Integrative Genomic Analysis Identifies Isoleucine and CodY as Regulators of Listeria monocytogenes Virulence

Lior Lobel¹, Nadejda Sigal¹, Ilya Borovok¹, Eytan Ruppin²,³, Anat A. Herskovits¹*

¹ The Department of Molecular Microbiology and Biotechnology, The George S. Wise Life Sciences Faculty, Tel Aviv University, Tel Aviv, Israel, ² The Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel, ³ The Blavatnik School of Computer Sciences, Tel Aviv University, Tel Aviv, Israel

Abstract

Intracellular bacterial pathogens are metabolically adapted to grow within mammalian cells. While these adaptations are fundamental to the ability to cause disease, we know little about the relationship between the pathogen’s metabolism and virulence. Here we used an integrative Metabolic Analysis Tool that combines transcriptome data with genome-scale metabolic models to define the metabolic requirements of Listeria monocytogenes during infection. Twelve metabolic pathways were identified as differentially active during L. monocytogenes growth in macrophage cells. Intracellular replication requires de novo synthesis of histidine, arginine, purine, and branch chain amino acids (BCAAs), as well as catabolism of L-riboflavin and glycerol. The importance of each metabolic pathway during infection was confirmed by generation of gene knockout mutants in the respective pathways. Next, we investigated the association of these metabolic requirements in the regulation of L. monocytogenes virulence. Here we show that limiting BCAA concentrations, primarily isoleucine, results in robust induction of the master virulence activator gene, prfA, and the PrfA-regulated genes. This response was specific and required the nutrient responsive regulator CodY, which is known to bind isoleucine. Further analysis demonstrated that CodY is involved in prfA regulation, playing a role in prfA activation under limiting conditions of BCAAs. This study evidences an additional regulatory mechanism underlying L. monocytogenes virulence, placing CodY at the crossroads of metabolism and virulence.

Introduction

Intracellular bacterial pathogens have developed sophisticated mechanisms to enter eukaryotic cells and replicate within them. These mechanisms involve bacterial proteins that overcome host defense strategies and barriers as well as nutritional limitations. Intracellular pathogens are generally categorized into two groups based on the compartment where they replicate, the vacuole/phagosome or the host cell cytosol. Each intracellular niche presents unique nutritional challenges demanding that bacteria exhibit specific metabolic adaptations to proliferate successfully. Vacuolar pathogens, such as Legionella pneumophila and Mycobacterium tuberculosis, actively modify their compartment via secretion of effector proteins that enrich the vacuole with nutrients to support growth; nevertheless growth rate in the vacuole is much slower than in rich media. In contrast, intracellular cytosolic bacterial pathogens, such as Listeria monocytogenes, Shigella flexneri and Burkholderia pseudomallei manage to exploit their niche such that growth rates resemble growth in rich media [1]. Little is known about the metabolic adaptations that enable intracellular cytosolic pathogens to grow rapidly or if such adaptations affect virulence. A better understanding of how these bacteria overcome nutritional limitations will give insight into cytosol nutrient composition and could facilitate development of drugs against intracellular pathogens.

L. monocytogenes is a Gram-positive facultative intracellular bacterial pathogen and the causative agent of listeriosis in humans, a disease with a variety of clinical manifestations including meningitis and abortion [2]. L. monocytogenes infects phagocytic and non-phagocytic cells, using surface expressed proteins called internalins, which bind and induce bacterial uptake by endocytosis [3]. Upon entry, L. monocytogenes escapes from the phagosome/vacuole into the host cytosol by producing the pore-forming hemolysin toxin, listeriolysin O (LLO, encoded by the hly gene), and two additional phospholipases [4–6]. Once in the host cytosol, L. monocytogenes multiplies rapidly and expresses the surface protein, ActA, which recruits the host actin polymerization machinery to propel the bacteria in the cytosol and facilitate spread from cell to cell [7,8]. All known virulence factors involved in internalization, vacuolar escape and cell-to-cell spread are co-regulated by the major virulence activator, PrfA [9].

L. monocytogenes uses several carbon sources during intracellular growth, but primarily glycerol, di-hydroxyacetone and phosphorylated carbohydrates (such as glucose 1-phosphate), indicating the availability of these substrates in the cytosolic niche [10–12]. Glycerol uptake is mediated by a glycerol permease, whereas phosphorylated sugars are transported via the specialized hexose-phosphate transporter, Hpt. Both systems are induced intracellularly and are important for bacterial replication [11,13]. It is well established that carbon metabolism during intracellular growth is
Intracellular bacterial pathogens have developed sophisticated mechanisms to invade and replicate within eukaryotic cells. For successful replication, pathogens have adapted metabolically to the intracellular niche. While this adaptation is fundamental to the ability to cause disease, we know little about pathogen’s intracellular metabolism and its association with virulence. In this study we took a global approach that combines computational and experimental methods to decipher the intracellular metabolic requirements of the human bacterial pathogen Listeria monocytogenes. We identified 12 metabolic pathways to be differentially active during infection in comparison to growth in rich lab media. We validated the essentiality of the active pathways for L. monocytogenes intracellular replication. Pathways included: biosynthesis of histidine, arginine, purine, and branch chain amino acids (BCAAs), as well as the catabolism of L-ribose and glycerol. Next we analyzed whether the requirement for these nutrients associates with virulence. We found that limiting concentrations of BCAAs, primarily of isoleucine, results in robust induction of the bacterial virulence state, a response that is dependent on the isoleucine responsive regulator, CodY. CodY was responsible for the up-regulation of the major virulence regulator of L. monocytogenes, PrfA. This study supports the premise that pathogens metabolism and virulence are closely interlinked.

Linked directly to the virulence of L. monocytogenes, Carbohydrates transported via the phosphoenolpyruvate phosphotransferase system (PTS) (such as glucose and cellobiose) repress the activity of PrfA whereas non-PTS carbon sources (such as glycerol and glucose 1-phosphate that are available in the host cytosol) induce PrfA activity, resulting in elevated expression of virulence genes [reviewed in: [14] and [15]]. Based on these observations, it was proposed that non-PTS sugars might serve to signal L. monocytogenes of its intracellular location.

Various additional metabolic pathways were indicated as important for intracellular replication of L. monocytogenes, including several amino acid biosynthesis pathways such as the branch chain amino acid (BCAA) and arginine pathways [16,17], the common aromatic compounds biosynthesis (shikimate) pathway, as well as the synthesis and uptake of thiamine (vitamin B12) [18,19]. A special adaptation of L. monocytogenes to the cytosolic niche is its ability to obtain the co-factor lipoate from the host, as it cannot be synthesized by the bacteria. Listerial expression of a lipoate ligase, LplA1, enables the co-factor to be derived from host lipoylpptides [20]. As for nitrogen sources, it is thought that L. monocytogenes utilizes ammonium, arginine and ethanolamine [21,22]. The latter is highly abundant in mammalian cells as it is the breakdown product of phosphatidylethanolamine. The ability to use ethanolamine as a nitrogen and/or carbon source is linked to the pathogenesis of several bacteria, such as Salmonella, Enterococcus and Clostridium [22]. Like Salmonella, listerial genomes encode the ethanolamine utilization pathway [23]. Taken together, these various adaptations highlight the complex metabolic requirements of intracellular growth.

Recent advances in genomic sequencing and development of constraint-based metabolic models enable now the reconstruction of genome scale metabolic networks of different organisms. This approach enables a better comprehension of the complete metabolic network of an organism under different growth conditions as well as the prediction of essential metabolic genes [24,25]. These remarkable advances have prompted several studies of the metabolic networks of pathogens during infection, with the goal of characterizing potential drug targets [26–30]. Lately, as part of the SEED project an automated genome-scale metabolic model of L. monocytogenes metabolism has been developed [31] that comprises a stoichiometric matrix of reactions and metabolites representing the organism’s entire metabolic network. In this study we used this metabolic model to analyze the global metabolic state of L. monocytogenes during infection.

To model the metabolic state of an organism under a given set of conditions, the availability of nutrients needs to be simulated. This information is mostly unknown for complex systems, but a specific metabolic state can be specified by integrating relevant transcriptome, proteome or metabolome (“omics”) data into the metabolic model. A computational tool that performs this data integration was recently developed, the integrative Metabolic Analysis Tool (iMAT) [32]. Briefly, taking into account omics data, iMAT predicts flux activity that is stoichiometrically consistent across the metabolic network in a global manner. It determines a subset of reactions to be confidently active or inactive, while leaving pathways that have alternative flux distributions as unknown due to the existence of isozymes or alternative metabolic pathways. By integrating the omics data with a metabolic model encompassing the pertaining biochemical knowledge, iMAT provides a more comprehensive and accurate prediction of flux activity, reflecting the effects of possible post-transcriptional and post-translational regulations that go beyond the information embedded in the raw gene expression data [33]. For example, consider a metabolic pathway composed of three enzymes that are highly active in a given condition. The transcription of only one of the enzymes is up regulated, while the transcription of the other two enzymes remains unchanged. Conventional bioinformatic pathway enrichment analysis based solely on transcriptional data will fail to denote this pathway as highly active, however iMAT analysis could be more successful, if the pathway’s activation results in a global network flux distribution that is best consistent with the overall gene expression input (Figure 1).

In this study we applied iMAT analysis to transcriptome data and a genome scale metabolic model of L. monocytogenes to better understand the bacterial metabolic requirements during growth in macrophage cells. Using this approach several metabolic pathways were predicted active or inactive during intracellular growth, potentially reflecting the metabolites availability within these cells. We experimentally deleted key genes in the pathways predicted to be active, validating their contribution to L. monocytogenes intracellular replication and studied the association of the corresponding metabolites in regulation of L. monocytogenes virulence genes.

Results

Conventional transcriptome-based pathway enrichment analysis of metabolic pathway changes during intracellular growth

The global transcriptional profiles of L. monocytogenes (strain 10403S) growing in two different conditions were assessed using whole genome microarray analysis. RNA was extracted from L. monocytogenes grown either inside macrophages (at 6 hours post infection, representing cytosolic replicating bacteria) or in brain heart infusion (BHI) broth (mid log phase). Transcription levels in intracellular bacteria were designated as relative to those in bacteria growing in BHI. Gene expression was coded as 1, −1 or 0 for each gene, indicating a minimum of twofold increase, decrease, or no change, respectively, in transcript levels during intracellular growth.
growth versus growth in BHI (Table S1). Next, we retrieved the list of metabolic reactions of *L. monocytogenes* from the automated genome scale metabolic model reconstruction pipeline [31]. This model consists of 1254 reactions and 816 metabolic genes, encompassing 102 SEED metabolic pathway annotations [34]. Genes in the transcriptome array were assigned to metabolic reactions and their expression levels were converted into reaction activity based on the model’s built-in gene-reactions mapping matrix (i.e. enzymatic complexes and isozymes). We then applied a standard hypergeometric test for pathway enrichment taking into account each metabolic reaction observed to change (up or down) to determine which pathways were enriched for altered activities. A metabolic pathway significantly enriched with up-regulated reactions was considered highly active, while a metabolic pathway enriched with down-regulated reactions was considered inactive (Table 1). In agreement with previous studies of metabolic pathways that contribute to *L. monocytogenes* growth during *in vitro* infection, we identified arginine biosynthesis, branched chain amino acids (BCAA) biosynthesis and glycerol utilization as pathways highly active during intracellular replication [10,11,16]. In addition to the known pathways, our analysis predicted two additional pathways as induced intracellularly: the histidine biosynthesis and L-rhamnose utilization pathways. Along with these highly active pathways, we identified two metabolic pathways to be specifically down regulated during intracellular growth: the formaldehyde assimilation genes and the bacterial fatty acids biosynthesis pathway (FASII).

### Model-based iMAT analysis of metabolic pathway changes during intracellular growth

Next, we applied the iMAT algorithm to predict metabolic flux activity during intracellular growth of *L. monocytogenes*. We defined a simulated medium that best represents the cytosolic environment (Table S2), successfully yielding a predicted *in silico* generation time of 55 min/gen, similar to the one determined experimentally (57 min/gen). iMAT returns a three-valued output, rating the predicted activity of each reaction as confidently active, confidently inactive, or unknown. This output was analyzed by computing the hypergeometric enrichment score of each pathway based on the predicted tri-valued flux activity of each of its reactions (this time determined by iMAT in a global, model based manner) (Table 1). In line with the literature and our conventional gene expression analysis, the arginine, histidine and BCAA biosynthesis pathways together with L-rhamnose and glycerol utilization pathways were predicted to be highly active during intracellular growth. In addition, however, iMAT analysis identified two other metabolic pathways as active intracellularly:
the common aromatic compounds biosynthesis (shikimate) pathway and the de novo purine biosynthesis pathway. Notably, the importance of the de novo purine biosynthesis pathway was highlighted by iMAT predictions of metabolites that are imported or exported during intracellular growth (based on the global fluxes and directionality of reactions) (Table S3). Indeed, iMAT predicted uptake of all nucleotides from the host, except for adenosine, explaining the need for de novo purine biosynthesis. Furthermore, iMAT designated five metabolic pathways as inactive/down regulated: the FASII biosynthesis pathway, in line with our conventional gene expression analysis; the mevalonate and the alternative isoprenoids biosynthesis pathways (in agreement with [35]); and two newly identified pathways, the heme biosynthesis and pantothenate-coenzyme A biosynthesis pathways. Statistical analysis of iMAT accuracy versus conventional gene expression analysis, based on a literature database of metabolic pathways that were reported for their role in L. monocytogenes intracellular growth (Table S4), revealed that iMAT predictions are more accurate (P-Value = 0.0001 vs. P-Value = 0.052, hypergeometric distribution test). The precision and recall for iMAT analysis are P = 0.583, R = 0.5, compared to that of the gene expression analysis P = 0.428, R = 0.357. Overall iMAT analysis resulted in a predicted metabolic shift in 12 metabolic pathways upon growth of L. monocytogenes within macrophages, five of which were missed by conventional gene expression analysis. Notably, both methods predicted the novel contribution of histidine biosynthesis and rhamnose utilization to L. monocytogenes intracellular replication.

Experimental validation of the predicted metabolic pathways induction during intracellular growth

Next, we decided to validate the essentiality of all the metabolic pathways predicted by iMAT analysis to be active during intracellular growth, except for the glycerol utilization pathway that has already been studied in great detail [10,11]. We chose key listerial enzymes in the active pathways and assessed directly their transcription levels during intracellular growth, as a validation for the microarray data, as well as the consequences of non-polar in-frame deletions of the respective genes. In total, six genes encoding key enzymes from six different metabolic pathways were subjected to further study (Table 2). As depicted by the metabolic map of L. monocytogenes all the chosen genes are central and cannot be compensated (Figure 2A). The genes transcription during intracellular growth of L. monocytogenes in macrophage cells versus growth in BHI medium was assessed using quantitative real-time PCR (RT-qPCR). All six metabolic genes were up-regulated during intracellular growth whereas spoD and bglA, two control genes, remained unchanged. As another control the transcription level of fabF gene (ins2201), part of the FASII pathway that was predicted to be down-regulated intracellularly, was analyzed and indeed this gene was shown to be down-regulated during intracellular growth in macrophage cells in comparison to growth in BHI media (Figure 2B). fabF, tglC and argD were highly induced, at least 64 fold, while purH, hisC and araE were induced 2–8 fold (Figure 2B). These enhanced transcriptional levels confirmed that indeed the six pathways are induced and active during L. monocytogenes intracellular growth. Next, we knocked out each one of the selected genes in L. monocytogenes 10403S strain and analyzed the growth capabilities of the resulting six mutants (Table 2). We first tested whether the mutants are indeed defective in their corresponding metabolic pathways. Mutants were grown in minimal defined medium (MDM) [36] and in MDM media that was specifically depleted of the cognate pathway metabolite (Figure S1). As shown in Figure S1, while most mutants grew normally in MDM media (except for AaroE and ApurH mutants) each one demonstrated a growth defect when the metabolite of their target pathway was depleted. As expected, introducing

### Table 1. Metabolic pathways predicted to be differentially active during L. monocytogenes intracellular growth by standard bioinformatics analysis and iMAT analysis.

| Metabolic pathway             | Standard bioinformatics analysis (P-value) | iMAT analysis (P-value) | Prediction  | References related to L. monocytogenes virulence |
|-------------------------------|-------------------------------------------|-------------------------|-------------|-----------------------------------------------|
| Arginine biosynthesis         | 1.25E-05                                  | 2.14E-07                | Highly active | [16]                                           |
| Branch-chain amino acids biosynthesis | 3.52E-10                                  | 7.68E-12                | Highly active | [16,17]                                       |
| Histidine biosynthesis        | 1.25E-05                                  | 3.68E-09                | Highly active |                                                |
| L-Rhamnose utilization        | 0.001                                     | 2.27E-05                | Highly active | [57]                                           |
| De novo purine biosynthesis   | NP                                        | 5.02E-04                | Highly active | [17]                                           |
| Glycerol utilization          | 8.77E-06                                  | 6.74E-04                | Highly active | [10,11]                                       |
| Common aromatic compounds biosynthesis | NP                                        | 9.51E-03                | Highly active | [17,18]                                       |
| Fatty acids biosynthesis FASII | 0.014                                     | 1.86E-17                | Inactive     |                                                |
| Pantothenate and CoA biosynthesis | NP                                        | 1.73E-06                | Inactive     |                                                |
| Mevalonate pathway for isoprenoids biosynthesis | NP                                        | 2.09E-09                | Inactive     | [35]                                           |
| Alternative pathways for isoprenoids biosynthesis | NP                                        | 1.35E-03                | Inactive     | [35]                                           |
| Heme and siroheme biosynthesis | NP                                        | 1.85E-03                | Inactive     |                                                |
| Formaldehyde assimilation: Ribulose monophosphate pathway | 0.002                                     | NP                      | Inactive     |                                                |

* Based on hypergeometric enrichment test.

**References related to L. monocytogenes virulence**
The corresponding genes complemented the growth defects of these mutants. When tested for growth in BHI, all mutants, with the exception of \textit{AarE}, grew like WT bacteria, demonstrating that these metabolic pathways are not essential for growth in rich medium (Figure S2). Next, we assessed the contribution of the predicted pathways to intracellular growth of \textit{L. monocytogenes}. Macrophage cells were infected with the different mutants and intracellular growth was measured. As shown in Figure 3A, all the metabolic mutants replicated less efficiently in macrophage cells in comparison to WT bacteria or to the complemented strains (Figure 3A and Figure S3). The intracellular growth of \textit{AarE} was highly impaired, however as shown before this gene is also required during growth in rich medium (Figure S2 and [18]). To exclude the possibility that the metabolic mutants are defective in phagosomal escape, we performed a microscope based escape assay, and demonstrated that all mutants were able to escape the macrophages phagosomes like WT bacteria, with the exception of \textit{AarE} that had a minor defect (Figure 3B). Taken together, these results validate that each of the six predicted pathways is required for intracellular replication of \textit{L. monocytogenes}, and importantly, establish for the first time a role for histidine biosynthesis and rhamnose catabolism during \textit{L. monocytogenes} intracellular growth.

### Metabolic requirements influence the expression of major virulence genes

Our analyses established that four anabolic pathways are specifically important during \textit{L. monocytogenes} growth in macrophage cells, namely the BCAAs, histidine, arginine and purine biosynthesis pathways. This finding surmises that limited amounts of these nutrients are available in the host cytosol. Given that the abundant availability of non-PTS sugars in the host cytosol is suspected to serve as a signal of intracellular location (see introduction), we investigated if low availability of BCAAs, histidine, arginine and purine might serve similarly as an intracellular signal that modulate the transcription of the virulence genes. Therefore, we tested whether the regulation of these four pathways is linked to the regulation of virulence genes. To this end, WT \textit{L. monocytogenes} was grown in BHI, minimal defined medium (MDM) and MDM with reduced concentrations (10-fold less) of arginine, histidine, adenine and BCAAs \textit{(i.e.} isoleucine, leucine and valine\textit{), which aimed to mimic the intracellular free amino acids concentration [37–39]. The transcription levels of corresponding metabolic genes and virulence genes \textit{(purH, ilvC, hisC and argD) were} then analyzed at mid-log phase. The expression of the metabolic genes \textit{(purH, ilvC, hisC and argD)} was induced during growth in MDM relative to growth in BHI rich medium (Figure 4A), indicating that MDM already contains limiting concentrations of their cognate nutrients. Growth in MDM with ~10-fold reduced concentrations of arginine, histidine, adenine, isoleucine, leucine and valine resulted in a prolonged lag phase (Figure S4) and in further induction of the BCAA biosynthesis pathway, as shown by increased expression of the \textit{ilvC} gene (Figure 4A). Remarkably, while growth in MDM resulted in moderate up-regulation of the virulence genes compared to BHI, limiting further the concentrations of arginine, histidine, adenine and BCAAs triggered robust induction of all three virulence genes (Figure 4B). This dramatic response was specific to nutrients synthesized by the intracellularly active metabolic pathways, as growth in MDM with reduced concentrations of other amino acids such as phenylalanine and tryptophan had no effect on expression of the virulence genes (Figure 4B).

Next we tested whether the induction of the virulence genes requires low concentrations of all or some of the identified nutrients. When WT \textit{L. monocytogenes} was grown in MDM depleted of each nutrient or combinations of nutrients, we found that limited concentrations of BCAAs is the signal for inducing expression of virulence genes (Figure 4C). Among the three BCAAs, isoleucine was found to be the most important for induction of the virulence genes, while leucine and valine presented only a minor contribution (Figure 4D). To explore the dynamic of this response we followed the induction of \textit{hly} gene \textit{(encoding for LLO toxin)} during growth in BHI, MDM and MDM with reduced concentration of isoleucine, using \textit{a lux} reporter system. Wild type bacteria were conjugated with an integrative plasmid containing \textit{lux} operon under the regulation of the \textit{hly} promoter \textit{(pPL2-P\_lux)} [40]. Bacteria were subjected to growth in the different media and parallel measurements of optical density (\textit{O.D.\_\text{600 mm}}) and luminescence were taken. As shown in Figure 5A, a robust induction of \textit{hly} promoter was observed during logarithmic phase under limiting concentrations of isoleucine. Notably, this response was not observed during stationary phase in any of the tested conditions, suggesting that a specific regulatory mechanism involves the activation of the virulence genes under low concentrations of isoleucine (Figure 5A–5B). In Gram-positive bacteria, including \textit{L. monocytogenes}, a nutrient responsive regulator that binds directly isoleucine is known and named CodY. CodY, when bound to isoleucine \textit{(and/or to GTP)} represses genes that are required for adaptation to nutrient limitation [41–44]. Thus, we next examined whether CodY is involved in the regulation of \textit{L. monocytogenes} virulence genes under low isoleucine concentrations.

**Table 2.** The genes selected for knock out encode key enzymes in the metabolic pathways predicted to be active during intracellular growth.

| Gene name | LMRG identifier (Imo identifier) | Enzyme description | Pathway |
|-----------|----------------------------------|--------------------|---------|
| \textit{aroE} | 01070.6 (Imo1923) | 3-phosphoshikimate 1-carboxyvinyltransferase | Common pathway of aromatic compounds biosynthesis |
| \textit{purH} | 02506.9 (Imo1765) | IMP cyclohydrolase | \textit{De novo} purine biosynthesis |
| \textit{ilvC} | 02420.6 (Imo2849) | Rhamnulokinase | L-rhamnose utilization |
| \textit{hisC} | 01072.6 (Imo1925) | Histidinol-phosphate aminotransferase | Histidine biosynthesis |
| \textit{argD} | 01379.6 (Imo1588) | Acetylornithine aminotransferase | Arginine biosynthesis |
| \textit{ilvC} | 01134.6 (Imo1986) | Ketol-acid reductoisomerase | Branched chain amino acids biosynthesis |

\textit{doi:10.1371/journal.pgen.1002887.t002}
A ΔcodY complete gene deletion mutant was generated, conjugated with pPL2-Pₕlylux plasmid and tested under similar conditions. Interestingly, deletion of codY gene did not result in activation of hly transcription, as predicted by its role as a repressor, rather the induction of the hly promoter under isoleucine limiting conditions was strongly dependent on CodY (Figure 5C). In this experiment the hly promoter was activated only in WT bacteria grown in MDM with low concentrations of isoleucine. Similarly, prfA, the master virulence activator gene and actA, the actin-polymerizing gene, were also not induced in the ΔcodY mutant in comparison to WT bacteria. This phenotype was complemented by introducing a copy of codY gene to the ΔcodY mutant, using the chromosomal integrative pLIV2 plasmid (pLIV2-codY; under the control of an IPTG inducible promoter) (Figure 5D).

In light of these findings, we tested whether CodY has a similar role in the regulation of some relevant metabolic genes. To this end, the transcription levels of ivoC and parH genes were analyzed...
Figure 5. CodY regulates the transcription of virulence and metabolic genes under limiting concentrations of BCAAs. A. Relative luminescence measurements (RLU) indicating activation of \textit{hly} promoter under growth of WT \textit{L. monocytogenes} (harboring pPL2-P\textit{lux} plasmid) in BHI, MDM and MDM with low concentration of isoleucine. B. Optical density measurements of the same cultures of WT \textit{L. monocytogenes} containing pPL2-P\textit{lux} plasmid, growing in BHI, MDM and MDM with low concentration of isoleucine. The results represent 3 independent experiments (N = 3). Error bars indicate a standard error of the mean. C. Relative luminescence measurements indicating activation of \textit{hly} promoter in \textit{D. codY} mutant and WT bacteria during growth in MDM and MDM with low concentration of isoleucine. The results represent 3 independent experiments (N = 3). Error bars indicate a standard error of the mean. D. RT-qPCR analysis of \textit{prfA} and \textit{actA} transcription levels during growth in MDM with low levels of Metabolic Requirements Control Virulence.
in the 

A

mutant and WT bacteria grown under varying concentrations of BCAAs. Notably, we observed that while \( \text{ibc} \) and \( \text{purH} \) were highly induced when BCAAs were limiting (250 and 15-fold induction, respectively), this induction was largely dependent on CodY. However, under high BCAAs concentrations (i.e. in BHI), the 

A

mutant also exhibited up-regulation of \( \text{ibc} \) and \( \text{purH} \), but to a much lesser extent than under conditions of BCAAs depletion alone (50-and 3-fold induction, respectively) (Figure 5E). Overall, these findings indicate that while CodY and BCAAs generally repress the expression of metabolic genes, under BCAA limitation CodY is primarily involved in activation of both metabolic and virulence genes. Finally, analysis of the intracellular growth of the 

A

mutant in macrophage cells demonstrated that CodY is required for 

L.

monocytogenes efficient intracellular replication, a phenotype that was complemented by in trans expression of 

codI

gene (pLIV2-codi) (Figure 5F).

CodY plays a positive role in 

prfA

transcription specifically from 

prfA

PIP2 promoters

To study further whether CodY regulation of the virulence genes (i.e., \( \text{hly} \) and \( \text{actA} \)) is direct or mediated by PrfA, the induction of both \( \text{hly} \) and \( \text{prfA} \) promoters by CodY was assessed using the lux reporter system. \( \text{prfA} \) transcription is initiated from two distinct regions, one proximal encoding two overlapping promoters named P1 and P2, and a second distal region encoding the P3 promoter located up-stream of plcA-prfA genes (Figure 6A) [45]. While the latter requires PrfA itself for co-transcription of plcA-prfA [45], the transcription from P1P2-prfA promoters is known to be PrfA-independent (Figure 6A) [46,47]. Both \( \text{prfA} \) promoter regions were cloned up-stream to the lux operon in the pL2-lux plasmid, resulting in pPL2-P1P2-prfAlux and pPL2-P3-plcA-prfAlux. Next, these plasmids and pPL2-P3lux were introduced, separately, to 

ApfA

, 

A

codY

and WT bacteria and promoter activities were measured under BCAAs limiting conditions (i.e., during growth in MDM media with low concentrations of BCAAs). Notably, while induction of \( \text{hly} \) and P3 promoters required both PrfA and CodY (Figure 6B–6C), the induction of P1P2-prfA promoters was dependent solely on the 

codI

gene (Figure 6D). These observations led us to conclude that CodY plays a positive role in the regulation of PrfA expression via the P1P2 promoters, while its role in the regulation of \( \text{hly} \) and 

plcA

-prfA genes is mediated by PrfA itself. Overall, the data presented here clearly demonstrate that CodY is a critical factor controlling PrfA expression linking 

L.

monocytogenes metabolism and virulence.

Discussion

In this study we examined the metabolism of the human bacterial pathogen 

L.

monocytogenes during infection using both a standard bioinformatics pathway enrichment analysis and an integrative computational analysis of transcriptome data using the IMAT tool. This analysis yielded the prediction of metabolic pathways that are highly active and inactive during intracellular growth of 

L.

monocytogenes, inferring the nutrients availability within the cytosolic niche. We validated the contribution of the predicted active pathways to 

L.

monocytogenes growth by generating gene deletion knockouts and monitoring replication rates of these mutants in primary macrophages cells. Importantly, we used this information to search for metabolic requirements that control 

L.

monocytogenes

virulence. We found that low concentrations of BCAAs signal the bacteria for its intracellular location and that the isoleucine responsive regulator, CodY, is responsible for the up-regulation of 

L.

monocytogenes virulence genes under these conditions.

The metabolism of 

L.

monocytogenes during intracellular growth differs from its metabolism when growing in rich media. Specifically, this study revealed 12 metabolic pathways to be differentially active during infection. In accordance with previous reports the glycerol utilization pathway was identified as active during intracellular growth [10,17]. However, it is known that 

L.

monocytogenes can utilize additional carbon sources and most likely switch between them according to cytosolic availability. During residence in the host cytoplasm, 

L.

monocytogenes scavenge for amino acids and vitamins. While most amino acids are acquired from the host [48], we confirmed, and in the case of histidine discovered, that several amino acid biosynthesis pathways are important for bacterial growth during infection. The requirement for arginine biosynthesis by 

L.

monocytogenes likely reflects the low concentrations...
of this amino acid in host cytosol, as this amino acid is conditionally essential in mammalian cells [49]. In addition, arginine biosynthesis could serve to generate a nitrogen pool, since arginine can be degraded into citrulline and ammonia by arginine deiminase [21,50]. The requirements for BCAA and histidine biosynthesis could be explained by the fact that these amino acids are not produced by human cells and therefore cytosolic concentrations could be limiting for pathogenic bacteria [49,51]. Support for this premise comes from studies of another cytosolic pathogen, *Burkholderia pseudomallei*, similarly found to require the BCAA and histidine biosynthesis pathways for intracellular growth [52,53].

We show here that *L. monocytogenes* requires an active histidine biosynthesis pathway for efficient intracellular growth. The HisC enzyme functions also in the synthesis of aromatic amino acids; however these amino acids were shown to be non-essential for *L. monocytogenes* intracellular replication [18]. Briefly, although the common aromatic compounds biosynthesis pathway was considered initially to be essential for *L. monocytogenes* intracellular growth, an elegant study by Goebel and colleagues delineated that the growth defect of *avo* mutants during infection is not due to the lack of aromatic amino acids, but rather to a requirement for menaquinone synthesis. Menaquinone takes part in the respiratory electron chain and this finding explained why the common aromatic compounds biosynthesis pathway is essential during aerobic growth of *L. monocytogenes* in rich medium but not under anaerobic conditions [18]. Notably, these observations point indirectly to the presence of oxygen in the host cytosol at a level sufficient for aerobic respiration. In accordance, iMAT analysis predicts the uptake of O2 and efflux of CO2 by *L. monocytogenes* during intracellular growth (Table S3).

The requirement for an active purine (adenine) biosynthesis pathway during *L. monocytogenes* intracellular growth is in accordance with a similar constraint demonstrated by other intracellular pathogens, such as *Brucella melitensis*, *Mycobacterium tuberculosis*, *Bacillus anthracis* and *Burkholderia pseudomallei*, and suggests limiting concentrations of purines in mammalian cells [53–56]. Of note, iMAT analysis identified the *de novo* purine biosynthesis pathway as induced during infection whereas conventional gene expression analysis failed to do so, implying of post-transcriptional up-regulation in this pathway. The iMAT prediction was validated as the *ΔpurH* mutant displayed impaired growth in macrophages.

In addition to the discovery that histidine biosynthesis is required for *L. monocytogenes* intracellular growth, we show here for the first time that the rhamnose utilization pathway is induced and important during infection. This finding relates to an old observation that rhamnose fermentation could serve as a marker to distinguish between pathogenic and non-pathogenic isolates of *L. monocytogenes* [57]. As animals do not synthesize rhamnose, it is unlikely that rhamnose utilization provides an alternative carbon source during intracellular bacterial growth. However, rhamnose is an important and abundant carbohydrate in the cell wall of many bacterial species, including *L. monocytogenes* [58,59], and accordingly is involved in several cell envelope processes. Indeed, an *Escherichia coli* *AvhaB* mutant displays higher resistance to cell wall antibiotics [60], supporting the hypothesis that rhamnose catabolism and turnover affect cell wall composition. Notably, a *Bacillus anthracis* mutant defective in dTDP-rhamnose synthesis was shown to adhere less to macrophages [61] and subsequently, it was reported that the macrophage CD14-Mac1 complex binds rhamnose residues and promotes bacterial internalization [62]. In this study we established that the *L. monocytogenes* *rhaB* gene encoding rhamnulose kinase is transcriptionally up regulated a 100-fold during intracellular growth. In *L. monocytogenes* rhamnose residues are known to decorate teichoic acids and were suggested to be antigenic determinants [58]. In light of our finding that the rhamnose utilization pathway is induced during infection, it is tempting to speculate that during intracellular growth *L. monocytogenes* modifies its teichoic acids to evade recognition and avoid triggering the innate immune system. The observed metabolic shift in rhamnose catabolism could directly affect the composition of the cell wall and in this way, bacterial survival during infection. Future studies are necessary to delineate why this pathway is actively required during *L. monocytogenes* intracellular growth. Overall, our findings that several amino acid biosynthesis pathways are required for growth of *L. monocytogenes* in macrophages imply that these essential metabolites might be limited in the host cytosol or that the bacteria do not transport them efficiently during intracellular growth. Differentiation between these possibilities requires further studies. It will also be important to discover if these nutrients are limiting in the absence of infecting bacteria or alternatively, are actively depleted from macrophage cytosol as a mechanism to prevent bacterial replication.

Pathogenic bacteria must express virulence factors to exploit nutrients within their host therefore it is not surprising that metabolism and virulence are closely linked. This intimate relationship is best demonstrated by the finding that intracellular carbon sources trigger up regulation of *PrfA* [12,63], and thus promote escape into the cytosol and spread to neighboring cells. Here we provide an additional regulatory mechanism connecting intracellular metabolism and virulence, i.e., that low availability of BCAAs, and primarily of isoleucine, leads to the induction of *PrfA* and in turn, of *PrfA*-regulated genes. The isoleucine binding transcription regulator, CodY, is directly linked to this response and, as shown, responsible for a positive regulation of *prfA*. Notably, biosynthesis of BCAAs lies at the crossroads of bacterial metabolism. The synthesis of valine and leucine requires the α-keto acid pyruvate and an amino group from glutamate, and thus is dependent on both glycolysis and the TCA cycle. Furthermore, isoleucine is synthesized from α-ketobutyrate, which is required for sulfur metabolism. Thus BCAA levels indicate the overall carbon, nitrogen, and sulfur metabolic status of the bacteria [64]. In other Gram-positive bacteria *CodY* was demonstrated to regulate genes involved in diverse processes such as adaptation to starvation, sporulation, biofilm and virulence [43,65]. Although initial studies indicated a role for *CodY* as a general repressor (when bound to GTP and/or BCAA), later reports in *Bacillus subtilis* and *Streptococcus pneumoniae* have demonstrated that it can also function as a transcriptional activator [66–68]. Accordingly, in some Gram-positive pathogens the expression of virulence genes was shown to be diminished in *CodY* null mutants [68–72]. In *L. monocytogenes*, *CodY* was shown to repress genes involved in amino acid metabolism, nitrogen assimilation and sugar uptake, and was suggested to affect virulence via a functional association with *RelA* [42]. Our data support the notion that *CodY* plays a role as repressor of metabolic genes in the presence of BCAAs. However, in this study we show that in the absence or under trace amounts of BCAAs, *CodY* is important for the activation of virulence genes and metabolic genes necessary for intracellular growth. We demonstrated that *CodY* specifically affects the expression of *prfA* via the proximal P1P2 promoters. It is still unclear whether *CodY* directly interacts with the *prfA* promoters or that this effect is mediated indirectly by another *CodY*-regulated protein. In summary, we propose that there are several regulatory connections between *L. monocytogenes* intracellular metabolism and virulence, with BCAAs and carbon source availability playing a key role in the cytosolic nutrient signature that signals the bacteria for their intracellular location.
This study is the first to address directly the ability of iMAT to serve as a tool to study the metabolic requirements of microorganisms in their natural habitat. We establish that iMAT analysis provides a more comprehensive overview of the activity of the metabolic network of an organism in a given condition. Yet, model-based analyses such as iMAT still suffer from limitations, primarily due to incomplete annotation. In addition, metabolic virulence factors like Hpt and LplA1 [13,20] can be overlooked, as these are typically not co-regulated with a metabolic pathway. Nevertheless, the approach we presented here can be applied to other bacterial pathogens to reveal novel insights concerning pathogen specific metabolic pathways, and expand our understanding of host-pathogen metabolic interactions. This study highlights the concept that bacterial pathogens have not only acquired dedicated metabolic virulence factors but also changed the regulation of their core metabolism to be able to grow inside their hosts and trigger the virulent response in the right time; further support for the emerging view that pathogen metabolism and virulence are closely interlinked.

Materials and Methods

Ethics statement

The use of animals in this study was limited to preparation of bone marrow derived macrophages from mice. Experimental protocols were approved by the Tel Aviv university Animal Care and Use Committee (L-09-008) according to the Israel Welfare Law (1994) and the National Research Council guide (Guide for the Care and Use of Laboratory Animals 2010).

Bacterial strains and growth media

*L. monocytogenes* 10403S was the wild type strain (WT) and served as the parental strain when generating gene deletion mutants (Table 2). *E. coli* XL-1 Blue (Stratagene) was used for vector propagation and *E. coli* SM-10 strain [73] was used for plasmid conjugation to *L. monocytogenes*. The full list of the strains and plasmids used in this study are described in Table S5. Primers used in this study are described in Table S6. *L. monocytogenes* bacteria were grown at 37°C with agitation in brain heart infusion (BHI) as rich medium or in minimal defined medium (MDM), which is identical to the improved minimal medium (IMM) described in [36]. MDM medium includes: phosphate buffer, 0.41 mg/ml magnesium sulfate, 10 mg/ml glucose, 100 μg/ml of each amino acid (i.e., leucine, isoleucine, valine, methionine, arginine, histidine, tryptophan, glutamate, cysteine and phenylalanine), 600 μg/ml glutamine, 0.5 μg/ml biotin, 0.5 μg/ml riboflavin, 20 μg/ml ferric citrate, 1 μg/ml para-aminobenzoic acid, 5 mg/ml lipoic acid, 2.5 μg/ml adenine, 1 μg/ml thiamine, 1 μg/ml pyridoxal, 1 μg/ml calcium pantothenate and 1 μg/ml nicotinamide. For growth under limiting concentrations of nutrients, MDM was freshly made with 10-fold less of the indicated nutrients: BCAs, histidine, arginine, adenine, phenylalanine and tryptophan (resulting in a final concentration of 10 μg/ml for amino acids and 0.25 μg/ml for adenine).

Bacterial infection of macrophages

Bone marrow derived macrophages (BMDM) were used for infection experiments and were isolated from 6–8 week old female C57/BL6 mice (Harlan laboratories) as described previously [74]. BMDM were cultured in DMEM based media supplemented with 20% fetal bovine serum, sodium pyruvate (1 mM), L-glutamine (2 mM), b-Mercaptoethanol (0.05 mM), and M-CSF (1929-conditioned medium). Approximately 8×10⁶ *L. monocytogenes* were used to infect 2×10⁶ macrophage cells seeded in a 60 mm Petri dish, resulting in 1–2 bacteria per cell. Thirty minutes after infection, macrophage monolayers were washed three times with PBS and fresh media added. At 1 hour post infection (h.p.i.) gentamicin (50 μg/ml) was added to limit bacterial extracellular growth. Intracellular growth was evaluated as follows. Macrophages were seeded on 13 glass cover slips in a 60 mm plate. At each time point three cover slips were removed and transferred to 2 ml of double-distilled sterile water, which released intracellular bacteria. Then serial dilutions of this 2 ml were plated on BHI plates and colony-forming units (CFUs) counted the next day. Phagosomal escape assay was performed as previously described [75], WT and metabolic mutants of *L. monocytogenes* expressing GFP (pPL2-GFP integrative plasmid) [65] were used to infect BMDMs on 20 mm slides. Cells were fixed at 2.5 h.p.i. with 4% paraformaldehyde-PBS and permeabilized with Triton X-100. Actin was stained with rhodamine phalloidin (Biotium), and DNA with DAPI containing Vectashield mounting media. Images were taken using Zeiss LSM 510-META confocal microscope.

Bacterial RNA purification and microarray analysis

RNA was harvested from bacteria growing mid-exponentially in BHI medium and from bacteria growing inside macrophages 6 h.p.i. (at which time the bacteria are cytosolic) as described previously [76]. Briefly, bacteria were harvested by filtration and the filters were frozen rapidly in liquid nitrogen. Later, filters were washed and bacterial RNA isolated using phenol-chloroform extraction. Bacterial RNA was amplified using MessageAmp™ II Bacteria Prokaryotic RNA Kit (Ambion). Microarray analysis was performed as described previously [74]. *L. monocytogenes* microarrays were printed at the Microarray Core Facility of University of California San-Francisco, using the *L. monocytogenes* oligo set designed and provided by The Institute for Genomic Research (TIGR) [76]. Statistical analysis was performed using the Statistical Analysis of Microarrays (SAM), with the false discovery rate set to 1%. Experiments were performed in duplicates. The values represent two biological repeats (N = 2).

Quantitative real-time PCR analysis

For validation of microarray data the intracellular transcription levels of bacterial metabolic genes were analyzed using real-time quantitative PCR (RT-qPCR) at 6 h.p.i. For the analysis of metabolic genes and virulence genes during growth in BHI medium, MDM and MDM with limiting concentrations of nutrients, RNA was harvested in mid log growth phase at O.D.₆₀₀ of 0.35. In all cases precultures were grown in MDM media overnight prior to the experiments. One microgram (1 μg) of RNA was reverse transcribed to cDNA using the High Capacity reverse transcription kit (Applied Biosystems). RT-qPCR was performed on 10 ng of cDNA using SYBER Green in a Step-one Plus real time PCR system (Applied Biosystems). The transcription level of each metabolic gene was normalized to that of a reference gene: 16S rRNA in the intracellular experiments and *spod* mRNA in the minimal medium experiments. Statistical analysis was performed using the StepOne™ V2.1 software. RT-qPCR primers are described in Table S6.

Analysis of metabolic pathways (gene expression and iMAT)

For the gene expression analysis, gene expression levels from the microarray analysis were discretized to 1, 0 or -1 for each gene
based on a twofold change in expression in intracellular bacteria vs. BHI grown bacteria. The metabolic reactions and pathways of L. monocytogenes were obtained from the genome scale automated metabolic model [31] using PERL scripts. Given the gene expression measurement, the activity state of each enzyme/reaction was determined based on the gene-reaction mapping embedded in the metabolic model, in a standard manner. Pathway-based enrichment was computed in a standard bioinformatic manner using a hypergeometric distribution, assigning an enrichment P-value for each pathway for being highly active or inactive. For iMAT analysis, the discretized gene expression levels were incorporated into the metabolic model to predict a set of high and low activity reactions (including post-transcriptional regulation predictions). The iMAT algorithm was described elsewhere [33]. Briefly, a mixed integer linear programming (MILP) problem is solved in order to find a steady-state reaction flux distribution that maximizes the number of reactions whose activity is consistent with their expression state. Subsequently, this list of active/inactive reactions was again analyzed for pathway enrichment in a standard manner, as above. The uptake reactions composing the simulated medium were selected from the pool of available exchange reactions present in the metabolic model, based on a systematic literature search in the Human Metabolome Database. The upper bounds on the set of uptake reactions selected (i.e. allowed import fluxes, measured in mmol h⁻¹ g⁻¹ dry weight, Table S2) were calibrated based on in silico flux balance analysis simulations of bacterial replication, best representing the experimentally observed bacterial generation time. In both analyses, a correction for multiple hypotheses was done using the false discovery rate (FDR) method. Metabolic pathways with less than 3 experimental observed bacterial generation time. In both analyses, a correction for multiple hypotheses was done using the false discovery rate (FDR) method. Metabolic pathways with less than 3

Supporting Information

Figure S1 A. Growth of WT Listeria monocytogenes and metabolic mutants in MDM. B. Growth of WT L. monocytogenes, ΔhisC and ΔhisC+pPL2-hisC strains in MDM without histidine. C. Growth of WT L. monocytogenes, ΔilvC and ΔilvC+pPL2-ilvC strains in MDM without BCAAs. D. Growth of WT L. monocytogenes, ΔargD and ΔargD+pPL2-argD strains in MDM without arginine. E. Growth of WT L. monocytogenes, ΔparH and ΔparH+pPL2-parH strains in MDM without adenosine. F. Growth of WT L. monocytogenes, ΔalaB and ΔalaB+pPL2-alaB strains in MDM with 10 mg/ml of L-rhamnose instead of D-glucose. The results represent 3 independent experiments (N = 3). Error bars represent standard error of the mean.

(TIF)

Figure S2 Growth of WT L. monocytogenes and the metabolic mutants in the rich lab media BHI. The results represent 3 independent experiments (N = 3). Error bars represent standard error of the mean. The ΔaroE mutant that exhibited a growth defect was complemented by introducing the aroE gene on pPL2.

(TIF)

Figure S3 Intracellular growth curves of the metabolic mutants and their complementation strains. A. Intracellular growth curves of WT L. monocytogenes, ΔparH and ΔargD mutants and their complemented strains. B. Intracellular growth curves of WT L. monocytogenes, ΔaroE and ΔilvC mutants and their complemented strains. The results are representative of 3 independent experiments (N = 3). Error bars represent standard error of the mean.

(TIF)

Figure S4 Growth curves of WT L. monocytogenes in BHI, MDM and in MDM with reduced (Low) concentrations (10 fold less) of Arg, His, Ade and BCAAs. Precultures were grown in MDM overnight. For RNA extraction, the bacteria were harvested at O.D. of 0.3, which in all cases represented logarithmic growth. The results represent two independent experiments (N = 2). Error bars represent standard error of the mean.

(TIF)

Table S1 Gene expression analysis of intracellular L.m. vs. BHI grown L.m.

(XLSX)

Table S2 The modified metabolic model of L. monocytogenes and stimulated medium used in this study.

(XLSX)

Table S3 Metabolites confidently predicted by iMAT to be imported or exported by intracellular L. monocytogenes.

(XLSX)

Table S4 Literature based “gold standard” of metabolic pathways that are essential or non-essential to L. monocytogenes intracellular growth.

(XLSX)

Table S5 List of strains and plasmids used in this study.

(XLSX)

Table S6 List of Primers used in this study.

(XLSX)
Acknowledgments

We thank Daniel Portnoy for allowing us to use the microarray experiments and the UCSF core facility. We are grateful to Colin Hill for giving us the pPl2-Plux plasmid. We thank Hadas Zur for her help with the iMAT analysis and Yair Aharonywitz and Martin Kupiec for critical review of the manuscript.

Author Contributions

Conceived and designed the experiments: LL ER AAH. Performed the experiments: LL NS AAH. Analyzed the data: LL NS IB AAH. Wrote the paper: LL AAH.

References

1. Ray K, Martemy B, Sansonetti PJ, Tang CM (2009) Life on the inside: the intracellular lifestyle of cytotoxic bacteria. Nat Rev Microbiol 7: 333–340.

2. Swaminathan B, Gerner-Smidt P (2007) The epidemiology of human listeriosis. Microbes Infect 9: 1236–1243.

3. Birke H, Sabat C, Personnic N, Cossart P (2007) Internalins: a complex family of leucine-rich repeat-containing proteins in Listeria monocytogenes. Microbes Infect 9: 1156–1166.

4. Cossart P, Vicente MF, Merougal J, Baquero F, Perez-Diaz JC, et al. (1998) Listeriolysin O is essential for virulence of Listeria monocytogenes: direct evidence obtained by gene complementation. Infect Immun 57: 3629–3636.

5. Smith GA, Marquis H, Jones S, Johnston NC, Portnoy DA, et al. (1995) The two multispanning membrane proteins EspA and EspB are necessary for invasion of epithelial cells. J Bacteriol 179: 1291–1297.

6. Kathariou S, Metz P, Hof H, Goebel W (1987) Tn916-induced mutations in the actA gene product, a surface protein. Cell 68: 521–531.

7. Tlhelyi LG, Portnoy DA (1989) Actin filaments and the growth, movement, and spread of the intracellular bacterial parasite, Listeria monocytogenes. J Cell Biol 109: 1597–1608.

8. Kocks C, Gouin E, Tabouret M, Berche P, Ohayon H, et al. (1992) L. monocytogenes-induced actin assembly requires the actA gene product, a surface protein. Cell 68: 521–531.

9. Dussurget O, Pizarro-Cerda J, Cossart P (2004) Molecular determinants of Listeria monocytogenes virulence. Annu Rev Microbiol 58: 567–610.

10. Freitag NE, Portnoy DA (1994) Dual promoters of the Listeria monocytogenes prfA gene are required for intracellular replication in macrophages. Mol Microbiol 10: 133–142.

11. Chico-Calero I, Suarez M, Gonzalez-Zorn B, Scortti M, Slaghuis J, et al. (2002) Identification of Listeria monocytogenes genes contributing to intracellular replication by transposon tagging. Mol Microbiol 45: 119–130.

12. Chico-Calero I, Suarez M, Gonzalez-Zorn B, Scortti M, Slaghuis J, et al. (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: 431–436.

13. Freitag NE, Portnoy DA (1999) The Listeria monocytogenes prfA gene is required for intracellular replication in macrophages. Mol Microbiol 34: 997–1008.

14. Edwardson W, Dandekar T, Heesemann J, Goebel W (2005) SigB-dependent in vitro transcription of prfA and some newly identified genes of Listeria monocytogenes. J Bacteriol 187: 536–540.

15. Freitag NE, Portnoy DA (1999) Characterization of leucine aminopeptidase from human liver. J Biol Chem 274: 2039–2045.

16. Bron PA, Monk IR, Coer SC, Hill C, Gahan CG (2006) Novel luciferase reporter system for in vitro and organ-specific monitoring of differential gene expression in Listeria monocytogenes. Appl Environ Microbiol 72: 2876–2884.

17. Shivers RF, Sonenshein AL (2004) Activation of the Bacillus subtilis global regulator CodY by direct interaction with branched-chain amino acids. Mol Microbiol 53: 99–115.

18. Schauer K, Stolz R, Bacher A, et al. (2008) Carbon metabolism of Listeria monocytogenes growing inside macrophages. Mol Microbiol 69: 1000–1017.

19. Joseph B, Merino S, Stoll R, Schar J, Umeshka KR, et al. (2008) Glycerol metabolism and PrfA activity in Listeria monocytogenes. J Bacteriol 190: 3412–3430.

20. Kocks C, Gouin E, Tabouret M, Berche P, Ohayon H, et al. (1992) L. monocytogenes-induced actin assembly requires the actA gene product, a surface protein. Cell 68: 521–531.

21. Joseph B, Merino S, Stoll R, Bacher A, et al. (2008) Carbon metabolism of Listeria monocytogenes growing inside macrophages. Mol Microbiol 69: 1000–1017.

22. Kocks C, Gouin E, Tabouret M, Berche P, Ohayon H, et al. (1992) L. monocytogenes-induced actin assembly requires the actA gene product, a surface protein. Cell 68: 521–531.

23. Buchrieser C, Rusniok C, Kunst F, Cossart P, Glaser P (2003) Comparison of the genome sequences of Listeria monocytogenes and Listeria innocua: clues for the evolution and pathogenicity. FEMS Immunol Med Microbiol 35: 207–213.

24. Oberhardt MA, Palsson BO, Papin JA (2009) Applications of genome-scale metabolic reconstructions. Mol Syst Biol 5: 320.

25. Price ND, Reed JL, Palsson BO (2004) Genome-scale models of microbial cells: elucidating the consequences of constraints. Nat Rev Microbiol 2: 106–117.

26. Kim HU, Kim SY, Jeong H, Kim TY, Kim JI, et al. (2011) Integrative genome-scale metabolic analysis of Vibrio vulnificus for drug targeting and discovery. Mol Syst Biol 7: 460.
52. Atkins T, Prior RG, Mack K, Russell P, Nelson M, et al. (2002) A mutant of Burkholderia pseudomallei, auxotrophic in the branched chain amino acid biosynthetic pathway, is attenuated and protective in a murine model of melioidosis. Infect Immun 70: 5290–5294.

53. Pállta S, Breitback K, Hein N, Fehlhaber B, Schulze J, et al. (2006) Identification of Burkholderia pseudomallei genes required for the intracellular life cycle and in vivo virulence. Infect Immun 74: 3576–3586.

54. Drazeck ES, Houng HS, Crawford RM, Hadfield TL, Hoover DL, et al. (1995) Deletion of purC attenuates Brucella melitensis 16 M for growth in human monocyte-derived macrophages. Infect Immun 63: 3297–3301.

55. Jenkins A, Cote G, Twerdná H, Merkel J, Buzou J, et al. (2011) Role of purine biosynthesis in Bacillus anthracis pathogenesis and virulence. Infect Immun 79: 153–166.

56. Jackson M, Phalen SW, Lagranderie M, Ensergueix D, Chavarot P, et al. (1999) Bacillus anthracis spore internalization by macrophages but not virulence in guinea pigs. Microb Pathog 38: 1–12.

57. Groves RD, Welshimer HJ (1977) Separation of pathogenic from apathogenic Listeria monocytogenes by three in vitro reactions. J Clin Microbiol 5: 559–565.

58. Kamisango K, Fujii H, Okumura H, Saiki I, Araki Y, et al. (1983) Structural and immunonochemical studies of teichoic acid of Listeria monocytogenes. J Biochem 93: 1401–1409.

59. Eugster MR, LM (2011) Rapid Analysis of Listeria monocytogenes Cell Wall Teichoic Acid Carbohydrates by ESI-MS/MS. PLoS ONE 6: doi:10.1371/journal.pone.0021500

60. Duo M, Hou S, Ren D (2008) Identifying Escherichia coli genes involved in intrinsic multidrug resistance. J Microbiol 5: 559–565.

61. Bozue JA, Parthasarathy N, Phillips LR, Cote CK, Fellows PF, et al. (2005) Construction of a rhamnose mutation in Bacillus anthracis affects adherence to macrophages but not virulence in guinea pigs. Microb Pathog 38: 1–12.

62. Oliva C, Turnbough CL, Jr., Kearney JF (2009) CD14-Mac-1 interactions in Burkholderia pseudomallei growth in human monocyte-derived macrophages. Infect Immun 77: 4437–4445.

63. Preis H, Eckart RA, Godiapik RK, Heidrich N, Brandt S (2009) CodY activates transcription of a small RNA in Bacillus subtilis. J Bacteriol 191: 5446–5457.

64. Hendriksen WT, Bootma HJ, Eteva H. A. M. Hoogenboezem T, de Jong A, et al. (2008) CodY of Streptococcus pneumoniae link between nutritional gene regulation and colonization. J Bacteriol 190: 590–601.

65. Shivers RP, Dineen SS, Sonenschein AL (2006) Positive regulation of Bacillus subtilis ackA by CodY and CcpA: establishing a potential hierarchy in carbon flow. Mol Microbiol 62: 811–822.

66. Lauer P, Chow MY, Loessner MJ, Portnoy DA, Calendar R (2002) Identification of Teichoic Acid Carbohydrates by ESI-MS/MS. PLoS ONE 6: doi:10.1371/journal.pone.0021500

67. Glomski IJ, Gedde MM, Tsang AW, Swanson JA, Portnoy DA (2002) The Listeria monocytogenes hemolysin has an acidic pH optimum to compartmentalize flow. Mol Microbiol 62: 811–822.

68. Hendriksen WT, Bootma HJ, Eteva H. A. M. Hoogenboezem T, de Jong A, et al. (2008) CodY of Streptococcus pneumoniae link between nutritional gene regulation and colonization. J Bacteriol 190: 590–601.

69. Dineen SS, McBride SM, Sonenschein AL (2010) Integration of metabolism and virulence by Clostridium difficile CodY. J Bacteriol 192: 5350–5362.

70. van Schaik W, Chateau A, Dilles MA, Copper JY, Sonenschein AL, et al. (2009) The global regulator CodY regulates toxin gene expression in Bacillus anthracis and is required for full virulence. Infect Immun 77: 4437–4445.

71. Chateau A, van Schaik W, Sin A, Acher W, Fouart A (2011) CodY regulation is required for full virulence and heme iron acquisition in Bacillus anthracis. FASEB J 25: 4445–4456.

72. Kreth J, Chen Z, Ferretti J, Malke H (2011) Counteractive balancing of transcriptional expression involving CodY and CovRS in Streptococcus pyogenes. J Bacteriol 193: 4153–4165.

73. Simon R, U. Priefer, and A. Pühler. (1983) A broad host range mobilization system for in vitro genetic engineering: transposon mutagenesis in Gram negative bacteria. Bio/Technology 1: 784–791.

74. Herskovits AA, Auerbuch V, Portnoy DA (2007) Bacterial ligands generated in a phagosome are targets of the cytosolic innate immune system. PLoS Pathog 3: e51. doi:10.1371/journal.ppat.0030051

75. Glomski IJ, Gedde MM, Tsang AW, Swanson JA, Portnoy DA (2002) The Listeria monocytogenes hemolysin has an acidic pH optimum to compartmentalize flow. Mol Microbiol 62: 811–822.

76. Hendriksen WT, Bootma HJ, Eteva H. A. M. Hoogenboezem T, de Jong A, et al. (2008) CodY of Streptococcus pneumoniae link between nutritional gene regulation and colonization. J Bacteriol 190: 590–601.

77. Leber JH, Crimmins GT, Raghavan S, Meyer-Morse NP, Cox JS, et al. (2008) Distinct TLR- and NLR-mediated transcriptional responses to an intracellular pathogen. PLoS Pathog 4: e6. doi:10.1371/journal.ppat.0040006

78. Lauer P, Hanssen B, Lemmens EE, Liu W, Luckett WS, et al. (2008) Constitutive Activation of the PrfA regulon enhances the potency of vaccines based on live-attenuated and killed but metabolically active Listeria monocytogenes strains. Infect Immun 76: 3742–3753.

79. 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: 4177–4186.