The global redox-responsive transcriptional regulator Rex represses fermentative metabolism and is required for *Listeria monocytogenes* pathogenesis

Short Title: Rex regulation and pathogenesis

Cortney R. Halsey¹, Maureen K. Thomason¹, Rochelle C. Glover¹, Michelle L. Reniere^{1*}

Department of Microbiology, University of Washington School of Medicine, Seattle, Washington, USA.

* Corresponding author

Email: reniere@uw.edu

ABSTRACT

The Gram-positive bacterium *Listeria monocytogenes* is the causative agent of the foodborne disease listeriosis, one of the deadliest bacterial infections known. In order to cause disease, *L. monocytogenes* must properly coordinate its metabolic and virulence programs in response to rapidly changing environments within the host. However, the mechanisms by which *L. monocytogenes* senses and adapts to the many stressors encountered as it transits through the gastrointestinal (GI) tract and disseminates to peripheral organs are not well understood. In this study, we investigated the role of the redox-responsive transcriptional regulator Rex in *L. monocytogenes* growth and pathogenesis. Rex is a conserved canonical transcriptional repressor that monitors the intracellular redox state of the cell by sensing the ratio of reduced and oxidized nicotinamide adenine dinucleotides (NADH and NAD⁺, respectively). Here, we demonstrated that *L. monocytogenes* Rex represses fermentative metabolism and is therefore required for optimal growth in the presence of oxygen. We also show that Rex represses the production of virulence factors required for survival and invasion of the GI tract, as a strain lacking *rex* was more resistant to acidified bile and invaded host cells better than wt. Consistent with these results, Rex was dispensable for colonizing the GI tract and disseminating to peripheral organs in an oral listeriosis model of infection. However, Rex-dependent regulation was required for colonizing the spleen and liver, and *L. monocytogenes* lacking the Rex repressor were nearly sterilized from the gallbladder. Taken together, these results demonstrated that Rex functions as a repressor of fermentative metabolism and suggests a role for Rex-dependent regulation in *L. monocytogenes* pathogenesis. Importantly, the gallbladder is the bacterial reservoir

36 during listeriosis, and our data suggest redox sensing and Rex-dependent regulation are
37 necessary for bacterial survival and replication in this organ.

AUTHOR SUMMARY

Listeriosis is a foodborne illness caused by *Listeria monocytogenes* and is one of the deadliest bacterial infections known, with a mortality rate of up to 30%. Following ingestion of contaminated food, *L. monocytogenes* disseminates from the gastrointestinal (GI) tract to peripheral organs, including the spleen, liver, and gallbladder. In this work, we investigated the role of the global redox-responsive regulator Rex in *L. monocytogenes* growth and pathogenesis. We demonstrated that Rex derepression coordinates expression of genes necessary in the GI tract during infection, including fermentative metabolism, bile resistance, and invasion of host cells. Accordingly, Rex was dispensable for colonizing the GI tract of mice during an oral listeriosis infection. Interestingly, Rex-dependent regulation was required for bacterial replication in the spleen, liver, and gallbladder. Taken together, our results demonstrate that Rex-mediated redox sensing and transcriptional regulation are important for *L. monocytogenes* metabolic adaptation and virulence.

INTRODUCTION

To successfully colonize different niches, bacteria must be able to rapidly sense and respond to environmental changes. The Gram-positive bacterium *Listeria monocytogenes* is an excellent example of this adaptability. As a saprophyte and intracellular pathogen, *L. monocytogenes* must coordinate its metabolic and virulence programs to transition from life in nature to the mammalian host where it causes the foodborne disease listeriosis. Following ingestion of contaminated foods by the host, *L. monocytogenes* contends with acid stress in the stomach and acidic bile in the small intestine before descending to the cecum where it traverses the intestinal barrier [1]. Traveling via the lymph or blood, *L. monocytogenes* disseminates to the spleen and liver where it replicates intracellularly. The intracellular lifecycle requires *L. monocytogenes* to quickly escape the oxidizing vacuolar compartment to replicate in the highly reducing environment of the cytosol and then spread cell-to-cell [2–5]. From the liver, the bacteria enter the gallbladder and replicate extracellularly to very high densities and then reseed the intestinal tract upon bile secretion [6–8]. Bile itself is antimicrobial, acting as a detergent that disrupts bacterial membranes and denatures proteins [9]. Although *L. monocytogenes* virulence determinants have been investigated for decades, the vast majority of studies injected the bacteria intravenously rather than infecting mice through the natural foodborne route [10,11]. Therefore, the mechanisms by which *L. monocytogenes* senses and adapts to the many stressors of the host environment during oral infection are not well understood.

Given its ability to replicate in diverse environmental niches, it is critical for *L. monocytogenes* to appropriately modify its metabolism in response to the changing

extracellular surroundings. Reduced and oxidized nicotinamide adenine dinucleotides (NADH and NAD⁺, respectively) play important roles in many biological processes and are therefore key molecules for sensing the intracellular redox state. [12]. For example, during aerobic respiration the NADH:NAD⁺ ratio is kept low as NADH is oxidized to NAD⁺ by the electron transport chain (ETC). In hypoxic environments or when the ETC is inhibited, NADH levels become elevated and NAD⁺ is no longer available to fuel carbon oxidation for growth. Therefore, the NADH:NAD⁺ ratio is the primary indicator of the metabolic state of a cell.

The transcriptional repressor Rex monitors the intracellular redox state of the cell by directly sensing the NADH:NAD⁺ ratio and repressing target genes when this ratio is low [13,14]. An increase in relative NADH abundance following reduced respiration results in Rex dissociating from DNA and derepression of target genes [15–18]. Rex is widely conserved across Gram-positive bacteria and while there is considerable variability in the identity of Rex-dependent genes among organisms, Rex generally functions to regulate metabolic pathways involved in NAD⁺-regeneration, such as fermentation [13,14].

L. monocytogenes encodes a Rex protein that shares 65% and 56% identity with homologues in *Bacillus subtilis* and *Staphylococcus aureus* [19]. We hypothesized *L. monocytogenes* Rex may be important during infection to sense the changing environment and regulate metabolic pathways accordingly. In this study, we identified Rex-dependent transcriptional changes in *L. monocytogenes* and demonstrated a role for Rex regulation during oral listeriosis.

RESULTS

Transcriptomics identifies Rex-regulated genes

To investigate the role of Rex in *L. monocytogenes*, we generated a Δrex mutant via allelic exchange and analyzed the Rex-dependent transcriptome under standard growth conditions. RNA sequencing (RNA-seq) was performed on RNA harvested from mid-log and stationary phase cultures of wild type (wt) and Δrex strains grown aerobically in brain heart infusion (BHI) broth (S1-S4 Tables). We did not observe dramatic growth phase-dependent differences in Rex-dependent regulation so here we focus on the stationary phase results for simplicity. In the Δrex mutant, 196 transcripts were significantly increased in abundance at least two-fold ($p < 0.01$), indicating these genes are repressed by Rex during aerobic growth (Table 1 and S1 Table). Some of the most dramatically increased transcripts were involved in fermentative metabolism, including those encoding alcohol dehydrogenase (*lap*), pyruvate formate lyase (*pflA* and *pflBC*), and lactate dehydrogenase (*ldhA*) (Table 1). Unexpectedly, transcripts encoding virulence factors involved in bile resistance (*bsh*, bile salt hydrolase) and host cell invasion (*inlAB*, internalin A and B) were in greater abundance in the Δrex mutant, indicating Rex-dependent regulation may impact virulence.

Table 1. Rex-repressed genes-of-interest.

LMRG	Lmo	Gene	Function	Fold change in Δrex
LMRG_01332	Imo1634	<i>lap</i>	bifunctional acetaldehyde-CoA/alcohol dehydrogenase	342.30
LMRG_00859	Imo1407	<i>pflC</i>	pyruvate formate-lyase 1-activating enzyme	88.30
LMRG_00858	Imo1406	<i>pflB</i>	formate acetyltransferase	59.84
LMRG_00046	Imo0355	<i>frdA</i>	fumarate reductase flavoprotein subunit	85.24
LMRG_01064	Imo1917	<i>pflA</i>	formate acetyltransferase	77.91
LMRG_01979	Imo2717	<i>cydB</i>	cytochrome d ubiquinol oxidase subunit II	19.05

LMRG_01980	Imo2716	<i>cydC</i>	ABC transporter	17.62
LMRG_01981	Imo2715	<i>cydD</i>	ABC transporter	15.76
LMRG_01978	Imo2718	<i>cydA</i>	cytochrome bd-I oxidase subunit I	13.99
LMRG_01659	Imo2173	-	sigma-54-dependent transcriptional regulator	16.62
LMRG_00127	Imo0434	<i>inlB</i>	internalin B	10.97
LMRG_00126	Imo0433	<i>inlA</i>	internalin A	10.26
LMRG_01801	Imo2447	-	Rgg/GadR/MutR family transcriptional regulator	10.76
LMRG_02012	Imo0912	-	formate transporter	10.71
LMRG_01217	Imo2067	<i>bsh</i>	bile acid hydrolase	5.57
LMRG_02632	Imo0210	<i>ldhA</i>	lactate dehydrogenase	3.30

Highlighted genes are predicted to be in an operon [20].

In silico promoter analysis of genes exhibiting Rex-dependent regulation was performed to determine potential Rex binding sites using the *Bacillus subtilis* Rex consensus sequence [14]. We identified potential Rex binding sites in the promoter regions of 48 genes and/or operons repressed by Rex (S5 Table). Specifically, we identified putative Rex binding sites upstream of *lap*, *pflBC*, and *pflA*, indicating Rex likely repress fermentative metabolism directly. Rex binding sites were also predicted upstream of *bsh* and *inlAB*, further suggesting the involvement of Rex in virulence gene regulation.

In the absence of *rex*, 110 transcripts were less abundant during aerobic growth, indicating the presence of Rex is required to fully activate these genes (S3 Table). As Rex is a canonical transcriptional repressor, we hypothesize these changes are due to indirect effects. Indeed, promoter analysis did not identify any putative Rex binding sites in the promoters of genes activated by Rex, suggesting these changes are likely due to indirect Rex-dependent regulation.

Fermentative metabolism is repressed by Rex

Transcriptional analysis indicated that Rex-mediated repression functions to down-regulate fermentative metabolism during aerobic growth (Fig 1A). To verify the role of Rex in regulating metabolism, we first assessed growth of the wt and Δrex strains during both aerobic and anaerobic growth. A small, but significant, growth defect was observed for Δrex -deficient *L. monocytogenes*, beginning 4 hours post-inoculation into aerobic shaking flasks (Fig 1B). This defect was not due to a change in glucose uptake, as wt and Δrex consumed glucose at similar rates (Fig 1C). In contrast, the Δrex strain exhibited no growth defect when incubated anaerobically (S1A Fig), demonstrating Rex-dependent repression is dispensable during anaerobic growth.

To clarify the effect of Rex regulation on *L. monocytogenes* aerobic growth, extracellular metabolites were quantified 4 hours post-inoculation. The Δrex mutant secreted approximately 90% more lactate and ~55% more formate than wt and the complemented strain (Δrex pPL2.*rex*, Figs 1D and 1E). This was accompanied by a concomitant decrease in the primary aerobic by-product acetate (Fig 1F). These metabolite changes confirmed the transcriptional analysis showing increased transcript abundance of *ldhA*, *pflA*, and *pflBC* in the absence of *rex* and demonstrated that carbon-flux is being directed primarily towards lactate fermentation (Fig 1A). We were unable to determine if the increased expression of *lap* in Δrex impacted ethanol production, as this volatile metabolite evaporates in a shaking flask and could not be reliably quantified.

Results from the extracellular metabolite analysis led us to hypothesize that the aerobic growth defect exhibited by the Δrex strain is a result of aberrant carbon flux through fermentation. This would result in decreased intracellular ATP stores compared to wt aerobic growth which generates ATP through oxidative phosphorylation and the

ETC (Fig 1A). To test this hypothesis, we measured ATP concentrations 4 hours post-inoculation when the growth defect of Δrex becomes apparent. Indeed, the *rex*-deficient strain had 50% the amount of ATP compared to wt and the complemented strain (Fig 1G). Glucose consumption and extracellular metabolite profiles were similar between the wt and Δrex strains when incubated anaerobically, demonstrating that Rex is normally derepressed in this growth environment (S1B-G Fig). Taken together, these data indicate that *L. monocytogenes* Rex functions to repress fermentative metabolism in the presence of oxygen and a strain lacking *rex* is impaired for aerobic growth as a result of altered carbon-flux and decreased ATP production.

L. monocytogenes* Δrex is more resistant to acidified bile *in vitro

In addition to metabolic genes, the transcriptional profile revealed that Rex represses virulence determinants necessary in the host gastrointestinal (GI) tract, including bile salt hydrolase (*bsh*) and internalins A and B (*inlAB*). Bsh detoxifies bile, which is encountered during transit through the GI tract and colonization of the gallbladder [21–23]. To investigate the role of Rex in bile resistance, we first generated *L. monocytogenes* strains lacking *bsh* or both *rex* and *bsh*, and assessed their survival following a 24-hour exposure to porcine bile in BHI. *L. monocytogenes* colonizing the gallbladder would be exposed to bile at neutral pH [9], which we found to have no effect on the survival of any bacterial strain tested (Fig 2A). These results are consistent with published reports demonstrating that *L. monocytogenes* *bsh* is not required for survival in neutral bile [22,23]. In contrast, *L. monocytogenes* in the GI tract encounters acidified bile following its release from the gallbladder into the low pH environment of the duodenum

[9,24]. Acidified bile was highly bactericidal, resulting in a 2.5-log reduction in wt survival 24 hours post-inoculation (Fig 2A). This killing was dependent on bile, as all strains grew equally well in acidified BHI lacking bile (S2A Fig). *L. monocytogenes* lacking *bsh* was even more sensitive to acidified bile, exhibiting a 4-log decrease in survival (Fig 2A). As predicted, the Δrex mutant was more resistant to the toxic effects of acidified bile, exhibiting only a ~9-fold reduction in CFU. Trans-complementation of *rex* returned the susceptibility to similar levels as wt. Moreover, the $\Delta rex \Delta bsh$ double mutant displayed similar susceptibility as the Δbsh mutant (Fig 2A), indicating the increased survival of the Δrex mutant is dependent upon increased *bsh* transcription.

L. monocytogenes in the GI tract would experience not only acidified bile, but also a hypoxic environment. Therefore, we next evaluated bile toxicity in anaerobic growth conditions and observed enhanced bactericidal properties, with most strains exhibiting a 5-log reduction in viability after 24 hours (Fig 2B). The only exception was Δrex , which displayed a 20-fold increase in survival compared to wt, although this difference was not statistically significant. The $\Delta rex \Delta bsh$ mutant survived identically to the wt and Δbsh strains, indicating that the increase in survival of Δrex was dependent on *bsh*. These data suggested that transcription of *bsh* is also increased in Δrex when grown anaerobically. As Rex is typically derepressed during anaerobic growth, we predict increased *bsh* transcription in Δrex is likely the result of activation by unknown factors. Taken together, these results demonstrated that *L. monocytogenes* lacking the Rex repressor are more resistant to acidified bile due to increased *bsh* expression and this phenotype is most pronounced during aerobic growth.

The role of Rex in the intracellular lifecycle of *L. monocytogenes*

The intracellular lifecycle of *L. monocytogenes* begins with entry into host cells by phagocytosis or bacterial-mediated invasion, followed by replication within the cytosol, and cell-to-cell spread via actin polymerization [2]. RNA-seq revealed that transcripts encoding InlA and InlB were increased in the Δrex mutant, leading to the hypothesis that Rex regulates invasion of non-phagocytic cells via receptor-mediated endocytosis. Specifically, InlA and InlB mediate invasion of epithelial cells and hepatocytes by engaging the host receptors E-cadherin and Met, respectively [5,25]. To investigate the effects of increased *inlAB* transcription in the Δrex mutant, we measured bacterial adherence and invasion of Caco-2 human intestinal epithelial cells and Huh7 human hepatocytes. Adherence to host cells was evaluated by enumerating CFU 1 hour post-infection. To measure invasion, cells were treated with gentamicin 1 hour post-infection and intracellular bacteria were enumerated 2 hours post-infection. We observed a dramatic increase in adherence of the Δrex strain to both cell types and this was completely dependent on *inlAB* expression, as adherence was not significantly different between the $\Delta inlAB$ and $\Delta rex \Delta inlAB$ strains (Figs 3A and 3C). In addition, Δrex exhibited significantly greater invasion of Caco-2 cells in an InlAB-dependent manner (Fig 3B). The increased adherence of Δrex to Huh7 hepatocytes did not translate to a significant increase in internalization by these cells (Fig 3D). Together, these results demonstrated that *L. monocytogenes* Δrex invades human intestinal epithelial cells and adheres to human hepatocytes better than wt as a result of increased *inlAB* transcription.

After invading host cells via receptor-mediated endocytosis or phagocytosis, *L. monocytogenes* replicates intracellularly and spreads cell-to-cell using actin-based

motility [2–5]. To investigate the role of Rex regulation in these facets of pathogenesis, we first measured intracellular growth in several relevant cell types. We found that the Δrex strain replicated intracellularly at the same rate as wt in activated bone marrow-derived macrophages, Huh7 human hepatocytes, and TIB73 murine hepatocytes (Figs 4A-C). These results suggested that Rex-regulated promoters are de-repressed in wt *L. monocytogenes* during intracellular growth and therefore, deleting the Rex repressor had no effect on growth.

Next, cell-to-cell spread was evaluated via plaque assays in which a monolayer of cells is infected and both intracellular growth and intercellular spread are measured over 3 days [26]. The Δrex mutant formed plaques ~10% larger than those formed by wt *L. monocytogenes* in L2 fibroblasts and formed plaques ~5% smaller in TIB73 hepatocytes (Fig 4D), indicating that Rex regulation is not required for cell-to-cell spread. Taken together, these data demonstrated that *L. monocytogenes* lacking the Rex repressor has an advantage at early stages of infection, as Δrex displayed increased adherence and invasion of host cells. However, Rex is dispensable for intracellular growth and cell-to-cell spread in all cell types analyzed.

Rex is required for virulence in a murine oral model of infection

We hypothesized that during oral infection of *L. monocytogenes*, Rex repression is alleviated due to the hypoxic environment of the GI tract. Rex derepression would not only up-regulate fermentative metabolism for energy production in this environment but would also increase transcription of bile resistance factors and invasion factors required for successful infection of the GI tract. To test the ability of Δrex to survive in the murine

GI tract, 6- to 8-week old female Balb/c mice were orally infected with 10^8 CFU of wt or Δrex and housed in cages with elevated wire bottoms to limit reinoculation by coprophagy [27,28]. Prior to infection, mice were treated with streptomycin for 48 hours to increase susceptibility to oral *L. monocytogenes* infection [8,27,28]. Changes in body weight were recorded throughout the infection as a global measurement of disease severity [8]. Mice infected with wt *L. monocytogenes* lost ~8% of their initial body weight throughout the 4 day infection (Fig 5A). In contrast, mice infected with Δrex lost only ~3% of their initial weight 3 days following infection and returned to their initial weight by 4 days post-infection (Fig 5A). These results indicated mice infected with Δrex experienced less severe disease than mice infected with wt following oral infection.

To determine bacterial burden, organs and feces were harvested, homogenized, and plated every 24 hours. Specifically within the GI tract, we analyzed the small intestinal tissue, intestinal contents, cecum, and feces. Similar bacterial loads were observed between wt and Δrex in the GI tract and feces throughout the infection, indicating that Rex-mediated transcriptional repression is dispensable for the GI phase of infection, as predicted (Figs 5B-E). Rex was also not required for dissemination from the GI tract to internal organs, as evidenced by the similar bacterial burdens between wt and Δrex in the spleen, liver, and gallbladder 1 day post-infection (Figs 5F-H). However, as the infection progressed, we observed a significant attenuation of Δrex compared to wt. Bacterial burdens in the spleens of mice infected with Δrex were decreased compared to wt by approximately 2-logs on day 2 and 1-log on days 3 and 4 of the infection (Fig 5F). Similarly, there was a 1-log decrease in Δrex CFU in the liver 4 days post-infection (Fig 5G). The most dramatic attenuation was observed in the gallbladder, with Δrex decreased

271 4- and 6-logs compared to wt at 3 and 4 days post-infection, respectively (Fig 5H). This
 272 significant defect was surprising, as the Δrex mutant survived similarly to wt when
 273 exposed to bile at neutral pH, as would be encountered in the gallbladder (Fig 3). Taken
 274 together, these results confirmed our hypothesis that Rex-dependent repression is
 275 dispensable for colonization and invasion of the GI tract during oral infection, suggesting
 276 Rex is normally derepressed in this anaerobic environment. In addition, the infection
 277 studies revealed that Δrex is able to disseminate to internal organs in the early stages of
 278 infection. Surprisingly, Rex-dependent regulation was required for replication in the
 279 spleen and liver and essential for surviving and colonizing the gallbladder after oral
 280 infection.

DISCUSSION

In this study, we investigated the role of the redox-responsive transcriptional regulator Rex in *L. monocytogenes*. Transcriptional and *in silico* promoter analyses identified dozens of genes likely to be directly repressed by Rex *in vitro*. We demonstrated that Rex derepression induces fermentative metabolism, resulting in decreased ATP production and impaired aerobic growth of *L. monocytogenes* lacking *rex*. We also present evidence that Rex directly regulates virulence factor production during infection. *In vitro* studies revealed that Δrex is more resistant to acidified bile in a Bsh-dependent manner and that over-expression of *inlAB* in the Δrex mutant leads to increased invasion of host cells. Consistent with these results, we found that Rex is dispensable for colonizing the GI tract and disseminating to peripheral organs in an oral listeriosis model of infection. However, Rex was required for colonization of the spleen, liver, and gallbladder. This *in vivo* attenuation was not a result of impaired intracellular replication or cell-to-cell spread, as the Δrex mutant performed similar to wt in cell culture assays of infection. Taken together, our results indicate an important role for redox sensing and Rex-mediated transcriptional repression during *L. monocytogenes* infection.

Transcriptome analysis revealed that *L. monocytogenes* Rex regulates metabolic pathways similarly to what has been described in other Gram-positive bacteria [14–17]. Specifically, fermentative metabolic pathways were the most significantly changed in the Δrex mutant. We identified putative Rex binding sites in the promoters of 48 Rex-repressed genes and/or operons, including those involved in fermentation and virulence, indicating Rex likely binds and directly represses these genes. In contrast, genes activated by Rex lacked a Rex-binding site and we hypothesize they are indirectly

regulated. Further protein-DNA binding analysis is needed to elucidate the direct regulon of *L. monocytogenes* Rex.

The results herein demonstrate that *L. monocytogenes* Rex functions to repress fermentation during aerobic growth in order to maximize energy generation. We found the Δrex mutant over-expressed genes necessary for fermentative metabolism (*lap*, *ldhA*, and *pflBC/pflA*) and accordingly, produced more lactate and formate than wt when replicating aerobically. While acetate is the major end-product generated by wt *L. monocytogenes* during aerobic growth [29,30], we observed a concomitant decrease in acetate production by Δrex . Together, these results suggest that in the absence of Rex repression, there is an increased metabolic flux from pyruvate towards lactate and away from acetate production (Fig 2A). The increased LdhA activity to produce lactate funnels NADH away from the ETC, resulting in less ATP generation by respiration. Indeed, the *L. monocytogenes* Δrex strain exhibited an aerobic growth defect and produced half as much ATP as wt. Further, these metabolic and growth phenotypes were ameliorated during anaerobic growth when Rex repression is relieved and fermentation is required for growth. Taken together, these results demonstrated that *L. monocytogenes* Rex is necessary to repress fermentative metabolism in the presence of oxygen in order to efficiently produce ATP.

Our results additionally established a role for *L. monocytogenes* Rex in regulating production of virulence factors necessary during oral listeriosis. Specifically, Rex derepression increased expression of genes encoding the bile detoxifying enzyme Bsh and the internalin proteins InlA and InlB. Within the GI tract, *L. monocytogenes* encounters acidified bile that can disrupt bacterial membranes, dissociate membrane

proteins, and induce DNA damage and oxidative stress [9,31]. Bsh detoxifies conjugated bile acids and contributes to bacterial survival in the GI tract, which is evidenced by the wide distribution of homologous enzymes among commensal gut bacteria [21,22,32]. Also within the GI tract, *L. monocytogenes* invades intestinal epithelial cells and disseminates to peripheral organs. InlA and InlB mediate invasion of non-phagocytic cells by engaging the host cell receptors E-cadherin and Met, respectively [5,25,33]. We demonstrated that *rex*-deficient *L. monocytogenes* were significantly more resistant to acidified bile stress and were better able to invade intestinal epithelial cells. Together, our results showed that Rex-mediated derepression coordinates expression of genes necessary in the GI tract during infection, including fermentative metabolism, bile resistance, and invasion of host cells.

Following invasion of intestinal epithelial cells, *L. monocytogenes* disseminates via the lymph and blood to the spleen and liver where it replicates intracellularly and spreads cell-to-cell without entering the extracellular space [4]. We found that Rex was dispensable for intracellular replication in activated bone marrow-derived macrophages and hepatocytes. Furthermore, *L. monocytogenes* Δrex grew and spread cell-to-cell at the same rate as wt in both fibroblasts and hepatocytes. Combined, our *in vitro* results suggested that Rex is dispensable for the intracellular lifecycle and implied that *L. monocytogenes* lacking the Rex repressor may have an advantage during oral infection due to the upregulation of anaerobic metabolism, bile resistance, and invasion factors.

To investigate the role of *L. monocytogenes* Rex in pathogenesis, we took advantage of a recently optimized oral listeriosis model of murine infection [8,28]. We found that Rex was completely dispensable for colonizing the GI tract, suggesting that in

wt *L. monocytogenes*, Rex repression is fully relieved in this hypoxic environment. Further, within the first 24 hours of infection, Δ *rex* disseminated to the spleen, liver, and gallbladder similarly to wt. However, the Δ *rex* mutant was attenuated overall, as mice infected with this strain lost significantly less body weight than mice infected with wt *L. monocytogenes*. Four days post-infection, we observed a 10-fold decrease in bacterial burden in the spleens and livers of mice infected with Δ *rex* compared to wt-infected mice. Surprisingly, although bile was not toxic to *L. monocytogenes* at neutral pH, Δ *rex* was attenuated ~6-logs in the gallbladders 4 days post-infection. Other than bile stress, not much is known about impediments to bacterial proliferation in the gallbladder, despite its importance to *L. monocytogenes* pathogenesis. Early during infection, a few bacteria are released from lysed hepatocytes and transit through the common bile duct to colonize the lumen of the gallbladder where they replicate to very high densities [6–8]. After a meal, the gallbladder contracts and delivers bile along with a bolus of *L. monocytogenes* back into the small intestine where it can reseed the intestinal tract [34]. Thus, the gallbladder quickly becomes the primary reservoir of *L. monocytogenes* during infection [7]. Ongoing work is aimed at identifying the stressors present in the gallbladder which specifically inhibit Δ *rex* replication in this organ, which may have more broad implications for other bacterial pathogens that replicate in the gallbladder, such as *Salmonella* spp [35].

Redox homeostasis and bacterial pathogenesis are intricately tied, although the mechanisms are not entirely understood [36]. Rex-regulated metabolic pathways have been indirectly implicated in virulence in other pathogens. *S. aureus* Rex controls expression of lactate dehydrogenase, which is essential for bacterial survival when exposed to nitric oxide produced by phagocytes [17,37]. Similarly, *Clostridium difficile*

Rex regulates butyrate production, which induces toxin synthesis during gut colonization [38]. In contrast, we identified putative Rex-binding sites in the promoters of *inlAB* and *bsh*, suggesting that *L. monocytogenes* Rex directly regulates these virulence factors. Interestingly, *bsh* and *inlAB* are also regulated by the master virulence regulator PrfA and the stress-responsive alternative sigma factor SigB [21,39,40]. We predict these regulatory factors sense distinct or potentially overlapping environmental signals and converge on these virulence factors for appropriate and efficient regulation. Future research will investigate the crosstalk between these transcriptional regulators during pathogenesis and the variable redox environments encountered by *L. monocytogenes* during infection.

Overall, this work suggests a model in which *L. monocytogenes* Rex-regulated genes are derepressed in the anaerobic environment of the GI tract, thus upregulating fermentative metabolism and virulence factor production. However, following dissemination to internal organs, Rex is required to regulate factors critical for colonization and survival within the gallbladder. As the primary reservoir of *L. monocytogenes* during infection, identifying the factors required for gallbladder colonization is imperative for understanding *L. monocytogenes* pathogenesis.

MATERIALS AND METHODS

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All protocols were reviewed and approved by the Animal Care and Use Committee at the University of Washington (Protocol 4410-01).

Bacterial strains and culture conditions. *L. monocytogenes* mutants were derived from wild type strain 10403S [41,42] and cultured in brain heart infusion (BHI) broth at 37°C with shaking (220 rpm), unless otherwise stated. Antibiotics (purchased from Sigma Aldrich) were used at the following concentrations: streptomycin, 200 µg/mL; chloramphenicol, 10 µg/mL (*Escherichia coli*) and 7.5 µg/mL (*L. monocytogenes*); and carbenicillin, 100 µg/mL. Porcine bile (Sigma Aldrich) was dissolved in sterile BHI with streptomycin to ensure sterility. In cases where pH adjustments of media were carried out, 1N HCl was used and the pH was determined using VWR sympHony benchtop pH meter. *L. monocytogenes* strains are listed in S1 Table and *E. coli* strains are listed in S2 Table. Plasmids were introduced in *E. coli* via chemical competence and heat-shock and introduced into *L. monocytogenes* wt via trans-conjugation from *E. coli* SM10 [43].

Cell lines. Huh7 and Caco-2 are cancer cell lines derived from human males with hepatocellular carcinoma and colon adenocarcinoma, respectively. TIB73 is a spontaneously immortalized hepatocyte cell line from a normal BALB/c embryo liver.

Huh7, Caco-2, and TIB73 cell lines were obtained from Joshua Woodward (University of Washington) [44]. L2 fibroblasts were described previously [45]. Cell lines were grown at 37 °C in 5% CO₂ in phenol red-free Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated fetal bovine serum (FBS; 20% for Caco-2 cells) and supplemented with sodium pyruvate (2 mM) and L-glutamine (1 mM). For passaging, cells were maintained in Pen-Strep (100 U/ml) but were plated in antibiotic-free media for infections. Initial infection of TIB73 cells was carried out in DMEM with 0.1% FBS and replaced with 10% FBS in DMEM during gentamicin treatment.

Vector construction and cloning. To construct the Δrex mutant, ~700 bp regions upstream and downstream of *rex* were PCR amplified using *L. monocytogenes* 10403S genomic DNA as a template. PCR products were restriction digested and ligated into pKSV7-*oriT* [46]. The plasmid pKSV7x Δbsh was constructed via Gibson assembly using the NEBuilder HiFi DNA assembly master mix. Regions ~1000 bp upstream and downstream of *bsh* were amplified with linker regions identical to those flanking a *ccdB* toxin cassette in pKSV7x [47]. pKSV7x was PCR amplified and DpnI treated. The linearized vector and insert PCR products were combined in the NEB master mix and ligated according to manufacturer instructions. pKSV7 Δrex and pKSV7x Δbsh were transformed into *E. coli* and sequences were confirmed by Sanger DNA sequencing. Plasmids with the mutant Δrex and Δbsh alleles were introduced into *L. monocytogenes* via trans-conjugation and integrated into the chromosome. Colonies were purified on selective nutrient agar and subsequently cured of the plasmid by conventional methods [45]. Allelic exchange was confirmed by PCR. To construct the $\Delta rex\Delta bsh$ mutant,

pKSV7 Δ rex was trans-conjugated into *L. monocytogenes* Δ bsh and integrated into the chromosome as described above.

To construct the Δ rex Δ inlAB mutant, the mutant *rex* region was amplified from pKSV7 Δ rex, restriction digested, and ligated into the pLIM1 plasmid containing a PheS* counterselection marker (provided as a generous gift from Arne Rietsch, Case Western Reserve University). Sequences were confirmed by Sanger DNA sequencing. The plasmid was introduced into *L. monocytogenes* Δ inlAB via trans-conjugation and integrated into the chromosome as previously described [45,48]. Briefly, transconjugants were selected by growing on BHI containing streptomycin and chloramphenicol at 30 °C for 24 hours. A colony from this plate was re-streaked onto a fresh plate and incubated at 42 °C for 24-48 hours. A colony was re-steaked and grown at 42 °C two additional times to ensure integration of the pLIM1 Δ rex plasmid into the chromosome. One colony was inoculated into BHI broth and grown overnight at 30°C. The culture was diluted 10⁻⁴ and 100 μ l was plated on BHI agar supplemented with *p*-chloro-phenylalanine (18 mM) and incubated at 37 °C overnight. Colonies that grew on the counterselection plates were validated to be chloramphenicol-sensitive and confirmed by PCR.

Ectopic expression of genes in *L. monocytogenes* was carried out using pPL2 integration plasmids [49]. The plasmid for complementing Δ rex was constructed by PCR amplifying *rex* along with its predicted native promoter using *L. monocytogenes* 10403S genomic DNA as a template. Sequences were confirmed by Sanger DNA sequencing. The constructed pPL2 plasmid was trans-conjugated into *L. monocytogenes* Δ rex and integration was confirmed by antibiotic resistance.

RNA isolation. Nucleic acids were purified from bacteria harvested from broth culture as previously described [50]. Briefly, bacteria were grown overnight in BHI shaking at 37 °C and subcultured to an optical density at 600 nm (OD₆₀₀) of 0.02 into 25 mL BHI. After 4 and 7 hours of aerobic growth at 37 °C shaking, bacteria were mixed 1:1 with ice-cold methanol, pelleted, and stored at -80 °C. Bacteria were lysed in phenol:chloroform containing 1% SDS by bead beating with 0.1 mm diameter silica/zirconium beads. Nucleic acids were precipitated from the aqueous fraction overnight at -20 °C in ethanol containing sodium acetate (150 mM, pH 5.2). Precipitated nucleic acids were washed with ethanol and treated with TURBO DNase per manufacturer's specification (Life Technologies Corporation).

Transcriptomics. Ribosomal RNA was removed from total RNA samples using the RiboZero rRNA Removal kit, according to manufacturer's recommendations (Illumina, Inc., San Diego, CA, USA). Depleted samples were analyzed and sequenced by the Genomics & Bioinformatics Shared Resources at Fred Hutchinson Cancer Research Center as previously described [51]. Results were evaluated using CLC Genomics Workbench (Qiagen) and transcripts that were changed >2-fold ($p < .01$) were included in our analysis. In addition, the data were technically validated by measuring expression of 6 genes via quantitative RT-PCR (qPCR) and a correlation was confirmed ($R^2 = .92$). In Table 1, we included genes of interest and those known to be regulated by Rex in other organisms.

Growth curves. For aerobic growth in broth, the cultures were normalized to an OD₆₀₀ of 0.02 in 25 mL BHI in 250 mL flasks and were incubated with shaking at 37 °C. The OD₆₀₀ was measured every hour. For anaerobic growth in broth, filter sterilized BHI broth was degassed overnight in an anaerobic chamber. Media was transferred to 16 x 125 mm Hungate Anaerobic Tubes (Chemglass Life Sciences) inside the anaerobic chamber and the tubes were autoclaved. The OD₆₀₀ was normalized to an OD₆₀₀ of 0.02 in 10 mL BHI in the Hungate tubes and incubated at 37 °C, with OD₆₀₀ measurements every hour.

Measurement of bacterial metabolites. Bacteria from aerobic and anaerobic cultures were collected (1 mL aliquots) after 4 hours of growth and centrifuged at 13,000 x g for 2 min. The supernatants were removed, sterile filtered, and stored at -20 °C until use. Extracellular metabolites were determined using Roche Yellow Line Kits (R-Biopharm), according to the manufacturer's recommendation. Intracellular ATP concentrations were determined using a BacTiter-Glo kit (Promega) according to the manufacturer's protocol and normalized to optical units. Intracellular NAD⁺ and NADH concentrations were determined using a NAD⁺/NADH-Glo kit (Promega) according to the manufacturer's protocol and normalized to total protein concentrations using the Pierce BCA Protein Assay kit (Thermo Scientific).

Bile sensitivity assays. Overnight cultures were diluted 1:200 into BHI, BHI pH 5.5, BHI supplemented with 0.1% porcine bile, or BHI pH 5.5 supplemented with 0.1% porcine bile. Aerobic cultures were incubated for 24 hours at 37 °C shaking, followed by serial dilutions and plating on BHI agar to enumerate CFU. Anaerobic cultures were incubated

for 24 hours at 37 °C in closed containers containing anaerobic gas-generating pouches (GasPak EZ; BD), followed by serial dilutions and plating on BHI agar to enumerate CFU.

Bacterial adhesion and invasion assays. Caco-2, Huh7, or TIB73 cells were seeded 2.0 x 10⁵ cells per well in 24-well plates and washed twice in sterile PBS just prior to infection. Bacterial cultures were incubated overnight in BHI broth at 30 °C static and then washed twice with sterile PBS and resuspended in cell culture media. Bacteria were added to cell monolayers at a multiplicity of infection (MOI) of 10 for Caco-2 cells, 20 for Huh7 cells, or 50 for TIB73 cells. To measure bacterial adhesion and invasion, monolayers were washed with PBS twice after 1 hour of infection, lysed with 0.1% Triton X-100, and plated on BHI agar for bacterial enumeration. To measure bacterial invasion, monolayers were washed twice with PBS after 1 hour of infection and incubated with cell culture media containing gentamicin (50 µg/mL) for 1 hour. Monolayers were washed with PBS twice and lysed with 0.1% Triton X-100 and internalized bacteria were enumerated following plating on BHI agar [25,52].

Intracellular growth curves. Growth curves in bone marrow-derived macrophages (BMMs) were performed as previously described [51], with the following modifications. Briefly, BMMs were harvested as previously reported [53] and seeded at a concentration of 6 x 10⁵ cells per well in a 24-well plate the day before infection. BMMs were activated by incubating the monolayer with recombinant murine IFNγ (100 ng/mL, PeproTech) overnight and during infection. Overnight bacterial cultures incubated at 30 °C statically were washed twice with PBS and resuspended in warmed BMM media [45]. BMMs were

washed twice with PBS and infected at an MOI of 0.1. Thirty minutes post-infection, cells were washed twice with PBS and BMM media containing gentamicin (50 µg/mL) was added to each well. To measure bacterial growth, cells were lysed by addition of 250 µL cold PBS with 0.1% Triton X-100 and incubated for 5 min at room temperature, followed by serial dilutions and plating on BHI agar to enumerate CFU.

Growth curves in Huh7 and TIB73 cells were performed as previously described [44]. Briefly, Huh7 and TIB73 cells were seeded at a concentration of 2.0×10^5 cells per well in 24-well plates the day before infection. Overnight bacteria cultures incubated at 30 °C statically were washed twice with sterile PBS and resuspended in cell culture media. Huh7 and TIB73 cells were infected at an MOI of 20 or 50, respectively. Sixty minutes post-infection, cells were washed twice with PBS and cell culture media containing gentamicin (50 µg/mL) was added to each well. To measure bacterial growth, cells were lysed by addition of 250 µL cold PBS with 0.1% Triton X-100 and incubated for 5 min at room temperature, followed by serial dilutions and plating on BHI agar to enumerate CFU. Experiments were performed with technical replicates and repeated two times.

Plaque assays. Plaque assays were performed as previously described [26,45]. Briefly, 6-well plates were seeded with L2 fibroblasts or TIB73 cells at a density of 1.2×10^6 and 1.5×10^6 , respectively. Bacterial cultures were incubated overnight at 30 °C in BHI broth and were then diluted in sterile PBS (1:10 for L2 infections; 1:2 for TIB73 infections). L2 fibroblasts and TIB73 cells were infected with 5 µL or 10 µL of diluted bacteria, respectively. 1 hour post-infection, cells were washed twice with sterile PBS and agarose overlays containing DMEM and gentamicin were added to the wells. 2 days post-infection,

cells were stained with neutral red dye and incubated overnight. Plaques were imaged 72 hours post-infection and plaque area was quantified using Image J software [54].

Mice. Female BALB/c mice were purchased from The Jackson Laboratory at 5 weeks of age and used in experiments when they were 6-7 weeks old. All mice were maintained under specific-pathogen-free conditions at the University of Washington South Lake Union animal facility. All protocols were reviewed and approved by the Animal Care and Use Committee at the University of Washington (Protocol 4410-01).

Oral murine infection. Infections were performed as previously described [8,28,55–57]. Groups of 5 mice were placed in cages with wire flooring raised 1 inch to prevent coprophagy, and streptomycin (5 mg/mL) was added to drinking water 48 hours prior to infection. Food and water were removed 16 hours prior to infection to initiate overnight fasting. *L. monocytogenes* cultures were grown overnight in BHI broth at 30 °C static. The cultures were diluted 1:10 into fresh BHI broth and grown at 37 °C shaking for 2 hours. Bacteria were diluted in PBS and mice were fed 10^8 bacteria in 20 μ L via pipette. The inocula were plated and enumerated after infection to ensure consistent dosage between strains. Food and water were returned to cages following infection and mice were euthanized at 1, 2, 3, and 4 days post-infection. Livers, spleens, and feces were harvested and homogenized in 0.1% NP-40. Gallbladders were harvested and ruptured in 1 mL of 0.1% NP-40 with a sterile stick. The cecum sections were emptied, flushed with sterile PBS, and homogenized in 0.1% NP-40 buffer. The small intestines were cut lengthwise with sterile forceps and flushed with sterile PBS. Intestinal contents were resuspended in

574 PBS and intestinal tissue was homogenized in 0.1% NP-40. All organs were serial diluted
575 in PBS and plated on LB agar to enumerate CFU.

FIGURE LEGENDS

Figure 1. Fermentative metabolism is repressed by Rex during aerobic growth. A.

Model of aerobic and anaerobic central metabolic pathways in *L. monocytogenes*.

Enzymes repressed by Rex are denoted in purple text. Underlined metabolic end-

products were those differentially produced by Δrex compared to wt during aerobic

growth. LdhA, lactate dehydrogenase; PflABC, pyruvate formate lyase; Lap, alcohol

dehydrogenase; ATP, adenosine triphosphate; NAD(H), nicotinamide adenine

dinucleotide; ETC, electron transport chain; TCA, tricarboxylic acid cycle. B. Aerobic

growth of wt, Δrex , and the complemented strain measured by optical density (OD₆₀₀). C-

F. Glucose and extracellular metabolites were quantified 4 hours post-inoculation.

Glucose depletion (C) and production of lactate (D), acetate (E), and formate (F) were

determined and normalized to OD₆₀₀. G. Relative intracellular ATP concentration was

measured at 4 hours. In all panels, data are the means and standard error of the mean

(SEM) of three independent experiments. *p* values were calculated using a

heteroscedastic Student's *t* test. * *p* < 0.05; ** *p* < 0.01; n.s. *p* > 0.05.

Figure 2. Rex derepression promotes bacterial survival in acidified bile. Survival of

wt (black), Δbsh (gray), Δrex (blue), Δrex pPL2.*rex* (light blue) and $\Delta rex\Delta bsh$ (white)

normalized to the initial inoculum (dashed line = 1) and expressed as log-transformed

CFU per mL of culture. Strains were evaluated for survival 24 hours post-inoculation in

BHI supplemented with bile and acidified BHI supplemented with bile under aerobic (A)

and anaerobic (B) conditions. In both panels, data are the medians and ranges of three

independent experiments. p values were calculated using a heteroscedastic Student's t test. * $p < 0.05$; ** $p < 0.01$; n.s. $p > 0.05$.

Figure 3. Rex derepression promotes bacterial adherence to Caco-2 epithelial cells and Huh7 hepatocytes. The ability of *L. monocytogenes* strains to adhere to and invade Caco-2 epithelial cells (A and B) and Huh7 hepatocytes (C and D) was evaluated. Adherence was measured by enumerating CFU 1 hour post-infection (A and C). Bacterial invasion was measured 2 hours post-infection and 1 hour after adding gentamicin to kill extracellular bacteria (B and D). Adherence and invasion were normalized to the initial inocula and are reported as a percentage of wt. In all panels, data are the means and SEM of three independent experiments performed in duplicate. p values were calculated using a heteroscedastic Student's t test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; n.s. $p > 0.05$.

Figure 4. Intracellular growth and cell-to-cell spread are not impaired in Δrex . A-C. Intracellular growth kinetics of wt and Δrex in IFN- γ activated BMMs (A), Huh7 cells (B), and TIB73 cells (C). D. Plaque area in L2 fibroblasts and TIB73 hepatocytes, measured as a percentage of wt. Data in panels A and D are the means and SEM of three independent experiments. Data in panels B and C are the means and SEMs of two independent experiments performed in duplicate. In all panels, p values were calculated using a heteroscedastic Student's t test. * $p < 0.05$; n.s. $p > 0.05$.

Figure 5. Rex is required for full virulence in an oral listeriosis model. Female BALB/c mice were orally infected with 10^8 CFU of wt (black squares) or Δrex (blue circles) and the number of bacteria present in each tissue was determined over time. A. The body weights of the mice over time, reported as a percentage of body weight prior to infection. B-H. Mice were sacrificed each day and organs were harvested to enumerate bacterial burden. Each symbol represents an individual mouse (n=5 per group) and the solid lines indicate the median. Dashed lines indicate the limit of detection (l.o.d.). Data are representative of two independent experiments. Results are expressed as log-transformed CFU per organ or per gram of feces. *p* values were calculated using a heteroscedastic Student's *t* test. * *p* < 0.05; ** *p* < 0.01; *** *p* < 0.001.

ACKNOWLEDGEMENTS

The authors would like to thank Arne Rietsch (Case Western Reserve University) for the pLIM plasmid and Alex Pollock (University of Washington) for technical assistance. The Genomics and Bioinformatics Shared Resources at Fred Hutchinson Cancer Research Center is partially funded from Cancer Center grant NCI 5P30CA015704-43. Research in the Reniere lab is supported by NIH grant R01 AI132356. C.R.H. was supported by NIH grant T32AI055396. R.C.G. was supported by 5T32AI055396.

REFERENCES

1. Vázquez-Boland JA, Kuhn M, Berche P, Chakraborty T, Domínguez-Bernal G, Goebel W, *et al.* *Listeria* Pathogenesis and Molecular Virulence Determinants. Clin Microbiol Rev. 2001;14: 584–640. doi:10.1128/CMR.14.3.584-640.2001
2. Tilney, Lewis G. P Daniel A. Actin filaments and the growth, movement, and spread of the intracellular bacterial parasite, *Listeria monocytogenes*. J Cell Biol. 1989;109: 1597–1608.
3. Schnupf P, Portnoy DA. Listeriolysin O: a phagosome-specific lysin. Microbes and Infection. 2007;9: 1176–1187. doi:10.1016/j.micinf.2007.05.005
4. Freitag NE, Port GC, Miner MD. *Listeria monocytogenes* — from saprophyte to intracellular pathogen. Nat Rev Microbiol. 2009;7: 623–628. doi:10.1038/nrmicro2171
5. Radoshevich L, Cossart P. *Listeria monocytogenes*: towards a complete picture of its physiology and pathogenesis. Nat Rev Microbiol. 2018;16: 32–46. doi:10.1038/nrmicro.2017.126
6. Hardy J, Francis KP, DeBoer M, Chu P, Gibbs K, Contag CH. Extracellular Replication of *Listeria monocytogenes* in the Murine Gall Bladder. Science. 2004;303: 851–853. doi:10.1126/science.1092712
7. Zhang T, Abel S, Abel zur Wiesch P, Sasabe J, Davis BM, Higgins DE, *et al.* Deciphering the landscape of host barriers to *Listeria monocytogenes* infection. Proc Natl Acad Sci USA. 2017;114: 6334–6339. doi:10.1073/pnas.1702077114
8. Louie A, Zhang T, Becattini S, Waldor MK, Portnoy DA. A Multiorgan Trafficking Circuit Provides Purifying Selection of *Listeria monocytogenes* Virulence Genes. Miller SI, editor. mBio. 2019;10: e02948-19, /mbio/10/6/mBio.02948-19.atom. doi:10.1128/mBio.02948-19
9. Begley M, Gahan CGM, Hill C. The interaction between bacteria and bile. FEMS Microbiol Rev. 2005;29: 625–651. doi:10.1016/j.femsre.2004.09.003
10. Pitts MG, D’Orazio SEF. A Comparison of Oral and Intravenous Mouse Models of Listeriosis. Pathogens. 2018;7. doi:10.3390/pathogens7010013
11. D’Orazio SEF. Animal models for oral transmission of *Listeria monocytogenes*. Front Cell Infect Microbiol. 2014;4. doi:10.3389/fcimb.2014.00015
12. Mesquita I, Varela P, Belinha A, Gaifem J, Laforge M, Vergnes B, *et al.* Exploring NAD⁺ metabolism in host–pathogen interactions. Cell Mol Life Sci. 2016;73: 1225–1236. doi:10.1007/s00018-015-2119-4

13. Richardson AR, Somerville GA, Sonenshein AL. Regulating the Intersection of Metabolism and Pathogenesis in Gram-positive Bacteria. *Metabolism and Bacterial Pathogenesis*. American Society of Microbiology; 2015. pp. 129–165. doi:10.1128/microbiolspec.MBP-0004-2014
14. Ravcheev DA, Li X, Latif H, Zengler K, Leyn SA, Korostelev YD, *et al.* Transcriptional Regulation of Central Carbon and Energy Metabolism in Bacteria by Redox-Responsive Repressor Rex. *Journal of Bacteriology*. 2012;194: 1145–1157. doi:10.1128/JB.06412-11
15. Brekasis D, Paget MS. A novel sensor of NADH/NAD⁺ redox poise in *Streptomyces coelicolor* A3(2). *The EMBO Journal*. 2003;22: 4856–4865. doi:10.1093/emboj/cdg453
16. Wang E, Bauer MC, Rogstam A, Linse S, Logan DT, von Wachenfeldt C. Structure and functional properties of the *Bacillus subtilis* transcriptional repressor Rex. *Molecular Microbiology*. 2008;69: 466–478. doi:10.1111/j.1365-2958.2008.06295.x
17. Pagels M, Fuchs S, Pané-Farré J, Kohler C, Menschner L, Hecker M, *et al.* Redox sensing by a Rex-family repressor is involved in the regulation of anaerobic gene expression in *Staphylococcus aureus*. *Molecular Microbiology*. 2010;76: 1142–1161. doi:10.1111/j.1365-2958.2010.07105.x
18. Gyan S, Shiohira Y, Sato I, Takeuchi M, Sato T. Regulatory Loop between Redox Sensing of the NADH/NAD⁺ Ratio by Rex (YdiH) and Oxidation of NADH by NADH Dehydrogenase Ndh in *Bacillus subtilis*. *J Bacteriol*. 2006;188: 7062–7071. doi:10.1128/JB.00601-06
19. Ruhland BR, Reniere ML. Sense and sensor ability: redox-responsive regulators in *Listeria monocytogenes*. *Current Opinion in Microbiology*. 2019;47: 20–25. doi:10.1016/j.mib.2018.10.006
20. Wurtzel O, Sesto N, Mellin JR, Karunker I, Edelheit S, Bécavin C, *et al.* Comparative transcriptomics of pathogenic and non-pathogenic *Listeria* species. *Mol Syst Biol*. 2012;8: 583. doi:10.1038/msb.2012.11
21. Dussurget O, Cabanes D, Dehoux P, Lecuit M, the European Listeria Genome Consortium, Buchrieser C, *et al.* *Listeria monocytogenes* bile salt hydrolase is a PrfA-regulated virulence factor involved in the intestinal and hepatic phases of Listeriosis. *Mol Microbiol*. 2002;45: 1095–1106. doi:10.1046/j.1365-2958.2002.03080.x
22. Begley M, Sleator RD, Gahan CGM, Hill C. Contribution of Three Bile-Associated Loci, *bsh*, *pva*, and *btlB*, to Gastrointestinal Persistence and Bile Tolerance of *Listeria monocytogenes*. *IAI*. 2005;73: 894–904. doi:10.1128/IAI.73.2.894-904.2005

23. Dowd GC, Joyce SA, Hill C, Gahan CGM. Investigation of the Mechanisms by Which *Listeria monocytogenes* Grows in Porcine Gallbladder Bile. *Infect Immun*. 2011;79: 369–379. doi:10.1128/IAI.00330-10
24. Kochan TJ, Shoshiev MS, Hastie JL, Somers MJ, Plotnick YM, Gutierrez-Munoz DF, *et al*. Germinant Synergy Facilitates *Clostridium difficile* Spore Germination under Physiological Conditions. *mSphere*. 2018;3: e00335-18, /msphere/3/5/mSphere335-18.atom. doi:10.1128/mSphere.00335-18
25. Pizarro-Cerdá J, Kühbacher A, Cossart P. Entry of *Listeria monocytogenes* in Mammalian Epithelial Cells: An Updated View. *Cold Spring Harb Perspect Med*. 2012;2. doi:10.1101/cshperspect.a010009
26. Sun AN, Camilli A, Portnoy DA. Isolation of *Listeria monocytogenes* small-plaque mutants defective for intracellular growth and cell-to-cell spread. *Infect Immun*. 1990;58: 3770–3778.
27. Becattini S, Littmann ER, Carter RA, Kim SG, Morjaria SM, Ling L, *et al*. Commensal microbes provide first line defense against *Listeria monocytogenes* infection. *J Exp Med*. 2017;214: 1973–1989. doi:10.1084/jem.20170495
28. Ghanem ENB, Jones GS, Myers-Morales T, Patil PD, Hidayatullah AN, D’Orazio SEF. InlA Promotes Dissemination of *Listeria monocytogenes* to the Mesenteric Lymph Nodes during Food Borne Infection of Mice. *PLOS Pathogens*. 2012;8: 15.
29. Romick TL, Fleming HP, McFeeters RF. Aerobic and anaerobic metabolism of *Listeria monocytogenes* in defined glucose medium. *Appl Environ Microbiol*. 1996;62: 304–307.
30. Müller-Herbst S, Wüstner S, Mühlig A, Eder D, M. Fuchs T, Held C, *et al*. Identification of genes essential for anaerobic growth of *Listeria monocytogenes*. *Microbiology*. 2014;160: 752–765. doi:10.1099/mic.0.075242-0
31. Gahan CGM, Hill C. Gastrointestinal phase of *Listeria monocytogenes* infection. *Journal of Applied Microbiology*. 2005;98: 1345–1353. doi:10.1111/j.1365-2672.2005.02559.x
32. Jones BV, Begley M, Hill C, Gahan CGM, Marchesi JR. Functional and comparative metagenomic analysis of bile salt hydrolase activity in the human gut microbiome. *Proc Natl Acad Sci U S A*. 2008;105: 13580–13585. doi:10.1073/pnas.0804437105
33. Lamond NM, McMullen PD, Paramasvaran D, Visvabhrathy L, Eallanardo SJ, Maheswhari A, *et al*. Cardiotropic isolates of *Listeria monocytogenes* with enhanced vertical transmission dependent upon the bacterial surface protein InlB. *Infect Immun*. 2020; IAI.00321-20, iai;IAI.00321-20v1. doi:10.1128/IAI.00321-20

34. Hardy J, Margolis JJ, Contag CH. Induced Biliary Excretion of *Listeria monocytogenes*. Infect Immun. 2006;74: 1819–1827. doi:10.1128/IAI.74.3.1819-1827.2006
35. Gonzalez-Escobedo G, Marshall JM, Gunn JS. Chronic and acute infection of the gall bladder by *Salmonella* Typhi: understanding the carrier state. Nat Rev Microbiol. 2011;9: 9–14. doi:10.1038/nrmicro2490
36. Reniere ML. Reduce, Induce, Thrive: Bacterial Redox Sensing during Pathogenesis. J Bacteriol. 2018;200. doi:10.1128/JB.00128-18
37. Richardson AR, Libby SJ, Fang FC. A Nitric Oxide-Inducible Lactate Dehydrogenase Enables *Staphylococcus aureus* to Resist Innate Immunity. Science. 2008;319: 1672–1676. doi:10.1126/science.1155207
38. Bouillaut L, Dubois T, Francis MB, Daou N, Monot M, Sorg JA, *et al.* Role of the global regulator Rex in control of NAD⁺-regeneration in *Clostridioides* (*Clostridium*) *difficile*. Mol Microbiol. 2019;111: 1671–1688. doi:10.1111/mmi.14245
39. Kazmierczak MJ, Mithoe SC, Boor KJ, Wiedmann M. *Listeria monocytogenes* σ B Regulates Stress Response and Virulence Functions. J Bacteriol. 2003;185: 5722–5734. doi:10.1128/JB.185.19.5722-5734.2003
40. Scotti M, Monzó HJ, Lacharme-Lora L, Lewis DA, Vázquez-Boland JA. The PrfA virulence regulon. Microbes and Infection. 2007;9: 1196–1207. doi:10.1016/j.micinf.2007.05.007
41. Bishop DK, Hinrichs DJ. Adoptive transfer of immunity to *Listeria monocytogenes*. The influence of in vitro stimulation on lymphocyte subset requirements. J Immunol. 1987; 139: 2005–2009.
42. Bécavin C, Bouchier C, Lechat P, Archambaud C, Creno S, Gouin E, *et al.* Comparison of Widely Used *Listeria monocytogenes* Strains EGD, 10403S, and EGD-e Highlights Genomic Differences Underlying Variations in Pathogenicity. mBio. 2014;5: e00969-14. doi:10.1128/mBio.00969-14
43. Simon R, Priefer U, Puhler A. A Broad Host Range Mobilization System for *in vivo* Genetic Engineering: Transposon Mutagenesis in Gram-Negative Bacteria. Biotechnology. 1983. doi: 10.1038/nbt1183-784
44. McFarland AP, Burke TP, Carletti AA, Glover RC, Tabakh H, Welch MD, *et al.* RECON-Dependent Inflammation in Hepatocytes Enhances *Listeria monocytogenes* Cell-to-Cell Spread. mBio. 2018;9: e00526-18, /mbio/9/3/mBio.00526-18.atom. doi:10.1128/mBio.00526-18
45. Reniere ML, Whiteley AT, Portnoy DA. An In Vivo Selection Identifies *Listeria monocytogenes* Genes Required to Sense the Intracellular Environment and

- 780 Activate Virulence Factor Expression. PLoS Pathog. 2016;12.
781 doi:10.1371/journal.ppat.1005741
- 782 46. Camilli A, Tilney LG, Portnoy DA. Dual roles of *plcA* in *Listeria monocytogenes*
783 pathogenesis. Mol Microbiol. 1993;8: 143–157.
- 784 47. Whiteley AT, Ruhland BR, Edrozo MB, Reniere ML. A Redox-Responsive
785 Transcription Factor Is Critical for Pathogenesis and Aerobic Growth of *Listeria*
786 *monocytogenes*. Infect Immun. 2017;85. doi:10.1128/IAI.00978-16
- 787 48. Argov T, Rabinovich L, Sigal N, Herskovits AA. An Effective Counterselection
788 System for *Listeria monocytogenes* and Its Use To Characterize the Monocin
789 Genomic Region of Strain 10403S. Appl Environ Microbiol. 2017;83: e02927-16,
790 e02927-16. doi:10.1128/AEM.02927-16
- 791 49. Lauer P, Chow MYN, Loessner MJ, Portnoy DA, Calendar R. Construction,
792 Characterization, and Use of Two *Listeria monocytogenes* Site-Specific Phage
793 Integration Vectors. J Bacteriol. 2002;184: 4177–4186. doi:10.1128/JB.184.15.4177-
794 4186.2002
- 795 50. Reniere ML, Whiteley AT, Hamilton KL, John SM, Lauer P, Brennan RG, *et al.*
796 Glutathione activates virulence gene expression of an intracellular pathogen.
797 Nature. 2015;517: 170–173. doi:10.1038/nature14029
- 798 51. Cesinger MR, Thomason MK, Edrozo MB, Halsey CR, Reniere ML. *Listeria*
799 *monocytogenes* SpxA1 is a global regulator required to activate genes encoding
800 catalase and heme biosynthesis enzymes for aerobic growth. Mol Microbiol.
801 2020;114: 230–243. doi:10.1111/mmi.14508
- 802 52. Burkholder KM, Bhunia AK. *Listeria monocytogenes* Uses Listeria Adhesion Protein
803 (LAP) To Promote Bacterial Transepithelial Translocation and Induces Expression of
804 LAP Receptor Hsp60. IAI. 2010;78: 5062–5073. doi:10.1128/IAI.00516-10
- 805 53. Sauer J-D, Sotelo-Troha K, von Moltke J, Monroe KM, Rae CS, Brubaker SW, *et al.*
806 The *N*-Ethyl-*N*-Nitrosourea-Induced *Goldenticket* Mouse Mutant Reveals an
807 Essential Function of *Sting* in the *In Vivo* Interferon Response to *Listeria*
808 *monocytogenes* and Cyclic Dinucleotides. Infect Immun. 2011;79: 688–694.
809 doi:10.1128/IAI.00999-10
- 810 54. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of Image
811 Analysis. Nat Methods. 2012;9: 671–675.
- 812 55. Bou Ghanem EN, Myers-Morales T, Jones GS, D’Orazio SEF. Oral Transmission of
813 *Listeria monocytogenes* in Mice via Ingestion of Contaminated Food. JoVE. 2013;
814 50381. doi:10.3791/50381
- 815 56. Jones GS, Bussell KM, Myers-Morales T, Fieldhouse AM, Bou Ghanem EN,
816 D’Orazio SEF. Intracellular *Listeria monocytogenes* Comprises a Minimal but Vital

- 817 Fraction of the Intestinal Burden following Foodborne Infection. Infect Immun.
818 2015;83: 3146–3156. doi:10.1128/IAI.00503-15
- 819 57. Jones GS, D’Orazio SEF. Monocytes are the predominate cell type associated with
820 *Listeria monocytogenes* in the gut but they do not serve as an intracellular growth
821 niche. J Immunol. 2017;198: 2796–2804. doi:10.4049/jimmunol.1602076
- 822

A

bioRxiv preprint doi: <https://doi.org/10.1101/2021.02.16.431404>; this version posted February 16, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

B

C

D

E

F

G

Fig 1

Fig 2

Fig 3

Fig 4

Fig 5