Title

L-cysteine acquisition mechanisms in Listeria monocytogenes

Author

Berude, John C

Publication Date

2023

Peer reviewed|Thesis/dissertation

L-cysteine Acquisition Mechanisms in *Listeria monocytogenes*

By

John Christopher Berude

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Molecular and Cell Biology

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Daniel A. Portnoy, Chair
Professor Jeffery Cox
Assistant Professor Denis Titov
Associate Professor Kathleen Ryan

Fall 2023

Abstract

L-cysteine Acquisition Mechanisms in *Listeria monocytogenes*

by

John Christopher Berude

Doctor of Philosophy in Molecular and Cell Biology

University of California, Berkeley

Professor Daniel A. Portnoy, Chair

Listeria monocytogenes is a Gram-positive facultative intracellular pathogen that is the causative agent of listeriosis in humans and other mammals. *L. monocytogenes* leads a biphasic lifestyle, growing as a saprophyte in the environment and transitioning to an intracellular lifecycle upon ingestion by mammalian hosts. During infection, *L. monocytogenes* must transition its metabolism to adapt to the unique nutritional landscape of the host cytoplasm, as well as selectively induce expression of virulence genes that facilitate intracellular growth and cell-to-cell spread. *L. monocytogenes* is a genetically tractable, easily manipulated, and rapidly growing bacterium that is a model organism for studying bacterial pathogenesis and host cell biology alike.

Virulence gene expression in *L. monocytogenes* is controlled by the master virulence regulator PrfA, whose expression and activity are highly regulated at multiple levels. One of the primary regulators of PrfA activity is allosteric activation by the tripeptide glutathione (GSH), which is produced in *L. monocytogenes* by the bifunctional glutathione synthase GshF. GSH is an important redox-active molecule that maintains the cytoplasmic reducing environment, and the amino acid L-cysteine is the rate-limiting substrate for GSH synthesis and is also essential for bacterial growth. Unlike many bacteria, *L. monocytogenes* is believed to be auxotrophic for L-cysteine and must import exogenous cysteine to support growth and virulence. While much is known about L-cysteine acquisition mechanisms in *L. monocytogenes*, there are still many gaps in our knowledge. First, it is known that GSH is enriched in the host cytoplasm and represents an attractive source of GSH for PrfA activation and a rich source of cysteine *in vivo*. Previous work suggests that *L. monocytogenes* can utilize exogenous GSH for PrfA activation and growth, but no GSH importers or other GSH utilization mechanisms have been identified. Second, though it is known that *L. monocytogenes* lack the genes for sulfate assimilation and is unable to synthesize L-cysteine from sulfate, the bacteria contain a conserved CysE/CysK two-step L-cysteine biosynthetic pathway whose function in *L. monocytogenes* is unknown.

In this work, we identified the first known GSH importer in *L. monocytogenes* and established a role for the CysE/CysK pathway in supporting bacterial growth on limited inorganic sulfur sources. Through bioinformatic analysis of known GSH importers, we identified a homolog of the GsiABCD GSH importer in *E. coli* that is comprised of the *L. monocytogenes* Ctp ABC transporter complex and the OppDF ATPases of the Opp oligopeptide importer. Here, we demonstrated that the Ctp complex is a high-affinity GSH importer that imports both reduced GSH and its oxidized counterpart GSSG and is required for growth at physiologically relevant concentrations of these substrates in the absence of other L-cysteine sources. We observed that OppDF is required for GSH import in an Opp-independent manner, and these data support a model where Ctp and OppDF form a complex for GSH import that supports *L. monocytogenes* growth and pathogenesis. Additionally, we demonstrated that *L. monocytogenes* utilizes the inorganic sulfur sources thiosulfate and H_2S to support growth in a CysK-dependent manner in the absence of other L-cysteine sources.

The results of this work open new avenues of inquiry into mechanisms of L-cysteine acquisition in *L. monocytogenes* and the roles of GSH and inorganic sulfur sources in pathogenesis. First, our observations suggest the role of alternative GSH utilization mechanisms that may be relevant *in vivo* and further work is required to identify these mechanisms and their contribution to pathogenesis. Second, even though thiosulfate and H_2S are present at uniquely high concentrations in the mammalian intestine, it remains unclear if utilization of these inorganic sulfur sources is important for *L. monocytogenes* colonization of the host gut. Additionally, it is unclear if the ability to produce L-cysteine is the primary function of the CysE/CysK biosynthetic pathway in specific environmental and host niches, or if CysE and CysK are independently essential for other metabolic processes and L-cysteine production is an adventitious outcome. And finally, these findings suggest a pathoadaptive role for partial L-cysteine auxotrophy in *L. monocytogenes*, where locally high GSH or thiosulfate/H_2S concentrations may signal arrival to distinct host niches.

Dedication

To everyone who has shared in the making of my life's story,
And to all the wonderful people who are yet to be a part of it.

I am so grateful for you all.

Thank you.

Table of Contents

List of Figures

Chapter 1

Chapter 2

List of Tables

Chapter 2

Acknowledgements

When asked about how to decide authorship for scientific publications, my advisor Dan shared his golden rule with me:

"Would this paper exist if that person had never been born?"

I quite liked that. It spoke to me.

As I reflect on my time at Berkeley and the sum of my life's journey thus far, it is clear to me that my story has been shaped by many hands. While I may be the first author of my life's story, there are many more authors who have contributed and deserve to be recognized as well.

There are those who have always been a part of my story and who will continue to be so until our last breathes, those whose love and support are as tenacious as the granite of my beloved Sierra Nevada mountains. These people are *family*.

There are those who have come and gone, and those that are here now but will go, all having left an indelible mark nonetheless. These people are *friends*, *teachers*, *mentors*, *colleagues*, *acquaintances*, *partners*, *coworkers*, and sometimes even *family*. Whoever they are and whatever role they played, they were a part of my journey, and I think they deserve some credit too.

And then there are those who just showed up one day and refused to leave. People who burst into my storyline unannounced and now they're so invested and interested that they're not leaving until the credits roll and the screen goes black. They just won't allow it any other way. These are the *true friends*, and as such, they are *family*.

Whoever you are on this list of authors in my life's story, know that I am grateful for you and the impact you have had, and continue to have, on my story. I am grateful for all the love, kindness, and support, and I can only hope that I had a positive impact on your journey as well. So, without further ado, let's get into the specifics and recognize some of these important people. You all have been so generous with me, and I think it's time I returned the favor.

The Portnoy Lab: This has been my landing spot for the past five years, my work home if you will, and I am overjoyed that it has been filled with such wonderful people. **Victoria**, **Andrea**, **Bere**, **Mariya**, **Ted**, **Preethi**, and old lab members **Ying**, **Raf**, **Alex**, **Brittney**, **Sam**, and **Bret**: thank you for not only being fantastic colleagues and incredible scientists, but also for being such warm and loving friends as well. Through our time together, we have shared far more than just science, and it has been a joy to work alongside you all. I am incredibly grateful for you all.

Dan: Well Dan, it's been a journey. I know that I haven't always made the best impression, but I'm so grateful that you were able to see potential in me and didn't once

give up on me, even though you could have many times. I spent much of my time in graduate school as a disillusioned young scientist, and that prompted much hardship and tension in the years to follow. Thank you for bringing me into the lab and showing me how real science is done, and for coaching me through the process of making and sharing discoveries. Thank you for not treating me like just some cog in the machine and for seeing me as a fellow human being. I chose your lab for a lot of reasons, but the most important of which was that you struck me as a human being and incredible mentor. I don't think I would have survived anywhere else. I'm so grateful for your mentorship and continued support, and I am proud to have trained with you. Thank you, Dan, for everything.

My Mentors: There are simply so many people that deserve credit here, so I'll try to stick to the most pertinent ones.

First and foremost, I want to thank my professors **Doug Latch** and **Joe Langenhan** from my time at Seattle University. You both were instrumental in exposing me to science and scientific research, and I am overjoyed that we have grown into lifelong friends as well and can share in such joys as music, food, libations, and mountain climbing. **Michelle DuBois** is another mentor and Seattle U professor who changed the course of my life, introducing me to the world of molecular biology and fostering my deep interest in it, as well as recognizing my potential and advocating ferociously for it. And all while trading the stiffness of the collegiate academic setting for the warmth of human experience. Thank you.

Thank you also to **Dan Gottschling**, who brought me into his lab as a technician at the Fred Hutch. Thank you for bringing me into the lab and introducing me to the world of real-life research and pushing me think like a scientist. Graduate school would have been a lot harder without your mentorship, and I am eternally grateful for it.

My Family: If there's one group that has been essential to my storyline, it's been my family. **Mom**, **Dad**, and **Ian**: thank you, for everything. Thank you for believing in me and pushing me to be my best self, thank you for suffering me as I learned and grew, thank you for sharing in my successes and for making me a part of yours, and most importantly, thank you for just being there for me. I wouldn't be where I am now without you all, and I am eternally grateful for you all. **Grandpa Jack** also deserves great thanks for pushing a young and often obstinate me to think critically and creatively, and for imbuing me with a sense of rebelliousness, free thinking, and healthy disobedience. You helped me loosen up and taught me that some rules can and should be broken. We broke some rules, didn't exactly get away with it, and we're all the better for it now. I might be breaking a rule by thanking so many people and writing such a long Acknowledgements section, but it feels like the right thing to do. You're partially responsible, and I think we might just get away with it. Thank you. And last but certainly not least, thank you to **Marty and Paul** and the extended **Berude and Gelinas families** for always being there for me, lifting me up, and for continually teaching me what it means to be a kind and loving human in this world.

The California Family: I simply wouldn't have made it to where I am now without you guys. I am incredibly grateful to **Nate**, **Mai**, and **Big Pig**, who were instrumental in helping me transition into graduate school life in California and have always been there for me throughout my journey. Thank you for all the love, support, and friendship. I am also grateful for all the wonderful people in my incredible California mountain community: the Lassen crew (**Joe**, **Billy**, **Greg**, **Erik**, **Jake**, **Mark**, **Russ**, **Phil**), **Sidney**, **Bryson**, and **Alex**. You all welcomed me in and made this place feel like home, and it's been a joy to learn from you all and grow alongside you through the years. You all are a special bunch, and it's a pleasure to be a part of each other's lives.

The Washington Family: Back to where it all began! I've been so lucky to have such an incredibly rich community of family, friends, and friends who are now family that have stuck with me for a long time and have seen me through some serious life transitions and growth. You all made it so hard to leave, but now I know that I've never really been gone. I am so grateful for my dudes **Jordan**, **Kirk**, **Kyle**, **Pat**, and **Nick**. Through our fast friendship, wild mountain adventures, and endless shenanigans, you all quickly grew to be family, and as such, it's been a joy to welcome **Lauren**, **Lindsey**, **Katie**, and **Kate** in as well. It's been a pleasure sharing in life with you all, and I can't wait to keep sharing it for a whole lot longer. I love you boys.

This section also wouldn't be complete without thanking **Katie Deets**, without whom I would have never ended up at Berkeley, and perhaps wouldn't have pursued graduate school at all. You led by example, worked hard, and followed your dreams, and I picked up the torch and followed in those footsteps. I owe you more thanks than I think either of us know.

Sofia: And then there is Sofia. I don't even know where to start. If I had to name a single event that changed my life forever, it would be meeting you. Because of you, I know more about life and love and family and what it genuinely means to be human than I ever thought possible. Because of you, I not only survived graduate school, but I have emerged from these years as a fundamentally better human being. Thank you so much for being my best friend, confidante, adventure buddy, and support system throughout these grad school years. Thank you for your patience and love, even when I was incapable of doing the same for you. Thank you for giving so much of yourself, for seeing my potential and pushing me to be a better person. Thank you for standing up for what is right, even when doing so was so incredibly hard. Thank you for showing me the fundamental value of seeking peace, joy, happiness, contentment, and balance in life. Thank you for bringing me into your wonderful family and sharing them with me. Through my time with you and the entire **D'Aniello family**, I have learned so much about what it truly means to be family and love unconditionally, and I am so grateful to have shared the time together that we have. You are a special bunch, and I am beyond grateful for every one of you. I am a better person for you all. There's so much more that I could say here, Sofia, but I think you know it all already. Thank you, Sofia. I am so, so grateful for you.

John Christopher Berude
Curriculum Vitae

EDUCATION

University of California, Berkeley Berkeley, CA
PhD, Molecular and Cell Biology December 2023
L-cysteine acquisition mechanisms in *Listeria monocytogenes*
Daniel A. Portnoy (Chair), Jeffery Cox, Denis Titov, Kathleen Ryan

Seattle University Seattle, WA
B.S. Cell and Molecular Biology, *summa cum laude*, Core Honors Program June 2013

RESEARCH EXPERIENCE

University of California, Berkeley Berkeley, CA
Doctoral Candidate, Professor Daniel A. Portnoy August 2017 – present

- Discovered a glutathione-specific importer in the bacteria pathogen *Listeria monocytogenes*
- Elucidated the functionality of the CysE/CysK L-cysteine biosynthetic pathway in *Listeria monocytogenes*
- Studied mechanisms of L-cysteine acquisition in the bacteria pathogen *Listeria monocytogenes*, using mammalian tissue culture and mouse models

Fred Hutchinson Cancer Research Center Seattle, WA
Research Technician I/II, Dr. Daniel E. Gottschling June 2014 – December 2016

- Contributed to studies on replicative aging in the budding yeast *Saccharomyces cerevisiae*, focus on the genetic basis of lipid droplet accumulation during aging

Seattle University Seattle, WA
Research Assistant, Professor Douglas E. Latch June 2010 – June 2013

- Developed extraction, isolation, and analytical methods for detection of pyrethroid pesticides in aquatic environments

AWARDS & SCHOLARSHIPS

National Science Foundation – Graduate Research Fellowship (2017)
Seattle University – John S. Ju Award (2013)
Seattle University – Environmental Chemistry Award (2013)
Seattle University – Outstanding Organic Chemistry Student Award (2011)
Seattle University – Bannan Scholarship (2011)
Seattle University – President's Scholarship (2009)

PUBLICATIONS

Berude JC, Kennouche P, Reniere ML, Portnoy DA. *Listeria monocytogenes* utilizes glutathione and limited inorganic sulfur compounds as a source of essential L-cysteine. *Biorxiv* 2023 (*Infection & Immunity*, *in review*)

Rodriguez R, Cambell-Kruger N, Gonzalez Camba J, **Berude J**, Fetterman R, Stanley S. MarR-Dependent Transcriptional Regulation of *mmpSL5* Induces Ethionamide Resistance in Mycobacterium abscessus. *Antimicrobial Agents and Chemotherapy*, 2023

Anaya-Sanchez A, Feng Y, **Berude JC**, Portnoy DA. Detoxification of methylglyoxal by the glyoxalase system is required for glutathione availability and virulence activation in Listeria monocytogenes. PLoS Pathogens, 2021

Felcyn JR, Davis JC, Tran LH, **Berude JC**, Latch DE. Aquatic photochemistry of isoflavone phytoestrogens: degradation kinetics and pathways. *Environmental Science & Technology*, 2012

TEACHING & MENTORING EXPERIENCE

Head Instructor, QB3 Undergraduate Lab Fundamentals Bootcamp (2023)
Instructor, QB3 Undergraduate Lab Fundamentals Bootcamp (2022, 2021)
Graduate Student Instructor, Bacterial Pathogenesis Discussion Section (SPH262, Spring 2020)
Graduate Student Instructor, Bacterial Pathogenesis Lecture (MCB103, Spring 2020)
Graduate Student Instructor, Immunology Laboratory (MCB150L, Fall 2018)

Graduate Research Rotation Mentor, Portnoy Lab (Fall 2018, Winter 2019, Winter 2020)
Master's Research Mentor, Portnoy Lab (Fall 2018 – Spring 2019)
Undergraduate Research Mentor, Portnoy Lab (Winter 2019 – Spring 2022)

SCIENCE COMMUNICATION

Lead Editor, QB3 Undergraduate Lab Fundamentals Bootcamp Manual (2021-2022)
Participant and Author, QB3-Berkeley's Science Writing for the Public (2021)

Chapter 1

An introduction to *Listeria monocytogenes*

1.1 *Listeria monocytogenes*, from saprophyte to intracellular pathogen

Listeria monocytogenes is a Gram-positive facultative intracellular pathogen and is the causative agent of listeriosis in humans and other mammals (1, 2). *L. monocytogenes* is typically a saprophytic bacterium that grows ubiquitously in the soil, and most cases of listeriosis result from ingestion of contaminated food products such as processed meats, dairy products, and fruits and vegetables. Infection with *L. monocytogenes* predominantly causes systemic disease in immunocompromised individuals, such as pregnant or elderly individuals (1-4). Once ingested, extracellular bacteria transit through the digestive track and invade host intestinal epithelial cells, where they transition to an intracellular lifecycle (1, 5). During this phase of growth, invading bacteria replicate in the host cell cytosol and spread intracellularly from cell-to-cell (1).

In addition to *L. monocytogenes'* relevance to human health, *L. monocytogenes* is a genetically tractable, easily manipulated, and rapidly growing bacterium, which makes it a useful model organism for studying bacterial pathogenesis, host-pathogen interactions, and host cell biology (5, 6). Because many bacterial pathogens exploit conserved host pathways, work in *L. monocytogenes* has expanded our understanding of other clinically relevant pathogens, such as *Salmonellae, Chlamydiae*, and *Mycobacterium tuberculosis* (7, 8). Further, insights in *L. monocytogenes* have contributed to discoveries in host cell biology and enabled our broader understanding of immunology and cancer biology (8, 9).

1.2 Virulence genes and the intracellular lifecycle

During infection, *L. monocytogenes* must adapt from its saprophytic lifecycle to an intracellular one to successfully colonize the host intracellular niche. Thus, the two key elements of *L. monocytogenes* pathogenesis are the bacteria's ability to transition their metabolism to utilize unique nutrients that are available in the host cytosol and the expression of virulence factors that facilitate host cell invasion, intracellular growth, and cell-to-cell spread.

L. monocytogenes possesses several key virulence factors that are critical for the intracellular phase of the bacterial lifecycle (6, 10, Figure 1.1). First, *L. monocytogenes* expresses a set of internalins (InlA, InlB) that promote bacterial adherence to host epithelial cells and hepatocytes and facilitate invasion (11). Next, bacteria secrete a cholesterol-dependent cytolysin (LLO) and two phospholipases (PLCs) that perforate and degrade the membranes of the phagolysome, enabling *L. monocytogenes* to escape into the host cytosol (12-14). Once in the cytosol, bacteria express a hexose phosphate transporter (Hpt) that enables the uptake of phosphorylated glucose and other hexoses, which are one of the major carbon sources available in host cytosol (15). Cytosolic bacteria also express ActA, which promotes polymerization of host actin and allows bacteria to spread cell-to-cell via the formation of actin "rocket tails" and evade degradation by host autophagy machinery (16-18).

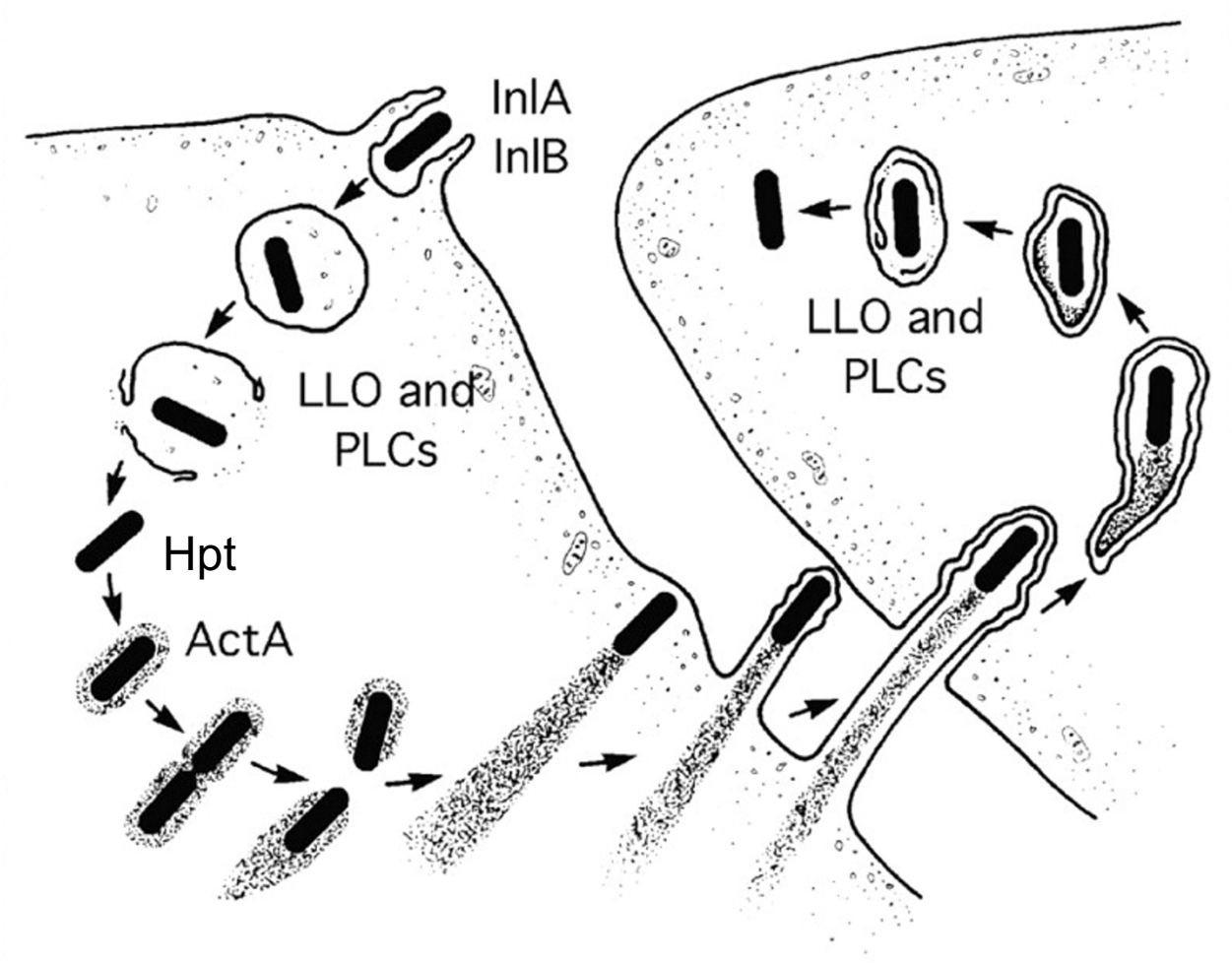


Figure 1.1. The intracellular lifecycle of *Listeria monocytogenes*. *L. monocytogenes* utilizes multiple virulence factors that facilitate host cell invasion (InlA, InlB), escape from the phagolysosome (LLO, PLCs), grow intracellularly (Hpt), and spread cell-to-cell (ActA). Adapted from (6).

1.3 PrfA, the master virulence regulator

The master virulence regulator in *L. monocytogenes* is PrfA, which selectively turns on expression of virulence genes during infection and is required for intracellular growth, as bacteria lacking the *prfA* gene are over 100,000-fold less virulent than wild-type (WT) bacteria in a murine infection model (10, 19-22). On the flipside, proper spatiotemporal control of PrfA activity is critical for bacterial fitness, as constitutive activation of PrfA in extracellular bacteria reduces fitness (23, 24). PrfA regulates expression of the virulence genes in its regulon by binding to the PrfA-box upstream of the target genes and recruiting RNA polymerase to promote transcription (22, 25, Figure 1.2).

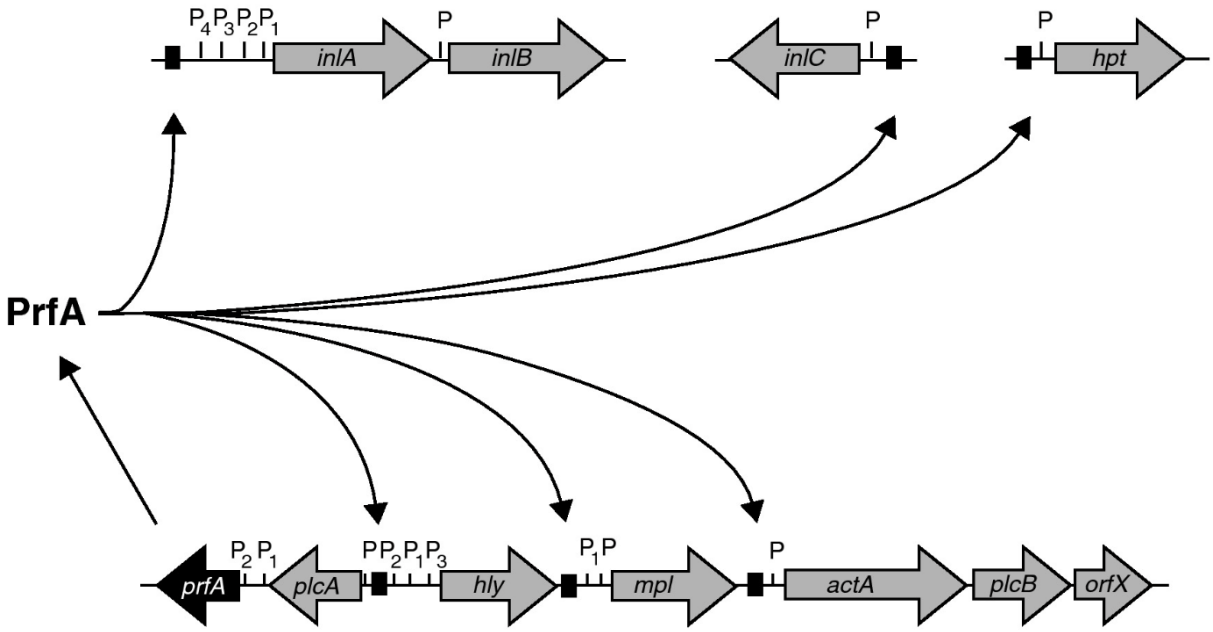


Figure 1.2. PrfA-mediated regulation of virulence genes in *L. monocytogenes*. The PrfA protein binds to PrfA-boxes upstream of virulence genes to promote transcription. Adapted from (22).

To ensure proper expression of virulence genes during infection, PrfA itself is subject to multiple levels of regulation. Transcription of the *prfA* gene is tightly regulated and intricately controlled, with *prfA* expression negatively regulated by the PrfA protein and positively regulated diverse stress sensors, such as sigmaB and CodY (10). PrfA protein levels are also controlled post-transcriptionally by an RNA thermosensor in the 5' UTR of the *prfA* mRNA that prevents translation of the PrfA protein at lower temperatures (<30^{o}C) and permits translation at host physiological temperatures (~37^{o}C) (10, 26-28).

While PrfA controls the expression of virulence genes in its regulon, we have long understood that not all genes are regulated equally. We now know that there are "early" genes and "late" genes in the PrfA regulon whose transcription depends on both the strength of the PrfA-box and the activation state of PrfA itself. Early genes include genes required for vacuolar escape, such as *hly* (which encodes LLO) and *plcA* (which also supports PrfA production via transcription of a bicistronic *plcA-prfA* transcript), while the rest are considered late genes that are required for intracellular growth and cell-to-cell spread (29, 30). The principal difference between transcription of these genes is that early genes have a strong PrfA-box that allows for binding of inactive PrfA while late genes feature a weaker PrfA-box that requires allosteric activation of PrfA to facilitates transcription (25, 30, 31). While allosteric activation of PrfA has been known to be required for transcription of late genes for many years, it was not until relatively recently that the identity of this allosteric activator was uncovered.

1.4 Glutathione as an allosteric activator of PrfA

PrfA is a member of the Crp/Fnr family of transcriptional regulators that are canonically regulated by small molecules at the post-translational level. Recent work by Reniere et al and others identified this allosteric activator to be the low-molecular weight thiol glutathione (GSH) (32, 33). GSH is a tripeptide that is comprised of glycine, glutamine, and cysteine. Upon binding to PrfA, GSH induces a conformational change that enables PrfA binding to its cognate DNA sequences and results in the expression of virulence genes (33).

GSH is an important molecule in many biological systems, where its canonical role is to detoxify reactive oxygen and electrophilic species and maintain a cytoplasmic reducing environment (34, 35). Because of its importance in cell biology, GSH is produced by all eukaryotic cells and Gram-negative bacteria, and typically is present at high concentrations (~1-10mM) in the cytoplasm of these cells (34, 36). Gram-positive bacteria, on the other hand, typically do not produce GSH and instead produce and use alternative low molecular weight thiols, such as bacillithiol and mycothiol, to combat reactive oxygen and electrophilic species and to maintain a cytoplasmic reducing environment (37, 38). Thus, *L. monocytogenes* is unusual in that it is a Gram-positive bacterium that utilizes GSH as a cytosolic redox buffer and cofactor for pathogenesis and can produce its own GSH via the bifunctional glutathione synthase, GshF (39). While it has been previously suggested that *L. monocytogenes* can import exogenous GSH, bacterial production of GSH is essential for full PrfA activation, and deletion of the *gshF* gene results in a >80% reduction in ActA levels and a 100-fold decrease in virulence in a murine IV infection model (32, 40). It was not until very recently that WT *L. monocytogenes* was shown to be able utilize GSH as a sole L-cysteine source (41).

1.5 L-cysteine in *L. monocytogenes* growth and pathogenesis

L-cysteine is an essential nutrient for *L. monocytogenes* growth and pathogenesis. L-cysteine is one of two sulfur-containing amino acids and the sulfhydryl moiety enables it to readily engage in redox chemistry and serve as a substrate for iron-sulfur cluster biogenesis. Thus, L-cysteine is vital for synthesis of redox-active proteins. L-cysteine also forms the core component of many redox buffering molecules like GSH and is the rate-limiting component for synthesis of these molecules (34, 42, 43). As such, L-cysteine availability is a key mediator of PrfA activation and virulence gene expression and is an essential nutrient for virulence and growth alike.

Despite the importance of L-cysteine in *L. monocytogenes*, it has been observed for over three decades that *L. monocytogenes* is auxotrophic for L-cysteine. While most bacteria make their own L-cysteine de novo from sulfur-containing substrates, *L. monocytogenes is* unable to make its own L-cysteine and requires exogenous cysteine sources to grow (44-46). These observations suggest that *L. monocytogenes* uses local L-cysteine availability to titrate GSH production and PrfA activation. Thus, to better understand virulence gene activation and pathogenesis in *L. monocytogenes*, it is necessary to understand the mechanisms that underlie L-cysteine acquisition.

1.6 Sources of nutritional L-cysteine and acquisition mechanisms

1.6.1 L-cysteine

Reduced L-cysteine is the most logical source of nutritional cysteine as the raw building block for protein synthesis and GSH production. However, reduced L-cysteine is not thought to be a major nutritional source of cysteine in aerobic environments, which represent the majority of environments in which *L. monocytogenes* is found. Reduced L-cysteine is unstable in oxic environments due to the high reactivity of the free thiol group, which can engage in Fenton chemistry and damage cellular structures by the production of reactive oxygen species (47, 48). This unique and damaging chemistry of free reduced L-cysteine requires cells to maintain very low intracellular cysteine concentrations, and any incoming L-cysteine must either be quickly consumed, degraded, or exported. It is not surprising then that there are very few known specific importers of reduced L-cysteine, and that the majority of reduced L-cysteine uptake is thought to be through non-specific polar amino acid importers (48-50).

There are a couple notable exceptions to this lack of specific L-cysteine importers. Recently, an L-cysteine-specific importer and desulfidase, *cyuPA*, were discovered in *E. coli* that facilitate uptake and degradation of reduced L-cysteine in anaerobic environments (50). Additionally, it has been proposed that reduced L-cysteine is imported by the Ctp complex in *L. monocytogenes*. The Ctp complex is an ABC transporter that consists of a substrate-binding domain (CtaP) and two transmembrane permeases (CtpP1 and CtpP2) but lacks dedicated ATPases to power import (51, 52). However, the complex was originally annotated as an oligopeptide importer and the current evidence to suggest that the Ctp complex is a dedicated, reduced L-cysteine-specific importer is not especially convincing, so it remains unclear if reduced L-cysteine is the primary or adventitious substrate for the Ctp complex.

1.6.2 L-cystine

L-cystine is the oxidized form of L-cysteine, consisting of two L-cysteine residues joined by a disulfide bond, and is one of the primary sources of nutritional cysteine in aerobic environments. Because L-cystine is so prevalent in oxic environments, most bacteria have evolved dedicated L-cystine import mechanisms, with most bacteria harboring both an L-cystine-specific ATP-driven ABC transporter and an ion-driven transporter (53). Because imported L-cystine is rapidly reduced to potentially damaging L-cysteine in the reducing environment of the cytoplasm, the expression of these importers is highly regulated (53, 54).

While no ion-driven L-cystine importers have been identified in *L. monocytogenes*, the bacteria do contain an intact ABC transporter, TcyKLMN. Recent work has shown that the TcyKLMN importer is required for growth on L-cystine *in vitro*, supports GSH production and PrfA activation, and that expression of TcyKLMN is regulated by the master regulator of cysteine metabolism, CymR, and its regulatory partner CysK (41).

1.6.3 Inorganic sulfur

Inorganic sulfur sources are one of the two principal substrates that bacteria use in the *de novo* production of L-cysteine. In this pathway, inorganic sulfur sources such as sulfate are imported and converted to free sulfide by enzymes of the sulfate assimilation pathway, and this free sulfide is then condensed with L-serine and acetyl-CoA to form L-cysteine via the canonical CysE/CysK two-step biosynthetic pathway (55). Exogenous sulfate is sufficient to support growth in the absence of other L-cysteine sources in bacteria that are competent for these two pathways.

Curiously, *L. monocytogenes* does not contain a functional sulfate assimilation pathway and is unable to grow on sulfate in the absence of other L-cysteine sources. *L. monocytogenes* has also been reported to be unable to use the alternative inorganic sulfur source thiosulfate (45, 46, 56). This is a curious observation, as *L. monocytogenes* harbors an intact CysE/CysK two-step biosynthetic pathway and it is known that thiosulfate can often be used as an alternative sulfide source for L-cysteine production, raising questions about the function of the CysE/CysK pathway in *L. monocytogenes*.

1.6.4 L-methionine

The sulfur-containing amino acid L-methionine is the other major substrate for *de novo* L-cysteine production in bacteria, and bacteria are known to use the reverse transsulfuration pathway to convert L-methionine to L-cysteine (57). However, some *L. monocytogenes* strains, including 10403S, are unable to grow on L-methionine as a sole sulfur source, suggesting that they either lack the necessary genes or contain defective genes (46).

1.6.5 Oligopeptides

Oligopeptides are another important source of nutritional cysteine in bacteria and are the richest source of amino acids in both the environment and the host cytosol (58, 59). *L. monocytogenes* relies on the Opp ABC importer complex to acquire nutritive oligopeptides, and it was recently shown that import of cysteine-containing oligopeptides was sufficient to support PrfA activation (60, 61). Host proteins have been reported to contain ~2-4x more cysteine that plant or microbial proteins, suggesting that the peptide signature of the habitat might play a role in PrfA activation and pathogenesis (61, 62).

1.6.6 Glutathione and oxidized glutathione

GSH and its oxidized counterpart, GSSG, are rich sources of L-cysteine that are commonly utilized by bacteria and are present at uniquely high concentrations in the host cytoplasm (34). Because GSH is one of the most abundant sources of cysteine in the host environment, numerous pathogenic bacteria, such as *E. coli*, *F. tularensis*, *S. mutans*, *S. pneumoniae*, *S. pyogenes*, and *S. aureus*, have been reported to utilize host GSH through GSH-specific importers and/or extracellular degradation by secreted γ-

glutamyl transpeptidases (63-69). Despite these observations in other pathogenic bacteria and the importance of GSH in PrfA activation and virulence, no GSH-specific acquisition mechanisms have been identified in *L. monocytogenes*, though previous work has suggested that *L. monocytogenes* is able to utilize host derived GSH for PrfA activation and GSH was recently found to support *L. monocytogenes* growth in the absence of other L-cysteine sources (32, 40, 41).

1.7 Outstanding questions

Considering the importance of L-cysteine *L. monocytogenes* growth and pathogenesis alongside our current understanding of L-cysteine acquisition mechanisms, we were left with two major outstanding questions. First and foremost, we wanted to know if *L. monocytogenes* can utilize GSH and GSSG for growth, and if so, what the mechanisms that underlie utilization are. Second, we wanted to know what the function of the intact CysE/CysK two-step L-cysteine biosynthetic pathway in *L. monocytogenes* and if it could support growth on limited inorganic sulfur sources.

Chapter 2

Listeria monocytogenes utilizes glutathione and limited inorganic sulfur compounds as sources of essential L-cysteine

Sections of this chapter were published in:

Berude JC, Kennouche P, Reniere ML, Portnoy DA. *Listeria monocytogenes* utilizes glutathione and limited inorganic sulfur compounds as a source of essential L-cysteine. *Biorxiv* 2023 (*Infection & Immunity, in review*)

2.1 Summary of results

The amino acid L-cysteine is essential for growth in all forms of life, and it must either be synthesized or taken up from the environment. While most bacteria can synthesize L-cysteine directly, it has been observed that *L. monocytogenes* is unable to produce its own L-cysteine and must rely on uptake of nutritive L-cysteine sources for growth and pathogenesis. While the acquisition mechanisms for some nutritive L-cysteine sources have been identified in *L. monocytogenes*, we lack an understanding of if and how the bacteria utilize the prominent L-cysteine source glutathione (GSH) and its oxidized counterpart GSSG, as well as the function of the intact CysE/CysK L-cysteine biosynthetic pathway in *L. monocytogenes*. Here, we found that *L. monocytogenes* utilizes diverse L-cysteine sources for growth, including GSH, GSSG, and the inorganic sulfur sources H_2S and thiosulfate. We demonstrate that the Ctp complex and the OppDF ATPases are required for growth on GSH and GSSG *in vitro*, and that the Ctp/OppDF complex is a high-affinity importer of GSH and GSSG that is required for full virulence in a murine IV infection model. Additionally, we show that *L. monocytogenes* utilizes the inorganic sulfur sources H_2S and thiosulfate for growth, and that growth on these substrates is dependent on CysK and the canonical two-step biosynthetic pathway. Together, these findings suggest a pathoadaptive role for the partial cysteine auxotrophy in *L. monocytogenes*, where locally high GSH/GSSG or thiosulfate/H_2S concentrations may signal arrival to distinct host niches.

2.2 Introduction

L. monocytogenes is a Gram-positive food-borne pathogen and the causative agent of listeriosis in humans and other mammals (1, 2). This saprophytic bacterium grows ubiquitously in soil and typically encounters mammalian hosts through contaminated food products (3). *L. monocytogenes* is a facultative intracellular pathogen, and bacteria invade host epithelial cells in the gut upon ingestion. Invading bacteria traverse the intestinal epithelium and can cause systemic disease in immunocompromised individuals, such as pregnant or elderly individuals (4). During infection, *L. monocytogenes* must adapt from a saprophytic lifestyle to an intracellular one, shifting its metabolism to better colonize the host intracellular niche. Thus, two factors are central to *L. monocytogenes* pathogenicity: the ability to utilize host nutrients for intracellular growth and the expression of virulence factors to facilitate pathogenesis.

The master virulence regulator PrfA is essential for *L. monocytogenes* pathogenesis, as it controls expression of virulence factors necessary for intracellular growth and cell-to-cell spread (22). PrfA is a member of the CRP family of transcriptional regulators that are canonically regulated by allosteric binding of small molecules. Work by Reniere et al and others found that PrfA activity is allosterically controlled by the binding of the low molecular weight thiol glutathione (GSH) (32, 33). GSH is a tripeptide, comprised of glycine, glutamine, and cysteine, and the canonical roles of GSH in cells is to maintain a cytoplasmic reducing environment and detoxify reactive oxygen and electrophilic species (34, 35). Because of its importance in cellular physiology, GSH is produced by all eukaryotic cells and Gram-negative bacteria. Notably, GSH is abundant in the cytoplasm of mammalian host cells where it is present at 1-3mM in most cells and

up to 10mM in hepatocytes (34, 36). While most Gram-positive bacteria produce alternative low molecular weight thiols, *L. monocytogenes* is unusual in that it is one the few Gram-positive species that utilizes GSH as a cytosolic redox buffer and can produce GSH through the bifunctional glutathione synthase, GshF. Bacterial GSH production is essential for full PrfA activation, though previous work has implied that *L. monocytogenes* can also utilize exogenous GSH for PrfA activation (32, 40).

The amino acid L-cysteine is the rate-limiting component for GSH synthesis and is essential for bacterial growth, where it serves as an amino acid for protein synthesis, an essential component of cytosolic redox buffering systems, and a source of inorganic sulfur for iron-sulfur cluster biogenesis (34). Common sources of nutritional cysteine include the oxidized cysteine species L-cystine, cysteine-containing peptides, GSH and its oxidized counterpart GSSG, L-methionine, and inorganic sulfur sources like sulfate. To obtain L-cysteine from the environment, bacteria have evolved numerous mechanisms for cysteine acquisition and biosynthesis. In *L. monocytogenes*, L-cystine is imported via the ABC transporter TcyKLMN, which is regulated by the master regulator of the intracellular cysteine pool, CymR, and its regulatory partner, CysK (41). Cysteine-containing peptides are imported by the ABC oligopeptide transporter OppABCDF and the peptide signature of the habitat is speculated to be important for intracellular growth and proper spatiotemporal control of PrfA activation in the cytoplasm of host cells (61).

Reduced L-cysteine is not thought to be a major source of nutritional cysteine in oxic environments because of its high reactivity in aerobic environments and ability to engage in damaging Fenton chemistry (47, 48). Because of this chemistry, intracellular cysteine concentrations are maintained at very low levels and there are few known specific transporters for reduced L-cysteine, with most uptake ascribed to non-specific import via polar amino acid importers (48-50). In *L. monocytogenes*, reduced L-cysteine has been proposed to be imported by the Ctp ABC transporter, which is comprised of the substrate-binding protein CtaP and the permeases CtpP1 and CtpP2 (51, 52). Considering that the Ctp complex is annotated as an oligopeptide import system, it remains unclear if L-cysteine is the primary substrate for this complex or if import is adventitious.

In addition to importing nutritive cysteine-containing substrates, bacteria can also synthesize L-cysteine from inorganic sulfur sources and L-methionine. Inorganic sulfur sources such as sulfate are commonly imported and converted to H_2S via the sulfate assimilation pathway, and H_2S and thiosulfate serve as the primary sulfur sources for L-cysteine biosynthesis via the canonical CysK-dependent two-step biosynthetic pathway (55). While *L. monocytogenes* has an intact L-cysteine biosynthetic pathway, it lacks the genes for sulfate assimilation and is unable to grow on sulfate and has not been observed to grow on thiosulfate (45, 46, 56). L-methionine is the second of the two sulfur-containing amino acids, and it is commonly converted to L-cysteine via the transsulfuration pathway. However, some strains of *L. monocytogenes* lack the genes for transsulfuration and are not able to grow on L-methionine as a sole sulfur source, including 10403S (46).

Despite the above observations, nothing is known about the mechanisms for exogenous GSH import and utilization or the function of the intact L-cysteine biosynthetic pathway in *L. monocytogenes*. GSH and GSSG are the most abundant

sources of L-cysteine in the host cytosol, which is the preferred host niche for *L. monocytogenes*, and other pathogenic bacteria such as *E.* coli, *F. tularensis*, and *S. mutans* are known to utilize host GSH for growth and virulence by either importing these molecules directly or by degrading them via the action of secreted γ-glutamyl transpeptidases (63-65, 67). Previous work has suggested that *L. monocytogenes* is able to import GSH for PrfA activation, as treatment with exogenous GSH is sufficient to activate PrfA in a *gshF* mutant (40). These observations suggest that the locally high GSH concentrations in the host cytosol could be a cue for bacterial arrival to the intracellular niche. Similarly, H_2S and thiosulfate are present at relatively high concentrations in the lumen of the intestine (1-3mM) and could similarly serve as a cue for bacterial arrival to the host environment (43, 70, 71).

2.3 Results

2.3.1 *L. monocytogenes* utilizes diverse sulfur sources for growth *in vitro*, including GSH, GSSG, and thiosulfate

To test the ability of WT *L. monocytogenes* to grow on different exogenous L-cysteine sources, we performed broth growth assays in chemically defined synthetic media (LSM) containing all amino acids except L-cysteine and supplemented with either L-cystine or another exogenous L-cysteine source (Figure 2.1). We observed normal growth of WT bacteria in LSM supplemented with either 0.5mM L-cystine or 1mM L-cysteine. Bacteria grown in LSM lacking all L-cysteine sources (LSM-cys) doubled once, presumably due to the presence of sufficient L-cysteine from overnight growth, but then failed to grow further. Notably, the LSM-cys growth condition included 0.67mM L-methionine and ~2mM sulfate, confirming previous genetic and experimental evidence that WT 10403S lacks the ability to synthesize L-cysteine from these sources using the reverse transsulfuration and sulfate assimilation pathways, respectively (45, 46, 56, 72). We also observed growth on the Arg-Gly-Asp-Cys (RGDC) oligopeptide as a sole L-cysteine source, which is consistent with previous reports (61). Growth kinetics of WT *L. monocytogenes* on RGDC were slightly altered relative to growth on L-cystine, demonstrating a longer lag phase, comparable log growth rate, and the same saturation density after 24 hours. While other exogenous L-cysteine sources were used at concentrations that provided 1mM equivalent of L-cysteine, the RGDC peptide was provided at 0.32mM L-cysteine equivalent as previously described due to saturation of the Opp oligopeptide importer (61).

While previous studies on PrfA activation have implied that *L. monocytogenes* can import and utilize exogenous GSH to activate PrfA (32, 40), growth on GSH or its oxidized counterpart, GSSG, as a sole L-cysteine source has not been studied. Here, we found that WT *L. monocytogenes* utilized both GSH and GSSG for growth in the absence of other L-cysteine sources and that growth kinetics match those of growth on both L-cystine and L-cysteine. Further, we also observed that WT *L. monocytogenes* grows readily on the inorganic sulfur compound thiosulfate in the absence of other L-cysteine sources, displaying similar growth kinetics to L-cystine, L-cysteine, GSH, and GSSG. This observation is contrary to previous observations and suggests that *L. monocytogenes* is not a complete L-cysteine auxotroph as previously suggested (44-46).

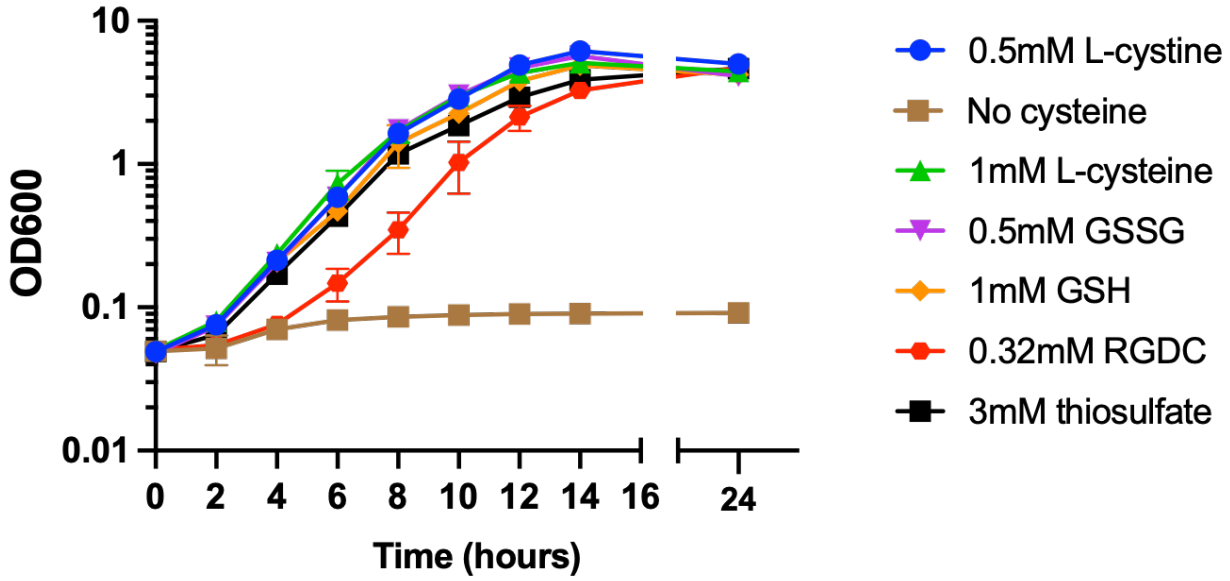


Figure 2.1. *L. monocytogenes* utilizes diverse L-cysteine sources for growth *in vitro*. Broth growth of WT *L. monocytogenes* in cysteine-free media supplemented with the indicated L-cysteine source. Bacteria were grown overnight in LSM supplemented with 0.5mM L-cystine. Cultures were washed once in LSM lacking L-cysteine and inoculated into media supplemented with various physiologically relevant L-cysteine sources. Growth was determined by OD_{600} at the indicated timepoints. Data representative of three independent replicates, error bars represent SD.

2.3.2 H_2S and thiosulfate utilization requires the enzyme CysK

To better understand the mechanism(s) of thiosulfate utilization in *L. monocytogenes*, we considered the role of the two-step L-cysteine biosynthetic pathway in facilitating growth on thiosulfate. The L-cysteine two-step biosynthetic pathway is a highly conserved pathway in bacteria that involves the conversion of L-serine to L-cysteine by two enzymes: CysE and CysK (Figure 2.2A). In the first step, CysE combines L-serine and acetyl-CoA to form the intermediate O-acetylserine (OAS). In the second step, CysK condenses OAS with sulfide or thiosulfate to form L-cysteine (55). These two genes are in distinct genomic loci in *L. monocytogenes*, with *cysE* present as part of a cysteine-responsive operon and *cysK* residing at a separate genomic locus (Figure 2.2B).

Typically, sulfide for this pathway is generated by import and reduction of sulfate by the sulfate assimilation pathway in sulfur-reducing bacteria (73), though reduced H_2S can be acquired from the environment through passive transport across the cell membrane (74). Thiosulfate is generated by the oxidation of H_2S in aerobic environments and is typically imported by members of the sulfate assimilation pathway or alternative thiosulfate-specific and inorganic ion importers (75). Previous genetic and experimental evidence has shown that WT 10403S *L. monocytogenes* is unable to utilize exogenous sulfate or thiosulfate to produce L-cysteine, however it has been noted that WT *L. monocytogenes* harbors an intact CysE/CysK two-step biosynthetic pathway (41, 45, 46, 56).

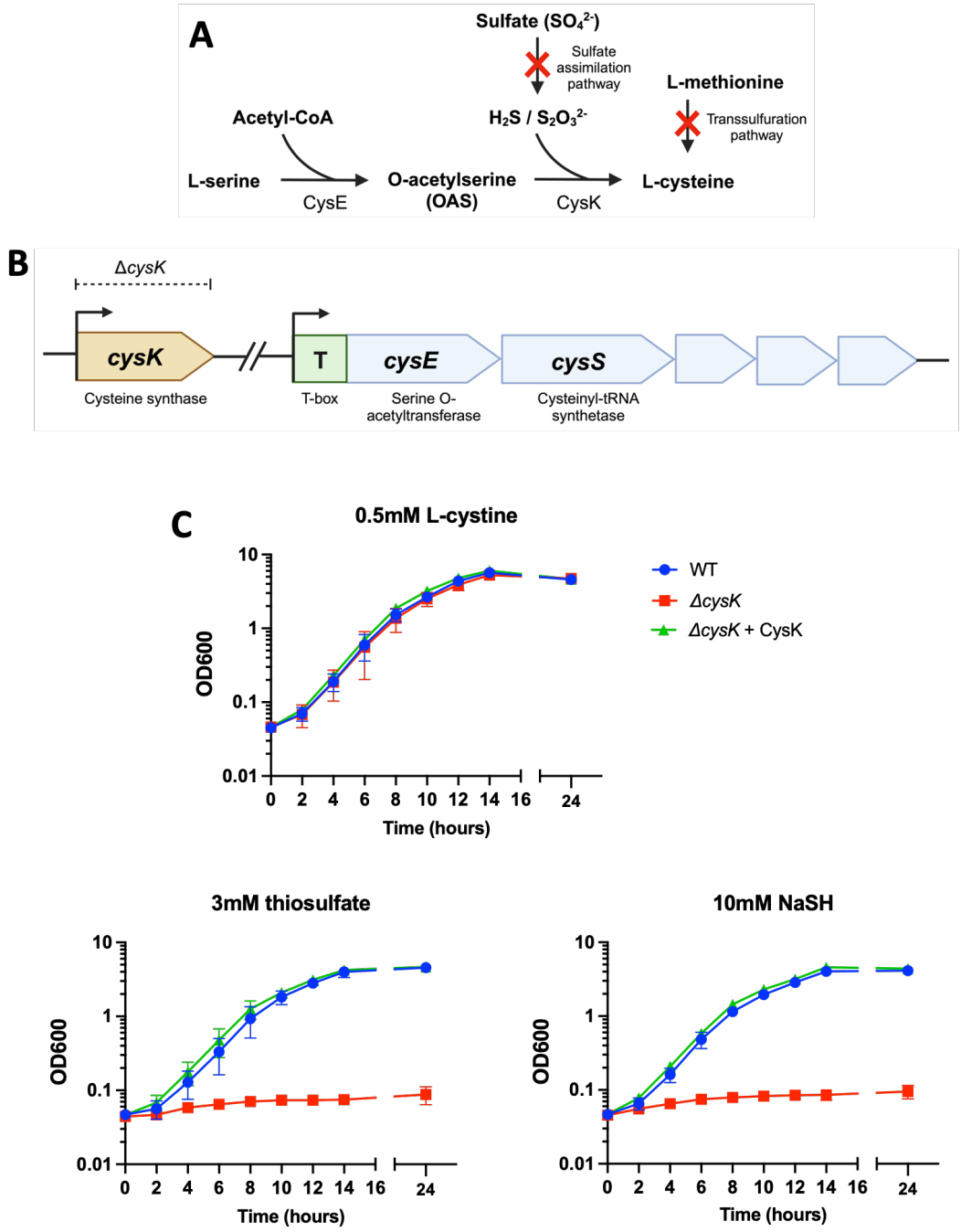


Figure 2.2. CysK is required for *L. monocytogenes* **growth on thiosulfate and H_2S.** A) Schematic of the two-step L-cysteine biosynthetic pathway in *L. monocytogenes* from L-serine and inorganic sulfur, including notable non-functional pathways in *L. monocytogenes*. B) Genomic organization of genes of the two-step biosynthetic pathway. C) Broth growth of the *cysK* mutant and complement in LSM supplemented with either thiosulfate or the H_2S generator NaSH. Strains were grown overnight in LSM media containing 0.5mM L-cystine, washed once in LSM lacking L-cysteine, and inoculated into media containing either L-cystine or the indicated inorganic sulfur source as the sole sulfur source. Growth was determined by OD_{600} at the indicated timepoints. Data representative of three independent replicates, error bars represent SD.

To test the role of the CysE/CysK two-step biosynthetic pathway, we generated a *∆cysK* mutant and tested its growth on thiosulfate and H_2S in the absence of other L-cysteine sources (Figure 2.2C). Neither the ∆*cysK* mutant nor the complemented strain displayed a defect when grown on L-cystine as a sole L-cysteine source. However, we observed that the ∆*cysK* mutant did not grow on either 3mM thiosulfate or 10mM sodium hydrosulfide (NaSH), which is a potent H_2S donor. These defects were rescued in both conditions by the complementation with the native *cysK* gene, demonstrating that the CysE/CysK two-step biosynthetic pathway is functional in *L. monocytogenes* and enables growth on a limited number of inorganic sulfur sources. The concentrations of thiosulfate and NaSH used represent the lowest concentrations that yielded similar growth kinetics in WT *L. monocytogenes* to the 0.5mM L-cystine control condition (J.C. Berude and D.A. Portnoy, unpublished data). Notably, 10mM NaSH was necessary to achieve similar growth kinetics of WT *L. monocytogenes* as with 3mM thiosulfate or 0.5mM L-cystine, likely due to the off-gassing of H_2S generated by the hydrosulfide ion in solution.

Next, we sought to determine if the CysE/CysK biosynthetic pathway plays a role in virulence gene activation in *L. monocytogenes* during infection. Human tissues are known to produce micromolar quantities of H_2S (76, 77), however concentrations of up to 1-3mM are present in the anoxic lumen of the intestine (71). The H_2S concentration in the gut is uniquely high and is toxic to colonic epithelial cells, and thus H_2S is rapidly converted to thiosulfate in the oxic environment of the outer intestinal lumen (70, 71). This reaction generates similarly high concentrations of thiosulfate (1-3mM) and suggests that these substrates might support *L. monocytogenes* growth and virulence in the gut. We observed no detectable defect in virulence of the ∆*cysK* mutant in a murine intravenous infection model (Supplemental figure 2.1), which is a measure of intracellular growth and virulence *in vivo*. We also did not observe a defect in growth of the *cysK* mutant in a murine oral competition assay, in which mice were orally infected with a 1:1 ratio of erythromycin (Erm)-sensitive WT and Erm-resistant *cysK*::Tn bacteria in a ∆*hly* background, which prevents intracellular infection and restricts bacteria to extracellular growth in the lumen of the intestine (Supplemental figure 2.2). Both WT and *cysK*::Tn Erm-resistant bacteria displayed a slight and equivalent defect in growth relative to Erm-sensitive WT bacteria, which suggests that the slightly low ratio of *cysK*::Tn/WT is due to lower plating efficiency of the Erm resistant strain and is not related to mutation of the *cysK* gene (78). Together, these observations suggest that the CysE/CysK two-step pathway does not play a significant role in growth and virulence of *L. monocytogenes* in the diverse nutritional landscape of the intestinal lumen.

2.3.3 The Ctp/OppDF complex imports GSH and GSSG

Next, we sought to elucidate the mechanism underlying GSH and GSSG import in *L. monocytogenes*. The GsiABCD ABC transporter is a known GSH-specific importer in *E. coli*, and a BLAST search for these genes identified putative homologs in *L. monocytogenes* that included the genes of the Ctp ABC transporter and the OppDF ATPases from the Opp complex (Table 2.1). The Ctp operon consists of three genes: a substrate-binding domain (*ctaP*) and two permeases (*ctpP1* and *ctpP2*) but lacks dedicated ATPase domains (Figure 2.3A). The Opp operon encodes a conserved oligopeptide importer that includes a substrate-binding domain (*oppA*), two permeases

(*oppB* and *oppC*), and two ATPases (*oppD* and *oppF*) (Figure 2.3A). In *L. monocytogenes*, the Opp complex is important for growth on oligopeptides. Previous work with the OppDF ATPases has suggested that they may play an important role in PrfA activation and intracellular growth, though it remains unclear if the core Opp complex (OppABC) is also required (60, 61). Additionally, even though the Ctp ABC transporter was initially annotated as a peptide importer in *L. monocytogenes*, it has been suggested that the complex is involved in high-affinity import of reduced L-cysteine (51, 52).

	% homology to *E. coli* GSH ABC importer GsiABCD subunits				**Annotated function**
Lm proteins	GsiA	GsiB	GsiC	GsiD	*Lm* / *E. coli*
CtaP (Lmo0135)	-	26%	-	-	Substrate-binding
CtpP1 (Lmo0136)	-	-	38%	-	Permease
CtpP2 (Lmo0137)	-	-	-	40%	Permease
OppD (Lmo2193)	42%	-	-	-	ATPase
OppF (Lmo2192)	48%	-	-	-	ATPase

Table 2.1. Homology of *E. coli* GsiABCD proteins to *L. monocytogenes* Ctp and OppDF. Homology determined by BLASTp search. Listed homology scores for the top search hits only.

To determine if the Ctp and Opp complexes are involved in GSH and GSSG import, we generated mutants in both pathways and assessed broth growth in LSM supplemented with either L-cystine or GSSG as a sole L-cysteine source. As expected, we observed normal growth kinetics of all strains (WT, Δ*ctp*, Δ*ctpP1*, Δ*oppB*, Δ*oppDF*) in 0.5mM L-cystine (Figure 2.3B). WT *L. monocytogenes* displayed normal growth kinetics when grown in 0.5mM GSSG, though no growth was observed in the Δ*ctp*, Δ*ctpP1*, and Δ*oppDF* mutants, suggesting that the Ctp complex and OppDF ATPases are both required for growth on GSSG (Figure 2.3B). The Δ*oppB* mutant displayed normal growth kinetics on both L-cystine and GSSG, suggesting that the core Opp complex is not necessary for GSSG import (Figure 2.3B).

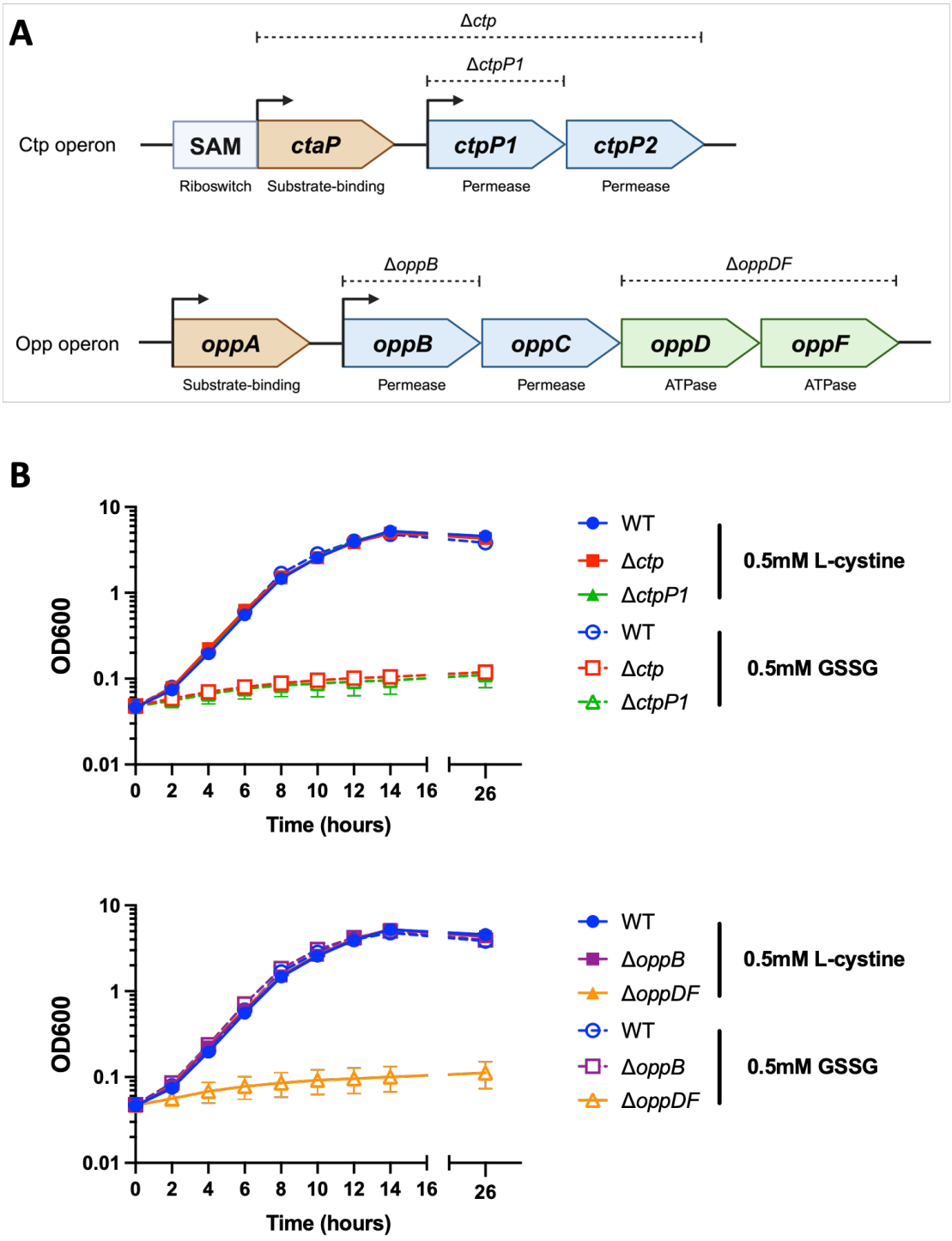


Figure 2.3. The Ctp operon and OppDF are required for growth on GSSG. A) Genomic organization of genes of the *ctp* and *opp* operons. Dashed lines denote specific mutants constructed. B) Broth growth of the *ctp* and *opp* mutants in LSM supplemented with GSSG. Strains were grown overnight in LSM media containing 0.5mM L-cystine, washed once in LSM lacking L-cysteine, and inoculated into media containing either L-cystine or GSSG as the sole L-cysteine source. Growth was determined by OD_{600} at the indicated timepoints. Data representative of three independent replicates, error bars represent SD.

To further elucidate the contributions of the Ctp and Opp complexes for growth on different L-cysteine sources, we tested the growth of mutants and their complements in saturation endpoint growth assays. As previously observed, WT *L. monocytogenes* displayed normal growth with 0.5mM L-cystine, 1mM L-cysteine, 0.5mM GSSG, 1mM GSH, and 0.32mM RGDC, and failed to grow in LSM lacking any L-cysteine source (Figure 2.4A). Mutants in the Ctp complex (Δ*ctp* and Δ*ctpP1*) displayed normal growth on L-cystine, L-cysteine, and RGDC, but were unable to grow on either GSH or GSSG, and growth on these substrates was recovered by complementation (Figure 2.4A). This result suggests that the Ctp locus is not required for growth on L-cysteine as previously reported, but instead for growth on GSH and GSSG. Deletion of Δ*oppDF* similarly impaired *L. monocytogenes* growth on GSH and GSSG but also eliminated growth on RGDC, which is consistent with the established role of the Opp complex for growth on oligopeptides. Growth of the Δ*oppDF* mutant on all three substrates could be recovered by complementation with *oppDF*, though complementation of the Δ*oppDF* mutant with either *oppD* or *oppF* alone did not rescue growth on GSH, GSSG, and RGDC, suggesting that both ATPase subunits are required for the proper function of both the Ctp and Opp complexes (Figure 2.4B). As previously observed, the Δ*oppB* mutant displayed no growth defect on either GSH or GSSG, but also exhibited a partial defect when grown on RGDC that was rescued by complementation (Figure 2.4C). To further explore the contribution of the Ctp complex to growth on short cysteine-containing peptides, we tested the growth of a Ctp/Opp double mutant. Consistent with observations with the Δ*oppDF* mutant, a Δ*ctpP1*/*oppB*::Tn grew like WT *L. monocytogenes* on L-cystine and L-cysteine, but was unable to grow on GSH, GSSG, or RGDC (Figure 2.4D). Complementation with *oppB* rescued growth on RGDC, but not GSH or GSSG, while complementation with *ctpP1* fully rescued growth on GSH and GSSG and partially rescued growth on RGDC. Consistent with the initial annotation of the Ctp locus as a peptide transporter, these observations suggest that the Ctp complex may also facilitate import of short cysteine-containing peptides.

Because LSM is a defined, synthetic medium that represents an artificial bacterial growth environment, we sought to determine if the Ctp complex plays a role in more complex growth conditions *in vitro*. Brain-Heart Infusion (BHI) medium is a rich, complex medium that is commonly used to cultivate fastidious pathogenic microorganisms like *L. monocytogenes*. The medium derives most of its nutrients from an infusion of host tissues, peptone, and glucose, with L-cysteine presumably present as GSH and/or GSSG, L-cystine, and as part of oligopeptides of various length. We tested the growth of our mutants in BHI and observed growth defects only in the Δ*oppDF* and Δ*ctpP1*/*oppB*::Tn double mutants, and not with the Δ*ctp*, Δ*ctpP1*, and Δ*oppB* single mutants (Supplemental figure 2.3). This result suggests that *L. monocytogenes* can acquire sufficient L-cysteine from either GSH/GSSG or oligopeptides, but that at least one of these import pathways is required for full *L. monocytogenes* growth in BHI. Taken together, these observations provide evidence of a novel GSH/GSSG-specific importer in *L. monocytogenes* that is comprised of the Ctp ABC transporter complex and the OppDF ATPases.

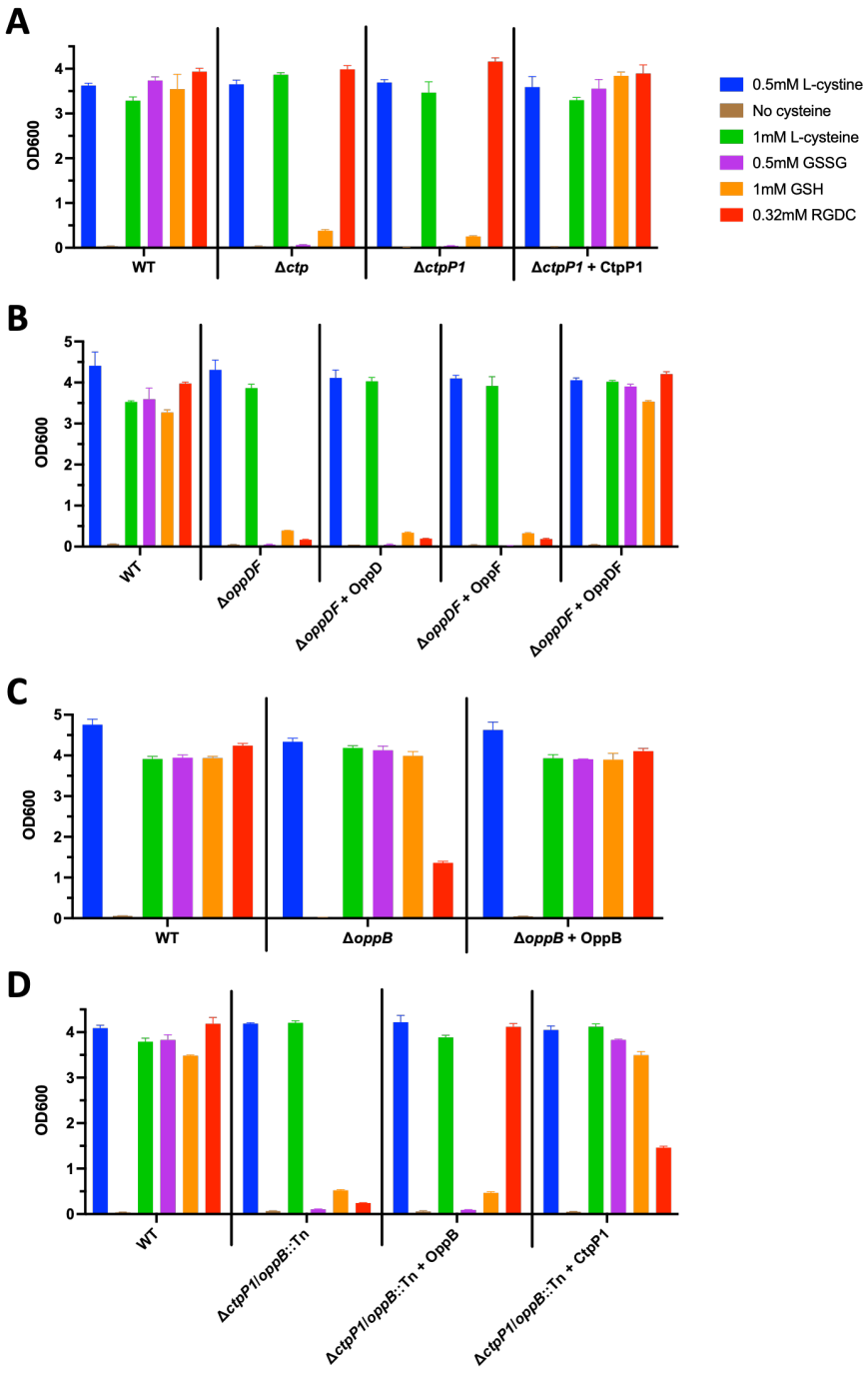


Figure 2.4. The Ctp operon and OppDF are required for growth on GSH and GSSG. Endpoint saturation broth growth of mutants and complements in cysteine-free LSM media supplemented with the indicated L-cysteine source. A) Broth growth of *ctp* mutants and complement. B) Broth growth of the *oppDF* mutant and complements. C) Broth growth of the *oppB* mutant and complement. D) Broth growth of the *ctpP1*/*oppB*::Tn double mutant and complements. Strains were grown overnight in LSM containing 0.5mM L-cystine, washed once in LSM lacking L-cysteine, and inoculated into media containing the indicated L-cysteine source as the sole L-cysteine source. Growth was determined by OD_{600} at the indicated timepoints. Data representative of three independent replicates, error bars represent SD.

2.3.4 The Ctp/OppDF complex is a high-affinity GSH/GSSG importer

To further elucidate the role of the Ctp/OppDF complex in GSH and GSSG import, we tested the affinity of the Ctp complex for both substrates in saturation endpoint growth assays. We observed that WT *L. monocytogenes* grew on as little at 5uM GSSG, with normal growth observed at concentrations at or above 50uM (Figure 2.5A). The Δ*ctp* mutant did not grow at any of the tested GSSG concentrations (1uM-5mM), suggesting that the Ctp complex is a high-affinity GSSG importer. Similarly, we tested *L. monocytogenes* growth on concentrations of GSH from 1uM-10mM, and we observed that WT *L. monocytogenes* grows on GSH concentrations as low as 50uM, with normal growth observed at or above 100uM (Figure 2.5B). The Δ*ctp* mutant did not grow in GSH concentrations at or below 0.5mM, with slight growth at 1mM, moderate growth at 2mM, and normal growth observed at 3mM GSH and above. Taken together, these data suggest that the Ctp complex is a high-affinity GSH importer, but that there are also alternative mechanisms of GSH acquisition in *L. monocytogenes*.

Because GSH is a tripeptide, we considered if the Opp complex may also import GSH and account for Ctp-independent growth on higher concentrations of GSH. Previous experiments with the Δ*ctpP1*/*oppB*::Tn double mutant strain demonstrated that complementation with *oppB* was not sufficient to recover growth on 1mM GSH (Figure 2.4D), suggesting that the Opp complex is not able to import GSH. To confirm this hypothesis, we tested growth of Ctp and Opp mutants in varying concentrations of GSH. Neither the ΔctpP1, ΔctpP1/*oppB*::Tn, or Δ*oppDF* mutants were able to grow on GSH concentrations of 1mM or less, and all demonstrated normal growth at 3mM GSH and above, suggesting that the Opp complex is not an alternate GSH importer (Figure 2.5C).

Overall, these observations suggest that the Ctp/OppDF complex functions as a high-affinity GSH/GSSG importer in *L. monocytogenes*, and that there is likely an alternative low-affinity GSH import mechanism that is independent of the Ctp and Opp complexes.

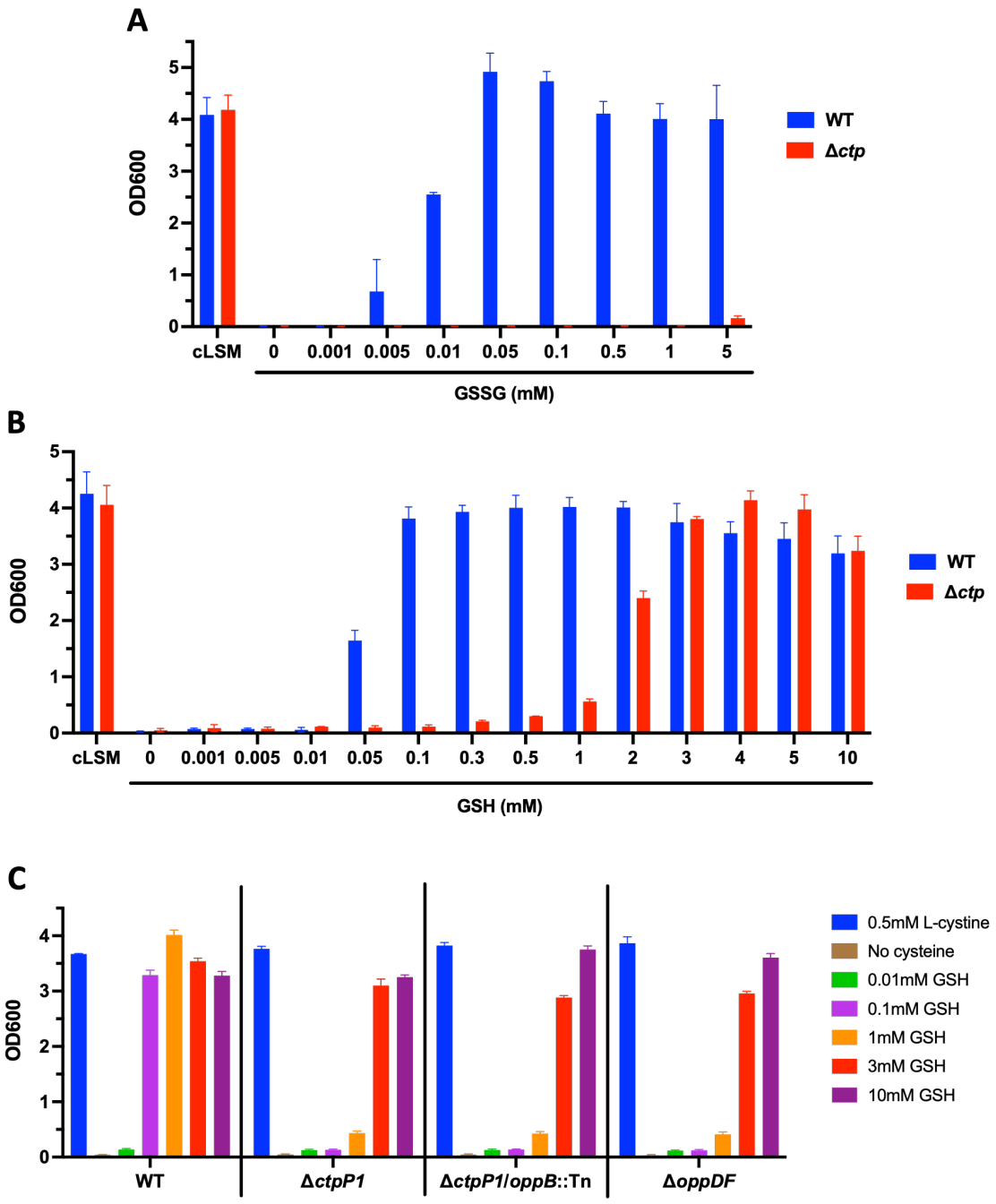


Figure 2.5. The Ctp complex is a high-affinity GSH/GSSG importer. Endpoint saturation broth growth of mutants and complements in cysteine-free LSM media supplemented with the indicated L-cysteine source. A) Broth growth of the *ctp* mutant in indicated concentrations of GSSG. B) Broth growth of the *ctp* mutant in indicated concentrations of GSH. All GSH media supplemented with 5mM TCEP. C) Broth growth of the *ctpP1*, *oppDF*, and *ctpP1*/*oppB*::Tn mutants on indicated concentrations of GSH. Strains were grown overnight in LSM containing 0.5mM L-cystine, washed once in LSM lacking L-cysteine, and inoculated into media containing the indicated L-cysteine source as the sole L-cysteine source. Growth was determined by OD_{600} at the indicated timepoints. Data representative of three independent replicates, error bars represent SD.

2.3.5 The Ctp and Opp complexes are required for virulence in a murine IV infection model

GSH is the most abundant source of L-cysteine in the mammalian cytoplasm, and imported GSH likely contributes directly to PrfA activation, virulence gene expression, and intracellular growth. Thus, the Ctp complex is of particular interest as a GSH/GSSG-specific importer and potential modulator of virulence in *L. monocytogenes*. Previous work has demonstrated that mutants in the Ctp complex exhibit variable defects in a murine IV infection model (51, 52), but it remains unclear if these defects are due to decreased PrfA activation needed for virulence, reduced access to nutritional L-cysteine needed for intracellular growth, or both. We sought to recapitulate these findings using an IV infection model and assess the relative contributions of the Ctp and Opp complexes to PrfA activation and nutrient acquisition *in vivo*.

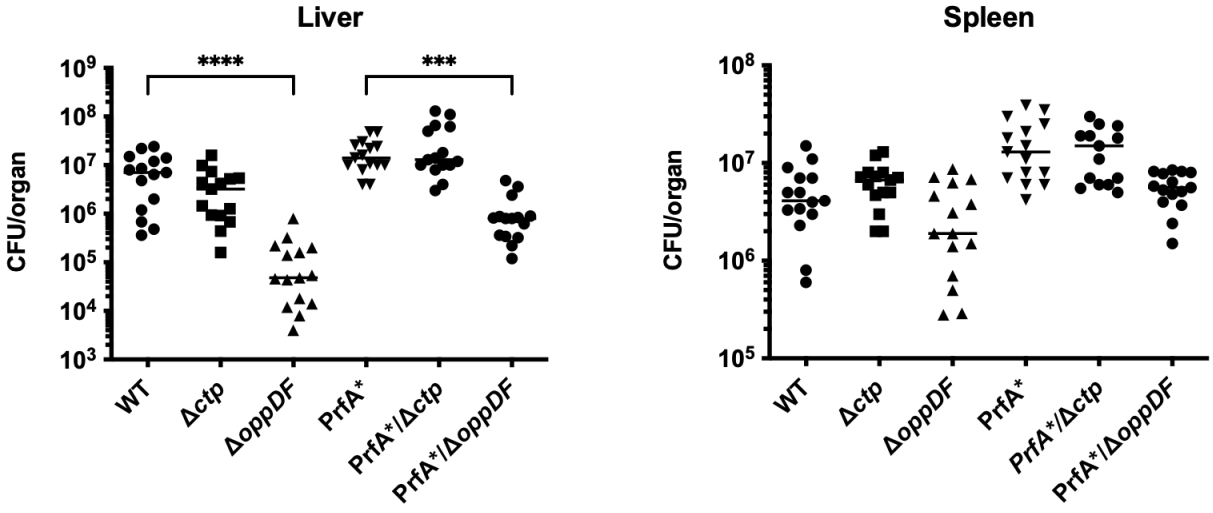


Figure 2.6. The Ctp/OppDF complex is required for full virulence in a murine IV infection model. 8-week old female CD-1 mice were intravenously infected with 10^5 CFU of indicated *L. monocytogenes* strains. Livers and spleens were harvested at 48 hours post infection and bacterial burden was measured by CFU. Data representative of three independent experiments. Statistical significance determined by Kruskal-Wallis test; ***P < 0.0002, ****P < 0.0001.

Contrary to previous findings, pooled data from three independent intravenous infection experiments did not reveal a significant impact on virulence with the Δ*ctp* mutant, suggesting that the Ctp complex alone is dispensable for growth and virulence activation *in vivo*. However, we observed a significant impact on virulence with the Δ*oppDF* mutant, which displayed a ~150-fold decrease in colony-forming units (CFU) in the livers of infected mice at 48 hours post-infection (Figure 2.6). To determine if these defects were due to a reduction in PrfA activation or a defect in nutrient acquisition, we compared the virulence defects of mutants in the WT 10403S background to those in the PrfA* background, which encodes a constitutively active form of PrfA that does not require allosteric binding of GSH for activation (32, 79). The ~150-fold virulence defect in the Δ*oppDF* mutant was reduced to ~15-fold in the PrfA* background, which suggests that the Ctp and Opp complexes together play a significant role in nutrient acquisition and virulence gene activation *in vivo*. Notably, the Δ*oppDF* mutant only had a significant

defect in livers of infected mice, though it appears to have a ~2-fold defect in spleens as well that is not rescued in the PrfA* background, further emphasizing the importance of OppDF in intracellular nutrient acquisition (Figure 2.6).

Overall, our data suggest that the Ctp complex is dispensable for virulence and nutrient acquisition *in vivo* but do not rule out the existence of an alternative redundant GSH importer. The data also suggest that the Ctp and Opp complexes are together required for growth and virulence *in vivo*.

2.4 Discussion

Listeria monocytogenes is a pathogen of humans and other mammals that alternates between a saprophytic and intracellular lifecycle, requiring the production and import of GSH to drive virulence gene expression and pathogenesis. Even though L-cysteine is required for GSH synthesis, *L. monocytogenes* is thought to be auxotrophic for L-cysteine, requiring import of exogenous L-cysteine sources to support growth and virulence (45, 46). Locally high GSH concentrations in the host cytosol have been posited to act as a signal for bacterial entry into the intracellular niche, as the binding affinity of PrfA for GSH has been reported to be ~4mM, which is well within the biologically relevant concentrations of the host cytoplasm (32, 40). Despite these observations, no GSH importers had been identified in *L. monocytogenes*. The results of this study demonstrate that the Ctp complex is a high-affinity GSH and GSSG importer in *L. monocytogenes*, and that *L. monocytogenes* is not auxotrophic for L-cysteine and can utilize limited inorganic sulfur sources to synthesize L-cysteine and support growth.

The Ctp ABC transporter operon consists of three subunits, a substrate-binding protein and two permeases, but lacks dedicated ATPase domains (Figure 2.3A). Our results indicate that the two ATPases from the Opp oligopeptide importer complex, OppDF, are also required for growth on GSH and GSSG independently of their role in the Opp complex (Figure 2.7A). Preliminary observations also suggest that the Ctp complex may import short cysteine-containing peptides at lower affinities, which is consistent with observations made of the GshT GSH importer in *Streptococcus mutans* that also facilitates growth on GSH derivatives (67). Despite the observation that the Ctp complex is a high-affinity GSH/GSSG importer, we observed growth of the *ctp* mutant on higher concentrations of exogenous GSH (Figure 2.5B), suggesting that an alternative, lower-affinity GSH acquisition mechanism also exists. The most likely candidates for this mechanism are either an additional yet-unidentified GSH importer or a secreted γ-glutamyl transpeptidase (GGT, Figure 2.7A), such as that utilized by *Francisella tularensis* (65). The importance of this alternative mechanism(s) to pathogenesis is accentuated by the observation that the *ctp* mutant does not display significant virulence defects in a murine IV infection model, and that the defects in the *oppDF* mutant are restricted to the liver where intracellular GSH concentrations are greatest (Figure 2.6, 36). Further work will be required to elucidate any additional GSH acquisition mechanisms.

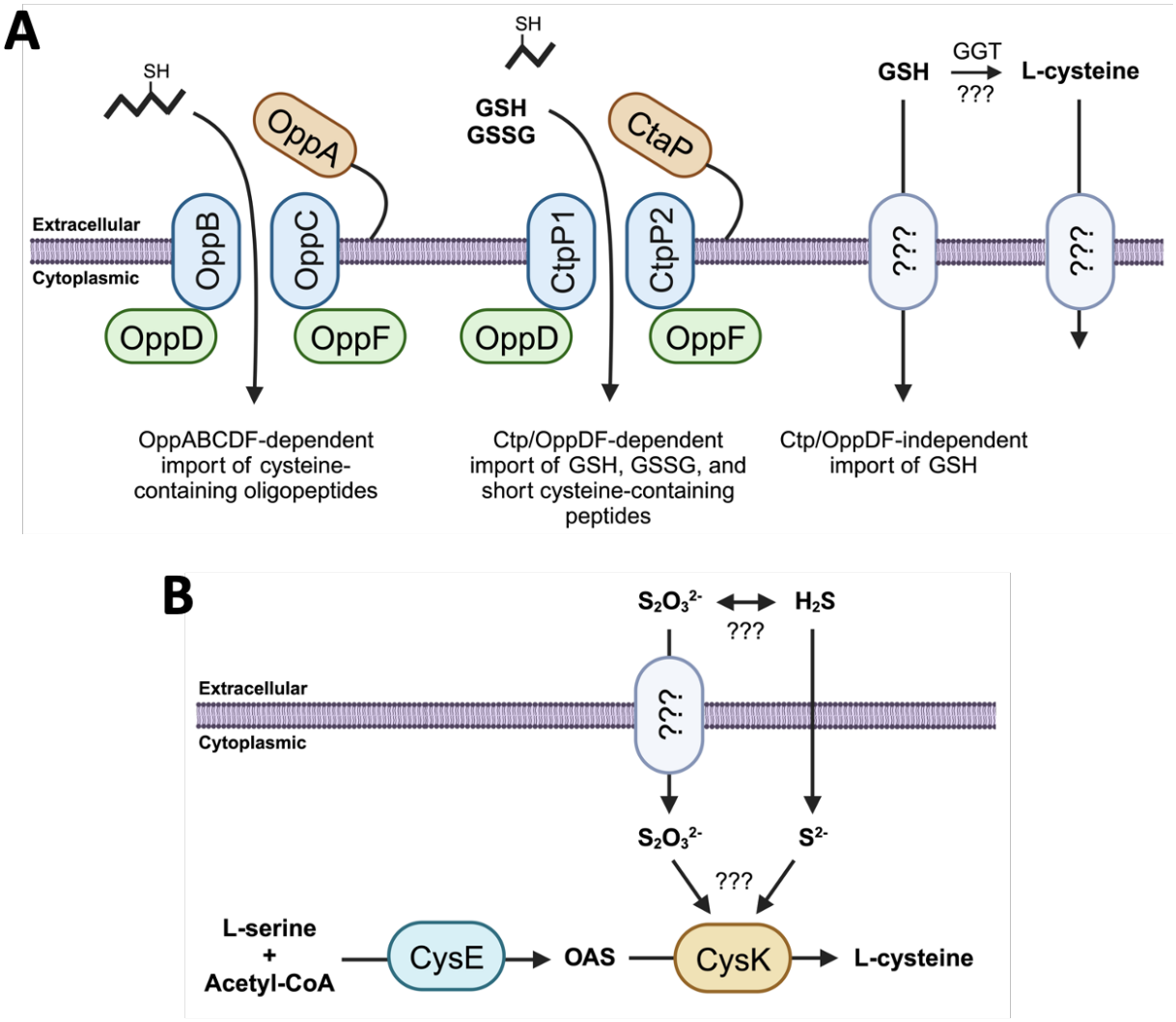


Figure 2.7. Model of mechanisms for *L. monocytogenes* growth on GSH, GSSG, thiosulfate, and H_2S. A) Cysteine-containing oligopeptides are imported by OppABCDF complex. GSH and GSSG are imported by the Ctp/OppDF complex with high affinity, and small cysteine-containing oligopeptides are likely imported with low affinity. An unknown GSH importer and/or a secreted GSH-specific ɣ-glutamyl transpeptidase (GGT) and L-cysteine importer likely also exist that support growth on higher concentrations of GSH. B) H_2S freely diffuses through the plasma membrane and/or thiosulfate is imported by an unknown mechanism, where they serve as sulfide donors for production of L-cysteine by the CysE/CysK two-step biosynthetic pathway. It is unclear if H_2S or thiosulfate is the preferred substrate for CysK. OAS: O-acetylserine, $S_2O_3^{2-}$: thiosulfate, H_2S: hydrogen sulfide.

Another novel finding of this work is that *L. monocytogenes* utilizes the inorganic sulfur sources thiosulfate and H_2S as an L-cysteine source. For decades, the consensus was that *L. monocytogenes* is auxotrophic for L-cysteine despite the presence of an intact and conserved two-step L-cysteine biosynthetic pathway (44-46, 56). Here, we show that the CysE/CysK two-step L-cysteine biosynthetic pathway is functional in *L. monocytogenes* and both exogenous thiosulfate and H_2S support growth

(Figure 2.7B). Because growth is only supported on limited inorganic sulfur sources, our results support a model of partial L-cysteine auxotrophy in *L. monocytogenes*. Despite our observations, it is not yet clear if or how *L. monocytogenes* imports thiosulfate, which is a charged molecule that requires facilitated import. Thiosulfate is canonically imported by members of the sulfate assimilation pathway, which is missing in *L. monocytogenes* (56, 80, 81), however import has also been shown to occur through independent importers and raises the possibility of identifying one or more importers in *L. monocytogenes* (75, 82). Alternatively, *L. monocytogenes* could potentially reduce thiosulfate to H_2S for passive import through the plasma membrane, however this is likely an unfavorable reaction in aerobic environments and little evidence exists to suggest this mechanism.

Another outstanding question is if the CysE/CysK pathway contributes to *L. monocytogenes* growth and pathogenesis, as concentrations of H_2S and thiosulfate are uniquely high in the intestinal lumen of mammals. Here, sulfur-reducing bacteria (SRBs) produce millimolar quantities of H_2S in the anoxic core of the intestine, which is oxidized to thiosulfate at the oxic-anoxic interface generated by oxygen species released by colonic epithelial cells (43, 70, 71, 76, 77). Bacterial pathogens are known to exploit these high concentrations of thiosulfate in the gut to facilitate colonization (83), suggesting that *L. monocytogenes* may have adapted to do so as well. Our results do not indicate that the CysE/CysK pathway alone is required for intestinal colonization in mice (Supplemental figure 2.2), however it is important to note that the lumen of the intestine is a complex nutrient-rich environment that contains many other exogenous L-cysteine sources. It is also important to consider that streptomycin treatment was used to reduce colonization resistance in the gut, but that the resulting dysbiosis could negatively impact populations of SRBs in the gut and alter the H_2S/thiosulfate landscape in the intestinal lumen in our experimental model, though little is known about the impacts of antibiotics on SRBs (84). Despite these observations, it is possible that the CysE/CysK pathway still contributes overall L-cysteine levels during infection, and high levels of these inorganic sulfur compounds in the intestine may represent an early host signal that primes *L. monocytogenes* for intracellular PrfA activation. More work is needed to determine the exact role of the CysE/CysK biosynthetic pathway in the dynamics of host colonization and pathogenesis.

Finally, this study calls into question the very nature of partial L-cysteine auxotrophy in *Listeria monocytogenes*. Auxotrophies constrain the interactions of bacteria with their environment, and bacterial pathogens are no different. While auxotrophies can arise through deleterious mutation, they often arise as specific adaptations to unique host or environmental conditions, suggesting a pathoadaptive role for these auxotrophies (85, 86). *L. monocytogenes* has a handful of well-characterized auxotrophies that are relevant to pathogenesis, namely lipoic acid and riboflavin. With lipoic acid, host scavenging appears to be essential for intracellular growth, suggesting an adaptation to a unique feature of the host environment (87). In the case of riboflavin, previous work has suggested that WT *L. monocytogenes* has lost the ability to synthesize a key riboflavin intermediate, 5-OP-RU, to enable evasion of bacterial recognition by host MAIT innate immune cells (88, 89). In both cases, the inability to produce these two essential metabolites confers a fitness advantage to *L. monocytogenes* in the host environment.

Curiously, L-cysteine auxotrophies in other pathogenic bacterial species (*S. enterica* and *E. coli*) confer a fitness advantage to these bacteria in certain *in vivo* niches (86), suggesting that the same may be true in *L. monocytogenes*. The pathogen *Staphylococcus aureus* is similarly partially auxotrophic for L-cysteine, lacking an intact sulfate assimilation pathway but can utilize limited inorganic sulfur sources like thiosulfate for growth, and recent work has highlighted the importance of L-cysteine acquisition mechanisms during infection (69, 90, 91). Considering the essential role of L-cysteine for growth and virulence gene expression in *L. monocytogenes*, it is compelling to suggest that partial L-cysteine auxotrophy is a pathoadaptive feature of *L. monocytogenes* metabolism, representing an adaptation to a unique host environment. Virulence gene activation is tightly regulated in *L. monocytogenes* and inappropriate activation during saprophytic growth results in considerable defects in bacterial fitness, highlighting the importance of proper spatiotemporal control of PrfA activation (23, 24). Together, these observations suggest that locally high L-cysteine concentrations in the host cytoplasm may serve as a vital host cue that signals bacterial arrival to their intracellular niche. Previous work lends support to this hypothesis, as the peptide signature of the habitat and the relatively higher concentrations of cysteine-containing peptides in the host cytoplasm have been postulated to directly impact GSH production and PrfA activation (61). The host cytoplasm contains uniquely high concentrations of GSH, and this activator of virulence and vital L-cysteine source represent an attractive and specific host signal that bacteria have arrived at their intracellular niche. The discovery of the first GSH-specific importer in *L. monocytogenes* and the ability to utilize limited inorganic sulfur sources for growth are significant steps in understanding the nature of partial L-cysteine auxotrophy and the spatiotemporal dynamics of virulence gene activation in the host-pathogen interactions of *Listeria monocytogenes*.

2.5 Materials and methods

Bacterial strains, plasmids, and growth conditions

All strains of *L. monocytogenes* used in this study were derived from the WT 10403S parental strain. Strains were cultured in chemically defined synthetic media (LSM) containing 200ug/mL streptomycin (Sigma-Aldrich) and 0.5mM L-cystine (Sigma-Aldrich) as the sole cysteine source. LSM was prepared as previously described (92). LSM containing the alternative cysteine sources reduced L-cysteine (Sigma-Aldrich), reduced glutathione (Sigma-Aldrich), oxidized glutathione (Sigma-Aldrich), RGDC oligopeptide (GenScript), sodium thiosulfate (Sigma-Aldrich), and sodium hydrosulfide (Sigma-Aldrich) was prepared by substituting L-cystine for the indicated concentration of each cysteine source. Media containing reduced L-cysteine or reduced glutathione were prepared fresh and supplemented with 2mM TCEP (Goldbio) prior to experimentation, except where otherwise stated. Media containing sodium hydrosulfide was prepared fresh immediately prior to experimentation to prevent off-gassing of volatile hydrogen sulfide gas. Additional antibiotics (Sigma-Aldrich) were used at the following concentrations: erythromycin (1ug/mL), carbenicillin (100ug/mL), tetracycline (2ug/mL), and chloramphenicol (7.5ug/mL for *L. monocytogenes* and 10ug/mL for *E. coli*).

Construction of in-frame deletion strains Δ*ctp*, Δ*ctpP1*, Δ*oppB*, Δ*oppDF*, and Δ*c*ysK was performed by allelic exchange using the temperature-sensitive vector

pKSV7 as previously described (93)). Nutrient-rich Brain Heart Infusion media (BHI, BD Difco) was used for bacterial growth during the allelic exchange protocol. To circumvent growth deficiencies of the Δ*ctp*, Δ*ctpP1*, and Δ*oppDF* mutants in BHI, mutants were grown in BHI supplemented with all 20 amino acids (Sigma-Aldrich) as in LSM media (0.1g/L, except L-glutamine at 0.6g/L).

Complementation of genes was performed by amplifying the native genes from WT 10403S *L. monocytogenes* and cloning into the site-specific pPL2 integrating vector containing the constitutively expressed pHyper promoter. Complementation vectors were conjugated into *L. monocytogenes* as previously described (94).

Broth growth assays

Broth growth assays were performed with *L. monocytogenes* strains grown overnight in LSM supplemented with 0.5mM L-cystine at 37°C with shaking (220rpm). Bacteria were washed once in synthetic media lacking an L-cysteine source (LSM-cys) and resuspended in an equal volume of LSM-cys. Washed bacteria were diluted to an optical density of 0.05 in LSM-cys containing the indicated cysteine sources and grown at 37°C with shaking. Growth was assessed spectrophotometrically by optical density at a wavelength of 600nm (OD_{600}). Broth growth curves were performed in 250mL flasks containing 35mL of media and OD_{600} measurements were taken every two hours for 14 hours and again at 24-26 hours as indicated. Saturation endpoint growth experiments were performed in 14mL round-bottom tubes containing 2mL of media and OD_{600} measurements were taken at 24-26 hours post-inoculation.

Mouse intravenous infections

Eight-week-old female CD-1 mice (Charles River Laboratories) were infected intravenously via the tail vein with 200uL PBS containing $1x10^5$ CFU of logarithmically growing *L. monocytogenes* as previously described (95). Bacteria used for infection were grown overnight in LSM supplemented with 0.5mM L-cystine and diluted 1:20 in fresh media to obtain logarithmically growing bacteria. Mice were euthanized 48 hours post-infection and spleens, livers, and gallbladders were collected, homogenized, and plated to enumerate CFUs per organ. Statistical significance was determined by Kruskal-Wallis test.

Mouse oral infection competition assay

Eight-week-old female C57BL/6J mice (Jackson Laboratories) were provided 5ug/mL streptomycin (Sigma-Aldrich) in drinking water 48 hours prior to infection as previously described (96). Mice were transferred to clean cages 18-24 hours prior to infection and the food source was removed to fast mice overnight. Bacteria used for infection (Δ*hly* and Δ*hly*/ErmR) were grown overnight in BHI and diluted 1:10 in fresh media to obtain logarithmically growing bacteria. At time of infection, bacteria were washed twice in PBS and inoculums containing $1x10^8$ each of Δ*hly* and Δ*hly*/ErmR (WT control or mutant) *L. monocytogenes* were prepared in 5uL PBS. A 3-mm piece of bread was inoculated with a total of $2x10^8$ CFU bacteria in PBS and covered with 3uL melted butter. Mice were fed a single piece of infected bread. Streptomycin-treated water was replaced with standard drinking water and food was restored at the time of inoculation. Fecal samples were collected every day post-infection for five days, weighed, and

resuspended in 1mL PBS by vortexing at 4°C for 10 minutes. Fecal samples were plated on BHI plates containing only streptomycin or streptomycin and erythromycin to enumerate CFUs of WT and erythromycin-resistant strains and the ratio of the two populations was calculated to determine the competitive fitness of mutant strains relative to WT 10403S.

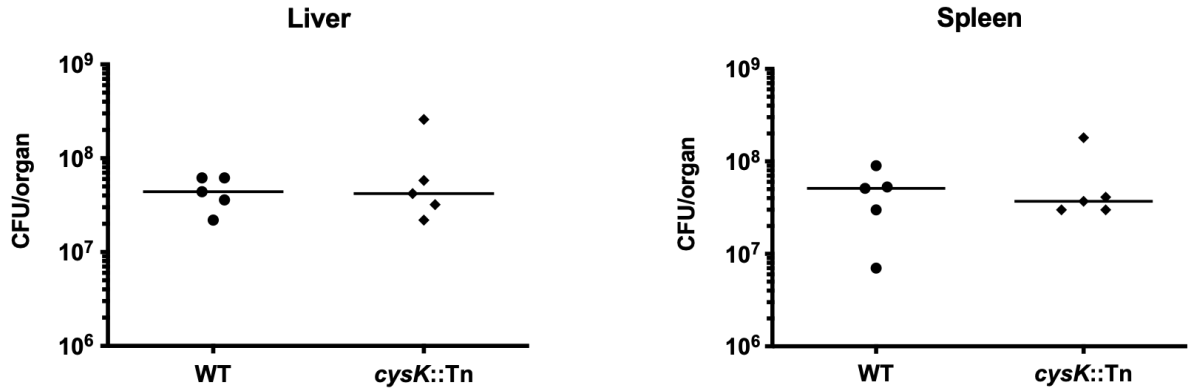
Animal Use Ethics Statement

Mice were maintained by University of California, Berkeley Office of Laboratory Animal Care personnel according to institutional guidelines. All animal studies were performed in accordance with the recommendation in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and university regulations. The protocols used in this study were reviewed and approved by the Animal Care and Use Committee at the University of California, Berkeley (AUP 2016-05-8811).

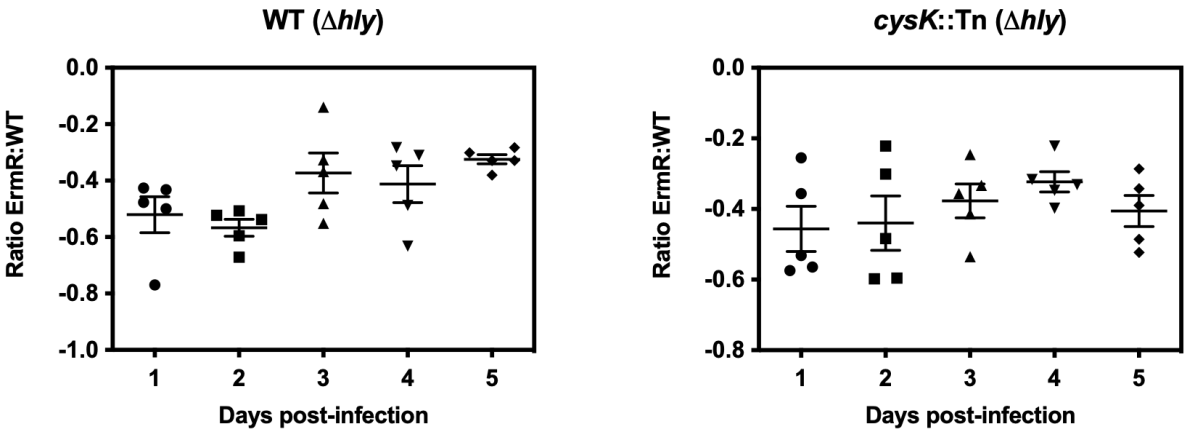
Statistical analysis

All statistical analysis was performed using GraphPad Prism version 10. *** indicates $P < 0.0002$, **** indicates $P < 0.0001$.

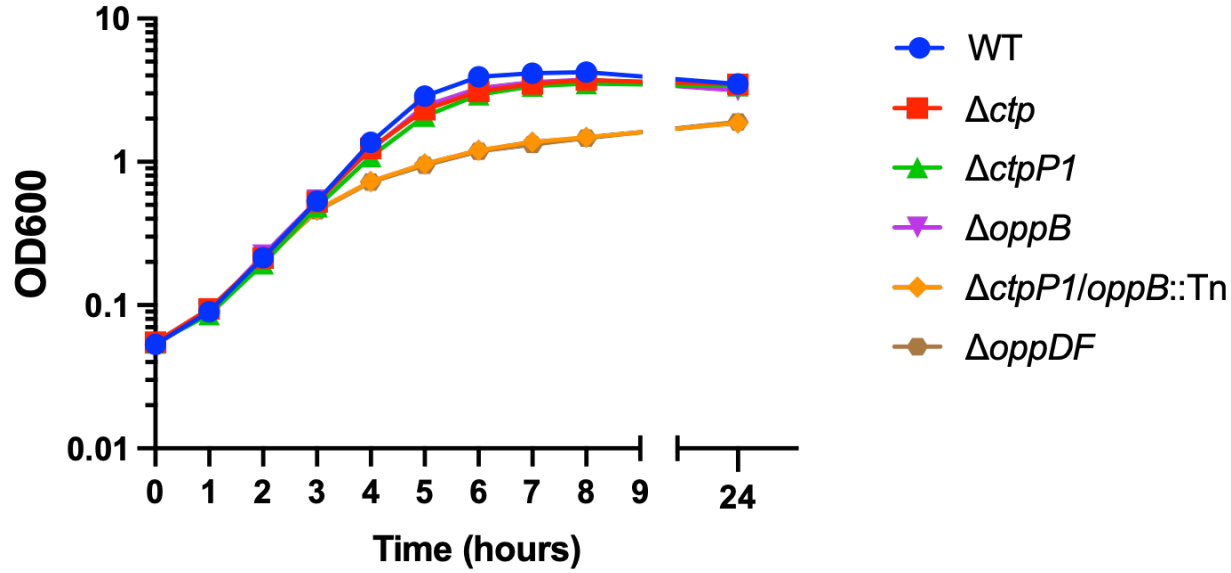
2.6 Supplemental figures



Supplemental figure 2.1. CysK is not required for virulence in a murine IV infection model. 8-week old female CD-1 mice were intravenously infected with 10^5 CFU of WT or *cysK*::Tn mutant *L. monocytogenes*. Livers and spleens were harvested at 48 hours post infection and bacterial burden was measured by CFU. Data representative of one replicate.



Supplemental figure 2.2. CysK is not required for bacterial fitness in a murine oral infection model. Streptomycin-treated 8-week old female C57BL/6J mice were orally infected with 10^8 CFU each of Δ*hly* and Δ*hly* containing the Erm resistance gene. Fecal pellets were collected every day for five days post-infection, CFUs were enumerated for total and Erm resistant bacterial populations, and the ratio of ErmR:WT bacteria was calculated. Data representative of one replicate.



Supplemental figure 2.3. OppDF and the Ctp/Opp double mutant demonstrate growth defects in complex rich media. Broth growth of the *ctp* and *opp* mutants in Brain-Heart Infusion (BHI) medium. Strains were grown overnight in LSM media containing 0.5mM L-cystine, washed once in BHI, and inoculated into BHI. Growth was determined by OD_{600} at the indicated timepoints. Data representative of three independent replicates, error bars represent SD.

Table 2.2. *Listeria monocytogenes* strains used in this study.

Strain	Strain number	Reference
Wild-type 10403S	DP-L184	(97)
cysK::Tn	DP-L7556	This study
Δ*cysK*	DP-L7557	This study
Δ*cysK* + pPL2-pHyper-CysK	DP-L7558	This study
Δ*ctp*	DP-L7559	This study
Δ*ctpP1*	DP-L7560	This study
Δ*ctpP1* + pPL2-pHyper-CtpP1	DP-L7561	This study
Δ*oppB*	DP-L6272	(98)
Δ*oppB* + pPL2-pHyper-OppB	DP-L7562	This study
Δ*oppDF*	DP-L7563	This study
Δ*oppDF* + pPL2-pHyper-OppD	DP-L7564	This study
Δ*oppDF* + pPL2-pHyper-OppF	DP-L7565	This study
Δ*oppDF* + pPL2-pHyper-OppDF	DP-L7566	This study
Δ*ctpP1*/*oppB*::Tn	DP-L7567	This study
Δ*ctpP1*/*oppB*::Tn + pPL2-pHyper-OppB	DP-L7568	This study
Δ*ctpP1*/*oppB*::Tn + pPL2-pHyper-CtpP1	DP-L7569	This study
Δ*hly*	DP-L2161	(99)
Δ*hly*/ErmR	DP-L4381	This study
Δ*hly*/*cysK*::Tn	DP-L7570	This study
PrfA*	DP-L5451	(100)
PrfA*/Δ*ctp*	DP-L7571	This study
PrfA*/Δ*oppDF*	DP-L7572	This study

Table 2.3. *Escherichia coli* strains used in this study.

Strain	Strain number	Reference
SM10 pKSV7x-*cysK*	DP-L7573	This study
SM10 pKSV7x-*ctp*	DP-L7574	This study
SM10 pKSV7x-*ctpP1*	DP-L7575	This study
SM10 pKSV7x-*oppDF*	DP-L7576	This study
SM10 pPL2x-pHyper-CysK	DP-L7577	This study
SM10 pPL2x-pHyper-CtpP1	DP-L7578	This study
SM10 pPL2x-pHyper-OppB	DP-L7579	This study
SM10 pPL2x-pHyper-OppD	DP-L7580	This study
SM10 pPL2x-pHyper-OppF	DP-L7581	This study
SM10 pPL2x-pHyper-OppDF	DP-L7582	This study

Table 2.4. Oligonucleotides used in this study.

Target gene	Forward primer	Reverse primer
pKSV7x vector	gctgcaggaggcagtgga	ggatccagcgccgct
pKSV7x-cysK 5’	atggggtccagcggcgctggatccaaagcgttagcctacgatggc	tcttcaaaattataaagcggtgaatttgcaattgtcattattaaagcactcc
pKSV7x-cysK 3’	ccgctttataattttgaagattaataatcagagagcc	gctcgctccactgcctcctgcagcccaccgcttcttctacttgc
pKSV7x-ctpP1 5’	ggtccagcggcgctggatccaagattcaacgactactttgacggc	gcgttttatgattgtttttagcataatttatctcct
pKSV7x-ctpP1 3’	aaaacaatcataaaacgccggattagggaaatggggtgagg	gctccactgcctcctgcagccgcgctctccttttgtgagc
pKSV7x-oppDF 5’	ggtccagcggcgctggatccgttaaaacctcgctcttccaaatcc	aaatggaaaagctattagaagctgaagctgctgcgaaataa
pKSV7x-oppDF 3’	ttctaatagcttttccatttttcctcacctctc	gctccactgcctcctgcagctcagccagcgcacattctg
pPL2x-pHyper vector	taatgaataaaacgaaaggctcagtcgaaag	gtcgactccctcctcgtgatac
pPL2x-pHyper-CysK	atcacgaggagggagtcgacatgacaattgcaaattcaatcactgatttaattgg	Gcctttcgttttattcattattaatcttcaaaattataaagcggcgtgc
pPL2x-pHyper-CtpP1	cacgaggagggagtcgacatgctaaaaacaatcataaaacgcgtattgc	ctttcgttttattcattatcaccccatttccctaatccgc
pPL2x-pHyper-OppB	atcacgaggagggagtcgacatggttaaatatacgttaaaaagagtattatatatgcttataacgt	gcctttcgttttattcattattattttctacctccagacacacggattcg
pPL2x-pHyper-OppD	atcacgaggagggagtcgacatggaaaagctattagaagttaaagatttaaatatttcattccac	gcctttcgttttattcattatcattctacctctacccctttcgc
pPL2x-pHyper-OppF	atcacgaggagggagtcgacatgactgaacaaagagaaaaattattagaaattcataatctaaagc	gcctttcgttttattcattattatttcgcagcagcttcagcagt
pPL2x-pHyper-OppDF	atcacgaggagggagtcgacatggaaaagctattagaagttaaagatttaaatatttcattccac	gcctttcgttttattcattattatttcgcagcagcttcagcagt

Chapter 3

Concluding remarks and future directions

3.1 Summary of results

In this study, we established the ability of *L. monocytogenes* to utilize GSH and GSSG for growth *in vitro* and identified a novel high-affinity GSH-specific ABC importer in *L. monocytogenes*. We showed that this importer is comprised of the Ctp complex and the OppDF ATPases, and we demonstrate that the Ctp importer is necessary and sufficient to support growth on exogenous GSH and GSSG *in vitro*. We observed that the Ctp complex is necessary for *L. monocytogenes* growth on all physiologically relevant concentrations of GSSG, but that it is only required for growth on GSH at concentrations below ~1mM. While the *oppDF* mutant, which knocks out both the Opp oligopeptide importer and the Ctp GSH importer, demonstrated a ~150-fold virulence defect in a murine IV infection model, we did not observe a virulence defect with the *ctp* mutant alone, suggesting that *L. monocytogenes* utilizes a redundant combination of GSH and cysteine-containing peptides to support growth and virulence in the host cytoplasm. When the *oppDF* mutant was tested in a PrfA* background, which harbors a constitutively active PrfA mutant that bypasses the need for GSH as an allosteric activator, the virulence defect in mice was reduced to ~15-fold, suggesting that the Ctp and Opp pathways together are required for both PrfA activation and growth in the host cytosol.

Further, we found that the intact CysE/CysK biosynthetic pathway is sufficient to support *L. monocytogenes* growth on the inorganic sulfur sources thiosulfate and H_2S, and we were the first to directly observe growth on these two substrates as a sole L-cysteine source. We observed that *cysK* is essential for growth on these two inorganic sulfur sources *in vitro*, but that a *cysK* mutant did not demonstrate any defects in either virulence or ability to colonize the host intestinal tract in a murine IV infection model and oral competition model.

3.2 Remaining questions and future directions

3.2.1 Determination of alternative glutathione utilization mechanisms

In the beginning of this work, we sought to identify and study mechanisms of L-cysteine acquisition that contribute to growth and pathogenesis in the bacterial pathogen *Listeria monocytogenes*. Our first task was to identify mechanisms of GSH acquisition and utilization. This was of interest considering the importance of GSH and L-cysteine in PrfA activation and virulence, the fact that the host cytoplasm is a rich source of GSH, and previous evidence that suggested that *L. monocytogenes* could import and/or utilize exogenous GSH for PrfA activation.

There are two mechanisms by which *L. monocytogenes* might utilize exogenous GSH. First, the bacteria could import exogenous GSH, where it could either be directly used for PrfA activation or degraded in the bacterial cytoplasm and the resulting L-cysteine could be used for subsequent GSH production via the bacterial GshF enzyme. In this scenario, exogenous GSH would be sufficient for growth and PrfA activation in a *gshF* mutant. It is also possible that imported GSH could not be used for PrfA activation directly and would first have to be degraded to L-cysteine by an intracellular γ-glutamyltransferase or protease before being remade into GSH by the bacterial GshF. In this case, exogenous GSH would be sufficient for growth, but not PrfA activation, in a

gshF mutant. Second, *L. monocytogenes* could secrete a γ-glutamyltransferase enzyme that degrades GSH extracellularly and allows the bacteria to import free reduced L-cysteine (or L-cystine in aerobic environments), which can be used to produce endogenous GSH for PrfA activation. Similarly, addition of exogenous GSH as a sole L-cysteine source would enable *L. monocytogenes* growth but not PrfA activation in a *gshF* mutant.

The results of this study, combined with previous observations, demonstrate that the first mechanism is at play in *L. monocytogenes*, but does not rule out the existence of the second. In this study, we found that *L. monocytogenes* can grow on GSH as a sole L-cysteine source, and we identified the Ctp complex as a GSH/GSSG-specific importer that is essential for growth on GSH and GSSG at physiologically relevant concentrations. This suggests that *L. monocytogenes* can degrade GSH into free L-cysteine in the bacterial cytoplasm, where it can then be utilized for protein synthesis and other essential uses. To further support our understanding, previous work by Reniere et al and Portman et al suggests that *L. monocytogenes* can directly utilize imported GSH for PrfA activation. These studies demonstrate that depletion of host GSH by BSO treatment is sufficient to eliminate bacterial ActA protein expression in a *gshF* mutant during infection, and they show that adding exogenous GSH to *gshF* mutant bacteria is sufficient to induce virulence gene expression that is comparable to that of a constitutively active PrfA* mutant (32, 40). While these observations strongly support a model where exogenous GSH is imported and directly utilized for PrfA activation and growth, they do not rule out the existence of a secreted γ-glutamyltransferase, such as is present in the bacterial pathogen *Francisella tularensis*, though more work is necessary to identify such an enzyme if it exists in *L. monocytogenes*.

Further, the results of this study suggest that there are alternative mechanisms for GSH import and utilization beyond the Ctp and Opp importers. Experiments with the *ctp* mutant indicated that the Ctp complex is not required for growth at GSH concentrations above ~2-3mM, which suggests the presence of a redundant GSH utilization system that functions at higher GSH concentrations. Additionally, the *ctp* mutant did not demonstrate a virulence defect in a murine IV infection model, further suggesting the presence of a redundant GSH utilization mechanism. While the nature of any redundant GSH utilization mechanisms is currently unknown, the discovery of the Ctp complex as a functional GSH importer greatly expands our ability to probe this question of alternative mechanisms.

For example, to determine if this alternative GSH utilization mechanism is another importer, we could knock out the *ctp* complex in a *gshF* background and measure PrfA activity in the presence of high levels of GSH. PrfA activation in this scenario would suggest that the mechanism in question is an importer, while a lack of PrfA activation would suggest the presence of a secreted γ-glutamyltransferase.

The most powerful line of inquiry, however, would be to use the *ctp* mutant to conduct genetic screens to identify redundant GSH utilization mechanisms. A simple screen would be to generate a transposon library in *ctp* mutant bacteria and look for transposon mutants that are not able to grow on high concentrations of GSH (3-10mM) as a sole L-cysteine source. This screen would provide insight into any additional genes required for GSH import and degradation. A secondary screen would be to generate a

transposon library in a *ctp*/*gshF* double mutant that harbors a PrfA activity reporter, such as the pActA-RFP or pActA-GUS constructs and look for a reduction in PrfA activity in transposon mutants when grown on high concentrations of GSH. The results of this screen would likely be more limited than the first, however, as only bacteria that can still utilize GSH for production of L-cysteine will grow, and thus this screen would only provide insight into putative secreted γ-glutamyltransferases. Another approach would be to conduct the same screen on high GSH in the presence of an alternative non-cysteine-containing L-cysteine source, such as thiosulfate, and this approach would allow for the identification alternative GSH importers as well. Identified mutants could then be tested for their specific roles in *in vitro* growth on exogenous GSH and *in vivo* virulence.

3.2.2 Role of the *de novo* L-cysteine biosynthetic pathway in *L. monocytogenes* growth and pathogenesis

In addition to identifying GSH utilization mechanisms, another goal of this study was to explore the role of the CysE/CysK two-step L-cysteine biosynthetic pathway in *L. monocytogenes*. In most bacteria, this pathway is critical to L-cysteine production from free sulfide supplied by the sulfate assimilation pathway. However, it has been long understood that *L. monocytogenes* lacks the necessary genes for sulfate import and assimilation and is thus not able to grow on inorganic sulfur sources like sulfate and thiosulfate as a sole source of L-cysteine (44-46).

This then raises the question of the role of the CysE/CysK pathway in *L. monocytogenes* physiology and pathogenesis, as the pathway would presumably still be functional if provided free sulfide. The most obvious of these substrates was H_2S itself, which is a direct sulfide donor and can freely cross membranes by passive diffusion. Despite previous observations, thiosulfate was also still an intriguing substrate to study, as previous work had identified thiosulfate as sulfide donor that could be directly used by either CysK or a homologous enzyme CysM to produce L-cysteine (55, 74). Further, it is known that both substrates are available in both the environment and the host, and thus could represent an important source of nutritional L-cysteine in these environments. In the host, H_2S is produced in millimolar quantities by sulfur-reducing bacteria in the anoxic core of the intestinal lumen and is then converted to millimolar quantities of thiosulfate in the more aerobic environment near the host epithelium (43, 71). H_2S and thiosulfate are similarly present in the environment, where decaying plant matter and sulfur-reducing bacteria produce H_2S in anaerobic environments that is oxidized to thiosulfate in the presence of oxygen (101, 102). Another surprising but potentially relevant source of thiosulfate in the environment is thiosulfate that is used to dechlorinate water during water treatment (103). Together, these observations provide compelling evidence that *L. monocytogenes* may utilize these inorganic sulfur sources in distinct niches.

The results of this study demonstrated that *L. monocytogenes* utilizes both H_2S and thiosulfate as a sole L-cysteine source in a CysK-dependent manner, but that the CysE/CysK pathway is dispensable for virulence in an IV infection model and bacterial growth in the intestinal lumen. This leaves us with several outstanding questions. First, it is still unclear whether CysK utilizes thiosulfate directly, or if thiosulfate must first be reduced to H_2S. In other bacteria, CysK utilizes free sulfide, while the homolog CysM

utilizes thiosulfate in the production of L-cysteine (55). *L. monocytogenes* lacks a CysM homolog, and CysK is required for growth on both substrates, which suggests that *L. monocytogenes* CysK may be able to utilize both substrates. Alternatively, *L. monocytogenes* could reduce thiosulfate to free sulfide via an unknown mechanism. It is also uncertain if and how thiosulfate would be imported into the *L. monocytogenes* cytosol. Canonically, thiosulfate import occurs via members of the sulfate assimilation pathway, which *L. monocytogenes* lacks, however other thiosulfate-specific and non-specific importers have been described, though none exhibit significant homology to genes in the *L. monocytogenes* genome (73, 75). To identify other genes involved in thiosulfate utilization, a couple of different screening methods are possible. A simple screen would be to generate a transposon library in a WT *L. monocytogenes* background and screen for mutants that are unable to grow on thiosulfate as a sole L-cysteine source. Another screen option would be to conduct a similar screen in a WT *L. monocytogenes* transposon library that harbors a PrfA activity reporter, such as pActA-RFP or pActA-GUS. Preliminary data has indicated that growing *L. monocytogenes* on increasing concentrations of thiosulfate result in increased GSH production and PrfA activation. This knowledge could be utilized for screening purposes, and transposon mutants could be screened for decreased PrfA reporter activity when grown on media containing a standard amount of L-cystine (0.5mM) and a high concentration of thiosulfate (>10mM). While both screens would be expected to produce mutants in *cysK*, they would likely also identify other genes involved in thiosulfate utilization, providing such systems are not redundant.

Further, while our results indicated that the CysE/CysK pathway is dispensable for growth in the intestinal lumen and virulence in an IV infection model, it is still unclear if the CysE/CysK pathway is sufficient to support growth and virulence in the host in the absence of other L-cysteine acquisition mechanisms. To determine this, we could make *L. monocytogenes* strains harboring combinations of knockouts in known L-cysteine acquisition mechanisms and compare growth and virulence to the same knockout strains lacking CysK as well. The ideal mutant would knock out oligopeptide import, L-cystine import, and GSH import (ΔoppB/Δ*ctp*/Δ*tcyK* or Δ*oppDF*/Δ*tcyK*). This strain could then be used in an oral competition assay with a mutant lacking *cysK* as well to determine if CysK is able to support *L. monocytogenes* intestinal colonization.

3.2.3 L-cysteine availability and spatiotemporal control of PrfA activation

Finally, a key aim of our work on L-cysteine acquisition mechanisms in *L. monocytogenes* has been to expand our understanding of the spatiotemporal dynamics of PrfA activation during infection. As previously discussed, proper spatiotemporal control of PrfA activation is essential for virulence and optimal bacterial fitness both in the environment and in the host, and we know a considerable amount about the complex regulatory network surrounding PrfA production and activation (10, 23, 24). While these diverse activating inputs include exposure to stressors and temperature changes that occur in the host environment, allosteric activation by GSH appears to be a dominant regulator of PrfA activity. Since GSH is present at high levels in the host intracellular niche and L-cysteine is the rate-limiting substrate for GSH production, it follows that GSH and broader L-cysteine availability are likely important factors in determining the spatiotemporal dynamics of PrfA activation.

Because of these observations, there has been much speculation about host intracellular GSH as an activating signal for PrfA and virulence gene expression during infection, and previous work has already suggested that increased L-cysteine availability through cysteine-rich peptides in the host cytoplasm may be a signal that results in increased GSH production and PrfA activity that drives transcription of late virulence genes required for intracellular growth and cell-to-cell spread (25, 30, 31, 32, 61). The identification of the Ctp complex as the first-known GSH importer in *L. monocytogenes* provides definitive evidence that the bacteria can utilize exogenous GSH and further suggests that increased L-cysteine availability in the host cytosol drives PrfA activation and virulence gene expression. While our work indicated that the Ctp complex alone is not necessary for virulence, it suggests that a combination of GSH and cysteine-containing peptides are required for full virulence. Additionally, while we identified the Ctp complex as a high-affinity GSH importer, our work suggests the presence of a redundant lower-affinity GSH acquisition mechanism as well that may be relevant in the context of infect. Further work is necessary to identify additional GSH utilization mechanisms in *L. monocytogenes* and will help us better understand the role of host GSH in PrfA activation and virulence.

3.2.4 Partial L-cysteine auxotrophy as a pathoadaptive feature of *L. monocytogenes* metabolism

A final lingering question presented by this work and a long-standing philosophical question in the field is the nature of partial L-cysteine auxotrophy in pathogenic *L. monocytogenes*. Prior to this work, the field held that *L. monocytogenes* was a complete L-cysteine auxotroph, unable to synthesize its own L-cysteine and requiring import of exogenous L-cysteine sources for growth and pathogenesis (44-46). Auxotrophies constraint the interactions of bacteria with their environment, and it follows that durable auxotrophies in bacterial pathogens arise from pathoadaptive mutations, as the auxotrophy better enables colonization of a distinct host niche (85, 86). Considering the importance of L-cysteine availability in modulating GSH production and PrfA activity and our current understanding of the host cytoplasm as a high-cysteine environment, it is reasonable to speculate that losing the ability to produce L-cysteine through the canonical pathways of sulfate assimilation and transsulfuration of L-methionine better enables *L. monocytogenes* to sense L-cysteine availability in its environment and tune PrfA activity accordingly.

In this model of *L. monocytogenes* L-cysteine auxotrophy, *L. monocytogenes* could tune PrfA activity based on L-cysteine availability in different environments. The host cytoplasm would thus represent a cysteine-rich environment, with a higher concentration of cysteine-containing peptides and millimolar quantities of GSH. The saprophytic environment would then represent a low-cysteine environment, where the primary L-cysteine sources are low-cysteine-containing plant- and microbial-derived peptides and oxidized L-cystine, whose import is heavily regulated to maintain very low levels of free intracellular reduced L-cysteine (41, 47, 48, 54, 61, 62, 104).

Considering this model, questions remain regarding the purpose of the intact CysE/CysK two-step biosynthetic pathway: is the pathway maintained to support growth in a distinct environmental niche or are the CysE and CysK enzymes independently important for *L. monocytogenes* metabolism and justify conservation of the pathway?

Evidence exists in support of both lines of reasoning. As previously discussed, the CysE/CysK pathway typically produces L-cysteine from assimilated sulfate, however our work indicated that *L. monocytogenes* utilizes the inorganic sulfur sources H_2S and thiosulfate in a CysK-dependent manner. Further, there are distinct niches during saprophytic growth and infection that may provide millimolar quantities of these substrates and can support bacterial growth in the absence of other L-cysteine sources. On the other hand, it is also known that CysE and CysK are independently important in other realms of *L. monocytogenes* metabolism. CysE, but not CysK, was recently shown to be required for *L. monocytogenes* growth in both broth and macrophages (105). Though the mechanism underlying this requirement for CysE remains unclear, the essentiality of CysE is likely due to the importance of its catalytic product, O-acetylserine, whose concentration is an important indicator of the intracellular cysteine pool in Gram-positive bacteria and has been proposed as an extracellular signaling molecule in *E. coli* (54, 104, 106). CysK is also known to have a secondary function as a sensor of the intracellular L-cysteine pool via O-acetylserine and complexes with the master regulator of cysteine metabolism, CymR, to regulate import of L-cystine (41, 54, 104). Thus, the functional importance of the CysE/CysK pathway is clear, though it remains uncertain if L-cysteine production from limited inorganic sulfur sources is the primary or adventitious role of this pathway. The inessentiality of CysK in the previously mentioned study suggests that L-cysteine production is an adventitious role of the pathway, however growth was assessed only in rich, complex media and macrophages, so more work is necessary to determine if the pathway offers *L. monocytogenes* an advantage in distinct H_2S- and thiosulfate-containing niches.

3.2.5 Concluding remarks

These are exciting times to be a microbiologist and scientist, especially in the *Listeria* field. While the Portnoy lab has spent the better part of the last four decades picking apart the minutiae of *L. monocytogenes* physiology and pathogenesis, it only becomes clearer with each passing day that there is still so much for us to learn. Every new technique and piece of information opens new avenues of inquiry into every aspect of bacterial biology, and the knowledge gleaned presents us with new ways to explore the biology of other bacterial pathogens and mammalian hosts alike. It is inspiring to see how the diligent work in the *Listeria* field has informed so many other successful inquiries into host cell biology and other pathogens, and it is even more inspiring to see the successful use of *L. monocytogenes* as a cancer vaccine in recent years. The anti-tumor application of *L. monocytogenes* and other bacteria is a fascinating concept, and the field is well on its way to developing *Listeria*-derived safe, efficacious cancer vaccines. The idea that bacterial pathogens that cause disease in humans can be harnessed to treat some of our most nefarious diseases is truly wild, and I hope to see these vaccines in more widespread use during my lifetime. I am proud to know that my work has contributed, even in the smallest of ways, to our overall understanding of a bacterium with such noble prospects.

While much about *L. monocytogenes* has been studied in the context of virulence, relatively little work has been done in parsing out unique elements of its metabolism. Prior to the discovery of *gshF* and the role of GSH production in PrfA activation and virulence, relatively little work had been done to explore the unique facets

of cysteine metabolism in *L. monocytogenes*. If nothing else, our work here emphasizes how little we know about such an important aspect of *L. monocytogenes* physiology and pathogenesis and how much more there is to learn. I can't help but wonder what other unique aspects of *L. monocytogenes* remain to be discovered and how we might be surprised at what we find. Parsing out metabolism is almost always a challenging and fickle endeavor, and it is always wise to use prior discoveries to inform us about where to direct our energies. In this fashion, the Portnoy lab has largely used virulence phenotypes to guide our inquiries into *L. monocytogenes* metabolism. This is a powerful (and fundable) approach, and it maximizes the reward for the effort applied. This approach has worked for many years and produced many valuable discoveries, and one would expect that this strategy will continue to work in the future. But in the spirit of basic science, I do hope that others dedicate time and energy to simply expanding our knowledge of all facets of *L. monocytogenes* metabolism. You never know what we might find.

We live in fascinating times.

References

1. Freitag NE, Port GC, Miner MD. 2009. Listeria monocytogenes - from saprophyte to intracellular pathogen. Nat Rev Microbiol 7:623-8.
2. Koopmans MM, Brouwer MC, Vázquez-Boland JA, van de Beek D. 2023. Human Listeriosis. Clin Microbiol Rev 36:e0006019.
3. Freitag NE. 2006. From hot dogs to host cells: how the bacterial pathogen Listeria monocytogenes regulates virulence gene expression. Future Microbiol 1:89-101.
4. Swaminathan B, Gerner-Smidt P. 2007. The epidemiology of human listeriosis. Microbes Infect 9:1236-43.
5. Radoshevich L, Cossart P. 2018. Listeria monocytogenes: towards a complete picture of its physiology and pathogenesis. Nat Rev Microbiol 16:32-46.
6. Portnoy DA, Auerbuch V, Glomski IJ. 2002. The cell biology of Listeria monocytogenes infection: the intersection of bacterial pathogenesis and cell-mediated immunity. J Cell Biol 158:409-14.
7. Vance RE, Isberg RR, Portnoy DA. 2009. Patterns of pathogenesis: discrimination of pathogenic and nonpathogenic microbes by the innate immune system. Cell Host Microbe 6:10-21.
8. Vance RE, Eichberg MJ, Portnoy DA, Raulet DH. 2017. Listening to each other: Infectious disease and cancer immunology. Sci Immunol 2.
9. Welch MD, Iwamatsu A, Mitchison TJ. 1997. Actin polymerization is induced by Arp2/3 protein complex at the surface of Listeria monocytogenes. Nature 385:265-9.
10. Johansson J, Freitag NE. 2019. Regulation of *Listeria monocytogenes* virulence. Microbiol Spectr 7.
11. Drolia R, Bhunia AK. 2019. Crossing the Intestinal Barrier via Listeria Adhesion Protein and Internalin A. Trends Microbiol 27:408-425.
12. Jones S, Portnoy DA. 1994. Characterization of Listeria monocytogenes pathogenesis in a strain expressing perfringolysin O in place of listeriolysin O. Infect Immun 62:5608-13.
13. Schuerch DW, Wilson-Kubalek EM, Tweten RK. 2005. Molecular basis of listeriolysin O pH dependence. Proc Natl Acad Sci U S A 102:12537-42.
14. Schnupf P, Portnoy DA. 2007. Listeriolysin O: a phagosome-specific lysin. Microbes Infect 9:1176-87.
15. Chico-Calero I, Suárez M, González-Zorn B, Scortti M, Slaghuis J, Goebel W, Vázquez-Boland JA, Consortium ELG. 2002. Hpt, a bacterial homolog of the microsomal glucose- 6-phosphate translocase, mediates rapid intracellular proliferation in Listeria. Proc Natl Acad Sci U S A 99:431-6.
16. Mitchell G, Ge L, Huang Q, Chen C, Kianian S, Roberts MF, Schekman R, Portnoy DA. 2015. Avoidance of autophagy mediated by PlcA or ActA is required for Listeria monocytogenes growth in macrophages. Infect Immun 83:2175-84.
17. Pillich H, Puri M, Chakraborty T. 2017. ActA of Listeria monocytogenes and Its Manifold Activities as an Important Listerial Virulence Factor. Curr Top Microbiol Immunol 399:113-132.

18. Mitchell G, Cheng MI, Chen C, Nguyen BN, Whiteley AT, Kianian S, Cox JS, Green DR, McDonald KL, Portnoy DA. 2018. *Listeria monocytogenes* triggers noncanonical autophagy upon phagocytosis, but avoids subsequent growth-restricting xenophagy. Proc Natl Acad Sci U S A 115:E210-E217.
19. Leimeister-Wächter M, Haffner C, Domann E, Goebel W, Chakraborty T. 1990. Identification of a gene that positively regulates expression of listeriolysin, the major virulence factor of listeria monocytogenes. Proc Natl Acad Sci U S A 87:8336-40.
20. Mengaud J, Dramsi S, Gouin E, Vazquez-Boland JA, Milon G, Cossart P. 1991. Pleiotropic control of Listeria monocytogenes virulence factors by a gene that is autoregulated. Mol Microbiol 5:2273-83.
21. Chakraborty T, Leimeister-Wächter M, Domann E, Hartl M, Goebel W, Nichterlein T, Notermans S. 1992. Coordinate regulation of virulence genes in Listeria monocytogenes requires the product of the prfA gene. J Bacteriol 174:568-74.
22. de las Heras A, Cain RJ, Bielecka MK, Vázquez-Boland JA. 2011. Regulation of Listeria virulence: PrfA master and commander. Curr Opin Microbiol 14:118-27.
23. Bruno JC, Freitag NE. 2010. Constitutive activation of PrfA tilts the balance of Listeria monocytogenes fitness towards life within the host versus environmental survival. PLoS One 5:e15138.
24. Vasanthakrishnan RB, de Las Heras A, Scortti M, Deshayes C, Colegrave N, Vázquez-Boland JA. 2015. PrfA regulation offsets the cost of Listeria virulence outside the host. Environ Microbiol 17:4566-79.
25. Böckmann R, Dickneite C, Middendorf B, Goebel W, Sokolovic Z. 1996. Specific binding of the Listeria monocytogenes transcriptional regulator PrfA to target sequences requires additional factor(s) and is influenced by iron. Mol Microbiol 22:643-53.
26. Leimeister-Wächter M, Domann E, Chakraborty T. 1992. The expression of virulence genes in Listeria monocytogenes is thermoregulated. J Bacteriol 174:947-52.
27. Renzoni A, Klarsfeld A, Dramsi S, Cossart P. 1997. Evidence that PrfA, the pleiotropic activator of virulence genes in Listeria monocytogenes, can be present but inactive. Infect Immun 65:1515-8.
28. Johansson J, Mandin P, Renzoni A, Chiaruttini C, Springer M, Cossart P. 2002. An RNA thermosensor controls expression of virulence genes in Listeria monocytogenes. Cell 110:551-61.
29. Freitag NE, Rong L, Portnoy DA. 1993. Regulation of the prfA transcriptional activator of Listeria monocytogenes: multiple promoter elements contribute to intracellular growth and cell-to-cell spread. Infect Immun 61:2537-44.
30. Reniere ML, Whiteley AT, Portnoy DA. 2016. An In Vivo Selection Identifies Listeria monocytogenes Genes Required to Sense the Intracellular Environment and Activate Virulence Factor Expression. PLoS Pathog 12:e1005741.
31. Deshayes C, Bielecka MK, Cain RJ, Scortti M, de las Heras A, Pietras Z, Luisi BF, Núñez Miguel R, Vázquez-Boland JA. 2012. Allosteric mutants show that PrfA activation is dispensable for vacuole escape but required for efficient spread and Listeria survival in vivo. Mol Microbiol 85:461-77.

32. Reniere ML, Whiteley AT, Hamilton KL, John SM, Lauer P, Brennan RG, Portnoy DA. 2015. Glutathione activates virulence gene expression of an intracellular pathogen. Nature 517:170-3.
33. Hall M, Grundström C, Begum A, Lindberg MJ, Sauer UH, Almqvist F, Johansson J, Sauer-Eriksson AE. 2016. Structural basis for glutathione-mediated activation of the virulence regulatory protein PrfA in Listeria. Proc Natl Acad Sci U S A 113:14733-14738.
34. Masip L, Veeravalli K, Georgiou G. 2006. The many faces of glutathione in bacteria. Antioxid Redox Signal 8:753-62.
35. Anaya-Sanchez A, Feng Y, Berude JC, Portnoy DA. 2021. Detoxification of methylglyoxal by the glyoxalase system is required for glutathione availability and virulence activation in Listeria monocytogenes. PLoS Pathog 17:e1009819.
36. Meister A, Anderson ME. 1983. Glutathione. Annu Rev Biochem 52:711-60.
37. Newton GL, Buchmeier N, Fahey RC. 2008. Biosynthesis and functions of mycothiol, the unique protective thiol of Actinobacteria. Microbiol Mol Biol Rev 72:471-94.
38. Newton GL, Rawat M, La Clair JJ, Jothivasan VK, Budiarto T, Hamilton CJ, Claiborne A, Helmann JD, Fahey RC. 2009. Bacillithiol is an antioxidant thiol produced in Bacilli. Nat Chem Biol 5:625-7.
39. Gopal S, Borovok I, Ofer A, Yanku M, Cohen G, Goebel W, Kreft J, Aharonowitz Y. 2005. A multidomain fusion protein in Listeria monocytogenes catalyzes the two primary activities for glutathione biosynthesis. J Bacteriol. 187:3839-47.
40. Portman JL, Dubensky SB, Peterson BN, Whiteley AT, Portnoy DA. 2017. Activation of the Listeria monocytogenes virulence program by a reducing environment. mBio 8.
41. Brenner M, Friedman S, Haber A, Livnat-Levanon N, Borovok I, Sigal N, Lewinson O, Herskovits AA. 2022. Listeria monocytogenes TcyKLMN Cystine/Cysteine Transporter Facilitates Glutathione Synthesis and Virulence Gene Expression. mBio 13:e0044822.
42. Janowiak BE, Griffith OW. 2005. Glutathione synthesis in Streptococcus agalactiae. One protein accounts for gamma-glutamylcysteine synthetase and glutathione synthetase activities. J Biol Chem 280:11829-39.
43. Imlay JA. 2019. Where in the world do bacteria experience oxidative stress? Environ Microbiol 21:521-530.
44. Premaratne RJ, Lin WJ, Johnson EA. 1991. Development of an improved chemically defined minimal medium for Listeria monocytogenes. Appl Environ Microbiol 57:3046-8.
45. Tsai HN, Hodgson DA. 2003. Development of a synthetic minimal medium for Listeria monocytogenes. Appl Environ Microbiol 69:6943-5.
46. Sauer JD, Herskovits AA, O'Riordan MXD. 2019. Metabolism of the Gram-Positive Bacterial Pathogen Listeria monocytogenes. Microbiol Spectr 7.
47. Park S, Imlay JA. 2003. High levels of intracellular cysteine promote oxidative DNA damage by driving the fenton reaction. J Bacteriol 185:1942-50.
48. Korshunov S, Imlay KRC, Imlay JA. 2020. Cystine import is a valuable but risky process whose hazards Escherichia coli minimizes by inducing a cysteine exporter. Mol Microbiol 113:22-39.

49. Zhou Y, Imlay JA. 2020. Escherichia coli K-12 Lacks a High-Affinity Assimilatory Cysteine Importer. mBio 11.
50. Zhou Y, Imlay JA. 2022. Escherichia coli Uses a Dedicated Importer and Desulfidase To Ferment Cysteine. mBio 13:e0296521.
51. Xayarath B, Marquis H, Port GC, Freitag NE. 2009. Listeria monocytogenes CtaP is a multifunctional cysteine transport-associated protein required for bacterial pathogenesis. Mol Microbiol 74:956-73.
52. Vaval Taylor DM, Xayarath B, Freitag NE. 2023. Two Permeases Associated with the Multifunctional CtaP Cysteine Transport System in Listeria monocytogenes Play Distinct Roles in Pathogenesis. Microbiol Spectr 11:e0331722.
53. Imlay JA. 2015. Transcription Factors That Defend Bacteria Against Reactive Oxygen Species. Annu Rev Microbiol 69:93-108.
54. Soutourina O, Poupel O, Coppée JY, Danchin A, Msadek T, Martin-Verstraete I. 2009. CymR, the master regulator of cysteine metabolism in Staphylococcus aureus, controls host sulphur source utilization and plays a role in biofilm formation. Mol Microbiol 73:194-211.
55. Fujishima K, Wang KM, Palmer JA, Abe N, Nakahigashi K, Endy D, Rothschild LJ. 2018. Reconstruction of cysteine biosynthesis using engineered cysteine-free enzymes. Sci Rep 8:1776.
56. Glaser P, Frangeul L, Buchrieser C, Rusniok C, Amend A, Baquero F, Berche P, Bloecker H, Brandt P, Chakraborty T, Charbit A, Chetouani F, Couvé E, de Daruvar A, Dehoux P, Domann E, Domínguez-Bernal G, Duchaud E, Durant L, Dussurget O, Entian KD, Fsihi H, García-del Portillo F, Garrido P, Gautier L, Goebel W, Gómez-López N, Hain T, Hauf J, Jackson D, Jones LM, Kaerst U, Kreft J, Kuhn M, Kunst F, Kurapkat G, Madueno E, Maitournam A, Vicente JM, Ng E, Nedjari H, Nordsiek G, Novella S, de Pablos B, Pérez-Diaz JC, Purcell R, Remmel B, Rose M, Schlueter T, Simoes N, et al. 2001. Comparative genomics of Listeria species. Science 294:849-52.
57. Wheeler PR, Coldham NG, Keating L, Gordon SV, Wooff EE, Parish T, Hewinson RG. 2005. Functional demonstration of reverse transsulfuration in the Mycobacterium tuberculosis complex reveals that methionine is the preferred sulfur source for pathogenic Mycobacteria. J Biol Chem 280:8069-78.
58. Farrell M, Hill PW, Farrar J, DeLuca TH, Roberts P, Kielland K, Dahlgren R, Murphy DV, Hobbs PJ, Bardgett RD. 2013. Oligopeptides represent a preferred source of organic N uptake: a global phenomenon? *Ecosystems.* 16:133–145.
59. Broughton RCI, Newsham KK, Hill PW, Stott A, Jones DL. 2015. Differential acquisition of amino acids and peptide enantiomers within the soil microbial community and its implications for carbon and nitrogen cycling in soil. *Soil Biol. Biochem.* 88:83–89.
60. Borezee E, Pellegrini E, Berche P. 2000. OppA of Listeria monocytogenes, an oligopeptide-binding protein required for bacterial growth at low temperature and involved in intracellular survival. Infect Immun 68:7069-77.
61. Krypotou E, Scortti M, Grundström C, Oelker M, Luisi BF, Sauer-Eriksson AE, Vázquez-Boland J. 2019. Control of Bacterial Virulence through the Peptide Signature of the Habitat. Cell Rep 26:1815-1827.e5.

62. Miseta A, Csutora P. 2000. Relationship between the occurrence of cysteine in proteins and the complexity of organisms. Mol Biol Evol 17:1232-9.
63. Parry J, Clark DP. 2002. Identification of a CysB-regulated gene involved in glutathione transport in Escherichia coli. FEMS Microbiol Lett 209:81-5.
64. Suzuki H, Koyanagi T, Izuka S, Onishi A, Kumagai H. 2005. The yliA, -B, -C, and -D genes of Escherichia coli K-12 encode a novel glutathione importer with an ATP-binding cassette. J Bacteriol 187:5861-7.
65. Alkhuder K, Meibom KL, Dubail I, Dupuis M, Charbit A. 2009. Glutathione provides a source of cysteine essential for intracellular multiplication of Francisella tularensis. PLoS Pathog 5:e1000284.
66. Potter AJ, Trappetti C, Paton JC. 2012. Streptococcus pneumoniae uses glutathione to defend against oxidative stress and metal ion toxicity. J Bacteriol 194:6248-54.
67. Vergauwen B, Verstraete K, Senadheera DB, Dansercoer A, Cvitkovitch DG, Guédon E, Savvides SN. 2013. Molecular and structural basis of glutathione import in Gram-positive bacteria via GshT and the cystine ABC importer TcyBC of Streptococcus mutans. Mol Microbiol 89:288-303.
68. Brouwer S, Jespersen MG, Ong CY, De Oliveira DMP, Keller B, Cork AJ, Djoko KY, Davies MR, Walker MJ. 2022. Streptococcus pyogenes Hijacks Host Glutathione for Growth and Innate Immune Evasion. mBio 13:e0067622.
69. Lensmire JM, Wischer MR, Kraemer-Zimpel C, Kies PJ, Sosinski L, Ensink E, Dodson JP, Shook JC, Delekta PC, Cooper CC, Havlichek DH, Mulks MH, Lunt SY, Ravi J, Hammer ND. 2023. The glutathione import system satisfies the Staphylococcus aureus nutrient sulfur requirement and promotes interspecies competition. PLoS Genet 19:e1010834.
70. Singh P, Teal TK, Marsh TL, Tiedje JM, Mosci R, Jernigan K, Zell A, Newton DW, Salimnia H, Lephart P, Sundin D, Khalife W, Britton RA, Rudrik JT, Manning SD. 2015. Intestinal microbial communities associated with acute enteric infections and disease recovery. Microbiome 3:45.
71. Dordević D, Jančíková S, Vítězová M, Kushkevych I. 2021. Hydrogen sulfide toxicity in the gut environment: Meta-analysis of sulfate-reducing and lactic acid bacteria in inflammatory processes. Journal of Advanced Research 27:55-69.
72. Nepal S, Kumar P. 2020. Growth, Cell Division, and Gene Expression of Escherichia coli at Elevated Concentrations of Magnesium Sulfate: Implications for Habitability of Europa and Mars. Microorganisms 8.
73. Kawano Y, Suzuki K, Ohtsu I. 2018. Current understanding of sulfur assimilation metabolism to biosynthesize L-cysteine and recent progress of its fermentative overproduction in microorganisms. Appl Microbiol Biotechnol 102:8203-8211.
74. Mathai JC, Missner A, Kügler P, Saparov SM, Zeidel ML, Lee JK, Pohl P. 2009. No facilitator required for membrane transport of hydrogen sulfide. Proc Natl Acad Sci U S A 106:16633-8.
75. Tanaka Y, Yoshikaie K, Takeuchi A, Ichikawa M, Mori T, Uchino S, Sugano Y, Hakoshima T, Takagi H, Nonaka G, Tsukazaki T. 2020. Crystal structure of a YeeE/YedE family protein engaged in thiosulfate uptake. Sci Adv 6:eaba7637.

76. Furne J, Saeed A, Levitt MD. 2008. Whole tissue hydrogen sulfide concentrations are orders of magnitude lower than presently accepted values. Am J Physiol Regul Integr Comp Physiol 295:R1479-85.
77. Karunya R, Jayaprakash KS, Gaikwad R, Sajeesh P, Ramshad K, Muraleedharan KM, Dixit M, Thangaraj PR, Sen AK. 2019. Rapid measurement of hydrogen sulphide in human blood plasma using a microfluidic method. Sci Rep 9:3258.
78. Tomich PK, An FY, Clewell DB. 1980. Properties of erythromycin-inducible transposon Tn917 in Streptococcus faecalis. J Bacteriol 141:1366-74.
79. Ripio MT, Domínguez-Bernal G, Lara M, Suárez M, Vazquez-Boland JA. 1997. A Gly145Ser substitution in the transcriptional activator PrfA causes constitutive overexpression of virulence factors in Listeria monocytogenes. J Bacteriol 179:1533-40.
80. Sirko A, Zatyka M, Sadowy E, Hulanicka D. 1995. Sulfate and thiosulfate transport in Escherichia coli K-12: evidence for a functional overlapping of sulfate- and thiosulfate-binding proteins. J Bacteriol 177:4134-6.
81. Hicks JL, Mullholland CV. 2018. Cysteine biosynthesis in Neisseria species. Microbiology (Reading) 164:1471-1480.
82. Mansilla MC, de Mendoza D. 2000. The Bacillus subtilis cysP gene encodes a novel sulphate permease related to the inorganic phosphate transporter (Pit) family. Microbiology (Reading) 146 (Pt 4):815-821.
83. Winter SE, Thiennimitr P, Winter MG, Butler BP, Huseby DL, Crawford RW, Russell JM, Bevins CL, Adams LG, Tsolis RM, Roth JR, Bäumler AJ. 2010. Gut inflammation provides a respiratory electron acceptor for Salmonella. Nature 467:426-9.
84. Karnachuk OV, Beletsky AV, Rakitin AL, Ikkert OP, Avakyan MR, Zyusman VS, Napilov A, Mardanov AV, Ravin NV. 2023. Antibiotic-resistant Desulfovibrio produces H_2S from supplements for animal farming. Microorganisms 11.
85. Sokurenko EV, Hasty DL, Dykhuizen DE. 1999. Pathoadaptive mutations: gene loss and variation in bacterial pathogens. Trends Microbiol 7:191-5.
86. Seif Y, Choudhary KS, Hefner Y, Anand A, Yang L, Palsson BO. 2020. Metabolic and genetic basis for auxotrophies in Gram-negative species. Proc Natl Acad Sci U S A 117:6264-6273.
87. O'Riordan M, Moors MA, Portnoy DA. 2003. Listeria intracellular growth and virulence require host-derived lipoic acid. Science 302:462-4.
88. Legoux F, Salou M, Lantz O. 2020. MAIT Cell Development and Functions: the Microbial Connection. Immunity 53:710-723.
89. Rivera-Lugo R, Light SH, Garelis NE, Portnoy DA. 2022. RibU is an essential determinant of Listeria pathogenesis that mediates acquisition of FMN and FAD during intracellular growth. Proc Natl Acad Sci U S A 119:e2122173119.
90. Lithgow JK, Hayhurst EJ, Cohen G, Aharonowitz Y, Foster SJ. 2004. Role of a cysteine synthase in Staphylococcus aureus. J Bacteriol 186:1579-90.
91. Lensmire JM, Dodson JP, Hsueh BY, Wischer MR, Delekta PC, Shook JC, Ottosen EN, Kies PJ, Ravi J, Hammer ND. 2020. The Staphylococcus aureus Cystine Transporters TcyABC and TcyP Facilitate Nutrient Sulfur Acquisition during Infection. Infect Immun 88.

92. Whiteley AT, Garelis NE, Peterson BN, Choi PH, Tong L, Woodward JJ, Portnoy DA. 2017. c-di-AMP modulates Listeria monocytogenes central metabolism to regulate growth, antibiotic resistance and osmoregulation. Mol Microbiol 104:212-233.
93. Camilli A, Tilney LG, Portnoy DA. 1993. Dual roles of plcA in Listeria monocytogenes pathogenesis. Mol Microbiol 8:143-57.
94. Lauer P, Chow MY, Loessner MJ, Portnoy DA, Calendar R. 2002. Construction, characterization, and use of two Listeria monocytogenes site-specific phage integration vectors. J Bacteriol 184:4177-86.
95. Rae CS, Geissler A, Adamson PC, Portnoy DA. 2011. Mutations of the Listeria monocytogenes peptidoglycan N-deacetylase and O-acetylase result in enhanced lysozyme sensitivity, bacteriolysis, and hyperinduction of innate immune pathways. Infect Immun 79:3596-606.
96. Louie A, Zhang T, Becattini S, Waldor MK, Portnoy DA. 2019. A Multiorgan Trafficking Circuit Provides Purifying Selection of Listeria monocytogenes Virulence Genes. mBio 10.
97. Bécavin C, Bouchier C, Lechat P, Archambaud C, Creno S, Gouin E, Wu Z, Kühbacher A, Brisse S, Pucciarelli MG, García-del Portillo F, Hain T, Portnoy DA, Chakraborty T, Lecuit M, Pizarro-Cerdá J, Moszer I, Bierne H, Cossart P. 2014. Comparison of widely used Listeria monocytogenes strains EGD, 10403S, and EGD-e highlights genomic variations underlying differences in pathogenicity. mBio 5:e00969-14.
98. Whiteley AT, Pollock AJ, Portnoy DA. 2015. The PAMP c-di-AMP Is Essential for Listeria monocytogenes Growth in Rich but Not Minimal Media due to a Toxic Increase in (p)ppGpp. [corrected]. Cell Host Microbe 17:788-98.
99. Jones S, Portnoy DA. 1994. Characterization of Listeria monocytogenes pathogenesis in a strain expressing perfringolysin O in place of listeriolysin O. Infect Immun 62:5608-13.
100. Miner MD, Port GC, Freitag NE. 2008. Functional impact of mutational activation on the Listeria monocytogenes central virulence regulator PrfA. Microbiology (Reading) 154:3579-3589.
101. Belila A, Abbas B, Fazaa I, Saidi N, Snoussi M, Hassen A, Muyzer G. 2013. Sulfur bacteria in wastewater stabilization ponds periodically affected by the 'red-water' phenomenon. Appl Microbiol Biotechnol 97:379-94.
102. Bhatnagar S, Cowley ES, Kopf SH, Pérez Castro S, Kearney S, Dawson SC, Hanselmann K, Emil Ruff S. 2020. Microbial community dynamics and coexistence in a sulfide-driven phototrophic bloom. *Environmental Microbiome.* 15, 3.
103. Ryon MG, Stewart AJ, Kszos LA, Phipps TL. 2002. Impacts on Streams from the Use of Sulfur-Based Compounds for Dechlorinating Industrial Effluents. *Water, Air, & Soil Pollution.* 136, 255–268.
104. Tanous C, Soutourina O, Raynal B, Hullo MF, Mervelet P, Gilles AM, Noirot P, Danchin A, England P, Martin-Verstraete I. 2008. The CymR regulator in complex with the enzyme CysK controls cysteine metabolism in Bacillus subtilis. J Biol Chem 283:35551-60.

105. Fischer MA, Engelgeh T, Rothe P, Fuchs S, Thürmer A, Halbedel S. 2022. *Listeria monocytogenes* genes supporting growth under standard laboratory cultivation conditions and during macrophage infection. Genome Res 32:1711-26.
106. Sturgill G, Toutain CM, Komperda J, O'Toole GA, Rather PN. 2004. Role of CysE in production of an extracellular signaling molecule in Providencia stuartii and Escherichia coli: loss of CysE enhances biofilm formation in Escherichia coli. J Bacteriol 186:7610-7.