTA TGT TTC TCC CTT CCA ATA CGC ATC. This fragment was ligated into the T7 expression vector pET-28(b) using NcoI and XhoI restriction sites that provided a C-terminal 6Xhistag. The plasmids were transformed into Rosetta *E. coli* BL21 and protein expression was performed as previously described [106]. Briefly, each mutant was growth to an optical density of 0.1, and protein expression was induced with 1 mM IPTG and was placed at 16°C with shaking for 12 hours. The bacteria were pelleted by centrifugation, resuspended in 20 mL lysis buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 5% glycerol, 0.1% β-mercaptoethanol, and 1 mM PMSF), and lysed with a cell disruptor (Branson). Cell debris was pelleted by centrifugation for 30 minutes followed by filtration. The supernatant was incubated with 1 mL Ni-NTA resin (Qiagen) for 1 hour at 4°C and then washed with 50 mL Wash 1 Buffer (lysis buffer with 20 mM imidazole) followed by 20 mL Wash 2 Buffer (lysis buffer with 50 mM imidazole). The bound protein was eluted using a step gradient method with elution buffers containing 50 mM Tris (pH 8.0), 150 mM NaCl, 5% glycerol and 200 mM, 300 mM or 500 mM imidazole. The fractions were assessed for purity by SDS-PAGE gel and fractions with higher than an estimated 95% purity were pooled and injected into a Slide-A-Lyzer Dialysis Cassette (Thermo Scientific) and incubated overnight at 4°C in dialysis buffer (50 mM Tris (pH 8.0), 150 mM NaCl). For storage, each protein aliquot received 5% glycerol and was flash frozen.

Enzymatic Synthesis of c-di-AMP

Recombinant DisA was expressed and purified as described previously (Hopfner, 2008). Purified DisA (0.6 μ M final) was added to a solution of ATP (1 mM), Tris (40 mM, pH 7.5), NaCl (100 mM), b-mercaptoethanol and MgCl₂ (20 mM). Reactions were incubated for 24 hours at 30°C. Subsequently, boiling the sample for 10 minutes precipitated protein. After cooling to room temperature, the sample was filtered and diluted 5-fold with de-ionized water. Nucleotide was applied to anion exchange resin (Q-sepharose, Pharmacia, 10 mL). Once adsorbed, the resin was washed with 5 bed volumes of de-ionized water. Elution of adsorbed nucleotide was accomplished by washing the resin with ammonium acetate (NH₄Ac, 2 M) until the A₂₆₀ in the eluate returned to baseline levels. Dissolved c-di-AMP was then speed vacuumed to dryness and then resuspended in water. This process was repeated two more times to ensure complete removal of NH₄Ac. Sample purity was assessed by HPLC analysis and confirmed to be >98 % based upon peak absorbance (data not shown).

Derivatization and detection of secreted c-di-AMP

To assess secretion of c-di-AMP, bacteria mutants were grown in defined minimal media [161] for 24 hours at 37°C with shaking. Bacteria were removed from the supernatant by centrifugation. 50µL samples of culture supernatants were combined with 50 μ L sodium acetate (1M, pH 4.5) and 5 μ L chloroacetaldehyde (4M) and incubated at 80°C for 20 minutes to derivatize all adenyl purine molecules, as described [162]. Samples were analyzed using an HPLC system (Agilent 1100 series) fitted with a Nova-Pak C₁₈ column (150 3.9 mm, 4 m, Waters) equipped with an Alltima C₁₈-LL guard column (5 m, Alltech). Solvent A consisted of sodium phosphate (30 mM), tetrabutylammonium bisulfate (5 mM, pH 6.0) and Solvent B contained 100% acetonitrile. The samples were eluted using an isocratic flow of 14% Solvent B over 14 minutes followed by a gradient from 14-40% B over 0.5 minutes, 40% B for 5 minutes, a return to 14% B over 0.5 minutes and 5 minutes at 14% B to re-equilibrate the column. The injection volume was 100 μ L and the flow rate was 0.7 mL/min. Excitation of N⁶etheno derivatized c-di-AMP was done at 278 nm and emission was monitored at 418 nm. Nucleotide was quantified by comparing fluorescent peak area to similarly derivatized standards of purified c-di-AMP. Concentrations of standards were determined spectrophotometrically based upon the absorbance at 259 nm ($e=30,000 \text{ M}^{-1} \text{ cm}^{-1}$).

PdeA Enzyme Activity

Reactions consisting of 20 uM c-di-AMP were incubated with 3 mM enzyme in Assay Buffer, consisting of Tris (100 mM,pH 8.0), potassium chloride (20 mM), manganese sulfate (0.5 mM) and were allowed to proceed for 30 minutes at 37°C prior to analysis using an HPLC system (Agilent 1100 series)Nova-Pak C₁₈ column (150 3.9 mm, 4 m, Waters) equipped with an Alltima C₁₈-LL guard column (5 m, Alltech). Solvent A consisted of sodium phosphate (30 mM), tetrabutylammonium bisulfate (5 mM, pH 6.0) and Solvent B contained 100% acetonitrile. The samples were eluted using a linear gradient from 5-12% Solvent B over 20 minutes followed by a gradient from 12-40% over 3 minutes. The injection volume was 100 μ L and the flow rate was 0.7 mL/min. c-di-AMP and pApA (Biolog) standards were run in each experiment.

Susceptibility to acid stress

Bacterial cultures were grown to stationary phase in BHI broth (OD=2.5) at 37°C with shaking, then the pH was adjusted to 2.5 with HCl. Viable cell counts were performed at the indicated time intervals by plating on LB plates.

Bacteriolysis in broth and in macrophages

A U153 transducing lysate was generated with donor strain DPL-967, which contains a Tn917 insertion that leads to constitutive expression of the contained lacZgene. Transduction of the c $\Delta dacA$ and wild-type L. monocytogenes strains was performed as described previously [163]. Broth lysis was assessed as the amount of β -galactosidase activity secreted into the broth supernatant during exponential growth. Overnight cultures of each strain were grown at 37°C with shaking. Cultures were back diluted 1:100 in fresh BHI media and grown to mid-log ($OD_{600}=0.5$). Bacteria were removed from the culture supernatant by centrifugation. Culture supernatants were then filter sterilized with syringe filters (Millex GP, 0.22 μ m, Millipore). The bacterial pellet was re-suspended in fresh BHI. Zirconia/Silica beads (Biospec Products Inc., $\sim 200 \ \mu$ L) were added to resuspended bacteria and vortexed for 10 minutes at 4°C. The lysed bacteria were then centrifuge for 5 minutes at 14,000 rpm to remove cellular debris and the beads. Dilutions of the cellular lysates of each strain (1-16 fold) were made. Diluted lysates (100 μ L) and sterile filtered culture supernatants (100 μ L) were separately mixed with Z-buffer $(100\mu L, 0.1 \text{ M Phosphate}, 0.01 \text{ M KCl}, 1 \text{ mM MgSO4}, 50 \text{ mM BME}, \text{pH 7.0})$ and ONPG (4 mg/mL in 0.1 M Phosphate, pH 7.0). Samples were placed in a 96-well plate and A_{420} was monitored at 37°C over time. b-galactosidase activity was calculated based on the rate of change in the A₄₂₀. Dilutions of bacterial lysates were used to generate a standard curve of lysis, with the 1:1 dilution of lysate equivalent to 100 % lysis and BHI equivalent to no lysis. Broth lysis was calculated based upon the activity observed in the culture supernatant relative to this standard curve.

To assess intracellular bacteriolysis, *L. monocytogenes* strains were engineered to carry the luciferase reporter plasmid, pBHE573 [98]. $5x10^5$ IFNAR^{-/-} macrophages were infected with at a multiplicity of infection of 5 bacteria per cell. At 1-hour post infection media was replaced with media containing 50 µg/mL gentamicin. Six hours post infection supernatants were removed, and cells were lysed with TNT lysis buffer (20 mM Tris, 200 mM NaCl, 1% triton [pH 8.0]) and transferred to 96-well plates. Luciferase

reagent was added to each well [112] and luminescence was measured by luminometer (VICTOR3, PerkinElmer; Waltham, MA).

Antibiotic susceptibility

Confluent lawns (approximately $2x10^7$ CFU) of bacteria were spread on BHI plates and allowed to dry. Sterile 7 mm diameter paper disks (Whatman 3MM) containing 5 µg cefuroxime, 5 µg ampicillin or 5 µg penicillin were placed on the plates. Following 24 hours of incubation at 37°C, the diameter of the zone of growth inhibition surrounding the disks was measured. Antibiotic susceptibility was also assessed by minimum inhibitory concentration. Briefly, stationary phase cultures were backdiluted 1:100 and used to inoculate fresh BHI media containing 2-fold serial dilutions of cefuroxime. Cultures were growth for 12 hours at 37°C in a SpectraMax Plus340 spectrophotometer (Molecular Devices; Sunnyvale, CA) and OD600 readings were taken every 15 minutes. The MIC was determined as the antibiotic concentration required to inhibit bacterial growth by 75%.

In vivo mouse infections

Prior to infection, bacterial strains are grown to stationary phase ($OD_{600}=1.2$) at 30°C, then back diluted and grown at 37°C with shaking until $OD_{600}=0.3-0.5$. Cultures were diluted in 1XPBS and used to intravenously infect female C57BL/6 mice between 6-8 weeks of age with a final inoculum of 10⁵ bacteria. At 48 hours post infection, mice are sacrificed and organs collected. Bacterial burdens were enumerated by plating organ homogenates on LB plates and incubated overnight.

IFN-β analysis by qRT-PCR

 $2x10^6$ macrophages from C57BL/6 mice were infected with a multiplicity of infection of one bacterium per cell in 6-well plates for 30 minutes. Monolayers were washed and media replaced. At one hour post-infection, gentamicin was added to the media at a final concentration of 50 µg/mL (Sigma). For vacuolar ATPase inhibition experiments macrophages were pretreated for 30 minutes with Bafilomycin A (Sigma) at a final concentration of 0.5 µM. Four hours post infection media was removed and macrophage RNA was isolated using the Ambion RNaqueous Kit according to the manufacturers instruction (Ambion). Samples were treated with TURBO Dnase (Ambion) for one hour at 37°C and RNA was precipitated overnight. As with qRT-PCR analysis of bacterial gene expression, RNA was reverse transcribed using iScript cDNA Synthesis Kit (Bio-Rad) and subjected to quantitative PCR using Sybr Fast qPCR kit (KAPA Biosystems) on an Mx3000P machine and analyzed using MxPro software (Stratagene). All macrophage *ifn-β* expression data is normalized to β -actin.

Lactate dehydrogenase release assay

Macrophage cell death was measured, as previously described (Sauer 2010). Briefly, $5x10^5$ macrophages were pretreated for 12-16 hours with 100 ng/mL Pam3CSK4 (Invivogen; San Diego, CA) then infected at a multiplicity of infection of 5 bacteria per cell. Thirty minutes post infection media was removed and replaced with media containing 50 µg/mL gentamicin and 100 ng/mL Pam3CSK4. Six hours post infection, supernatants were harvested and added to LDH detection reagent [109]. Absorbance at 490 nm was read on a SpectraMax Plus340 spectrophotometer (Molecular Devices; Sunnyvale, CA). Lysis values were calculated as percentage of cells lysed with 1% Triton X-100.

Figure 4-1



Figure 4-1: PdeA is a c-di-AMP phosphodiesterase. (A) Schematic representation of the site of transposon insertion in *pdeA*, marked with a triangle. (B) qRT-PCR analysis of IFN- β expression in bone marrow-derived macrophages infected with wild-type *L*. *monocytogenes* or *pdeA::Himar1* mutants. (C) HPLC detection of nucleotides following incubation of c-di-AMP in the absence (black bars) or presence (gray bars) of recombinant Δ 83PdeA protein. HPLC data is representative of more than three independent experiments. * indicates these values are statistically different with a *p* value <0.05 using a Student's t test.

Figure 4-2



Figure 4-2: HPLC detection of secreted c-di-AMP from *L. monocytogenes* mutants. Samples from the indicated strains were taken during stationary phase and c-di-AMP secretion was evaluated by HPLC following derivatization of the samples. Values are presented as the average of three independent measurements of samples from the indicated strains. Error bars represent the standard deviation from the mean. *indicates these values are statistically different compared to wild-type (WT), with a *p* value <0.05 using a Student's t test. ND = none detected.





Figure 4-3: Acid resistance and resuscitation-promoting factors/stationary phase-survival factors are up-regulated in *L. monocytogenes* $\Delta pdeA$ mutants. (A) Acid resistance of wild-type *L. monocytogenes* (circles) and $\Delta pdeA$ mutants (squares) was assessed by quantifying survival in BHI media adjusted to pH 2.5. Plot is representative of three independent experiments. Error bars represent the standard deviation of the mean of triplicates within the representative experiment. *indicates these values are statistically different from WT, with a *p* value <0.05 using a Student's t test. (B) Broth growth curves of indicated *L. monocytogenes* strains grown in BHI media at 37°C with shaking. (C) C57BL/6 mice were infected with 1x10⁵ CFU of wild-type *L. monocytogenes*, $\Delta lmo2522$, $\Delta lmo0186$, or $\Delta lmo2522\Delta lmo0186$ mutants. Organs were harvested at 48 h.p.i., and bacterial burden per spleen (closed circles) or liver (open circles) was enumerated. Median values are presented as horizontal lines.




Figure 4-4: c-di-AMP levels affect bacterial *in vitro* growth. Broth growth curves of *L. monocytogenes* strains grown in BHI media at 37°C with shaking. (A) Wild-type *L. monocytogenes* (circles) and $\Delta pdeA$ mutants (triangles) (B) PrfA* *L. monocytogenes* (circles) and PdeA-overexpression strain, PrfA* pINT *pdeA* (triangles). (C) Wild-type *L. monocytogenes* (circles) and c $\Delta dacA$ mutants +IPTG (triangles) and -IPTG (diamonds). (D) Wild-type *L. monocytogenes* (circles), c $\Delta dacA$ pLIV2::*disA* +IPTG (triangles) and -IPTG (diamonds). Each plot is representative of three independent experiments.

Figure 4-5

С



L. monocytogenes strain	MIC Cefuroxime (µg/mL)
WT	0.94-1.88
$\Delta p de A$	3.75-7.5
c∆dacA	<0.12
PrfA* G145S	1.88-3.75
PrfA* G145S pINT pdeA	0.94-1.88
$\Delta lmo2522$	0.94-1.88
$\Delta lmo0186$	0.94-1.88
$\Delta lmo2522\Delta lmo0186$	0.94-1.88



Figure 4-5: c-di-AMP affects bacterial cell wall stability. (A) Antibiotic susceptibility of indicated mutants as measured as zone of bacterial growth inhibition surrounding filter disk impregnanted with cefuroxime (5 µg), ampicillin (5 µg), or penicillin (5 µg). Values represent the average of more than three independent experiments and error bars are a standard deviation from that mean. (B) Susceptibility of indicated *L. monocytogenes* mutants to cefuroxime (5 µg/mL) as measured by disk diffusion zone of inhibition. (C) Cefuroxime susceptibility represented by minimum inhibitory concentration. (D) Broth growth curves in BHI media at 37°C with *L. monocytogenes* strains wild-type *L. monocytogenes* (circles), $\Delta lmo0927$ mutants (triangles), and $\Delta lmo0927$ pdeA::Himar1 mutants (diamonds). (E) In vitro bacteriolysis was measured as the amount of β -galactosidase activity in broth supernatant during logarithmic growth in BHI media (black bars) or BHI supplemented with 2% NaCl (gray bars) from the indicated strains. *indicates these values are statistically different, with a *p* value <0.05 using a Student's t test.



Figure 4-6: C-di-AMP levels affect L. monocytogenes ability to establish infection. (A) Representative intracellular growth of wild-type L. monocytogenes (circles), pdeA (triangles), or *pdeA*-overexpressing mutants (diamonds) in bone marrow-derived macrophages. Error bars represent the standard deviation from the mean of triplicates within the representative experiment. (B) C57BL/6 mice were infected with $1x10^{5}$ CFU of wild-type or *ApdeA* mutants. Organs were harvested at 48 h.p.i., and bacterial burden per spleen (closed circles) or liver (open circles) was enumerated. Median values are presented as horizontal lines (C) Representative intracellular growth of wild-type L. monocytogenes (circles) or dacA-conditional mutants in the presence (triangles) or absence (diamonds) of IPTG in bone marrow-derived macrophages. Error bars represent the standard deviation from the mean of triplicates within the representative experiment. (D) C57BL/6 mice were infected with 1×10^5 CFU of wild-type or *dacA*-conditional mutants in the absence or presence of 10 mM IPTG in the drinking water. Organs were harvested at 48 h.p.i., and bacterial burden per spleen (closed circles) or liver (open circles) was enumerated. Median values are presented as horizontal lines. *indicates these values are statistically different, with a *p* value <0.05.



Figure 4-7: *L. monocytogenes* mutants with altered c-di-AMP have increased detection by host innate immune pathways. (A) IFN- β induction was measured by qRT-PCR following a 4-hour infection of bone marrow-derived macrophages with the indicated strains. (B) IFN β was measured by qRT-PCR of infected macrophages with mock treatment (black bars) or Bafilomycin A pretreament (gray bars). (C) Cell death was measured by lactate dehydrogenase release following a 6-hour infection of bone marrow-derived macrophages with the indicated strains. (C) Bacteriolysis was measured by delivery and expression of luciferase from the reporter plasmid pBHE573 following 6-hour infection of IFNAR^{-/-} bone marrow-derived macrophages. Bacteriolysis data are represented relative to infection with the Holin/lysin-expressing strain. Data are presented as the average of at least three independent experiments, and the error bars represent the standard deviation from the mean. *indicates these values are statistically different with a *p* value <0.05 using a Student's t test.

Figure 4-8



Figure 4-8: *L. monocytogenes* c-di-AMP metabolism. Model of c-di-AMP metabolism in *L. monocytogenes* including synthesis by DacA, degradation by PdeA, and secretion by MDRs.

Concluding Thoughts

Cytosolic detection pathways and L. monocytogenes pathogenesis

The results of these studies have uncovered how *L. monocytogenes* activates two host cytosolic signaling pathways- the CSP induced by c-di-AMP as well as DNA, and inflammasomes stimulated by a number of microbial ligands of which DNA is most relevant for *L. monocytogenes* infection. Based on the work described in Chapter 3 in addition to other published studies, we know stimulation of inflammasomes is detrimental for *L. monocytogenes* pathogenesis [98,103,164]. The remaining question concerns the role of the CSP. As briefly described in Chapter 1, the role of Type 1 IFNs in bacterial infections is unclear. I typically only think of the CSP as inducing expression of Type 1 IFNs since IFN- β is the most highly upregulated gene, though several dozen other genes are similarly regulated and should also be explored.

There are a number of transposon mutants identified in the forward genetic screen that are currently unexplainable though their phenotypes are reproducible (see Tables 2-1,-2,-3). Further characterization may reveal additional mechanisms of stimulation of cytosolic signaling pathways or other bacterial components involved in regulating bacteriolysis or c-di-AMP production/secretion.

Cyclic di-AMP signaling

In my opinion, the finding that cyclic di-AMP is the CSP ligand secreted by *L.* monocytogenes was easily the biggest discovery from our lab during my 5.5 years. Members of this lab had been searching for this molecule since the initial observation that host cells transcriptionally respond differently to *L. monocytogenes* in the phagosome versus in the cytosol. This was an observation documented in Dan's personal lab notebook well before the notion of pattern recognition receptors caught on. Identification of the ligand was not initially discovered in our forward genetic screen, although we did isolate a mutation in what we later characterized as a c-di-AMP phosphodiesterase. C-di-AMP was found by biochemical analysis of other mutants that affected IFN- β production [74]. This emphasizes the power of a multi-disciplinary approach to science and will likely fuel future discoveries in microbial pathogenesis. The field needs people taking alternative approaches in conjunction with the classics, like forward genetics, to answer the many remaining questions. Not only did this solve the mystery of cytosolic detection of *L. monocytogenes*, this has opened a new avenue of research concerning the role of cdi-AMP signaling in *L. monocytogenes*.

Over twenty years ago, an analogous but unique signaling molecule, cyclic di-GMP, was discovered in *Gluconacetobacter xylinus* [165]. A PubMed search now returns over 300 hits for this bacterial small molecule. Many of the advances in the c-di-GMP field will likely accelerate the progress of understanding c-di-AMP signaling. Using other signaling molecules, like c-di-GMP, as a model, several general predictions can be made. For example, c-di-GMP can be degraded by specific phosphodieasterases into pGpG or directly to GMP. The presence of two distinct end products and different proteins involved in generating either suggests pGpG may be another cellular signal. Does a parallel process occur with c-di-AMP? At this time, only DHH/DHHA1 domains have been ascribed with c-di-AMP phosphodiesterase activity, although others likely exist.

Clearly, there must be molecular targets regulated by the molecule and likely a specific binding domain, like the PilZ domain binding of c-di-GMP [166]. We have

begun analyzing bacterial transcriptional profiles of mutants with altered c-di-AMP levels and identified numerous genes we believe may be regulated by the molecule, suggesting it may interact with a transcription factor(s). One attractive target, although there is no direct evidence, is PrfA, the master transcriptional regulator of many virulence genes. PrfA belongs to the cAMP receptor protein (CRP) family of bacterial transcription factors, which bind cAMP to control carbon utilization. Based on BLAST protein predictions, the *L. monocytogenes* genome does not encode an adenylate cyclase, suggesting it does not synthesize cAMP. This also raises the question whether c-di-AMP could fulfill a similar function. Interestingly, as discussed in Chapter 4, c-di-AMP appears to be required for the bacterial transition into the host cytosol, a step regulated by PrfA. Although by microarray analysis, PrfA-dependent genes were not differentially regulated in mutants with altered c-di-AMP levels.

In effort to identify targets of c-di-AMP or pathways that lead to the bacterial transcriptional response to high intracellular levels of the molecule, we constructed a transcriptional fusion. This reporter system used the promoter of one of the most highly upregulated genes, *lmo2522* (encodes a homolog of *B. subtilis* YocH) fused to a gus, which encodes β -glucoronidase. When $P_{lmo2522}$ is activated, β -glucoronidase is produced which degrades a colorimetric substrate 5-bromo-4-chloro-3-indolyl glucuronic acid (X-Glc). Transposon mutagenesis of an L. monocytogenes strain containing the P_{lmo2522}-gus fusion led to the identification of several genes that when disrupted affected activation of the *lmo2522* promoter. One of the more thought-provoking "hits" in this small scale screen was an insertion in the *iap* that encodes a secreted autolysin p60. There is some evidence in *B. subtilis* that *yocH* may be induced by a two-component system YycG/F and potentially a PASTA-domain containing Serine/Threonine kinase PrkC, either or both of which are predicted to bind specific peptidoglycan fragments [167]. This data suggests that p60 may be involved in generating a peptidoglycan fragment that is recognized by PrkC and signals through a pathway that may involve c-di-AMP. Other mutants identified in the $P_{lmo2522}$ -gus fusion screen harbored insertions in lmo0195 (a component of a putative ABC transporter), *lmo1081* (similar to a glucose-1-phosphate thymidyl transferase), and *lmo1813* (similar to phosphoglycerate dehydrogenase).

In addition to the direct targets of c-di-AMP, we have very little understanding of what regulates the activities of DacA and PdeA to control synthesis and degradation. Membrane localization of all the factors known to be involved in signaling (DacA, PdeA, MDRs) as well as their ties to cell wall stability suggest that perhaps cell wall stress or perturbations are sensed (perhaps by additional membrane proteins like DacB) and those signals relayed to DacA and/or PdeA. PdeA is predicted to have a PAS domain and highly modified GGDEF domain (GGDQV). Degenerate or modified GGDEF domains have been shown to function as regulatory domains by binding c-di-GMP [168,169,170]. It remains to be determined whether the GGDEF domain of PdeA can interact with c-di-AMP or c-di-GMP and affect activity of the DHH/DHHA1 domains. The c-di-AMP phosphodiesterase activity of PdeA can be competitively inhibited by the presence of ppGpp *in vitro*, similar to what has been shown with the homolog YybT (Witte CE and Portnoy DA, unpublished data)[106]. L. monocytogenes is also predicted to encode several intact putative GGDEF and EAL proteins, suggesting c-di-GMP may also function as a messenger molecule in L. monocytogenes. The presence of additional nucleotide signaling molecules potentially involved in aspects of stress response and

environmental adaptation raises questions as to whether there is cross talk or coordination between signaling pathways.

We propose that secretion of c-di-AMP through MDR transporters is a mechanism to rapidly decrease intracellular concentrations of c-di-AMP and adapt to a changing environment; though, the environmental signals that trigger release of the molecule are not understood. PdeA-deficient mutants represent a paradox: unlike mutants that affect MDR expression and consequently c-di-AMP secretion *in vitro* and *in vivo*, $\Delta pdeA$ mutants only secrete more c-di-AMP than wild-type *L. monocytogenes* in cells. These observations indicate there may be a specific signal during infection of a host cell that stimulates secretion of c-di-AMP. We hypothesize that stresses, such as vacuolar acidification, trigger release of c-di-AMP, potentially through activating MDR expression or activity, in order for the bacterium to mount the appropriate response to survive in the host cell.

Our results implicate c-di-AMP as a critical signaling molecule required for *L. monocytogenes* replication. As discussed in Chapter 4, genes encoding putative diadenylate cyclases in other organisms including *Mycoplasma* and *Streptococci* are predicted to be essential [171,172,173]. With such a central role in bacterial physiology, some bacteria are evolutionarily constrained to produce the molecule; as such, c-di-AMP may be an ideal PAMP that represents bacterial replication or a PAMP-*per vita* [174]. It remains to be determined whether cyclic dinucleotides are detected during infection with other pathogens known to activate the CSP.

Future of host-pathogen interactions

In the past decade, the study of host-pathogen interactions has been dominated by innate immune detection of pathogens, largely due to the identification of many host PRRs. Many of these questions have now been answered, and it's beginning to be appreciated how pathogen detection affects pathogenesis and establishment of immunity. I think the next important questions concern integrating our basic understanding of hostpathogen interactions into the complex picture of a real, natural host. I think infection models will diverge from single organism infections (ie. one strain of one microbe) and towards coinfection models and commensal-pathogen-host interactions. New deep sequencing technologies, metagenomic analyses, and many microbiota-transplantation studies have uncovered the composition and evolution of the microbiota as well as its impacts on the host [175]. The complex population of commensal microorganisms can both negatively and positively affect virulence of invading pathogens and is centrally involved in many aspects of host physiology. I predict that the future direction of this field will incorporate our knowledge of the development of immunity, how microbe populations interact with one another, and how all of these signals interplay during infection. During a natural infection, you may be infected by a single species of microbe or by multiple different microbes, in both cases the enormous population of commensals contribute to or detract from virulence of a pathogen and/or the host response and establishment of immunity. Several recent studies have demonstrated that inflammation by inflammasome activation controls the gut microbiota homeostasis, affecting colonization by pathogenic species and host disease [176,177]. Humans, as the most relevant host organism, are composed of at least 10-fold more bacterial cells than human

cells. How these populations interact with the host and invading pathogens to promote disease, or hinder it, will be critical in understanding an overall view of infection.

L. monocytogenes has been an exceedingly useful model pathogen from which we've learned an immense amount regarding basic aspects of cell biology, immunology, and pathogenesis. As most of the primary virulence factors have been identified and well characterized, the study of *L. monocytogenes* pathogenesis (and perhaps microbial pathogenesis as a whole) will have to evolve. Additionally, factors involved in organ-specific infection have also begun to be identified [153,178]. In my opinion, the next important direction will be pathogen metabolism. The ability to replicate and obtain necessary nutrients within a particular environment, such as a host, is pathogenesis at its most basic level. The first paper describing the incomplete TCA cycle of *L. monocytogenes* was published in 1971 [131], so basic metabolism may seem like a regression, but the advent of new tools and technologies will allow a comprehensive understanding how *L. monocytogenes* and other intracellular pathogens are able to replicate within the host.

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