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Virulence Factor Regulation in *Listeria monocytogenes*

By

Jonathan Lewis Portman

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Infectious Diseases and Immunity

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Daniel A. Portnoy, Chair
Professor Russell E. Vance
Professor Britt A. Glaunsinger
Professor Kathleen Collins

Summer 2017

Abstract

Virulence Factor Regulation in *Listeria monocytogenes*

by

Jonathan Lewis Portman

Doctor of Philosophy in Infectious Diseases and Immunity

University of California, Berkeley

Professor Daniel A. Portnoy, Chair

Listeria monocytogenes is a Gram-positive intracellular pathogen that is readily amenable to genetic manipulation and for which there are excellent *in vitro* and *in vivo* virulence models. These attributes have allowed a thorough examination of the molecular underpinnings of *L. monocytogenes* pathogenesis, however, there are still a number of major unresolved questions that remain to be answered. For example, it has been known for many years that *L. monocytogenes* rapidly changes its transcriptional profile upon access to the host cytosol, however the host cues and bacterial components that are involved in driving this change have remained continually unanswered. One large piece of evidence came when the long-sought co-factor for the primary virulence regulator, PrfA, was discovered to be the antioxidant tripeptide, glutathione. Glutathione was demonstrated to play a crucial role in the activation of PrfA *in vivo*— a finding that has since led to two important discoveries that are described herein. First, the activation of PrfA *in vitro* requires both exogenous glutathione and a metabolic licensing step that can be recapitulated by a chemically defined synthetic media. Second, glutathione also functions as a post-translational regulator of the pore-forming virulence factor, Listeriolysin O (LLO), by reversibly binding via an S-glutathionylation reaction and preventing membrane association of the LLO monomers. These discoveries elucidate numerous regulatory roles for glutathione during infection and describe how *L. monocytogenes* is able to sense and respond to critical host compartments to mount a successful infection.

Upon entry to the host cell cytosol, the facultative intracellular pathogen *Listeria monocytogenes* coordinates the expression of numerous essential virulence factors by allosteric binding of glutathione (GSH) to the Crp-Fnr family transcriptional regulator, PrfA. Here we report that robust virulence gene expression can be recapitulated by growing bacteria in a synthetic medium (iLSM) containing GSH or other chemical reducing agents. Bacteria grown under these conditions were 45-fold more virulent in an acute murine infection model and conferred greater immunity to a subsequent lethal challenge compared to bacteria grown in

conventional media. During cultivation *in vitro,* PrfA activation was completely dependent on intracellular levels of GSH, as a glutathione synthase mutant (Δ*gshF*) was activated by exogenous GSH but not reducing agents. PrfA activation was repressed in iLSM supplemented with oligopeptides, but suppression was relieved by stimulation of the stringent response. These data suggest that cytosolic *L. monocytogenes* interpret a combination of metabolic and redox cues as a signal to initiate robust virulence gene expression *in vivo.*

Cholesterol-dependent cytolysins (CDCs) represent a family of homologous pore-forming proteins secreted by many Gram-positive bacterial pathogens. CDCs mediate membrane binding partly through a conserved C-terminal undecapeptide, which contains a single cysteine residue. While mutational changes to other residues in the undecapeptide typically have severe effects, mutating the cysteine residue to alanine has minor effects on overall protein function. Thus, the function of this highly conserved reactive cysteine residue remains largely unknown. We report here that the CDC Listeriolysin O (LLO), secreted by the facultative intracellular pathogen *Listeria monocytogenes*, was post-translationally modified by a S-glutathionylation at this conserved cysteine residue, and that either endogenously synthesized or exogenously added glutathione was sufficient to form this modification. When recapitulated with purified protein *in vitro,* this modification completely ablated the activity of LLO, and this inhibitory effect was fully reversible by treatment with reducing agents. A cysteine-to-alanine mutation in LLO rendered the protein completely resistant to inactivation by S-glutathionylation and retained full hemolytic activity. A mutant strain of *L. monocytogenes* expressing the cysteine-to-alanine variant of LLO was able to infect and replicate within bone marrow-derived macrophages indistinguishably from wild-type *in vitro,* yet was attenuated 4-6 fold in a competitive murine infection model *in vivo*. This study suggests that S-glutathionylation may represent a mechanism by which CDC family proteins are post-translationally modified and regulated, and help explain an evolutionary pressure behind the highly conserved undecapeptide cysteine.

“No. Try not. Do, or do not. There is no try.” - Yoda

Dedication

To my parents, Kim and Roland,
my sisters, Megan and Whitney,
my beautiful wife, Tamara,
and my best friend, Alex.

Thank you for your endless support,
patience, and love. I wouldn't have
made it this far without each of you.

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My “Lab bros” – **Aaron**: No other person has remotely influenced my career like you have, and a short dissertation acknowledgement can’t convey how tremendously thankful I am for your friendship. Our time together made the lab, IDI, and all of Berkeley more instructive and definitely more fun. **Thomas**: Coming to lab everyday was only fun because of you and our constant banter (despite the stinky feet and merciless pun-ridicule). You helped me believe in my own scientific instincts, (even when no one else did), and I will always admire your independent and strategic thinking. **Michelle**: I never thought I’d ever have a dual science-nerd / gym-buddy until we met. Our 7am mornings at Crossfit were always a blast, and during the day you taught me most of my skills as a scientist. If I can ever manage 80% of your efficiency and organizational skills, I know I’m going to do alright.

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Jonathan Lewis Portman
Curriculum Vitae

EDUCATION

Ph.D. **University of California, Berkeley**
Infectious Diseases & Immunity, expected August 2017
Virulence Factor Regulation in Listeria monocytogenes
Daniel A. Portnoy (Chair), Russell E. Vance, Britt A. Glaunsinger, Kathy Collins

B.S. **University of California, Davis**, Cell Biology, June 2010

RESEARCH EXPERIENCE

Doctoral Research: Infectious Diseases & Immunity Graduate Group, UC Berkeley, 2011-Present (Research Advisor: Dr. Daniel A. Portnoy)
Identified a novel post-translational modification of the pore-forming virulence factor, Listeriolysin O, which potently inhibits activity of the protein when recapitulated *in vitro*
Discovered that inducing a strong reducing redox potential during *in vitro* culture of *L. monocytogenes* is sufficient to fully activate the master virulence regulator, PrfA
Described a novel mechanism of post-transcriptional regulation intrinsic to the aforementioned virulence factor Listeriolysin O that when disrupted leads to a 100,000 fold loss of virulence in an animal model of infection.

Doctoral Research Internship: Department of Infectious Diseases, Genentech, Summer 2016 (Research Advisor: Dr. Mireille Nishiyama)
Explored alternative models of bacterial stress response activation in *E. coli* utilizing proprietary antimicrobial compounds

Undergraduate Research: Environmental Toxicology, UC Davis, 2007-2010 (Research Advisor: Dr. Michael S. Denison)
Investigated the validity of a newly proposed alternative binding mechanism of the aryl hydrocarbon receptor

Undergraduate Research Internship: Department of Pathology and Immunology, UC Davis Medical Center, 2008 (Research Advisor: Dr. Sunny Lee)
Isolated hundreds of bacterial and fungal isolates from crude environmental samples to purify and evaluate secondary metabolites for potential therapeutic value as anti-cancer agents

AWARDS & ACHIEVEMENTS
Beattie Memorial Award— *For excellence in laboratory-based research, UC Berkeley SPH (2017)*
Conference Travel Award— *Graduate Division, UC Berkeley (2016)*
Student Leadership Award— *School of Public Health, UC Berkeley (2015)*
Berkeley Fellow— *UC Berkeley Distinguished Graduate Award recipient (2011)*
Co-Founder— *The Wellmen: A community-service and leadership student group (2009)*
Eagle Scout— *Boy Scouts of America (2006)*

PUBLICATIONS & PATENTS
Peer-Reviewed Publications
Chen, G. Y., McDougal, C. E., D'Antonio, M. A., **Portman, J. L**., & Sauer, J. D. (2017). A Genetic Screen Reveals that Synthesis of 1,4-Dihydroxy-2-Naphthoate (DHNA), but Not Full-Length Menaquinone, Is Required for *Listeria monocytogenes* Cytosolic Survival. *mBio*, *8*(2), e00119–17. http://doi.org/10.1128/mBio.00119-17

Portman, J.L., Huang, Q., Reniere, M.L., Iavarone, A.T., Portnoy, D.A. (2017). Activity of the pore-forming virulence factor Listeriolysin O is reversibly inhibited by naturally occurring S-glutathionylation. *Infection and Immunity.* http://doi.org/10.1128/IAI.00959-16

Barry, K.C., Fontana, M.F., **Portman, J.L**., Dugan, A.S., & Vance, R.E. (2013). IL-1α signaling initiates the inflammatory response to virulent Legionella pneumophila in vivo. *Journal of Immunology (Baltimore, MD: 1950)*, *190*(12), 6329–6339.

Manuscripts in Preparation
Portman, J.L., Dubensky, S.B., Whiteley, A.T., Portnoy, D.A. (2017). Activation of the *Listeria monocytogenes* virulence program by a reducing environment

Portman, J.L., Portnoy, D.A. (2017). Listeriolysin O regulates its own translation through a cis-acting RNA element located deep within the ORF

Patent
Methods and compositions for modulating *prfA* mediated virulence gene activation in *Listeria spp.* (Patent Pending) Inventors: Daniel A. Portnoy, **Jonathan L. Portman**. Filed: 9/2/2015

TEACHING & MENTORSHIP

Research Mentor
Huntly Morrison (1/2017-5/2017), *UC Berkeley*
Samuel Dubensky (7/2013-9/2015), *Bentley High School*
Emily Lockshaw (9/2013-6/2014), *UC Berkeley*

Instructor
Molecular and Cellular Basis of Bacterial Pathogenesis *(Spring 2014)*
Graduate level; weekly discussions of primary literature chosen by instructor; students graded based on contributions to weekly discussions and a large individual term-project.

Teaching Assistant
Bacterial Pathogenesis *(Spring 2014) (Instructor: Daniel A. Portnoy)*

Laboratory Teaching Assistant
Public Health Microbiology Lab *(Fall 2012) (Instructor: Kathy Loretz)*

LEADERSHIP

President, Infectious Diseases & Immunity Graduate Group (8/2014-8/2015)
Admissions Committee Member, Infectious Diseases & Immunity Ph.D. Program (8/2013-7/2014)
Event Coordinator, Infectious Diseases & Immunity Graduate Group (8/2012-7/2013)

SELECTED PRESENTATIONS

Gordon Research Symposium & Conference: Microbial Toxins & Pathogenesis (Waterville Valley, NH) July 8-15, 2016
Poster Presentation: Growth of *Listeria monocytogenes* in a chemically defined reducing media is sufficient to activate the master virulence regulator PrfA *in vitro*

Cold Spring Harbor Meeting: Microbial Pathogenesis & Host Response (Cold Spring Harbor, NY) Sep 8-12, 2015
Poster Presentation: Growth of *Listeria monocytogenes* in a chemically defined reducing media is sufficient to activate the master virulence regulator, PrfA, *in vitro*

Infectious Diseases & Immunity Seminar Series (UC Berkeley) Nov 17, 2014
Talk (60min): Mechanisms dampening the toxicity of the pore-forming toxin, Listeriolysin O

Bay Area Microbial Pathogenesis Meeting (UC San Francisco) March 29, 2014
Poster Presentation: Transcript Stability of Listeriolysin O is Critical for Virulence of *L. Monocytogenes*

Pathogenesis Super-Group Meeting (UC Berkeley) Feb 7, 2014
Talk (90min): Regulatory elements used by *Listeria monocytogenes* to restrict the cytotoxic activity of Listeriolysin O during infection

West Coast Bacterial Physiologists Meeting (Asilomar, CA) Dec 13-15, 2013
Poster Presentation: Transcript Stability of Listeriolysin O is Critical for Virulence of *L. monocytogenes*

Chapter 1

An introduction to *Listeria monocytogenes*

1.1 *Listeria monocytogenes,* an intracellular pathogen

Listeria monocytogenes is a Gram-positive, facultative intracellular pathogen responsible for approximately 2,500 illnesses and 500 deaths in the United States annually (*1*). It is also one of the most virulent food-borne pathogens, with 20 to 30 percent of clinical infections resulting in death (*1*). Aside from its clinical relevance, however, *L. monocytogenes* is a durable and fastidious bacterium ideally suited for study as a model organism to understand conserved pathways in more globally threatening intracellular pathogens such as *Mycobacterium tuberculosis*, *Salmonellae,* and *Chlamydiae* (*2*). As it encounters host cells during infection, *L. monocytogenes* is able to facilitate its own uptake, escape the phagocytic vacuole, replicate in the host cytosol, and then spread cell to cell by polymerizing host actin (*3*). To achieve full pathogenicity, *L. monocytogenes* must carefully regulate a number of key virulence factors at each of these stages to survive and evade the host immune response (*4*). In particular, proper spatiotemporal activity of both the primary virulence regulator, PrfA, and the cholesterol dependent cytolysin, Listeriolysin O (LLO), are essential for pathogenesis (*5*, *6*).

1.2 Determinants of virulence

L. monocytogenes employs a number of host-specific proteins in order to successfully invade, replicate, and spread during infection (Fig. 1.1). Internalin A (InlA) and B (InlB) are invasins that promote uptake of bacteria into non-phagocytic cells (*7*). The pore-forming toxin, LLO, along with two phospholipases (PI-PLC & PC-PLC), facilitate escape from the endocytic vacuole and access to the host cytosol (*8*-*10*). Following cytosolic access, the transcriptional regulator, PrfA, becomes activated and drives transcription of additional virulence factors including the hexose-phosphate transporter, Hpt, and the host-actin nucleating protein, ActA (*11*-*13*). These factors work in concert to allow the cyclical invasion, replication, and spread of *L. monocytogenes* throughout tissues in the host. While these and other virulence factors all contribute to a successful infection, interruptions to the expression or activity of LLO, PrfA, or ActA in particular are sufficient to cause significant fitness defects *in vivo*.

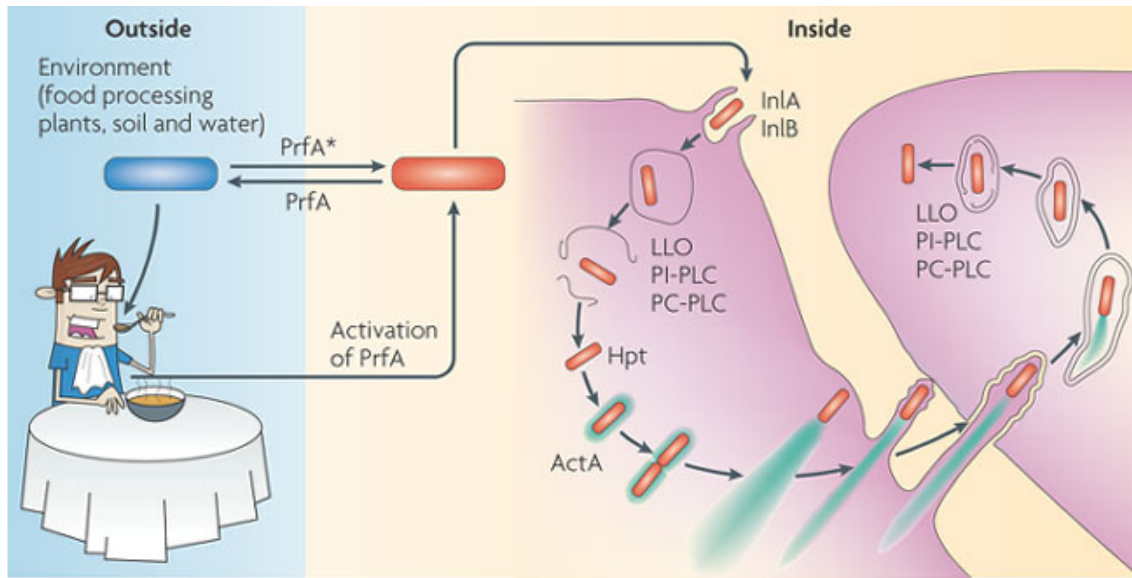

Figure 1.1 From saprophyte to intracellular pathogen
Artistic depiction of the switch from environmental saprophyte to intracellular pathogen via the activation of PrfA with the ensuing pathogenic lifecycle of *L. monocytogenes* as originally observed from electron micrographs (*14*). Selected virulence factors and the relevant stage they affect are noted in text. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Microbiology (*5*), copyright (2009).

1.3 Virulence factor regulation

The ability of *L. monocytogenes* to replicate intracellularly is a delicate balance between virulence and toxicity. For example, substitution of LLO for Perfringolysin O, a closely related homolog encoded by *Clostridium perfringens,* leads to massive host cytotoxicity and a profound loss of virulence (*15*). Investigation into the mechanisms preventing host toxicity by LLO has led to numerous discoveries including evidence for post-transcriptional regulation, an acidic pH optimum to restrict activity in the neutral cytosol, and host-mediated proteolytic degradation (*16*-*18*). Paradoxically, LLO is upregulated upon access to the host cell cytosol by PrfA which only increases the chance of host membrane damage (*19*, *20*). The lack of any observed toxicity despite this upregulation likely hints towards additional mechanisms of control that are yet to be identified.

PrfA is a Crp/Fnr family transcriptional regulator that directly and indirectly affects the transcription of over 100 target genes (*21*). Outside of cells, the transcriptional activity of PrfA is limited, and only becomes fully active upon vacuolar escape and access to the host cytosol (*22*). While numerous stimuli have been shown to influence the activity of PrfA, including temperature and carbon source availability, full activation of PrfA has yet to be recapitulated *in vitro* (*23*-*26*). Like other Crp-family transcription factors, PrfA is activated by the allosteric binding of a small molecule co-factor that induces conformational changes that promote

DNA-binding and transcriptional activation (*27*). For PrfA, this small molecule has been identified as the tripeptide antioxidant, glutathione (GSH) (*28*, *29*). GSH is synthesized by the bifunctional glutathione synthase, *gshF* that is known to be upregulated upon entrance to the host cytosol (*28*). Despite the identification of GSH as the co-factor for PrfA, traditional growth media supplemented with an excess of GSH remains insufficient to activate PrfA *in vitro* (J. L. Portman, unpublished data).

1.4 Redox and pathogenesis

The identification of GSH as the critical co-factor for PrfA *in vivo* has led to a surge of interest in the redox biology of *L. monocytogenes*. Redox reactions involve the transfer of electrons from one source (a reducing agent), to another (an oxidizing agent) based on their relative redox potentials (*30*). The effects of strong oxidizing agents (that scavenge electrons from other molecules) on bacteria are well studied and are typically antimicrobial, (e.g. hydrogen peroxide, household bleach, oxygen, ozone). Bacteria combat these compounds in part by consistently keeping a pool of low molecular weight thiols (LMWTs) in a reduced state that can donate electrons to, and thus detoxify, these compounds (*31*). LMWTs are sulfur containing small molecules like GSH, cysteine, acetyl-CoA, bacillithiol and mycothiol, which are thought to be functionally analogous in their primary role as redox buffers (*32*-*35*). *L. monocytogenes* synthesizes GSH as its predominant LMWT, however genetic depletion of this molecule leads to an unusually slight increase in sensitivity to oxidizing agents (*28*, *36*). In addition, the severe virulence defect of a mutant lacking GSH is fully rescued by an allele of PrfA that is locked into an active state, suggesting that the primary function of GSH involves virulence gene regulation, and not necessarily its canonical role as a redox buffer (*28*). The precise host-specific signal that leads to PrfA activation by GSH, however, remains unknown.

Chapter 2

Activation of the *Listeria monocytogenes* virulence program by a reducing environment

Sections of this chapter were submitted for publication as:
Portman, J.L., Dubensky, S.B., Whiteley, A.T., Portnoy, D.A. (2017). *Activation of the Listeria monocytogenes virulence program by a reducing environment*.

2.1 Summary of results

Intracellular pathogens are responsible for much of the worldwide morbidity and mortality from infectious diseases. These pathogens have evolved various strategies to proliferate within individual cells of the host and avoid the host immune response. All intracellular pathogens must access the host cell cytosol early during infection in order to establish their respective replicative niches. Determining how these pathogens sense and respond to the intracellular compartment to establish a successful infection is critical to our basic understanding of the pathogenesis of each organism, and for the rational design of therapeutic interventions. *Listeria monocytogenes* is a model intracellular pathogen with robust *in vitro* and *in vivo* infection models. Studying the host-sensing and downstream signaling mechanisms evolved by *L. monocytogenes* often describe themes of pathogenesis that are broadly applicable to less tractable pathogens.

Upon entry to the host cell cytosol, the facultative intracellular pathogen *Listeria monocytogenes* coordinates the expression of numerous essential virulence factors by allosteric binding of glutathione (GSH) to the Crp/Fnr family transcriptional regulator, PrfA. Here we report that robust virulence gene expression can be recapitulated by growing bacteria in a synthetic medium (iLSM) containing GSH or other chemical reducing agents. Bacteria grown under these conditions were 45-fold more virulent in an acute murine infection model and conferred greater immunity to a subsequent lethal challenge compared to bacteria grown in conventional media. During cultivation *in vitro,* PrfA activation was completely dependent on intracellular levels of GSH, as a glutathione synthase mutant (Δ*gshF*) was activated by exogenous GSH but not reducing agents. PrfA activation was repressed in iLSM supplemented with oligopeptides, but suppression was relieved by stimulation of the stringent response. These data suggest that cytosolic *L. monocytogenes* interpret a combination of metabolic and redox cues as a signal to initiate robust virulence gene expression *in vivo.*

2.2 Introduction

The facultative intracellular pathogen *Listeria monocytogenes* is the third leading cause of death from foodborne illness in the United States, with an estimated 1,600 cases leading to 260 deaths per year (*37*). This ubiquitous Gram-positive saprophyte is found in soil where it commonly contaminants produce and livestock products such as dairy milk (*38*). Upon ingestion by primarily immunocompromised individuals and pregnant women, bacteria traverse the intestinal epithelium and cause systemic infection, often leading to miscarriage, neonatal sepsis or meningitis (*39*). After invasion, a critical regulatory switch occurs during the transition from vacuole to cytosol when *L. monocytogenes* significantly remodels its transcriptional profile by activating the master virulence regulator, PrfA (*40*). Proper temporal expression of PrfA is critical for bacterial invasion and vacuolar escape, as inappropriate expression leads to a loss of fitness both in and out of the host (*5*, *41*).

PrfA is directly responsible for the transcription of 10 core virulence genes and indirectly affects the expression of over 140 others, many of which are essential for virulence (*40*, *42*). The activity of PrfA is tightly regulated and only becomes activated upon entry into cells. This strict regulation is responsible for transcript levels of the PrfA-dependent actin assembly-inducing protein, ActA, increasing over 200-fold in the host cytosol compared to broth cultures (*43*). While the precise cues that define the intracellular milieu are not described and complete activation of PrfA-mediated gene expression has not been recapitulated *in vitro*, it's clear that PrfA activity is allosterically activated by the small molecule, glutathione (GSH) (*28*, *29*). GSH is a tripeptide antioxidant canonically utilized by eukaryotes, cyanobacteria, and proteobacteria as a redox buffer to protect against oxidative damage (*32*). *L. monocytogenes* is unusual in that it also synthesizes GSH (*36*). During infection the expression of bacterial glutathione synthase (*gshF)* increases 10-fold, however it is still not appreciated why *gshF* is upregulated in host cells or why exogenous GSH is insufficient to activate PrfA in traditional broth culture (*28*) (J. L. Portman and D. A. Portnoy, unpublished data).

A number of diverse factors influence PrfA activity including temperature, osmolarity, and iron availability (*40*, *44*). Furthermore, perturbations to several metabolic pathways influence the activity of PrfA *in vitro* (*45*), including those that influence the pleiotropic metabolic repressor, CodY. CodY regulates hundreds of genes in response to nutrient starvation and directly interacts with the coding region of the *prfA* gene (*46*-*48*). In addition to sensing intracellular levels of branched-chain amino acids (BCAAs), the activity of CodY is also influenced by intracellular GTP pools that can be quickly depleted during starvation upon production of the nucleotide secondary messengers guanosine penta- and tetraphosphate (combined here and referred to collectively as (p)ppGpp) (*49*-*51*), in a process known as the stringent response. Due to the direct relationship between CodY and *prfA* (*46*), we hypothesized that manipulating CodY via (p)ppGpp through growth on specialized media may be required for PrfA activation by GSH *in vitro*.
Here we report that growth of *L. monocytogenes* in nutrient-limiting synthetic media is sufficient to allow robust activation of PrfA by exogenous glutathione and chemical reducing agents. Our findings help to unify a number of PrfA-related studies into a two-step activation model, and clearly demonstrate the potent regulatory role of metabolic signaling to virulence gene regulation in *L. monocytogenes*.

2.3 Results

2.3.1 Glutathione is sufficient to activate PrfA in synthetic but not rich media

In order to rapidly monitor PrfA activity under various conditions, a strain of *L. monocytogenes* was utilized that expresses red fluorescent protein (RFP) under the control of the tightly regulated PrfA-dependent promoter for the actin-polymerizing gene, *actA* (*43*, *52*, *53*). This transcriptional fusion was used to test the ability of exogenous GSH to activate PrfA during *in vitro* growth in various conditions and culture media. Growth of *L. monocytogenes* in all standard growth media showed negligible increases in fluorescence upon addition of 10mM GSH. However, growth in a defined synthetic media (iLSM), led to a higher basal level of fluorescence, as well as a robust increase of fluorescence in response to GSH (Fig. 1a). The level of PrfA activation seen in iLSM+GSH was equal to that seen with an allele of PrfA that is locked in the active conformation, PrfA(G145S) or PrfA* (*54*). To verify the transcriptional fusion was accurately reporting PrfA-dependent activity, direct mRNA quantification of the *actA* gene was performed using qRT-PCR (Fig. 1b) and protein secretion of another PrfA-regulated virulence factor, Listeriolysin O (LLO), was measured by western blots (Fig. 1c) (*8*, *11*). In all scenarios, the addition of GSH to iLSM was sufficient to induce PrfA activation to levels comparable to PrfA*.

To compare the effect of GSH to other inducers of PrfA reported in the literature, we adapted our synthetic media to test growth with glycerol, low concentrations of BCAAs, L-glutamine, the phosphosugar glucose-1-phosphate, and in the presence of the charcoal-like resin, XAD-4 (*24*, *26*, *46*, *55*-*58*). Consistent with previous reports, each of these growth conditions led to an increase in fluorescence from the reporter strain, but only iLSM+GSH induced activation equal to the PrfA* control (Fig. 1d).

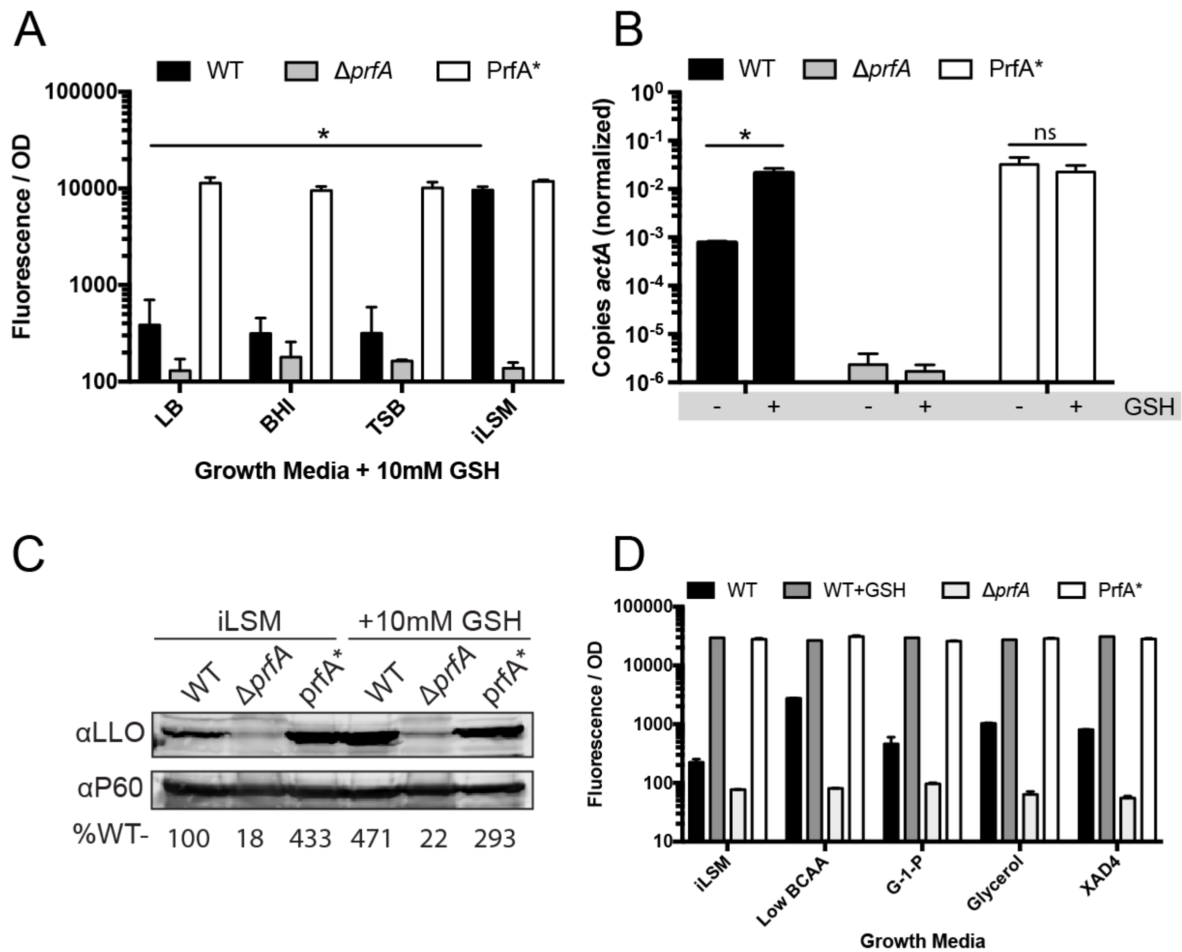

Figure 2.1. Exogenous glutathione is sufficient to activate PrfA *in vitro.*
(A) Strains of *L. monocytogenes* expressing RFP under the control of the PrfA-dependent *actA* promoter (P_{actA}_RFP) were grown in various media containing 10mM glutathione (GSH). * denotes a p-value of <0.05.
(B) Strains of *L. monocytogenes* were grown to mid-log in iLSM media in the presence or absence of 10mM GSH. Total RNA was harvested and transcript abundance of *actA* was quantified using RT-qPCR and normalized to the housekeeping gene, BglA. * denotes a *p*-value of <0.05.
(C) Strains of *L. monocytogenes* were grown to stationary phase in iLSM media in the presence or absence of 10mM GSH. Precipitated culture supernatants were separated by SDS-PAGE and probed for Listeriolysin O (LLO) by western blot. The autolysin P60 was also quantified and used as a loading control. The reported value is the abundance of LLO/P60 relative to WT in percentage.
(D) Strains of *L. monocytogenes* expressing P_{actA}_RFP were grown in iLSM or variations thereof with 20% standard BCAA (Low BCAA), glucose-1-phosphate substituted for glucose (G-1-P), glycerol substituted for glucose (Glycerol), or iLSM with 1%(w/v) Amberlite™ XAD-4 resin (XAD-4). Fluorescence was measured and normalized to the bacterial number using the respective OD600. * denotes a *p*-value of <0.05.

2.3.2 Nutritive oligopeptides potently inhibit PrfA activation by glutathione

In order to differentiate between the presence of PrfA activating components specific to iLSM versus the presence of inhibitory components in rich media, iLSM was mixed with rich media at different ratios and tested for the ability of exogenous GSH to activate PrfA. Consistent with the notion that rich media contains inhibitory molecules, approximately 8% of any of the three rich medias tested was sufficient to completely block activation of PrfA by GSH (Fig. 2a). To determine what components of rich media were responsible for inhibition, common ingredients of rich media, including tryptone, peptone, yeast extract, and casamino acids were tested. When added to iLSM+GSH at the concentrations found in standard LB medium, yeast extract and casamino acids had a negligible effect on PrfA activation, while tryptone and peptone potently repressed activation (Fig. 2b). These data suggested that oligopeptides found in tryptone and peptone, (and to a much lesser degree yeast extract and casamino acids), were primarily responsible for the potent inhibition of PrfA activation by GSH *in vitro.* To confirm that oligopeptides were sufficient for repression, synthetically derived hexapeptides containing repeats of glycine, alanine, leucine, or isoleucine with flanking lysines, (for solubility), were tested in iLSM+GSH (Fig. 2c). While the glycine and alanine containing peptides yielded significant but incomplete repression, peptides containing leucine and isoleucine potently inhibited PrfA activation. These data suggested that oligopeptides containing BCAAs are potently inhibitory to PrfA activation by GSH *in vitro* and are consistent with findings that limiting amounts of BCAAs in minimal media lead to increased basal levels of PrfA activity (*46*) (Fig. 1d).

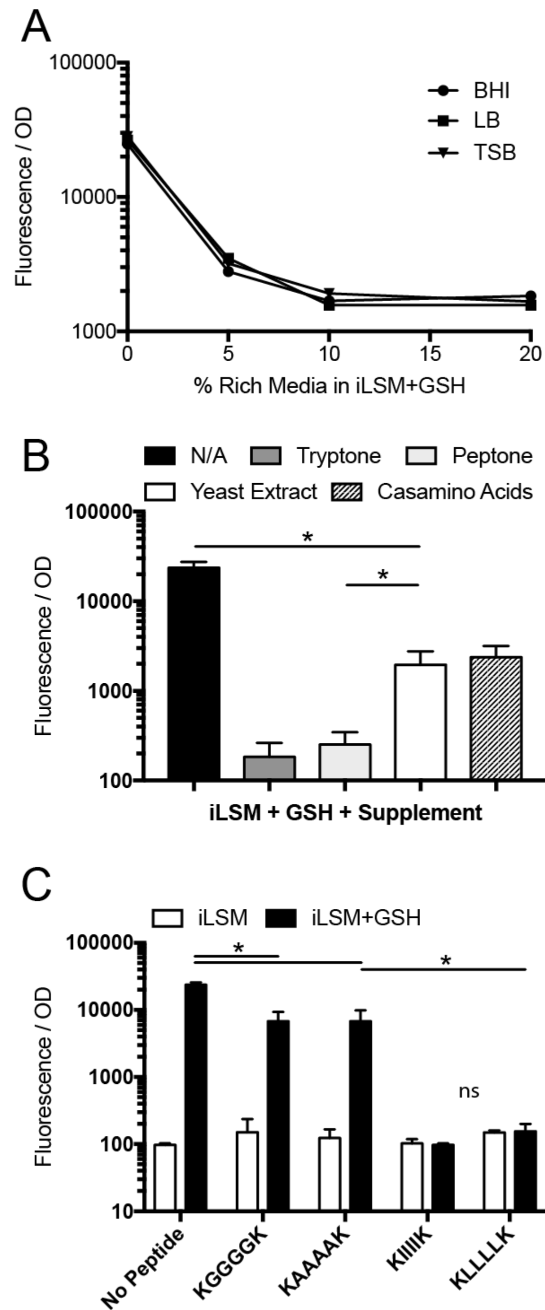

Figure 2.2. Oligopeptides inhibit activation of PrfA *in vitro.*
Wild-type bacteria expressing P_{actA}_RFP were grown in **(A)** a mixture of iLSM and rich media and 10mM GSH **(B)** iLSM with various supplements and 10mM GSH **(C)** iLSM with or without synthetic peptides and/or 10mM GSH. Fluorescence was measured and normalized to the bacterial number using the respective OD600. * denotes a *p*-value of <0.05.

2.3.3 Induction of the stringent response is sufficient to rescue PrfA activation by GSH in the presence of oligopeptides

The bacterial stringent response is a highly conserved, global regulatory system that responds to stress and is strongly induced during amino acid starvation (*59*). This response is propagated by the nucleotide secondary messenger, (p)ppGpp, which in Firmicutes is synthesized by the bifunctional synthase/hydrolase, RelA, and two accessory synthases, RelP and RelQ (*60*, *61*). Importantly, RelA is the only identified hydrolase for (p)ppGpp in *L. monocytogenes* and is thought to be the dominant stress-responsive synthase (*62*). Since CodY is canonically inactivated by high levels of (p)ppGpp during nutrient starvation, we reasoned that the addition of peptides to iLSM may prevent PrfA activation by promoting low levels of (p)ppGpp that prevent the inactivation of CodY and consequent PrfA activation. Therefore, artificially inducing the stringent response should reverse the inhibitory effect of peptides in iLSM supplemented with GSH. To induce the stringent response, DL-serine hydroxamate (SHX), which inhibits seryl-tRNA synthetase and causes accumulation of (p)ppGpp, was added to the growth media (*61*). During growth in iLSM, where levels of (p)ppGpp should be naturally elevated due to limited nutrients, SHX had no discernable effect on PrfA activity in any condition (Fig 3a). However, in iLSM media containing tryptone, the addition of SHX with GSH was sufficient to restore full activation of the fluorescent reporter (Fig. 3b).

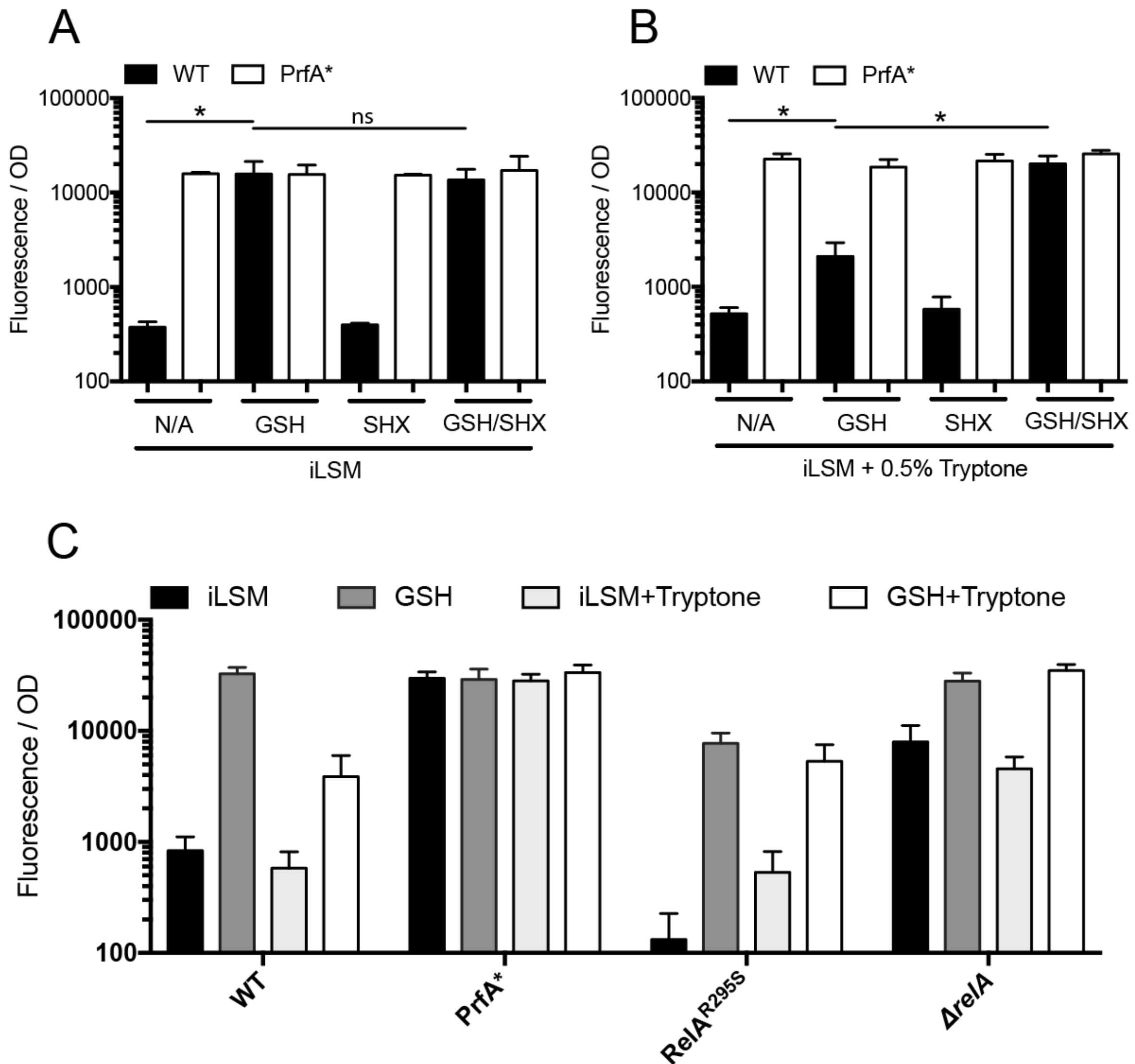

Figure 2.3. Increasing intracellular (p)ppGpp reverses the inhibitory effect of oligopeptides. Wild-type or PrfA* bacteria expressing P_{actA}_RFP were grown in **(A)** iLSM or **(B)** iLSM+0.5% Tryptone and supplemented with 10mM GSH, 2mg/mL DL-Serine Hydroxamate (SHX), or both. Fluorescence was measured and normalized to the bacterial number using the respective OD600. * denotes a *p*-value of <0.05.
(C) Strains of *L. monocytogenes* expressing P_{actA}_RFP were grown in iLSM in the presence or absence of 10mM GSH and 0.5% Tryptone. Fluorescence was measured and normalized to the bacterial number using the respective OD600.

Another strategy for artificially elevating levels of (p)ppGpp in *L. monocytogenes* is to utilize a *relA* deletion strain that lacks the only identified (p)ppGpp hydrolase (*relA)*, but retains the two remaining (p)ppGpp synthases, (*relP & relQ)* (*61*). In iLSM media with tryptone, the *relA* deletion strain was also rescued for PrfA activation by GSH, analogous to the wild-type strain treated with SHX (Fig. 3c). In contrast, a point mutation in the synthase domain of RelA that prevents

synthesis, yet allows hydrolase activity, RelA^{R295S}, exhibited lowered levels of PrfA activation by GSH in iLSM, and was no longer rescued by SHX in iLSM with tryptone (*61*, *63*). These data suggested that high levels of (p)ppGpp are necessary and sufficient to allow activation of PrfA by GSH.

2.3.4 Chemical reducing agents are sufficient to activate PrfA

Since the addition of glutathione to iLSM was sufficient to activate PrfA *in vitro*, we considered if other redox-related compounds would function similarly and tested iLSM supplemented with either a simple oxidant, (hydrogen peroxide), a thiol-specific oxidant, (diamide), or a chemical reducing agent, (tris(2-carboxyethyl)phosphine (TCEP)) (*64*, *65*). Neither hydrogen peroxide or diamide had any effect on PrfA activity, however the addition of TCEP was sufficient to fully activate PrfA (Fig. 4a). In fact, all chemical reducing agents including dithiothreitol and 2-mercaptoethanol activated PrfA similarly to TCEP (J. L. Portman, S. B. Dubensky, and D. A. Portnoy, unpublished data). In support of the requirement for glutathione as a co-factor for PrfA, a mutant lacking the bacterial glutathione synthase, *gshF*, was rescued by the addition of exogenous GSH, yet activation by TCEP was completely abrogated (Fig. 4b) (*28*, *29*).

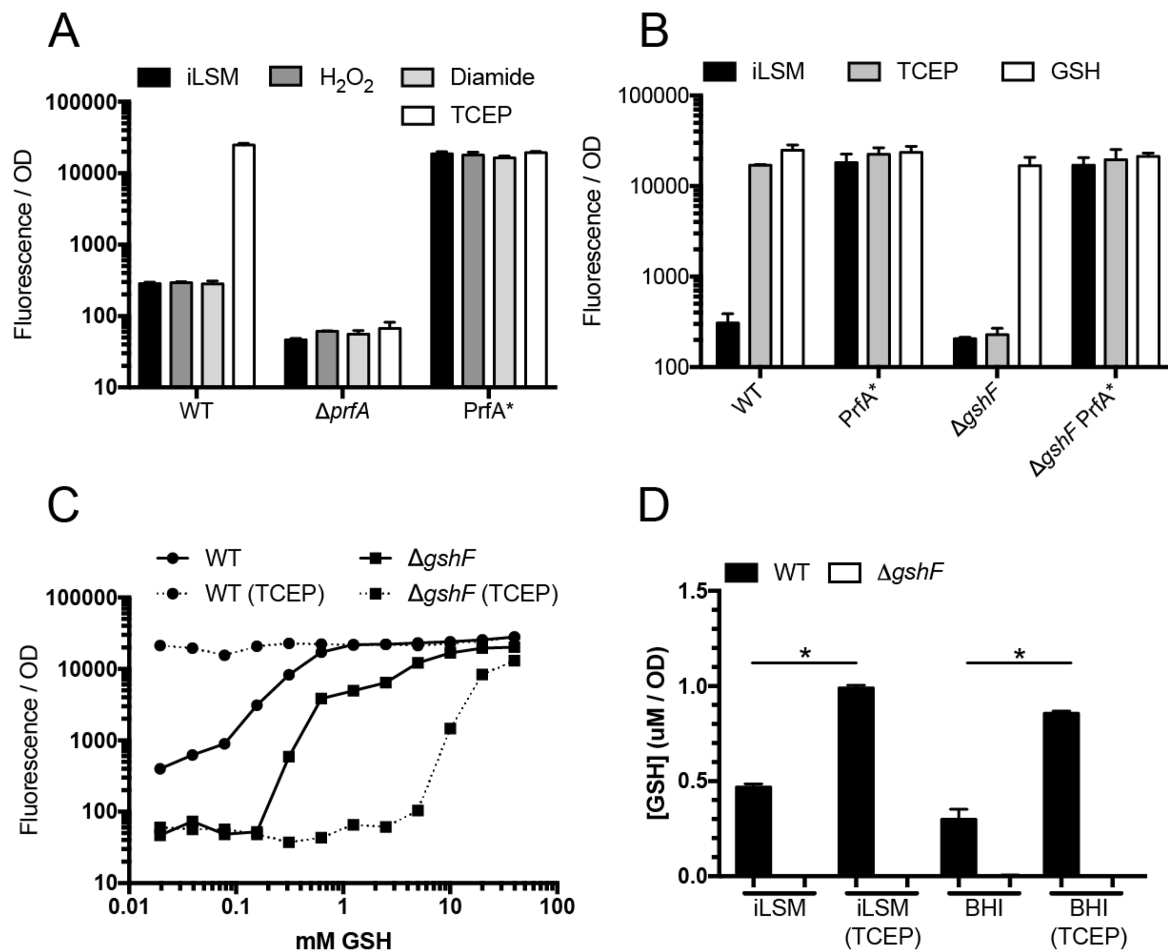

Figure 2.4. Reducing agents are sufficient to activate PrfA by increasing intracellular GSH.
(A/B) Strains of *L. monocytogenes* expressing P_{actA}_RFP were grown in iLSM in the presence or absence of 100 μM hydrogen peroxide (H_2O_2), 0.25mM Diamide, 2mM TCEP, or 10mM GSH. Fluorescence was measured and normalized to the bacterial number using the respective OD600.
(C) Wild-type or Δ*gshF L. monocytogenes* expressing P_{actA}_RFP were grown in iLSM in the presence or absence of 2mM TCEP and varying concentrations of GSH. Fluorescence was measured and normalized to the bacterial number using the respective OD600.
(D) Wild-type or Δ*gshF L. monocytogenes* expressing P_{actA}_RFP were grown in iLSM or BHI in the presence or absence of 2mM TCEP. Fluorescence was measured and normalized to the bacterial number using the respective OD600. * denotes a *p*-value of <0.05.

Although the overall requirement of GSH for PrfA is clear, what was unclear is whether a small amount of exogenous GSH would be sufficient to allow a ΔgshF strain to then respond to TCEP. To test this, GSH was titrated into a culture of wild-type and Δ*gshF* grown in iLSM in the presence or absence of TCEP and PrfA activity was monitored with our reporter strain (Fig. 4c). As expected, the wild-type strain showed a dose-dependent activation of the reporter by GSH that was bypassed by the addition of TCEP. In the Δ*gshF* mutant, high levels of exogenous GSH were sufficient to activate PrfA, however the addition of TCEP not only failed

to synergize with GSH, but prevented activation until higher levels of exogenous GSH were reached. These data suggested that while TCEP is sufficient to activate PrfA in a wild-type strain, it likely does so by influencing intracellular levels of GSH through an unknown mechanism.

Consistent with the requirement for glutathione as a co-factor for PrfA, in a mutant lacking the bacterial glutathione synthase (Δ*gshF*), GSH, but not TCEP, activated PrfA (Fig. 4b). This result implies that TCEP's induction of PrfA activation relates to an indirect effect on bacterial glutathione production. To test if chemical reducing agents induce accumulation of intracellular levels of GSH that may directly activate PrfA, intracellular levels of GSH were measured in bacterial strains grown in iLSM after treatment with reducing agents. Exogenous TCEP indeed led to increased levels of intracellular GSH in wild-type, but not Δ*gshF* bacteria (Fig. 4d). Elevated levels of intracellular GSH were also seen in bacteria grown in rich media despite an absence of PrfA activation in this condition (Fig. 1a). These data support a model where exogenous GSH or reducing agents lead to increased intracellular levels of GSH, however this increase is only sufficient for PrfA activation if the bacteria are grown in a defined medium.

2.3.5 Induction of PrfA prior to infection increases virulence and immunogenicity *in vivo*

L. monocytogenes with constitutively active alleles of PrfA are significantly more virulent compared to wild-type bacteria during an acute murine infection (*22*). However, it's unclear what stage(s) during infection the constitutively active allele confers an advantage (*5*). Utilizing iLSM and TCEP to activate PrfA in wildtype *L. monocytogenes* allows assessment of early contributions of a pre-activated PrfA to infection, while leaving the subsequent stages of infection unperturbed. Using an i.v. model of infection, *L. monocytogenes* grown in iLSM supplemented with TCEP had a 60- and 45-fold increase in median bacterial burdens in the liver and spleen, respectively, over untreated controls at 48hrs post-infection (Fig. 5a-b). There was no statistically significant difference in bacterial burdens between pre-activated wildtype bacteria (iLSM + TCEP) versus bacteria harboring the constitutively active allele of PrfA, PrfA*. These data suggested that the virulence advantage conferred by the PrfA* mutation occurred early during infection, likely by avoiding killing, facilitating invasion or escape from the primary vacuole.

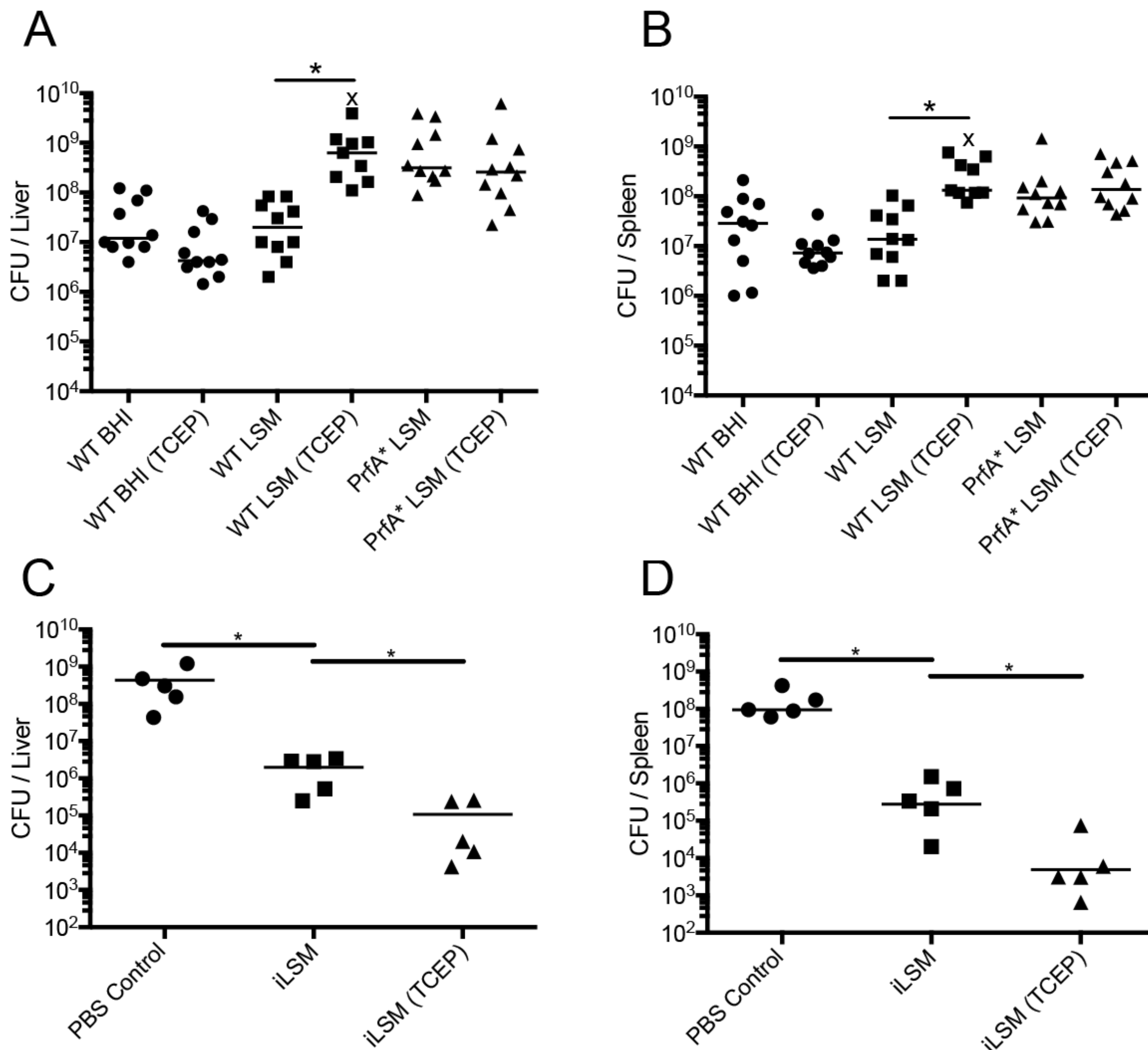

Figure 2.5. Pre-activation of PrfA leads to increased bacterial burden and protection *in vivo.*
(A/B) Mice were infected intravenously with 1 × 10^5 CFU of either wild-type or PrfA* bacteria grown in iLSM or BHI with or without 2mM TCEP prior to infection. After 48hrs, the final CFU per liver and spleen were enumerated as described in Materials and Methods. * denotes a *p*-value of <0.05.
(C/D) Mice were injected with a PBS control or 1 × 10^3 CFU of an attenuated strain of bacteria lacking the *actA* gene grown in iLSM in the presence or absence of 2mM TCEP prior to injection. 28-34 days later mice were challenged with 5 × 10^4 CFU of wild-type *L. monocytogenes* and bacterial burdens in the liver and spleen were enumerated 72 hours later as described in Materials and Methods. * denotes a *p*-value of <0.05.

Attenuated strains of *L. monocytogenes* are being developed as vectors for cancer immunotherapy due to their ability to elicit a robust CD8+ T-cell response (*66*-*68*). Increasing the immunogenicity of an attenuated vaccination strain has the potential to increase the effectiveness of these treatments, or allow comparable results with a lower inoculum. To see if pre-activation of PrfA might lead to enhanced immunogenicity in a vaccination model, an attenuated strain of *L. monocytogenes* (Δ*actA*) was grown in iLSM in the absence or presence of TCEP to

activate PrfA prior to vaccination. Low dose immunizations with 1000 bacteria were performed in C57BL/6 mice intravenously along with a PBS control. Thirty days after vaccination, mice were challenged intravenously with a lethal dose of wildtype bacteria, and after 48 hours, bacterial burdens in the livers and spleens were enumerated. Pre-treatment with TCEP, to activate PrfA, led to a 10 to 100-fold decrease in bacterial burdens in the liver and spleen, signifying a significant increase in vaccination efficacy (Fig. 5c-d).

2.4 Discussion

Listeria monocytogenes leads a biphasic lifestyle in which it alternates between environmental saprophyte and facultative intracellular pathogen of mammals. For decades, investigators have been intrigued by how the bacteria recognize and respond to the intracellular environment of the host (*5*, *40*, *44*, *69*, *70*). The results of this study are consistent with a two-stage mechanism leading to the activation of PrfA, the master transcriptional regulator of *L. monocytogenes* pathogenesis. First, a metabolic licensing step is required that can be recapitulated *in vitro* by growth in a defined synthetic media lacking oligopeptides. Second, a reducing agent is required to initiate complete activation of PrfA, likely by increasing intracellular levels of GSH, the allosteric activator of PrfA. These findings build upon and unify the literature describing virulence gene activation in *L. monocytogenes*, and further, describe a simple method for complete PrfA activation *in vitro*.

Upon entering a host cell, intracellular pathogens must couple the remodeling of their metabolism with the appropriate expression of virulence factors (*71*). In *L. monocytogenes,* the expression and activation of PrfA is influenced by many factors including temperature, carbon sources, L-glutamine, and BCAA levels (*23*, *46*, *56*), yet full activity cannot be recapitulated in complex media. The results of this study indicate that the concentration and composition of peptides in complex media block PrfA activation, with peptides containing BCAAs exerting the largest inhibitory effect. Our findings further suggest that inhibition is due to the levels of (p)ppGpp, as the inhibitory effect of peptides is reversed by induction of the stringent response. Although we have not directly measured the levels of (p)ppGpp during intracellular growth, these data suggest that that growth *in vivo* requires the stringent response as observed in many pathogens (*72*). However, since *L. monocytogenes* can acquire amino acids from host peptides (*73*), it is not yet clear what stress(ors) triggers the stringent response *in vivo*. Nevertheless, *L. monocytogenes* is able to replicate rapidly *in vivo* which implies that the cytosol contains sufficient nutrients for rapid growth. Among the many consequences of elevated (p)ppGpp, is inhibition of c-di-AMP-dependent phosphodiesterases leading to elevated levels of cyclic-di-AMP (*50*), which modulates central metabolism by inhibition of pyruvate carboxylase and controls osmoregulation (*74*). Therefore, it appears as though the regulation of *L. monocytogenes* metabolism and virulence regulation are inextricably linked.

Another consequence of *L. monocytogenes* intracellular growth is the induction of glutathione synthase and the production of glutathione, which is the allosteric activator of PrfA (*28*, *29*). *L. monocytogenes* is one of the few Gram-positive bacteria that synthesize glutathione where it is required for virulence, yet is dispensable in mutants in which PrfA is genetically locked into an active conformation (PrfA*) (*28*). Thus, it appears that the primary function of glutathione during infection is PrfA activation, and not its canonical role of maintaining redox homeostasis. This is not surprising since the cytosolic environment in which *L. monocytogenes* replicates is reducing (*75*). The observation that reducing agents, but not oxidizing agents, trigger PrfA activation suggests that *L. monocytogenes* has evolved to differentiate between the two redox stressors and utilize the uniquely reducing environment of the host cytosol as a spatial-temporal cue during pathogenesis.

There are a number of possible mechanisms to explain why the addition of reducing agents leads to an increase in intracellular GSH. In Gram-negative bacteria, addition of reducing agents is toxic because it inhibits periplasmic disulfide bond formation leading to extracytoplasmic stress (*76*). However, Firmicutes, like *L. monocytogenes,* contain very few proteins with disulfide bonds and are therefore relatively resistant to reducing agent toxicity (*77*). It is more likely that the addition of reducing agents impart reductive stress caused by altering the cellular NAD+/NADH balance, a condition that in Gram-positive bacteria activates a transcriptional response governed in part by the Rex transcription factor (*78*). We speculate that the addition of reducing agents activates Rex though an NAD+/NADH imbalance that either directly or indirectly leads to enhanced synthesis of GSH. Similarly, *in vivo*, the reducing nature of the host cell cytosol may lead to upregulation of *gshF* and consequent activation of PrfA.

The ability to fully activate PrfA *in vitro* allowed us to address the effect of pre-activation on the overall fitness of *L. monocytogenes in vivo.* We demonstrated that stimulation of the PrfA regulon prior to acute infection led to a significant increase in bacterial burden, and conferred enhanced protective immunity to subsequent lethal challenge. The increase in bacterial burdens and protective immunity is most easily explained by a boost in invasion, survival, and/or vacuolar escape. However, it is difficult to reconcile how pre-activation of PrfA translates to a 45-fold increase in virulence. It is possible that the initial increase in virulence gene expression allows the bacteria to invade an alternative subset of host cells that would otherwise normally restrict replication. Further study will be necessary to delineate the exact source of the growth advantage. However, regardless of mechanism, pre-activation of PrfA clearly makes attenuated strains of *L. monocytogenes* more immunogenic and therefore has significant clinical relevance as a method to enhance the efficacy of existing therapies that utilize *L. monocytogenes* as an immunogenic platform for the treatment of cancer (*79*).

Chapter 3

Activity of the pore-forming virulence factor Listeriolysin O is reversibly inhibited by naturally occurring S-glutathionylation

Sections of this chapter were published in:
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Doi:10.1128/IAI.00959-16

3.1 Summary of results

Cholesterol-dependent cytolysins (CDCs) represent a family of homologous pore-forming proteins secreted by many Gram-positive bacterial pathogens. CDCs mediate membrane binding partly through a conserved C-terminal undecapeptide, which contains a single cysteine residue. While mutational changes to other residues in the undecapeptide typically have severe effects, mutating the cysteine residue to alanine has minor effects on overall protein function. Thus, the function of this highly conserved reactive cysteine residue remains largely unknown. We report here that the CDC Listeriolysin O (LLO), secreted by the facultative intracellular pathogen *Listeria monocytogenes*, was post-translationally modified by a S-glutathionylation at this conserved cysteine residue, and that either endogenously synthesized or exogenously added glutathione was sufficient to form this modification. When recapitulated with purified protein *in vitro,* this modification completely ablated the activity of LLO, and this inhibitory effect was fully reversible by treatment with reducing agents. A cysteine-to-alanine mutation in LLO rendered the protein completely resistant to inactivation by S-glutathionylation and retained full hemolytic activity. A mutant strain of *L. monocytogenes* expressing the cysteine-to-alanine variant of LLO was able to infect and replicate within bone marrow-derived macrophages indistinguishably from wild-type *in vitro,* yet was attenuated 4-6 fold in a competitive murine infection model *in vivo*. This study suggests that S-glutathionylation may represent a mechanism by which CDC family proteins are post-translationally modified and regulated, and help explain an evolutionary pressure behind the highly conserved undecapeptide cysteine (*80*).

3.2 Introduction

Cholesterol-dependent cytolysins (CDCs) comprise a family of large oligomeric pore-forming toxins that are primarily secreted by pathogenic Gram-positive bacteria within the Firmicutes and Actinobacteria (*81*). The vast majority of CDC homologs are secreted as monomers that bind cholesterol-containing membranes, oligomerize, and then undergo significant conformational changes that allow for efficient pore-formation (*82*). Decades of study has led to a detailed understanding of the mechanism of action, as well as insight into the nuanced differences between how different pathogens employ their cognate CDC, however, a number of important questions remain unanswered.

One unresolved question relates to the original name given to this class of toxins, thiol-activated cytolysins (*83*). Although a few exceptions necessitated a change in nomenclature, this historical name still highlights a key feature of almost every known CDC— full *in vitro* activation of hemolytic activity requires pretreatment with reducing agent (*84*). This requirement for reduction has been attributed to a single cysteine residue residing in a highly conserved, tryptophan-rich undecapeptide positioned within the membrane-binding domain of the toxin. Yet, the intermediate oxidation state, disulfide, or modification that is sensitive to reducing agents has remained undetermined.

Recent work demonstrating the importance of glutathione in regulating the virulence of *Listeria monocytogenes* (*28*, *36*), combined with the discovery of S-bacillithiolated proteins in *Bacillus subtilis* (*33*, *85*), inspired us to seek the identity of any redox-sensitive modifications to the CDC-family hemolysin, listeriolysin O (LLO). LLO is an essential virulence factor secreted by the facultative intracellular pathogen *L. monocytogenes* that facilitates rapid bacterial escape from a phagocytic vacuole into the cytosol (*6*, *8*). Mechanisms that affect the pore-forming ability of LLO have previously been found to have profound effects on the overall virulence of the bacteria, thus making it an attractive model to evaluate the role of modifications (*15*, *17*, *86*). In addition, due to the highly conserved nature of CDCs, and the undecapeptide in particular, any newly identified modifications may lead to a better understanding of the reduction requirement for other CDCs. The results of this study show that LLO is rendered fully inactive by S-gluathionylation, and that a mutant of LLO that is unable to be modified (LLO^{C484A}) is slightly attenuated *in vivo.*

3.3 Results

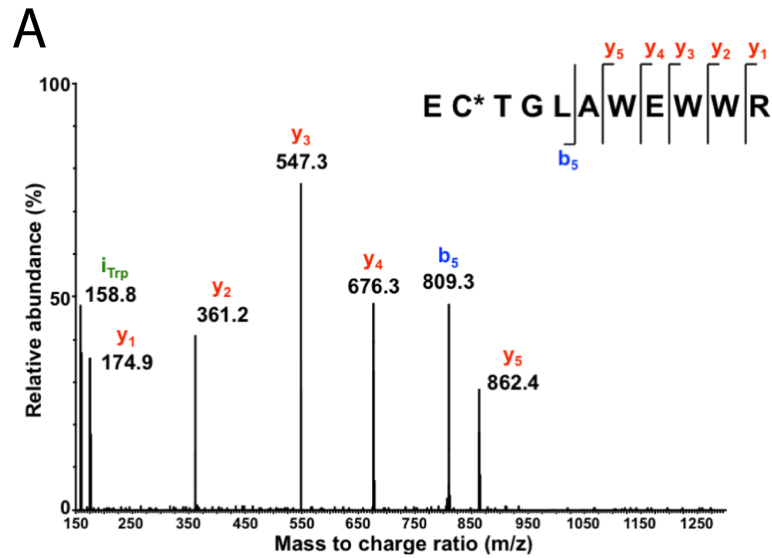
3.3.1 Identification of LLO post-translational modifications

In order to identify post-translational modifications of listeriolysin O (LLO), we performed tandem mass spectrometry analysis of tryptic digests of LLO samples concentrated from broth culture. Since a critical role for the tripeptide antioxidant glutathione (GSH) in *L. monocytogenes* pathogenesis had been recently characterized for its role in post-translationally activating virulence gene production (*28*, *36*), a number of these samples were prepared under native, non-reducing conditions to preserve any potential redox-sensitive modifications. Under these conditions, the single highly conserved cysteine residue (Cys484) of LLO was observed to be S-glutathionylated (Fig. 1). This modification was observed when WT *L. monocytogenes* was grown in a synthetic media lacking GSH and when a *L. monocytogenes* mutant incapable of producing glutathione (Δ*gshF*) was grown in GSH-replete media, demonstrating that the modification can result from either endogenous or exogenous GSH (Table 3.1). When the GSH-deficient mutant of *L. monocytogenes* was grown in the synthetic media lacking GSH, the sole LLO cysteine was S-cysteinylated (Table 3.1). Highlighting its propensity for oxidation, an unmodified “free” cysteine was never observed by mass spectrometry under non-reducing conditions.

Table 3.1. Comparison of LLO modifications under various conditions by MS/MS

L. monocytogenes Strain	*Growth Media (GSH+/-)*[b]	*Modification observed by MS/MS at LLO(Cys484)*[c]
10403S	BHI (GSH+)	S-glutathionylation
10403S	iLSM (GSH-)	S-glutathionylation
∆*gshF*[a]	BHI (GSH+)	S-glutathionylation
∆*gshF*	iLSM (GSH-)	S-cysteinylation

[a.] ∆*gshF* is a strain of 10403S lacking the bifunctional glutathione synthase, *gshF* (lmo2770), and is unable to synthesize glutathione.
[b.] Bacteria were grown in culture media either containing glutathione (BHI), or lacking glutathione (iLSM).
[c.] All modifications detected from any replicates are listed—LLO(Cys484) was never detected as unmodified.



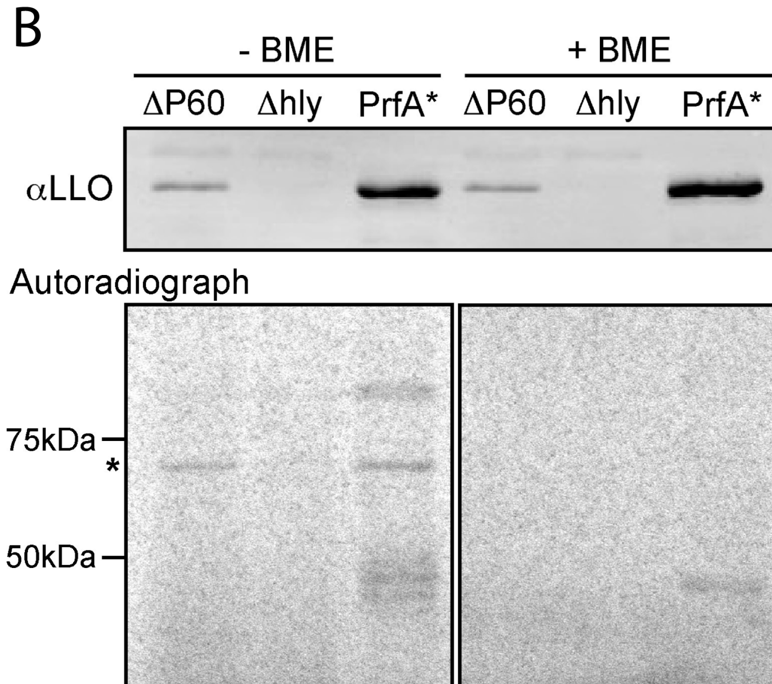


Figure 3.1. LLO is naturally S-glutathionylated at the conserved cysteine.
(A) Tandem mass (MS/MS) spectrum resulting from collision-induced dissociation (CID) of the triply charged, positive precursor ion at *m/z* = 581.2, which is due to the $[M+3H]^{3+}$ ion of the peptide, EC*TGLAWEWWR (listeriolysin O residues 483-493), where C* denotes a S-glutathionylated cysteine residue. Glutathionylation results in an increase in molecular mass of 305.068 Da. **(B)** Western blot and autoradiograph of secreted proteins from cultures of *L. monocytogenes* grown in the presence of radiolabeled glutathione, and separated by SDS-PAGE in the presence or absence of the reducing agent, BME. Δ*P60* is a strain of *L.monocytogenes* lacking the abundant autolysin, P60 (or IAP), that co-migrates with LLO and used here in combination with an LLO deletion strain (Δ*hly*) to confirm that the observed signal in the autoradiogram is due to modified LLO. The PrfA* mutant is a constitutively active mutant of the master virulence transcription factor, PrfA, and synthesizes more LLO. The asterisk denotes the band corresponding to LLO.

3.3.2 *In vitro* activity of modified LLO

To test the effect of S-glutathionylation on activity, LLO monomers were purified under standard reducing conditions then incubated with an optimized concentration of GSH and diamide to catalyze the synthetic S-glutathionylation of LLO monomers (LLO-SSG). Compared to unmodified LLO, the sheep red blood cells (sRBCs) incubated with LLO-SSG showed almost undetectable levels of lysis after 30 min at 37 °C (~1000x less active) (Fig. 2). The loss of activity was fully reversible by treatment with chemical reducing agent. A modified version of LLO in which the sole cysteine residue was mutated to an alanine residue, (LLO^{C484A}), retained nearly full activity compared to wild-type LLO and was completely resistant to inactivation by S-glutathionylation, confirming that S-glutathionylation of Cys484 was responsible for this loss of activity.

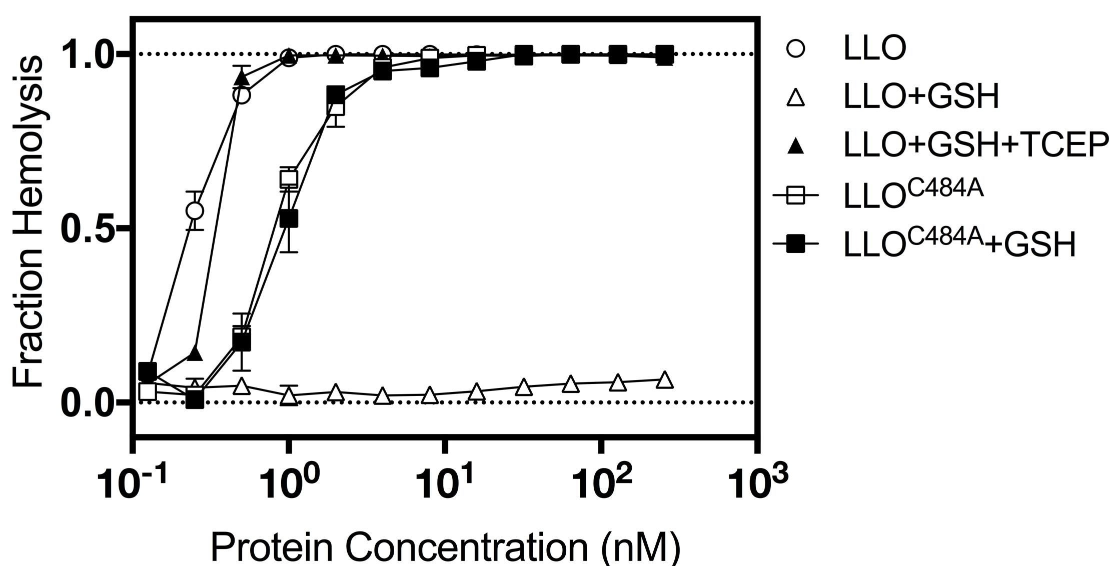


Figure 3.2. *In vitro* S-glutathionylation of LLO completely blocks hemolysis of sRBCs. Preparations of either purified LLO or cysteine-substituted LLO (LLO^{C484A}) are exposed to various pre-treatments and then mixed with sRBCs. The fraction of lysed sRBCs is quantified after 30 min and plotted versus concentration of LLO. Artificially S-glutathionylated LLO (open triangles) showed negligible lysis of sRBCs up to 300 nM, which is largely reversed upon further treatment with the reducing agent, TCEP (closed triangles).

Because the modified cysteine is located at the tip of the membrane-binding domain of LLO, we hypothesized that the S-glutathionylation of cysteine might physically prevent association of LLO with its target membrane. To test this hypothesis, LLO monomers that had been translationally fused to the fluorescent protein mCherry were compared for their ability to bind sRBCs at 4 °C before and after S-glutathionylation (Fig. 3). Consistent with the hemolysis data, S-glutathionylation rendered the LLO monomers unable to bind sRBCs up to 300 nM (i.e., a >35-fold loss of binding affinity). Treatment with the chemical reducing agent

TCEP completely reversed the loss of binding and the (LLO^{C484A}) mutant was immune to modification.

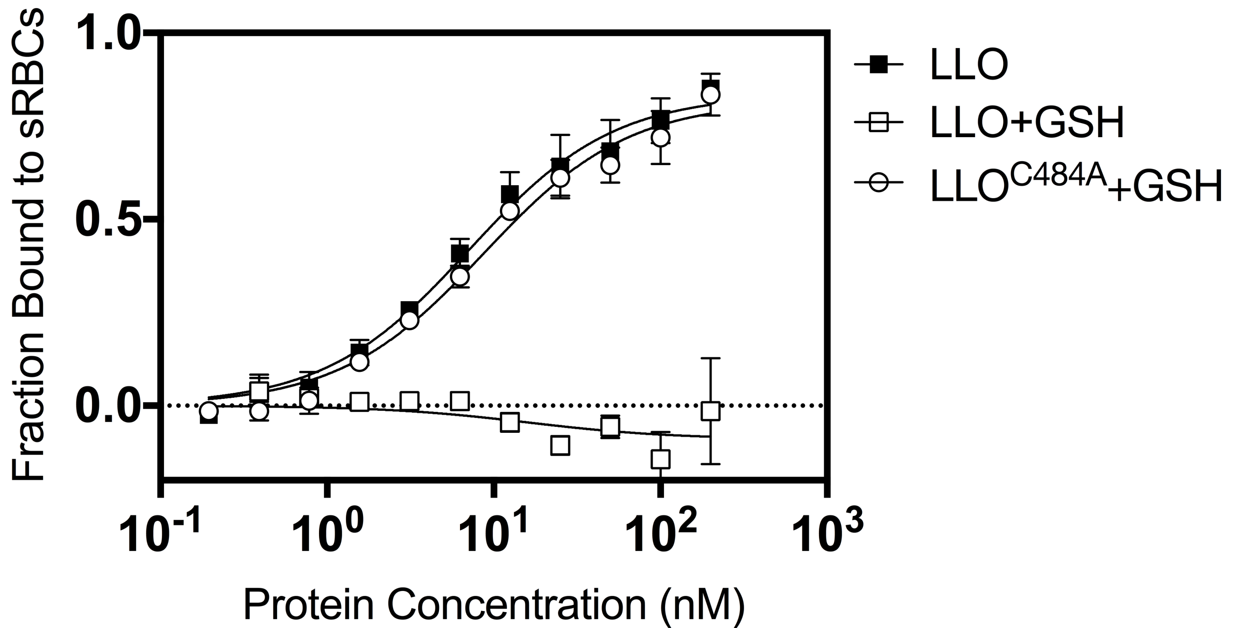


Figure 3.3. *In vitro* S-glutathionylated LLO is unable to bind erythrocytes.
In vitro S-glutathionylation of mCherry-tagged LLO results in complete loss of binding to sRBCs (open squares), as compared to the fully reduced control (closed squares). A cysteine-substituted mutant of LLO (LLO^{C484A}) retains activity after identical S-glutathionylation treatment.

3.3.3 *In vivo* results

The potent yet rapidly reversible negative effect of S-glutathionylation on the function of LLO led us to hypothesize that this modification might be critical for restricting pore-forming activity to appropriate spatial and/or temporal compartments during infection. To test this hypothesis, we performed a competitive infection to compare the virulence of a wild-type strain to a strain expressing the C484A LLO mutant (LLO^{C484A}). Forty-eight hours after an i.v. infection of CD-1 mice, the strain expressing LLO^{C484A} exhibited a statistically significant defect in the liver and spleen as compared to wild-type (Fig. 4B). While this defect suggests a role for Cys484 *in vivo*, we wanted to determine if there was another model of infection that would reveal a more specific and striking impact of losing this redox-sensitive switch of LLO. The LLO^{C484A} mutant, however, showed no significant growth defect in bone-marrow derived macrophages (Fig. 4A), or spreading defect in a monolayer of L2 fibroblasts (Fig. S2A). In addition, growth in resident peritoneal macrophages (Fig. S2B), *in vivo* oral infections (Fig. S2C), and *in vivo* non-competitive infections (Fig. S1) were tested for their abilities to potentially reveal a specific role for the S-glutathionylation of LLO, yet only the *in vivo* competitive index was sensitive enough to yield a statistically significant defect of the LLO^{C484A} mutant.

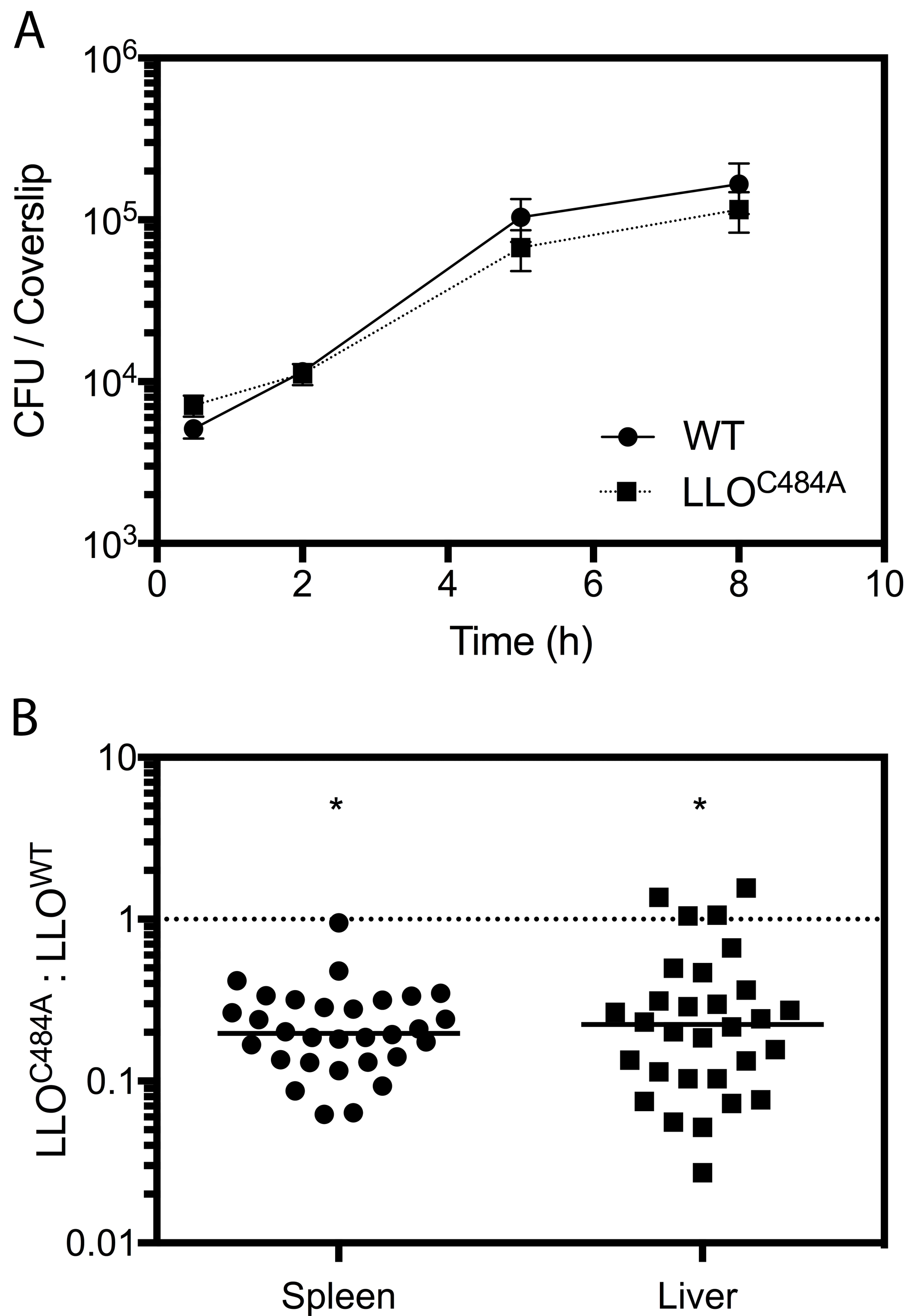


Figure 3.4. Glutathionylation of LLO is required for maximal virulence in an animal model of infection. **(A)** A mutant of *L. monocytogenes* expressing a cysteine-substituted variant of LLO (LLO^{C484A}) grows similarly to wild-type during *in vitro* infection of BMDMs. **(B)** The cysteine-substituted mutant of LLO (LLO^{C484A}) is attenuated for virulence compared to wild-type bacteria during *in vivo* competitive infection of mice. The median competitive index for the liver and spleen are 0.1976 and 0.2240, respectively, and significance was calculated by a one-sample t-test using Prism 7 (GraphPad); *p <0.0001.

3.4 Discussion

The sole cysteine residue within LLO is conserved among nearly every CDC homolog, yet the evolutionary pressure to retain such a highly reactive residue has remained a mystery. Although it is known that this cysteine confers the prototypical reduction requirement for almost all CDCs, the precise mechanism and biological relevance of this observation is still poorly understood (*87*, *88*). Previous studies have established that artificial chemical modification of this residue, such as alkylation, prevents binding and/or subsequent pore-formation of target membranes (*87*, *89*). The results of this study show that glutathione, derived from either endogenous or exogenous sources, S-glutathionylates LLO and reversibly ablates LLO activity.

The prevalence and importance of low molecular weight (LMW) thiols in bacterial species is becoming increasingly clear (recently reviewed (*90*)). For example, in *Salmonella typhimurium* more than two dozen proteins are either S-glutathionylated or S-cysteinylated, depending on the growth media (*91*). In *B. subtilis* and *Staphylococcus aureus*, many proteins are modified by the analogous LMW thiol bacillithiol, while *Mycobacterium tuberculosis* utilizes mycothiol (*34*, *92*). *L. monocytogenes* is rare among Gram-positive bacteria in that it can synthesize glutathione (*36*) and, as demonstrated here, utilize it as a redox-sensitive substrate for post-translational modifications. In agreement with previous studies (*93*), a variant of LLO that is unable to be S-glutathionylated (LLO^{C484A}) decreased the virulence of *L. monocytogenes in vivo*, despite having similar (or in one study, greater (*94*)) lytic activity.

The primary role of LLO is to mediate escape of *L. monocytogenes* from a potentially hostile phagocytic vacuole (*6*). It is possible that S-glutathionylation protects the cysteine from irreversible oxidation in a phagosome. However, the S-glutathionylation of LLO in this context would be expected to inhibit phagosomal escape, suggesting that either the bacteria or the host possess an oxidoreductase capable of removing the glutathione. Interestingly, macrophages possess a γ-interferon-inducible lysosomal thiol reductase (GILT, also known as Ifi30) that is capable of activating LLO *in vitro* (*95*). In addition, $GILT^{-/-}$ macrophages and mice are resistant to infection by wild-type *L. monocytogenes,* but not to a mutant secreting LLO^{C484A} (*95*). Combined with the data presented in this study, it is reasonable to suspect that the vacuolar-localized GILT is necessary to reduce S-glutathionylated LLO, however, we were unable to reproduce the *in vitro* growth defect of wild-type *L. monocytogenes* in $GILT^{-/-}$ macrophages (Fig S3). Nevertheless, the notion that S-glutathionylated LLO restricts its activity to specific host cells capable of removing this modification is appealing.

The discovery of this naturally occurring modification represents one of the first examples of an S-glutathionylated protein derived from a Gram-positive bacteria. In addition, these data suggest that S-thiolation of the conserved cysteine

of CDCs may be a conserved post-translational regulatory mechanism necessary for the optimal activity of different CDCs in their respective niches. Future studies with other CDC-secreting pathogens in infection models analogous to that of *L. monocytogenes* will be necessary to fully appreciate the regulatory importance of this modification.

Chapter 4

Concluding remarks and future perspectives

4.1 Summary of results

This study defined novel mechanisms of virulence gene regulation in *Listeria monocytogenes.* More specifically, two distinct forms of post-translational control were described that augment the intrinsic activity of PrfA and LLO by glutathione. First, we demonstrated that growth of *L. monocytogenes* in a synthetic nutrient media supplemented with glutathione is sufficient for full activation of PrfA and all consequent virulence factors. This represents the first example of complete virulence gene activation *in vitro*. The addition of exogenous peptides to the synthetic growth media blocks activation by glutathione, yet the effect is reversed by artificially increasing intracellular levels of (p)ppGpp. These data support a model of PrfA activation whereas when *L. monocytogenes* enters the host cell cytosol, a nutritive or yet unknown stress leads to an increase in intracellular (p)ppGpp that allows the activation of PrfA by glutathione. Further, the source of glutathione is either an increase in bacterially synthesized glutathione in response to the reducing environment of the cytosol, direct import of host glutathione, or a combination of the two. Further study will be necessary to determine how elevated levels of (p)ppGpp license PrfA activation by GSH and the biologically relevant source of glutathione *in vivo*. Second, LLO was identified as being post-translationally modified by glutathione at a highly conserved cysteine residue. This reversible modification leads to a loss of membrane binding and thus pore-forming activity *in vitro*. Mutation of this conserved cysteine residue left the LLO molecule functioning properly, but ablated the ability of glutathione to form the S-glutathionylation modification. This mutation led to a significant, albeit slight, attenuation of virulence *in vivo,* highlighting the regulatory importance of this modification during infection.

4.2 Remaining questions and future directions

4.2.1 S-glutathionylation of LLO

The purpose of the highly conserved cysteine within the undecapeptide of LLO and almost all cholesterol dependent cytolysins has been speculated on for decades, but never fully determined. Mutations to almost all residues within the critical undecapeptide lead to catastrophic loss of pore-forming activity, however, an alanine substitution at the cysteine residue has almost no effect. Why, then, would there be a selective pressure to retain such a reactive residue? What biological process is that cysteine important for? Our data suggests that this residue is the target of a post-translational modification that reversibly inhibits activity, which may be a critical feature for the pathogenesis of *L. monocytogenes.* The relatively minor loss of fitness in an *in vivo* virulence model, however, suggests this cysteine residue is relatively dispensable for virulence during acute i.v. infection. It is interesting to note that LLO is the only cholesterol dependent cytolysin secreted by an intracellular pathogen. All other homologs of LLO are secreted by extracellular pathogens like *Streptococcus pyogenes* and *Clostridium perfringens*— a distinction that may significantly affect how we determine the evolutionary role for the cysteine. An *in vivo* infection model using *S. pyogenes*, for example, would be useful to

determine if the cysteine-substituted analog of LLO was a greater detriment to virulence in extracellular pathogens.

4.2.2 PrfA activation *in vitro*

The relationship between PrfA and (p)ppGpp remains unclear. High levels of (p)ppGpp appear to be required for PrfA activation by GSH *in vitro*, however whether or not this interaction is direct or indirect is unknown. A strain of *L. monocytogenes* lacking all three known (p)ppGpp synthases (relA,relP,relQ), was still able to activate PrfA in the presence of GSH (J. L. Portman, data not shown), however, this strain grew extremely poorly and may have acquired suppressor mutations that affected the result. In addition, the reported role of CodY in the activation cascade of PrfA was not consistent with our results, further emphasizing the importance of future studies to verify the linkage between (p)ppGpp and PrfA.

Interestingly, multiple studies have now linked the regulation of (p)ppGpp with another secondary nucleotide messenger implicated in metabolic adaptation, cyclic di-adenosine monophosphate (c-di-AMP). C-di-AMP regulates carbon flux through the bacterial TCA cycle by inhibiting pyruvate carboxylase (PycA) during normal growth, but is also inexplicably secreted upon vacuolar escape and is a known agonist of the cytosolic innate immune sensor, STING(*96*, *97*). It is tempting to speculate that its secretion in the host cytosol is a consequence of increased intracellular (p)ppGpp levels from nutrient limitation that serves to quickly lower intracellular levels of c-di-AMP and drive metabolic reprogramming. Since this metabolic shift is absolutely necessary for efficient nutrient acquisition and virulence gene activation in the cytosol, it may explain the evolutionary pressure on *L. monocytogenes* to continue secreting c-di-AMP in the cytosol, despite detection by STING.

The true impact of these findings is not only limited to advancing our understanding of the interplay between metabolism and virulence gene regulation, but has the potential to drive significant practical applications as well. For example, these data serve as a proof-of-concept for the use of the *in vitro* activation of PrfA as a tool to enhance the efficacy of existing immunotherapies that utilize *L. monocytogenes* as an immunogenic platform for the treatment of human disease. The strength of the *actA* promoter could also be co-opted to drive the heterologous expression of proteins for commercial applications. An engineered strain of the closely related BSL-1 bacteria, *Listeria innocua*, for example, could be engineered to express PrfA and the desired protein fused to the promoter of *actA.* Exogenous glutathione or other reducing agent, then, should lead to robust expression and synthesis of the desired protein, without the fear of LPS contamination that plague Gram-negative expression platforms. Validation experiments would need to be performed to compare the yield of this setup to current Gram-positive expression systems.

4.3 Speculation into the future

Study into the pathogenesis of *L. monocytogenes* has enjoyed decades of success due to creative scientists working collaboratively using the latest tools molecular biology has to offer. As we pass the 30-year mark since Listeriolysin O was first cloned, it is difficult to imagine what the next 30 years might hold for the field. Technology within the field has been advancing exponentially for years— starting with the advent of PCR, through the development of next-gen sequencing platforms, and now modern CRISPR/Cas9 gene editing tools. Just 10 years ago, many of the tools in our modern genetic toolbox either didn't exist, or were too labor intensive and/or expensive to be realistic. Soon, most nucleic acid manipulation techniques will become obsolete as *de novo* synthesis becomes increasingly cost-effective. While something like *de novo* sequencing will significantly improve the speed and accuracy of which our genetic manipulations are implemented, advances like these will also increase the burden on scientists to elucidate increasingly complex problems in order to remain competitive. Many young scientists believe the older generations had it easy— the cloning of single genes often led to the publication of high impact papers, and thus, successful careers. To the young scientists that will regularly sequence dozens of genomes without commendation, this feeling is not surprising. However, the reason the sequencing of single genes used to warrant publication on their own is the techniques to do so at the time were very labor intensive and very challenging *technically*. While the burden on scientists to unravel increasingly complex mechanistic pathways is absolutely challenging, it is a *mental* challenge and less of a *technical* one. In many ways, the *mental* challenge is what makes science fun and interesting. Therefore, successful scientists of the future must appreciate the diminishing technical barriers and fully embrace the thrill of big, risky, hypothesis-driven research. What this means for *L. monocytogenes* is unclear, but I hypothesize that future studies will better elucidate the complex interplay between metabolism, redox, and virulence that is now coming to our attention.

Materials and Methods

Chapter 2

Ethics Statement

This study was carried out in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Research Council of the National Academy of Sciences (*98*). All protocols were reviewed and approved by the Animal Care and Use Committee at the University of California, Berkeley (AUP-2016-05-8811).

Bacterial Strains and Growth Conditions

All *L. monocytogenes* strains (See Table S1.) were derivatives of 10403S (*99*) cultured in brain-heart infusion (BHI, BD Biosciences) or a defined media specific for *L. monocytogenes* (*74*) at 37˚C, with shaking, and without antibiotics unless otherwise stated. Growth was measured by the optical density at a wavelength of 600 nm (OD600). Frozen bacterial stocks were stored at -80˚C in BHI + 40% glycerol. All chemicals were purchased from Sigma-Aldrich unless otherwise stated. Antibiotics were used at the following concentrations unless otherwise stated: streptomycin (200 µg/mL), chloramphenicol (7.5 µg/mL for *L. monocytogenes*, 10 µg/mL for *E. coli*).

Growth Supplements and Inhibitors

For purified peptides, 0.075 g hexapeptide (0.025% w/v final concentration, 49.7mM for KGGGGK, 44.7 mM for KAAAAK, and 34.4 mM for KIIIIK and KLLLLK) were dissolved in 3 mL iLSM and the LSM-peptide product was sterilized using a 0.2 µm filter. Specific peptides were purchased from Mimotopes (Australia) and have specifications as follows: KGGGGK (MW=502.57 g mol^{-1} >65% pure), KAAAAK (MW=558.68 g mol^{-1} >61% pure), KIIIIK (MW=727.00 g mol^{-1} >71% pure), KLLLLK (MW=727.00 g mol^{-1} >72% pure). DL-Serine hydroxamate was used where noted at a final concentration of 2mg/mL.

Fluorescence Reporter Assay

Strains of *L. monocytogenes* harboring the integrating plasmid pPL2 expressing RFP controlled by the *actA1p* promoter are grown overnight at 37C in iLSM, shaking (*52*). These cultures were diluted 1:10 into the noted media with any applicable supplements and grown at 37C shaking until an OD600 of approximately 2. 500 µL was taken from each culture and transferred into a clear 24-well flat-bottom plate and subsequently read for fluorescence intensity on a Tecan M1000 multi-plate reader with the following parameters: 560/580 (Ex/Em), bottom-read, optimal flashes, optimal gain. OD600 was taken in parallel with a handheld spectrophotometer for normalization.

Quantitative RT-qPCR of Bacterial Transcripts

Bacteria were grown overnight and subcultured 1:20 into 5 ml iLSM. Bacteria were harvested at an OD_{600} = 1.0 by the addition of an equal volume of RNAprotect

Bacteria Reagent (Qiagen). Bacteria were harvested by centrifugation and flash frozen in liquid nitrogen prior to RNA extraction. Bacteria were lysed in phenol:chloroform containing 1% SDS by vortexing with 0.1 mm diameter silica/zirconium beads (BioSpec Products Inc.). Nucleic acids were precipitated from the aqueous fraction overnight at −80 °C in ethanol containing 150 mM sodium acetate (pH 5.2). Precipitated nucleic acids were washed with ethanol and treated with TURBO DNase per manufacturer's instructions (Life Technologies Corporation). RNA was again precipitated overnight and then washed in ethanol. RT–PCR was performed with iScript Reverse Transcriptase (Bio-Rad) and quantitative PCR (qPCR) of resulting cDNA was performed with KAPA SYBR Fast using the manufacturer's recommended cycling parameters (Kapa Biosystems). Primers used for qPCR of *actA* transcript: *actA*_F: CGACATAATATTTGCAGCGAC, *actA*_R: TGCTTTCAACATTGCTATTAGG.

Immunoblot for LLO

Briefly, overnight cultures of bacteria in iLSM were diluted 1:10 into iLSM in the presence or absence of 10mM GSH and incubated for six hours at 37°C, shaking, then the bacteria were separated from the supernatant by centrifugation. The supernatant was treated with 10% v/v TCA for one hour on ice to precipitate protein. The protein pellet was washed twice with ice- cold acetone, followed by vacuum drying. The proteins were dissolved in LDS buffer (Invitrogen) containing 5% BME using a volume that normalized for OD_{600} of harvested bacteria, boiled for 10 minutes, and separated by SDS-PAGE. Primary antibodies were each used at a dilution of 1:5,000, including a rabbit polyclonal antibody against LLO, and a mouse monoclonal antibody against P60 (Adipogen). P60 is a constitutively expressed bacterial protein used as a loading control for secreted proteins (*100*). All immunoblots were visualized and quantified using Odyssey Imager and appropriate secondary antibodies from the manufacturer according the manufacturer's instructions.

Intracellular Glutathione Quantification

Reduced glutathione (GSH) and GSSG concentrations were measured by a commercial kit supplied by Cayman Chemical per the manufacturer's recommendation and as described previously (*101*). Briefly, bacteria were grown to mid-log in either iLSM or BHI and resuspended in PBS containing 1mM EDTA at a pH of 6.5. Bacteria were lysed by vortexing with 0.1 mm diameter silica/zirconium beads (BioSpec Products Inc.), and lysate was stored on ice. Samples were deproteinated with an equal volume of metaphosphoric acid and stored at -20C prior to quantification with the supplied kit.

In Vivo Infections

For acute infections, eight-to-twelve-week-old female C57BL/6 mice (The Jackson Laboratory) were infected intravenously with 1×10^5 colony-forming units (CFU) in 200ul of PBS as described previously (*102*, *103*) and organs were harvested 48

hours later. For immunization studies, mice were injected with 1×10^3 CFU in 200ul of PBS of an attenuated strain of *L. monocytogenes* harboring a deletion in the *actA* gene, then 28-34 days later mice were challenged with 5×10^4 CFU of wild-type *L. monocytogenes* in 200ul of PBS and organs were harvested 72 hours later. In both cases all bacteria were grown to an OD600 of approximately 0.5 in either iLSM or BHI in the presence or absence of 2mM TCEP. The bacteria were washed twice with PBS, suspended in a solution of 9% glycerol in PBS and then flash frozen in liquid nitrogen before storage at -80C. Prior to infection, frozen bacteria were thawed and diluted to the appropriate cell density in PBS and plated for enumeration in parallel with the infection to verify inoculum accuracy. To collect organs, the mice were euthanized and spleens and livers were harvested, homogenized in 5mls or 10mls IGEPAL CA-630 (Sigma), respectively, and plated for enumeration of bacterial burdens.

Statistical Analysis
Statistical analyses were carried out with the GraphPad Prism software (v.7.0a). Values plotted exponentially were transformed to base 10 logarithmic values before being used for statistical analyses. One-way ANOVA with Tukey's post-hoc test was used to compare groups, with only relevant comparisons noted on each figure for clarity.

Chapter 3

Ethics Statement
This study was carried out in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Research Council of the National Academy of Sciences (*98*). All protocols were reviewed and approved by the Animal Care and Use Committee at the University of California, Berkeley (MAUP R235-0815B).

Bacterial Strains and Growth
All *L. monocytogenes* strains (listed in Table S3) were derivatives of 10403S (*99*) cultured in brain-heart infusion (BHI, BD Biosciences) at 37˚C, with shaking, and without antibiotics unless otherwise stated. Growth was measured by the optical density at a wavelength of 600 nm (OD600) using a spectrophotometer. Frozen bacterial stocks were stored at -80˚C in BHI + 40% glycerol. All chemicals were purchased from Sigma-Aldrich unless otherwise stated. Antibiotics were used at the following concentrations unless otherwise stated: streptomycin (200 µg/mL), chloramphenicol (7.5 µg/mL for *L. monocytogenes*, 10 µg/mL for *E. coli*), erythromycin (1 µg/mL).

Identification of LLO S-glutathionylation by Tandem Mass Spectrometry
Cultures of *L. monocytogenes* were grown in either Incomplete Listeria Synthetic Medium (iLSM) (*74*) or brain-heart infusion broth (BHI, BD Biosciences) with

shaking at 37˚C until entrance into stationary phase. Bacteria were separated by centrifugation and the supernatant fluid was filtered prior to precipitating the proteins with 10% TCA. Samples were suspended in 1X LDS buffer (Invitrogen) without the addition of reducing agent and separated by SDS-PAGE. After Coomassie Blue staining, the LLO band (~59kDa) was excised, destained with 25mM ammonium bicarbonate in 50% acetonitrile, and in-gel digested with an equal volume of Trypsin Gold (Promega) at 12.5µg/mL in 80% acetonitrile with 25mM ammonium bicarbonate for 6-8 hours at 37˚C. The digested peptides were then extracted from the gel pieces by three successive 10-minute vortex treatments using a solution of 45% water, 50% acetonitrile, and 5% formic acid. The total extracted solution was reduced to 10µL by vacuum centrifugation and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). LC-MS/MS measurements were performed using an LTQ-Orbitrap-XL mass spectrometer that was connected in line with an UltiMate3000 RSLCnano liquid chromatograph (Thermo Fisher Scientific, Waltham, MA).

Natural Modification of LLO by ^{35}S-labeled Glutathione
Cultures of *L. monocytogenes* were incubated at 37°C in tryptic soy broth medium supplemented with ^{35}S-glutathione (4 µCi/mL) for five hours (Perkin-Elmer). Bacteria were removed by centrifugation and filtration prior to precipitation of supernatant proteins with TCA (10%) and separation by SDS-PAGE with or without 2-ME. The gel was dried and exposed to a phosphor-screen for four days before imaging on a Typhoon scanner (GE Life Sciences). Duplicate gels were transferred to a nitrocellulose membrane and analyzed for protein abundance by immunoblotting for LLO and P60 using previously described methods (*70*).

Intracellular Growth Curve
Wild-type or GILT-/- (Gift from Peter Cresswell) bone marrow-derived macrophages were harvested as previously described (*97*) and 3 x 10^6 cells were plated in 60 mm non-TC-treated Petri dishes. Cells were infected with a multiplicity of infection (MOI) of 0.1 and growth curves were performed as described previously (*8*).

Competitive Index In Vivo
Competitive indices were determined as previously described (*104*). Briefly, frozen culture stocks were thawed, grown to log phase in fresh BHI for 2 hours, and mixed in a 1:1 ratio. Female 6-8 week old CD-1 mice (Charles River) were infected with 1 x 10^5 CFU. Animals were sacrificed 48 hours later, and their spleens and livers were harvested. Spleens and livers were homogenized in 5 and 10 mL 0.2% NP-40, respectively, and homogenates were plated onto LB agar. At least 100 colonies per organ were replica plated or patched onto BHI agar containing erythromycin (2 µg/mL). Competitive indices were calculated by dividing the number of test strain CFU (erythromycin sensitive) by the number of reference strain CFU (erythromycin resistant).

Plasmid Encoding mCherry-LLO

The mCherry-LLO expression vector was constructed by cloning the gene encoding mCherry from pHpPL3-mCherry to pET29b-hly via Gibson assembly using the primers listed in Table S2 (*105*). The resulting protein had mCherry (26.7 kD) fused to the N-terminus of LLO. The primers and templates are listed in Table S2.

Protein Expression and Purification

Expression and purification of recombinant LLO was described previously (*17*). Specifically, *E. coli* expressing strains harboring expression plasmids (listed in Table S3) were grown at 37°C to stationary phase and inoculated into 1 L fresh LB media with chloramphenicol to an initial OD_{600} of 0.01. When OD_{600} of the culture reached 0.6~0.7, expression was induced by the addition of 0.5 mM of IPTG and maintained for 6 hours. To harvest the cells, bacteria were pelleted and resuspended in 35 mL cold Lysis Buffer (50 mM sodium phosphate, pH 8.0, 1 M NaCl, 20 mM imidazole, 10 mM 2-mercaptoethanol, 1 mM PMSF). Cells were lysed by sonication. The majority of target protein ended up in the supernatant after centrifugation. Two mL of Ni-NTA resin equilibrated in Washing Buffer (20 mM sodium phosphate, pH 7.4, 1 M NaCl) was added to the lysate supernatant and mildly stirred at 4 °C for 1 hour. The resin was packed in a column and washed with up to 1 L of Washing Buffer. LLO was eluted with Elution Buffer (20 mM sodium phosphate, pH 6.0, 1 M NaCl, 800 mM imidazole). Extra imidazole in eluate was removed by dialysis against Storage Buffer (20 mM sodium phosphate, 1 M NaCl, pH 6.0, 5 mM DTT, 1 mM EDTA). The protocol yielded over 50 mg of LLO protein per liter of media. mCherry-LLO proteins were purified by the same method.

Hemolysis Assay

The hemolysis assay was performed as previously described (*17*). Briefly, sheep red blood cells (sRBCs) were washed three times in PBS, and resuspended to 2% (vol/vol) in PBS at pH 5.5 (adjusted with HCl). Protein samples were pre-warmed in 0.1 mg/mL BSA, PBS in 96-well plate at 37 °C for 30 min; in the case of glutathionylated LLO, protein was treated with 0.2 mM glutathione (GSH), 0.5 mM diamide, and 0.1 mg/mL BSA in PBS. Where noted, LLO was subsequently reduced with 8mM TCEP for 30 min. After the pre-incubation, protein samples were serial diluted to target concentrations and mixed at 5:1 (vol/vol) ratio with 2% RBCs. The resulting mixtures were gently shaken and incubated at 37 °C for 30 min, followed by absorbance measurement at 600 nm using a SpectraMax M5 Microplate reader. The fractional hemolysis was calculated as $(A_{protein} - A_{buffer})/(A_{TX} - A_{buffer})$. $A_{protein}$ is the absorbance of protein samples. PBS buffer was mixed with cells (no LLO) to yield A_{buffer}, and complete hemolysis A_{TX} was achieved with 0.1% Triton X-100. Triplicate samples were assayed for each protein concentration.

In Vitro S-glutathionylation

Conditions for protein glutathionylation were explored with oxidants such as hydrogen peroxide and diamide. Diamide was chosen due to its rapid conversion

and little to no alteration in cell function (*64*). The optimized conditions were as follows: 10 μM LLO was incubated at 4°C overnight with 0.2 mM GSH and 0.5 mM diamide, in PBS. Excessive GSH and diamide were removed by dialysis against storage buffer without DTT. The resulting protein conjugate was verified with trypsin digestion and LC-MS/MS (*106*), and the percentage of glutationylated peptides were each estimated to be 99.7%.

mCherry-LLO Membrane Binding Assay

Sheep red blood cells (sRBCs) were resuspended to 20% (vol/vol) in PBS pH 7.4 after washes. mCherry-LLO was diluted in series and added to the sRBCs at 5:1 (protein:RBC) ratio. The mixture was incubated at 4 °C for 15 min and centrifuged. Fluorescence emission of supernatant was measured at 650 nm (excitation: 580 nm). The fraction of protein bound was calculated as $(F_0\text{-}F)/F_0$, where F_0 was the fluorescence of mCherry-LLO added to PBS buffer and F was the fluorescence of the protein added to RBCs.

Plaque Assay

Plaque assays in L2 murine fibroblasts were performed as previously described (*107*). Briefly, bacterial cultures were grown overnight at 30 °C, then washed and diluted 1:10 in sterile PBS. Six-well dishes containing 1.2×10^6 L2 cells per well were infected with *L. monocytogenes* for 1 h, then washed and overlaid with 3 ml of media containing 0.7% agarose and gentamicin (10 μg ml^{-1}) to prevent extracellular growth. After 3 days at 37 °C, an overlay containing gentamicin and neutral red dye (Sigma) was added and stained overnight. The plates were then scanned and analyzed with ImageJ software (*108*).

Virulence Experiments

Six-to-eight-week-old female CD-1 mice (The Jackson Laboratory) were either infected intravenously with 1×10^5 colony-forming units (CFU) in 200ul of PBS or orally with 1×10^9 CFU in contaminated bread, (as described previously (*109*)). Forty-eight hours post-infection the mice were euthanized and spleens and livers were harvested, homogenized in 5mls or 10mls 0.1% NP-40, respectively, and plated for enumeration of bacterial burdens. All animal work was done in accordance with university regulations. Protocols were reviewed and approved by the Animal Care and Use Committee at the University of California, Berkeley (MAUP# R235-0815B).

Intracellular Growth Curve of Peritoneal Macrophages

Resident peritoneal cavity cells were harvested from six-to-eight-week-old female C57BL/6 mice as described previously (*110*). The peritoneal exudates were then plated for 3hrs and peritoneal macrophages were enriched for by discarding non-adherent cells. These cells were then collected using cell-scrapers and growth curves were performed as described previously (*8*).

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Supplemental Information

Table S1. Bacterial strains used in Chapter 2

Strain Code	Parent Strain	Description	Reference
10403S	*L. monocytogenes*	Wild-type	(*99*)
DP-L4317	*L. monocytogenes*	Δ*prfA*	(*111*)
NF-L1177	*L. monocytogenes*	PrfA* (G145S)	(*112*)
DP-L6188	*L. monocytogenes*	Δ*gshF*	(*28*)
DP-L6508	*L. monocytogenes*	Wild-type + pPL2_P_{actA} RFP	(*52*)
DP-L6561	*L. monocytogenes*	Δ*prfA* + pPL2_P_{actA} RFP	This Study
DP-L6562	*L. monocytogenes*	PrfA* (G145S) + pPL2_P_{actA} RFP	This Study
DP-L6563	*L. monocytogenes*	Δ*gshF* + pPL2_P_{actA} RFP	This Study
DP-L6291	*L. monocytogenes*	$RelA^{R295S}$	(*61*)
DP-L6292	*L. monocytogenes*	Δ*relA*	(*61*)
DP-L3078	*L. monocytogenes*	Δ*actA*	(*13*)

Table S2. Primers used in Chapter 3

#	Sequence (5' to 3')	Construct	Vector Template	Insert Template	Pair
1	ctttaagaaggagatatacatatggt tagtaaaggtgaagaagat	mCherry-LLO-His_6	pET29b-*hly*	pHpL3-mCherry	2
2	gaatgcagatgcatccttcatatgttt atataattcatccataccacc	mCherry-LLO-His_6	pET29b-*hly*	pHpL3-mCherry	1
3	ctttaagaaggagatatacatatggt tagtaaaggtgaagaagat	mCherry-C484A-His_6	pET29b-*hly*^{C484A}	pHpL3-mCherry	4
4	gaatgcagatgcatccttcatatgttt atataattcatccataccacc	mCherry-C484A-His_6	pET29b-*hly*^{C484A}	pHpL3-mCherry	3

Table S3. Bacterial strains used in Chapter 3

Strain Code	Parent Strain	Description	Reference
10403S	*L. monocytogenes*	Wild-type	(*99*)
DP-L2261	*L. monocytogenes*	Δ*hly*	(*113*)
NF-L1177	*L. monocytogenes*	PrfA* (G145S)	(*112*)
DP-L6188	*L. monocytogenes*	Δ*gshF*	(*28*)
DP-L4351	*L. monocytogenes*	LLO^{C484A}	This Study
DP-L3903	*L. monocytogenes*	Wild-type (ErmR)	(*104*)
DP-E3570	*E. coli*	pET29b_*hly*	(*17*)
DP-E6560	*E. coli*	pET29b_*hly*^{C484A}	This Study
DP-E6502	*E. coli*	pET29b_*hly*_mCherry	This Study
DP-E6504	*E. coli*	pET29b_*hly*^{C484A}_mCherry	This Study

Figure S1.

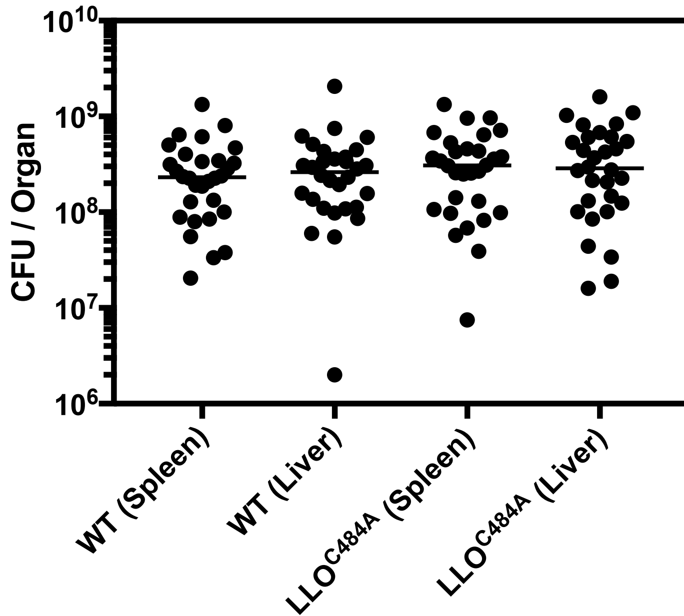
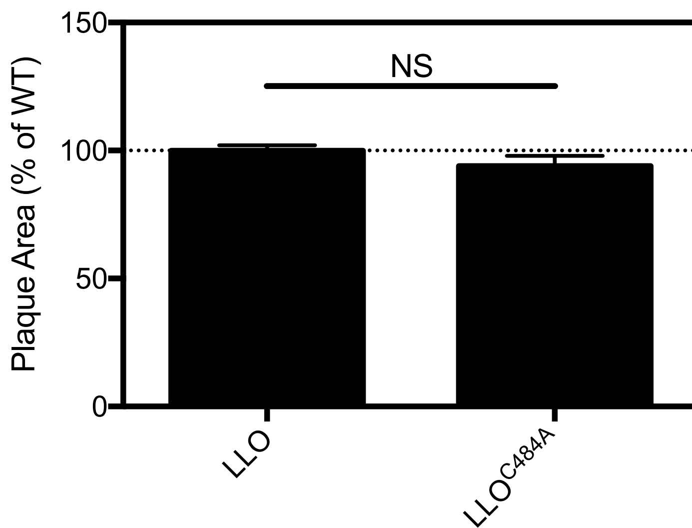


Figure S1. Non-competitive infection masks the virulence defect of LLO^{C484A} *in vivo.*
Mice were infected intravenously with 1 x 10^5 CFU of either wild-type or LLO^{C484A} mutant bacteria for 48hrs and the final CFU per liver and spleen were enumerated as described in Materials and Methods. Using this model, there is no statistically significant defect of the LLO^{C484A} mutant *in vivo,* highlighting the sensitivity of the competitive model and the mild virulence defect of the LLO^{C484A} strain.

Figure S2.

A.



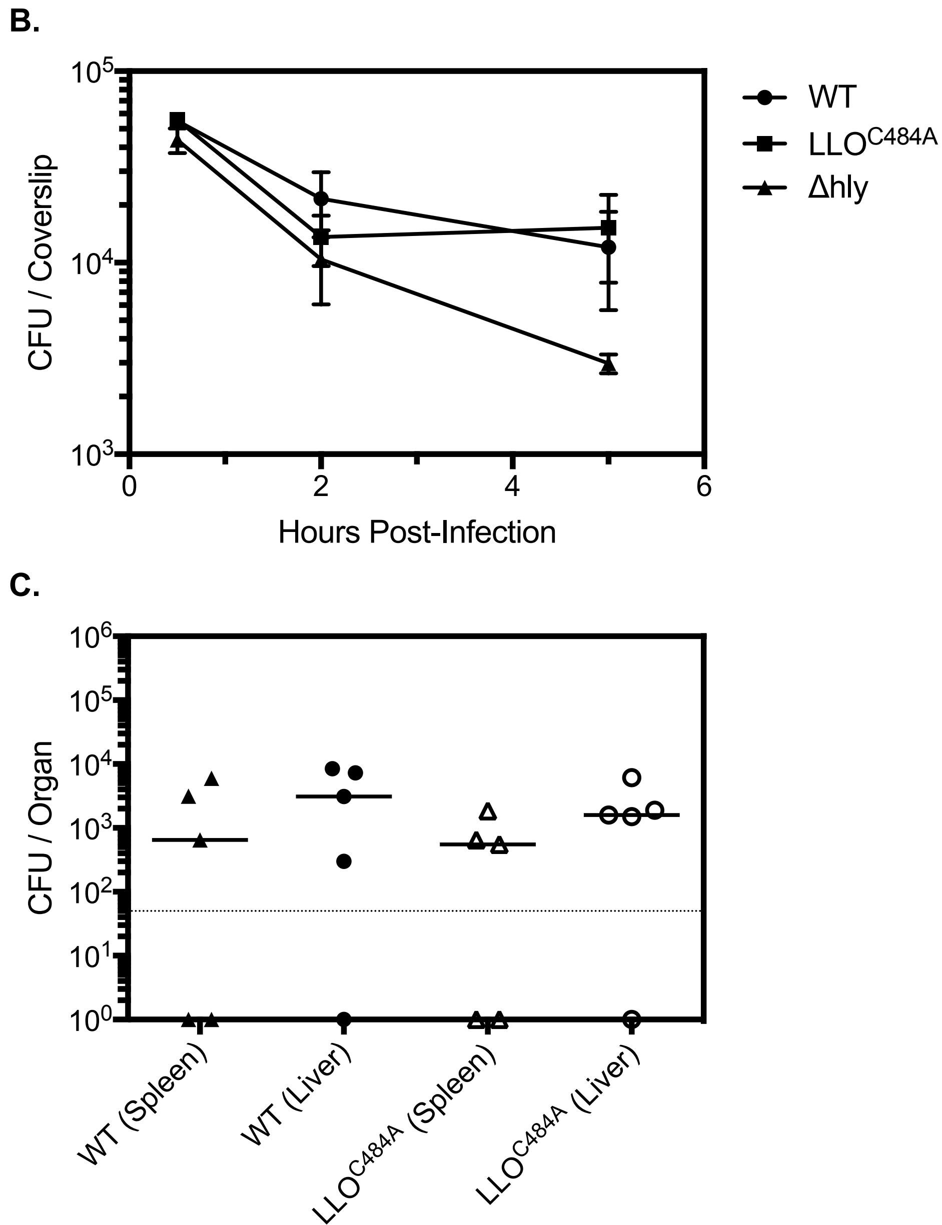


Figure S2. Glutathionylation of LLO is largely dispensable in alternative infection models. Several infection models were tested for their potential to accentuate the virulence defect of the LLO^{C484A} mutant. **(A)** *In vitro* plaque assays were performed to monitor cell-to-cell spread of *L.monocytogenes* through a monolayer of immobilized L2 fibroblasts. **(B)** Intracellular growth in naturally restrictive resident peritoneal macrophages was assessed as described in supplemental Materials and Methods. **(C)** An *in vivo* oral infection model using contaminated bread was performed as described in supplemental Materials and Methods. No statistically significant defects were observed compared to wild-type bacteria in these models.

Figure S3.

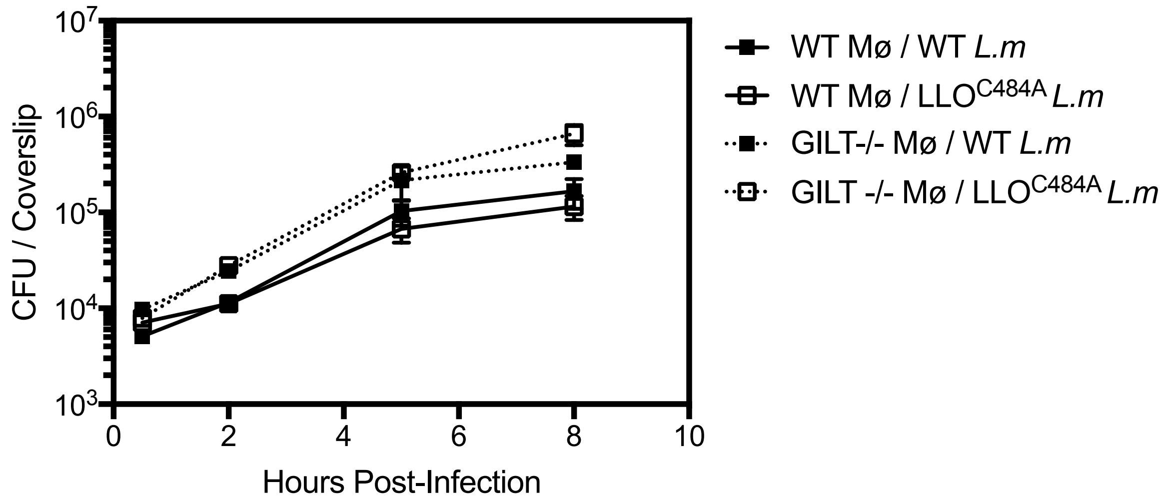


Figure S3. GILT is not required for efficient escape and replication of *L.monocytogenes* in bone-marrow derived macrophages.
Wild-type and LLO^{C484A} *L.monocytogenes* were used to infect either wild-type or *GILT*$^{-/-}$ bone-marrow derived macrophages *in vitro*. CFU per coverslip (~1.2 x 10^5 cells / coverslip) are reported at various time points after infection and representative of intracellular growth. Both strains of bacteria were able to efficiently replicate in *GILT*$^{-/-}$ BMMs.