RESEARCH ARTICLE

Systems Level Analyses Reveal Multiple Regulatory Activities of CodY Controlling Metabolism, Motility and Virulence in *Listeria monocytogenes*

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Abstract

Bacteria sense and respond to many environmental cues, rewiring their regulatory network to facilitate adaptation to new conditions/niches. Global transcription factors that co-regulate multiple pathways simultaneously are essential to this regulatory rewiring. CodY is one such global regulator, controlling expression of both metabolic and virulence genes in Gram-positive bacteria. Branch chained amino acids (BCAAs) serve as a ligand for CodY and modulate its activity. Classically, CodY was considered to function primarily as a repressor under rich growth conditions. However, our previous studies of the bacterial pathogen *Listeria monocytogenes* revealed that CodY is active also when the bacteria are starved for BCAAs. Under these conditions, CodY loses the ability to repress genes (*e.g.*, metabolic genes) and functions as a direct activator of the master virulence regulator gene, *prfA*. This observation raised the possibility that CodY possesses multiple functions that allow it to coordinate gene expression across a wide spectrum of metabolic growth conditions, and thus better adapt bacteria to the mammalian niche. To gain a deeper understanding of CodY's regulatory repertoire and identify direct target genes, we performed a genome wide analysis of the CodY regulon and DNA binding under both rich and minimal growth conditions, using RNA-Seq and ChIP-Seq techniques. We demonstrate here that CodY is indeed active (*i.e.*, binds DNA) under both conditions, serving as a repressor and activator of different genes. Further, we identified new genes and pathways that are directly regulated by CodY (e.g., *sigB*, *arg*, *his*, *actA*, *glpF*, *gadG*, *gdhA*, *poxB*, *glnR and fla genes*), integrating metabolism, stress responses, motility and virulence in *L. monocytogenes*. This study establishes CodY as a multifaceted factor regulating *L. monocytogenes* physiology in a highly versatile manner.

Author Summary

Bacterial pathogens sense multiple host-related metabolic signals that alert them of host localization and result in induction of virulence traits. The Gram-positive foodborne pathogen *Listeria monocytogenes* activates the transcription of its virulence genes in response to low levels of branch-chained amino acids (BCAAs). This phenomenon is dependent on the global transcription regulator CodY, which binds BCAAs as a ligand and this binding affects its regulatory functions. CodY is classically thought to function under rich growth conditions, when bound to its ligands, however we recently reported that CodY directly activates *L. monocytogenes* virulence when BCAAs are limited. Identifying this novel CodY activity prompt us to further investigate CodY functions under different growth conditions in a genome wide level. For this purpose, we set on analyzing CodY's regulon in *L. monocytogenes* in both rich and minimal growth conditions using genome-wide sequencing techniques. Remarkably, we identified for the first time a global regulatory role for CodY when BCAAs are limited, that are similar to those within the mammalian niche. Furthermore, our data establish CodY as a central regulator that integrates metabolism, motility, stress responses and virulence in *L. monocytogenes*.

Introduction

Listeria monocytogenes is a Gram-positive facultative intracellular pathogen transmitted by ingesting contaminated foods. *L. monocytogenes* causes a disease termed listeriosis associated with a mortality rate of up to 30%. Listeriosis typically manifests as a mild gastroenteritis in healthy people, however it can lead to meningitis in elderly and immunocompromised people and cause stillbirth in pregnant women [1]. The replicative niche of *L. monocytogenes* inside the host is within the cell cytosol [2]. The bacteria invade non-phagocytic host cells by expressing specialized proteins termed internalins that induce active internalization; in the case of phagocytic cells the bacteria are simply phagocytosed [3, 4]. Subsequently, *L. monocytogenes* rapidly escapes from the endosome/phagosome vacuole using primarily the listeriolysin O toxin (LLO), two phospholipases (PlcA and PlcB), and components of the competence system [5–8]. Having gained entry to the host cell cytosol *L. monocytogenes* replicates rapidly (at a growth rate similar to that exhibited in rich laboratory medium), and spreads from cell to cell using actin based motility, which is mediated by the virulence factor, ActA [9, 10]. Remarkably, all the above-mentioned virulence factors (and other factors) are positively regulated by PrfA, a Crp/Fnr like transcription regulator that is considered the master virulence activator of *L. monocytogenes* [11, 12].

The transition from saprophyte to deadly pathogen relies largely on *L. monocytogenes* sensing multiple host-specific signals that are transduced to trigger PrfA expression and activity [13]. For example, sensing of temperature, certain carbon sources (*e.g.*, glucose-1-phosphate), the availability of amino acids (*e.g.*, isoleucine), iron and glutathione, were all shown to trigger PrfA via different mechanisms, and thus activate downstream virulence genes [14–21]. These examples highlight that multiple mechanisms have evolved to sense various host-specific cues and metabolic signals that conjointly inform *L. monocytogenes* of its intracellular location and the need to switch to the virulent state. To better understand the metabolic environment within the host cell and the signals that activate *L. monocytogenes* virulence genes during intracellular growth, we previously performed a genome scale integrative study that combined transcriptome analysis and metabolic modeling *in silico* [18]. Various bacterial metabolic pathways were identified to be highly active during *L. monocytogenes* infection and contribute to bacterial

intracellular growth in macrophage cells. Notably, the biosynthesis of branch-chained amino acids (BCAAs) (*i.e.*, isoleucine, leucine and valine (ILV)) was highly induced in intracellularly grown bacteria, a pathway encoded by the *ilv* operon, suggesting that BCAAs may be limiting in macrophage cells. In light of this finding, we reasoned that sensing of metabolite availability within the host cell might alert the bacteria of their intracellular location and the need to activate the virulence state. Accordingly, we found that growing *L. monocytogenes* in minimal defined medium with limiting amounts of BCAAs indeed leads to robust activation of the virulence genes [18]. Under low concentrations of BCAAs, in particular of isoleucine, *prfA* and some of its downstream-regulated genes were highly expressed concomitantly with the BCAA biosynthesis pathway. These observations identified BCAAs as an important metabolic signal for *L. monocytogenes* within the mammalian niche.

Subsequently, we demonstrated that CodY, a global regulator and sensor of BCAAs, is responsible for the upregulation of *prfA* and the virulence genes under low BCAAs concentrations [17, 18]. CodY was found to bind directly within the coding sequence of the *prfA* gene (15 nucleotides down-stream the ATG start codon) and activate transcription, in turn leading to upregulation of virulence genes. These findings were surprising since CodY was thought to function primarily as a repressor and to bind DNA primarily under rich media conditions (*i.e.* under high BCAA concentrations) [17].

CodY is a Gram-positive specific global regulator that was discovered two decades ago in *Bacillus subtilis* as a general repressor of stationary phase genes, though now it is known to regulate many cellular processes [22–24]. CodY responds to cellular levels of BCAAs by directly binding these amino acids, an interaction that influences its structural conformation and activity [25–27]. In some Gram-positive bacteria CodY also binds GTP, however the effect of this interaction is not completely understood [28]. Initial studies indicated that CodY acts as a general repressor (when bound to GTP or BCAAs) of many metabolic genes including the BCAAs biosynthesis pathway. However, later studies in *B. subtilis* demonstrated that CodY also functions as an activator, in the presence of its ligand (*i.e.*, under high concentrations of BCAAs) [29, 30]. In *L. monocytogenes*, CodY was shown to repress genes involved in amino acid metabolism, nitrogen assimilation and sugar uptake under conditions of high BCAA concentrations [31]. A role for CodY in regulation of virulence was demonstrated in Gram-positive pathogens other than *L. monocytogenes*, including *Clostridium perfringens*, *Bacillus anthracis* and *Streptococcus pyogenes* where CodY was shown to activate indirectly the expression of certain virulence genes [32–35].

As mentioned above, before the present study, CodY activity was primarily documented under rich growth conditions, with the ability of CodY to bind DNA demonstrated in the presence of its regulatory ligand [25, 36]. However, our previous observation that CodY possesses a regulatory activity and DNA binding capacity also under minimal growth conditions, exhibiting limited amounts of BCAAs (as shown for the *prfA* gene), raised the possibility that CodY may bind DNA and function also when BCAAs are at low concentrations, maybe even in its unliganded form [17]. Support for this premise came from the observation that a CodY protein mutated within its BCAA-binding site (*i.e.*, harboring a R61A substitution within its GAF domain) loses the ability to repress metabolic genes under high BCAAs concentrations but retains the ability to activate *prfA* under low BCAAs concentrations [17].

To further delineate if indeed CodY possesses diverse activities under rich and minimal growth conditions and thus regulates different target genes, we took a system-level approach employing *L. monocytogenes* bacteria. The CodY regulon, in particular its direct target genes, were analyzed in bacteria grown in rich and minimal media (the latter containing low concentrations of BCAAs) using RNA-Seq and ChIP-Seq techniques. Notably, the data reveal that CodY retains multiple regulatory activities under both conditions, orchestrating the expression of metabolic, stress and virulence genes in a highly versatile manner.

Results

RNA-Seq transcriptome analysis defines the CodY regulon under both rich and minimal growth conditions

Before the present study, CodY's regulon and direct target genes were assessed under rich growth conditions (or that are rich in BCAAs), and thus a role for CodY was documented in the presence of its ligand isoleucine [33, 37–42]. To better decipher CodY's activity in relation to the availability of its regulatory ligand/s, we analyzed the CodY regulon under both rich and minimal growth conditions (the latter containing low levels of BCAAs) using the RNA-Seq technique. Wild-type (WT) and *ΔcodY L. monocytogenes* bacteria were grown in rich brain heart infusion (BHI, containing excess amounts of BCAAs > 800 μM) and low BCAAs minimal defined media (LBMM; containing ~80 μM of BCAAs). Total bacterial RNA was extracted at mid-logarithmic growth and subjected to deep sequence analysis using Illumina HiSeq 2500. Of note, these experimental conditions were chosen in concordance with our previous findings demonstrating that during growth in BHI, CodY effectively represses metabolic genes (*e.g.*, the *ilv* operon, the hallmark of CodY regulation), whereas during growth in LBMM this repression is relieved concomitantly with CodY activation of the *prfA* gene [17, 18]. As reported previously, WT and *ΔcodY* bacteria exhibit similar growth in LBMM medium, whereas *ΔcodY* bacteria grown in BHI displayed a slightly reduced growth as compared to WT bacteria (S1 Fig) [17]. RNA-Seq analysis was performed in triplicate and the reproducibility of the biological repeats was high (a mean R^2–0.956) (S2 Fig).

A total of 368 genes (~14% of *L. monocytogenes* genome) were found to be affected by CodY (directly and indirectly) under both conditions. Among these, 237 genes were upregulated and 131 genes were down regulated in the *ΔcodY* mutant in comparison to WT bacteria. In BHI medium, 334 genes were differentially regulated by CodY (directly and indirectly), in agreement with previous reports [37–43]. Under this condition, 111 genes were down regulated and 223 genes were upregulated in the *ΔcodY* mutant in comparison to WT bacteria (Fig 1A and 1C). Notably in LBMM, 181 genes were differentially regulated by CodY, among them 55 genes were down regulated and 126 were upregulated in the *ΔcodY* mutant, demonstrating for the first time a global regulatory role for CodY under low BCAAs conditions (Fig 1B and 1C). Notably, among all of the upregulated genes (237 genes), 112 (45%) were upregulated under both rich and minimal conditions, whereas among the down regulated genes (131 genes), 36 (~27%) were down regulated under both conditions (Fig 1B). Overall these findings indicate that CodY may serve as both a repressor and as an activator of genes under high and low BCAAs levels. Moreover, they demonstrate that another mode of CodY regulation might exist that is independent of BCAAs. The latter may be mediated by additional regulatory factors such as GTP. Applying manually curated criteria clustering and hierarchical clustering on all of the differentially regulated genes yielded 6 distinct gene clusters representing all modes of CodY regulation (Fig 1B and 1C and S1 Table). Specifically, 111 genes were identified to be repressed (cluster I) and 76 genes to be activated (cluster II) by CodY (directly and indirectly) exclusively under rich growth conditions, 14 genes were identified to be repressed (cluster III) and 19 genes to be activated (cluster IV) by CodY exclusively under minimal growth conditions, while 112 genes were identified to be repressed (cluster V) and 36 genes to be activated (cluster VI) by CodY under both rich and minimal growth conditions (clusters are listed in S1 Table). Taken together, the data highlight CodY's plasticity and ability to regulate genes in diverse spectrum of growth conditions.

Pathways and responses were identified manually and by functional enrichment analysis using the MIPS server (Fig 2 and S2 Table) [44]. Genes repressed by CodY under rich growth conditions are involved in amino acid metabolism and transport (*e.g.*, BCAAs and histidine
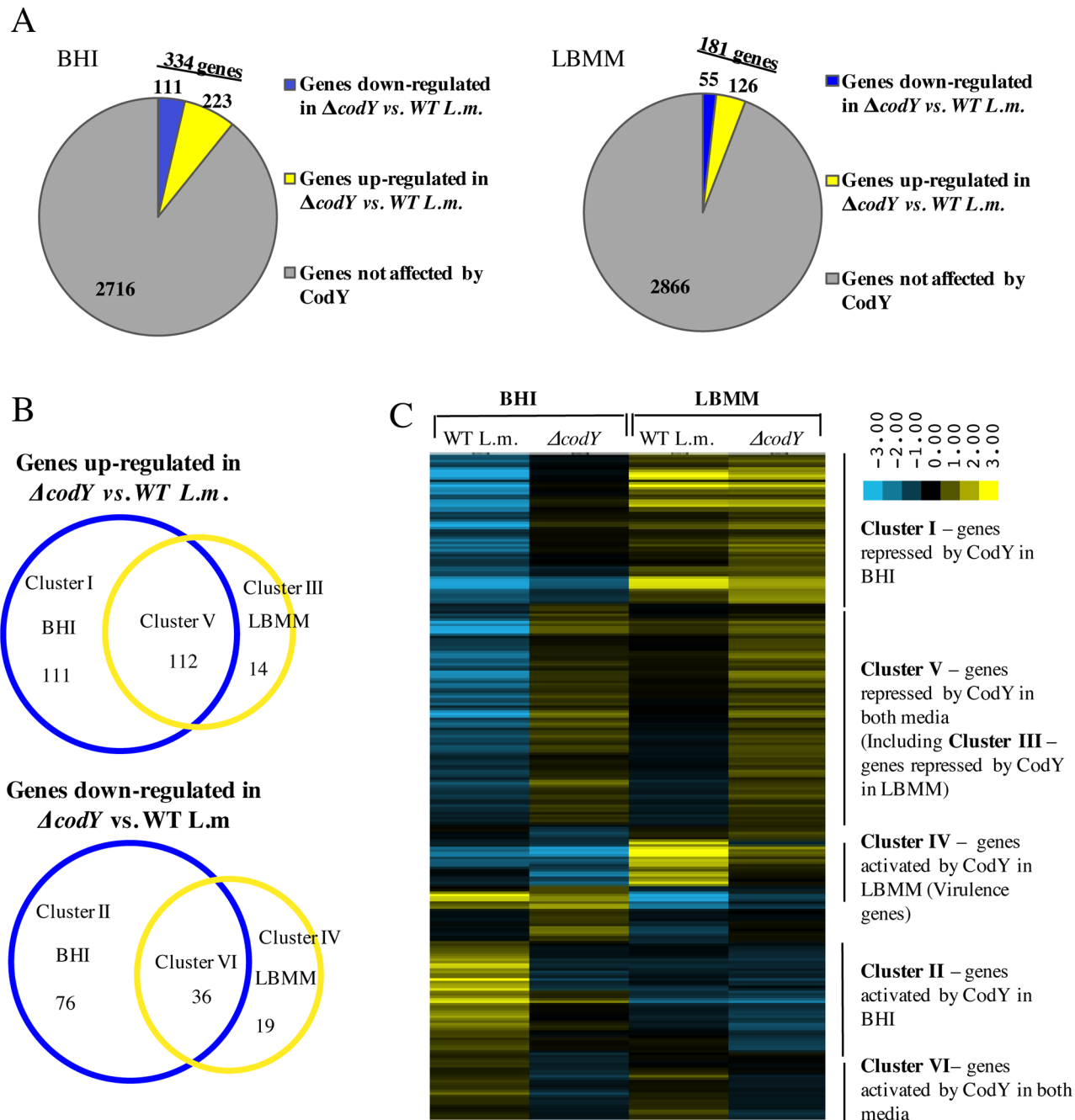
A

B

C

Fig 1. RNA-Seq analysis of WT and *ΔcodY L. monocytogenes* strains grown in BHI and LBMM media. A. Pie charts representing differentially transcribed genes in WT and *ΔcodY L. monocytogenes* bacteria grown in BHI or LBMM. **B.** Venn diagrams representing the different CodY regulated gene clusters in both BHI and LBMM media. **C.** Hierarchical clustering of differentially transcribed genes in WT and *ΔcodY L. monocytogenes* bacteria in both BHI and LBMM media.

biosynthesis pathways), peptide and sugar transport systems (*e.g.*, the OppC and OppF permeases and the glycerol uptake protein) and stress responses (*e.g.*, bile salt hydrolase (*bsh*), *clpC*, and heat shock proteins) (cluster I) (Fig 2 and S1 Table). Notably, this cluster includes several virulence-associated genes such as internalin A and B and the glycerol transporter and kinase, which are expressed during *L. monocytogenes* infection of mammalian cells [15, 18]. Several
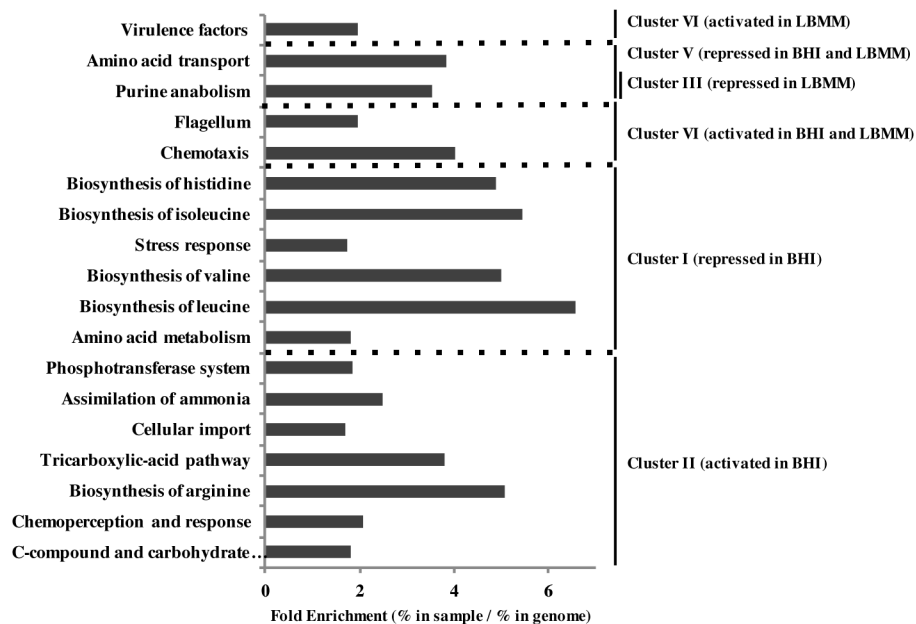
Fig 2. Functional enrichment analysis of CodY regulated genes. Categories functionally enriched in each cluster of CodY regulated genes, analyzed using the MIPS server [44]. All categories are significantly enriched (P-value <0.005).

metabolic genes/pathways were activated by CodY under this condition, such as arginine biosynthesis, assimilation of ammonia, certain PTS systems and enzymes of the tricarboxylic acid (TCA) pathway (cluster II) (Fig 2 and S2 Table).

Under minimal growth conditions, we identified *prfA* and its downstream virulence genes (*e.g.*, *hly*, *actA*, *plcA*, *plcB* and *mpl*) to be activated by CodY (cluster IV), which is in accordance with our previous results [17, 18] (S1 Table). Notably, all the other genes within this cluster (except for one; LMRG_01981) were previously found to be induced during *L. monocytogenes* infection of mammalian cells [18], implicating a potential role in *L. monocytogenes* virulence. Some of these genes mediate ammonium transport, nitrogen regulation, cell wall synthesis and certain PTS systems, while others represent conserved hypothetical genes. Of note, it is not known yet whether these genes are under the direct regulation of PrfA. Overall, these findings strengthen the premise that CodY plays an important role in the activation of *L. monocytogenes* virulence under low BCAAs conditions, which resemble the mammalian cytosolic niche. Under these conditions, we found CodY to repress genes involved in purine metabolism, and iron transport, as well as some genes that encode hypothetical proteins (Cluster III) (S1 and S2 Tables). Genes repressed by CodY under both rich and minimal conditions are involved in nitrogen metabolism, arginine deiminase, osmotic and salt stress responses, distinct PTS systems and encode amino acid transport proteins, which most likely reflects nutrient availability within the media and the growth conditions tested (cluster V). Genes/pathways activated by CodY under both conditions include various metabolic genes and transport systems, D-alanine dipeptide synthesis, as well flagella biosynthesis and chemotaxis (cluster VI) (S1 and S2 Tables). Notably, several transcription regulators and regulatory proteins were identified within the CodY regulon (distributed among the different clusters), such as sigma-B, GlnR, GntR and certain CRP/FNR transcription regulators, as well sigma-54 regulatory proteins, indicating a hierarchy in CodY gene regulation. Overall, the RNA–Seq analysis highlights the

breadth of CodY regulation in *L. monocytogenes* and its potential regulatory functions under varying metabolic environments.

ChIP-Seq analysis identifies genes directly regulated by CodY

To delineate which genes in the CodY regulon are directly regulated by CodY, a genome wide chromatin immunoprecipitation was performed in combination with DNA sequence analysis (ChIP-Seq) using Illumina HiSeq 2500. An *L. monocytogenes codY-6his* strain, in which the *codY* gene was replaced with a 6-histidine tagged *codY*, was grown in both BHI and LBMM media to mid-exponential phase. Bacteria were then cross-linked using formaldehyde and subjected to ChIP as described in the Materials and Methods. Of note, we have established previously that the CodY-6His protein functions similarly to the native CodY [17]. The ChIP-Seq analysis revealed 302 DNA regions bound by CodY under both conditions (270 in LBMM and 131 in BHI), with ~30% overlap (S3 Table). Among the 302 binding regions identified, 61 were mapped to transcriptional units (genes and operons) that were shown to be differentially regulated by CodY in the RNA-Seq analysis (corresponding to a total of 127 genes), and thus may represent *bona fide* targets of CodY under the tested conditions. Notably, 48 DNA regions were associated with transcriptional units regulated in LBMM (activated and repressed) and 33 DNA regions were associated with transcriptional units regulated in BHI (activated and repressed), whereas 20 DNA regions overlapped the two conditions (Fig 3A and S4 Table). Importantly, the data revealed that CodY directly regulates 33% of the genes within its regulon, and that it directly binds more regulatory regions under minimal growth conditions harboring low levels of BCAAs than under rich growth conditions, which is surprising given what is known about this regulator (see Discussion). Among the 302 CodY-binding regions identified, 71 (~23%) contained a putative CodY-box/s similar to those found in *Lactococcus lactis* and *B. subtilis* [37, 45], whereas among the 61 DNA regions that coincided with CodY regulated genes, 24 (~40%) exhibited a CodY-box, as determined by the MAST algorithm that predicts the presence of CodY binding motifs (Fig 3B and S3 Table). This observation suggests that CodY may employ additional binding sites or mechanisms to directly regulate genes. As expected, we found that the *ilvD* promoter region was bound specifically under high BCAA concentrations, whereas the virulence genes region was bound specifically under low BCAA concentrations; these data points serve effectively as positive controls for the ChIP-Seq dataset (Fig 3B and S4 Table).

By combining the data of the RNA-Seq and ChIP-Seq analyses, we were able to determine CodY direct-regulated genes, which further fall into the six described clusters. Under rich growth conditions, CodY directly represses the transcription of BCAAs, histidine, methionine, purine and riboflavin biosynthesis genes as well as the transcription of sigma B, *clpC*, glycerol uptake and phosphorylation, and a few general metabolic genes (cluster I). Under these same conditions, CodY directly activates the transcription of genes that encode for arginine biosynthesis enzymes, peptidoglycan deacetylation enzymes (on N-acytelglucosamine), and several PTS systems (cluster II) (S4 Table). Under minimal growth conditions, CodY directly represses purine biosynthesis, iron transport, a gene involved in pyrimidine biosynthesis and an amino acids permease (cluster III). Under the same conditions, CodY directly activates a cysteine transporter and a specific PTS system, in addition to the virulence regulator, *prfA* (cluster IV). Surprisingly, within this latter cluster we identified a novel CodY binding region upstream to the *actA* gene, which is responsible for *L. monocytogenes* intracellular actin based motility, and that is itself under the regulation of PrfA (S4 Table). Of note, this regulatory relationship represents an additional direct role for CodY in *L. monocytogenes* virulence. Under both rich and minimal growth conditions, CodY directly represses amino acids transport, PTS systems, genes

A

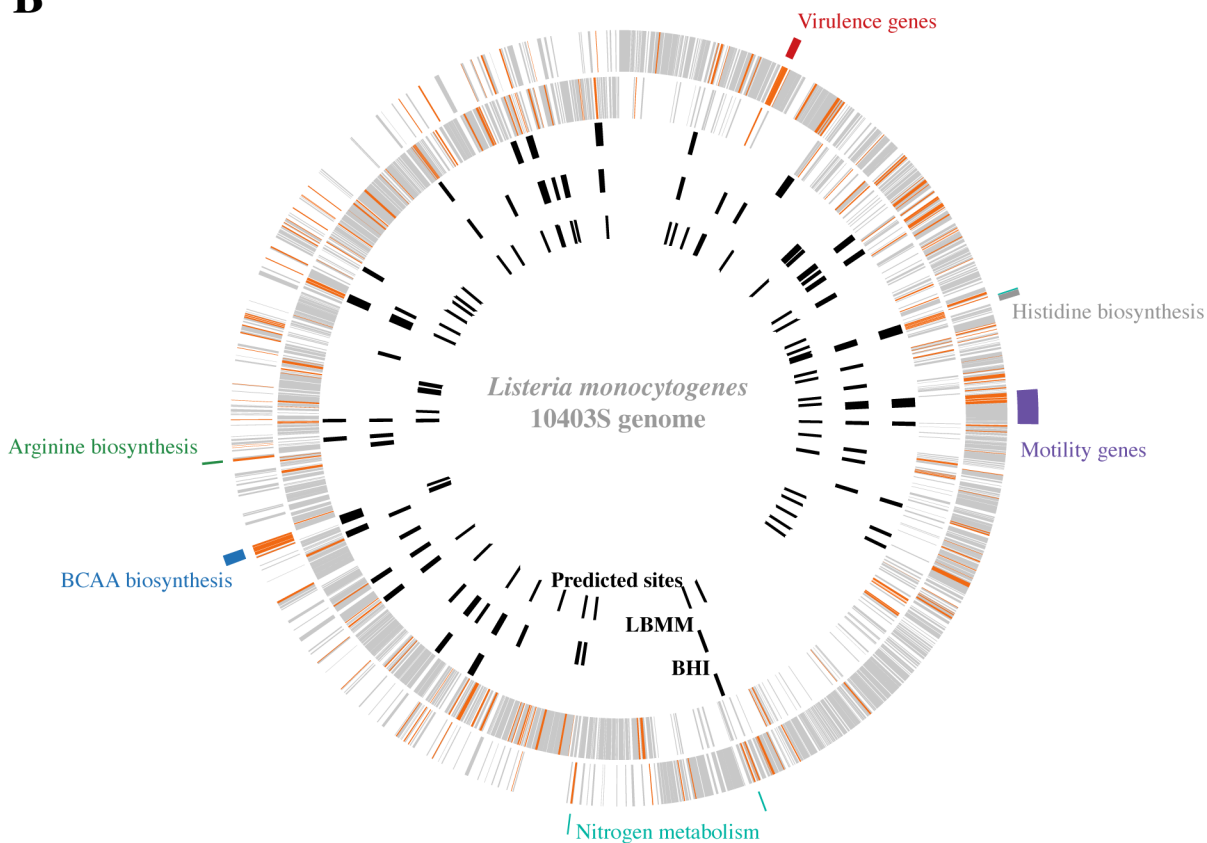**CodY binding sites associated with differentially regulated genes**
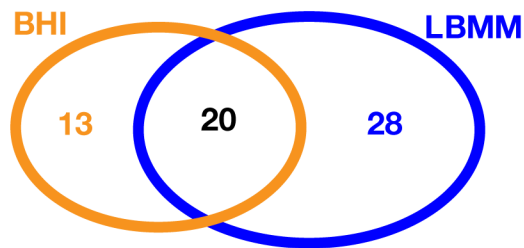
B

Fig 3. ChIP-seq analysis of CodY binding sites under growth in BHI and LBMM media. A. Venn diagram representing CodY binding sites associated with differentially expressed genes in BHI and LBMM. **B.** Genomic view of CodY binding sites in *L. monocytogenes* 10403S– the two outer circles represent the + and—strands of *L. monocytogenes* genome, each line is a gene. Orange lines represent CodY regulated genes, while grey lines represent genes unaffected by CodY (based on the RNA-Seq analysis). The third circle represents CodY binding sites associated with differentially expressed genes in BHI medium, while the forth circle represents CodY binding sites associated with differentially expressed genes in LBMM. The fifth circle represents CodY motifs associated with differentially expressed genes, identified by the MAST algorithm [46] using a CodY binding consensus sequence as an input based on previous experimental CodY-box analyses [36, 37]. The consensus sequence was generated using the MEME algorithm [47]. Only sites with E value < 10 and P value < 0.05 are shown.

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involved in nitrogen (*e.g.*, glutamate dehydrogenase, *gdhA* gene), pyruvate and lipids metabolism, and directly activates motility and chemotaxis genes, the GlnR regulator, other PTS systems and additional metabolic genes (S4 Table).

Analysis of direct regulation of genes by CodY

Next, as real-time quantitative PCR (RT-qPCR) analysis is still considered the gold standard in gene transcription analysis, we employed it together with ChIP RT-qPCR analysis to validate that CodY directly regulates representative genes from each cluster (clusters I-VI). To this end, we chose genes/operons that contain a putative CodY-box in their regulatory region (S3 Table and S3 Fig). From cluster I, comprising genes repressed by CodY in BHI, we chose the BCAAs and the histidine biosynthesis pathways (genes tested: *ilvD*, *ilvC*, *hisG*, *hisA* and *hisI*), as well the sigma B regulator (*sigB*) and the glycerol uptake transporter (*glpF*). From cluster II, comprising genes activated by CodY in BHI, we chose the arginine biosynthesis pathway and the glutamate decarboxylase gene (genes tested: *argH*, *argF* and *gadG)*. From cluster III, comprising genes repressed by CodY in LBMM, iron uptake genes were chosen (*e.g.*, *feoA*). From cluster IV, comprising genes activated by CodY in LBMM, *prfA* and *actA* genes were chosen. From cluster V, comprising genes repressed by CodY under both conditions, the *gdhA* gene was chosen [39, 48] and *poxB* gene encoding a pyruvate oxidase. From cluster VI, comprising genes activated by CodY under both conditions, flagella and motility genes were chosen (e.g., *motB*, *flhA* and *fliP*) as well the *glnR* gene, encoding the nitrogen metabolism regulator GlnR. In general, we found the RT-qPCR transcription profiles of the tested genes to be similar to those observed using RNA-Seq analysis. Genes predicted to be repressed by CodY were up regulated in the *ΔcodY* mutant in comparison to WT bacteria, whereas genes predicted to be activated by CodY were down regulated in the *ΔcodY* mutant (Fig 4A). The only exception is the observation that the *ilv* genes exhibit a higher transcriptional level in WT bacteria versus *ΔcodY* mutant during growth in LBMM. These results suggest that CodY further activates these genes when BCAAs concentrations drop significantly, a phenotype that was previously observed at [18].

ChIP RT-qPCR experiments were performed to verify direct binding of CodY to the regulatory regions of the chosen genes/operons (in the case of an operon the first gene of the operon was tested). Similar to the ChIP-Seq experiments, a CodY-6His variant was used to precipitate DNA fragments during *L. monocytogenes* growth in BHI and LBMM. Amplification of CodY binding regions upstream to the different genes/operons indeed verified that CodY binds all the tested regulatory regions (Fig 4B). In most cases, the binding of CodY correlated with the corresponding conditions in which CodY's regulatory activity was observed.

Next, to affirm that CodY regulation responds primarily to the availability of BCAAs, we repeated the RT-PCR and the ChIP RT-PCR experiments in bacteria grown in minimal defined medium containing high levels of BCAAs (HBMM, containing 800 µM of BCAAs) and compared it to BHI and LBMM conditions (Fig 4A and 4B). Interestingly, most of the representative genes were regulated in HBMM as in BHI ($R^2 = 0.81$), and were different from LBMM ($R^2 = 0.005$), indicating that indeed BCAAs represent the primary ligand of CodY under these conditions. An exception, were the genes of the histidine and the arginine biosynthesis pathways, which were regulated by CodY in BHI (repressed and activated, respectively), but not in HBMM, suggesting that in conjunction with CodY, additional factors (e.g., GTP) mediate the regulation of these pathways under rich conditions.

To further characterize the binding of CodY to regulatory regions of select genes/operons, an electrophoresis mobility shift analysis (EMSA) was performed using DNA probes comprising the upstream intergenic sequences plus a ~100 bp of the gene 5'-coding sequence. In accord with the ChIP data, we observed binding of CodY to all tested probes with varying affinities.
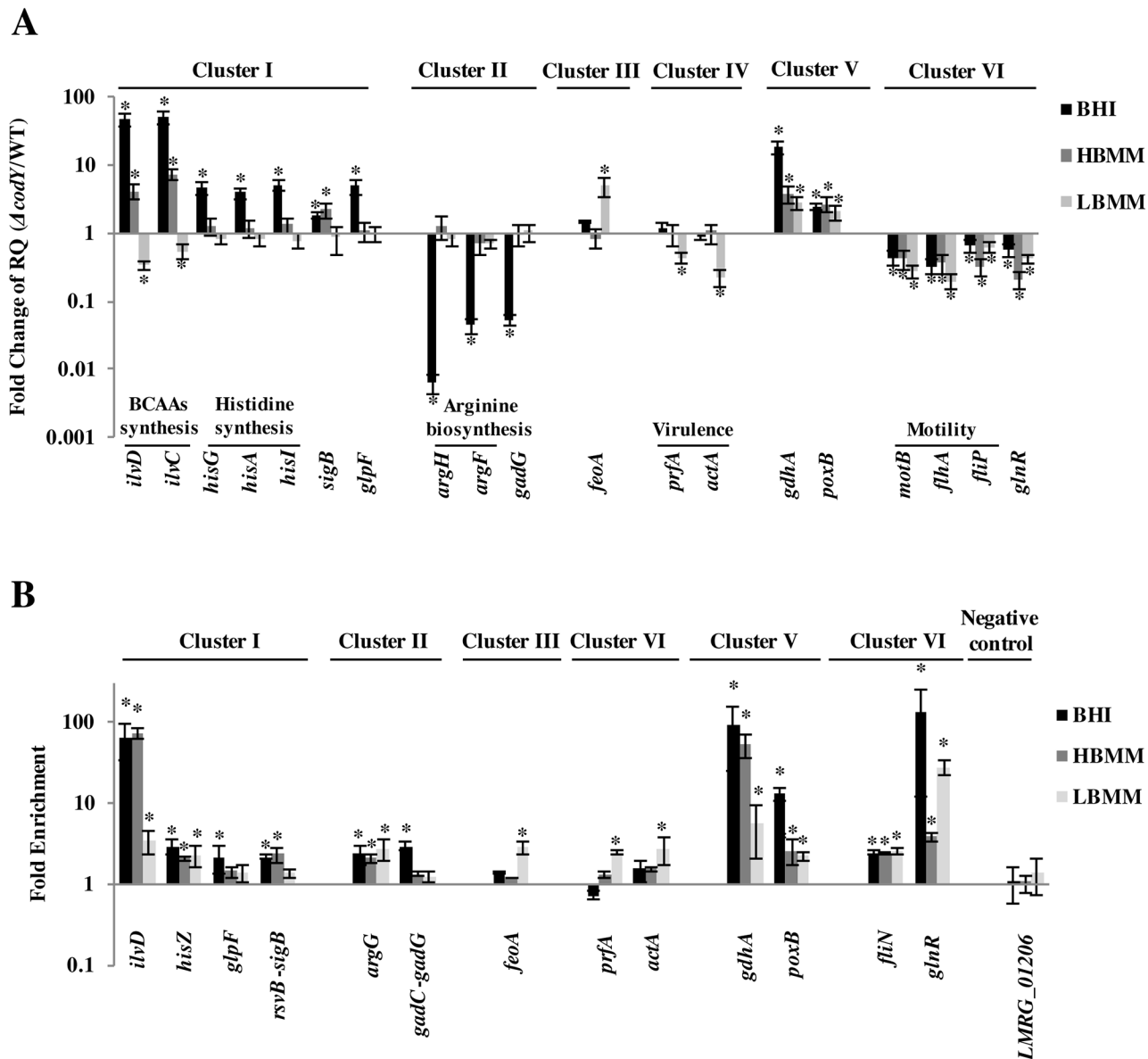
Fig 4. Validation of CodY-dependent differential transcription and CodY binding to representative genes from each cluster using RT-PCR and ChIP RT-PCR. A. RT-PCR transcription analysis of representative genes of each cluster presented by fold change values of mRNA relative quantity (RQ) in WT *vs.* Δ*codY* bacteria grown in BHI, HBMM and LBMM media. Results are average of at least 3 independent biological repeats. Transcription levels were normalized to the transcription of *rpoD*. Error bars represent standard error (SE). **B.** ChIP RT-PCR analysis of CodY binding to representative genes from each cluster (corresponding panel A) in bacteria grown in BHI, HBMM and LBMM media. Fold enrichment of CodY association with each one of the tested sequences was first normalized to two control sequences: *bglA* and *rpoD* (normalization to multiple control genes was done by StepOne software of Applied biosystems) and then to its no-ChIP control. Results are average of at least 3 independent biological repeats. Error bars represent standard error (SE). Asterisk (*) represents p-value < 0.05.

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Although EMSA reactions are not at equilibrium and should be considered qualitatively, apparent K_D values were derived as following: 150 ± 34 nM for *hisZ*, 151.5 ± 0.5 nM for *rbsV-sigB*, 114 ± 13 nM for *glpF*, 70 ± 11 nM for *argG*, 25 ± 13 nM for *gadC-gadG*, 90 ± 48 nM for *feoA*, 208 ± 19 nM for *actA*, 57 ± 3 nM for *gdhA*, 55 ± 2 nM for *poxB*, 74 ± 32 nM for *glnR* and 26 ± 9 nM for *fliN* (Fig 5 and S4 Fig). Of note, binding of CodY to the regulatory regions of genes specifically regulated in LBMM (Clusters III and IV) was measured in the absence of
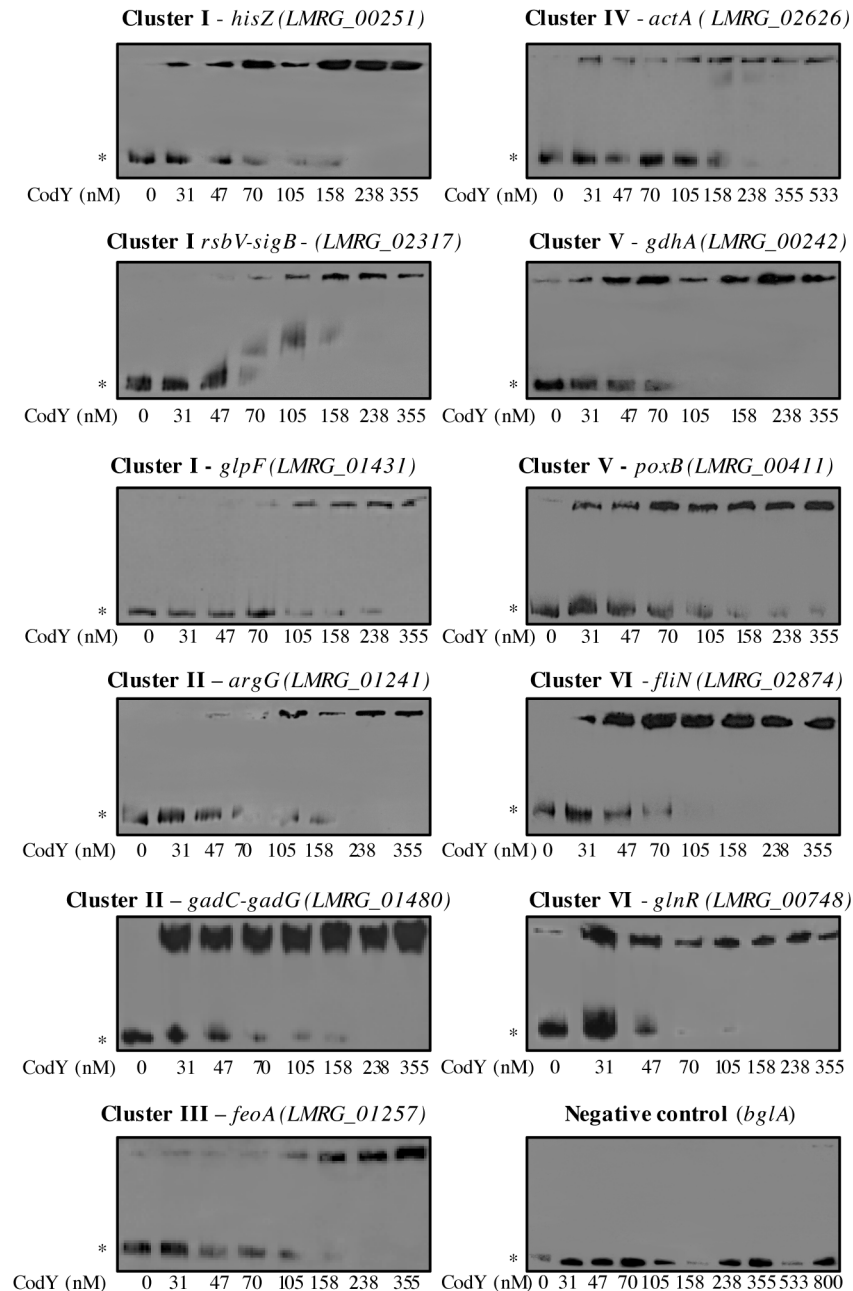
Fig 5. EMSA analysis of CodY binding to select genes from the different clusters. Binding of CodY to 11 regulatory regions of CodY regulated transcriptional units (*hisZ*, *rsbV*, *glpF*, *argG*, *gadC*, *feoA*, *actA*, *gdhA*, *poxB*, *glnR* and *fliN*) and a control probe (*bglA*). Primers used for amplification of DNA probes are described in S5 Table. Results are representative of at least 2 independent biological repeats. EMSA was performed with 10 mM BCAA for *hisZ*, *rsbV*, *glpF*, *argG*, *gadC gdhA*, *poxB*, *glnR* and *fliN*. For *feoA* and *actA* BCAAs were not added. Asterisks (*) point to unbound DNA probes.

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BCAAs. Taken together, these experiments corroborate CodY's ability to directly bind and regulate different genes in a versatile manner and identified *hisZ*, *rbsV-sigB*, *glpF*, *argG*, *gadC-gadG*, *feoA*, *actA*, *gdhA*, *poxB*, *glnR* and *fliN* as novel *L. monocytogenes* CodY direct target genes, representing metabolic and virulence genes.

CodY activates flagella biosynthesis in *L. monocytogenes* and facilitates bacterial attachment to mammalian cells

Finally, intrigued by the observation that CodY directly activates genes involved in flagella biosynthesis, we examined the ability of the *ΔcodY* mutant to swarm on soft agar plates. WT and *ΔcodY* bacteria were subjected to a swarming assay on plates containing BHI and LBMM media. In accordance with our findings, the *ΔcodY* mutant was found to be severely impaired in motility in comparison to WT bacteria on both media tested (Fig 6). On BHI plates, swarming regions with mean diameters of 1.07 ± 0.07 cm and 0.625 ± 0.06 cm were measured for WT and *ΔcodY* bacteria, respectively (Fig 6A), while on LBMM plates, mean diameters of 1.3 cm ± 0.09 cm and 0.76 ± 0.04 cm were measured for WT and *ΔcodY* bacteria, respectively
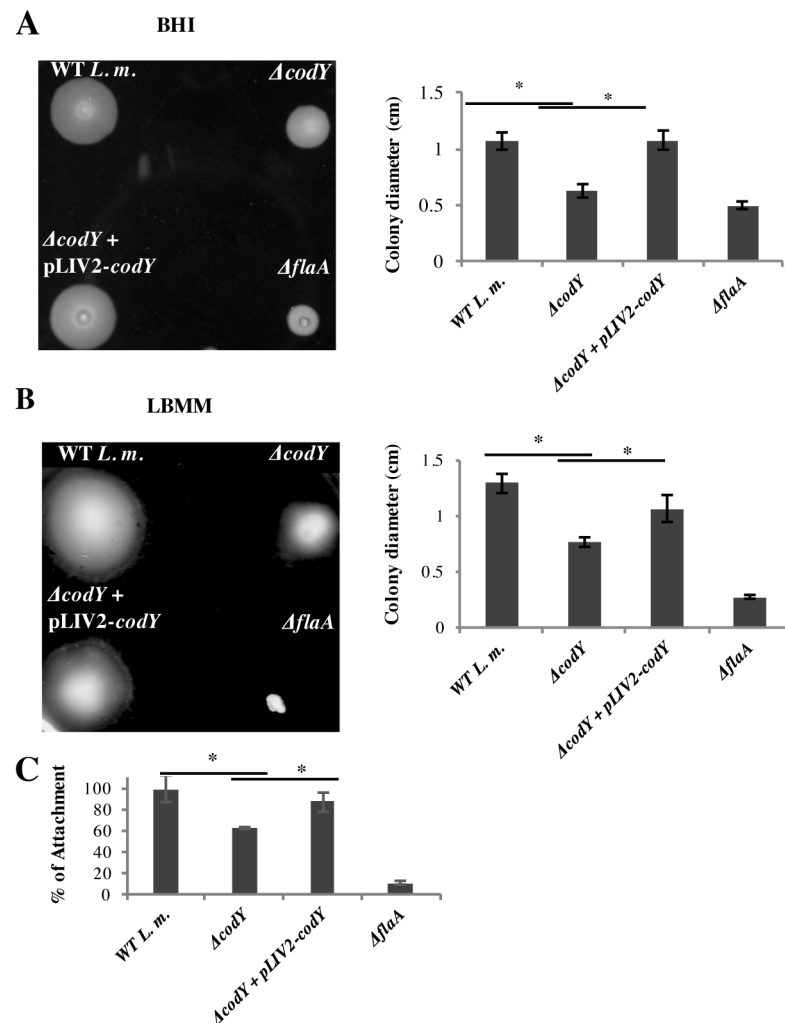

Fig 6. *ΔcodY* mutant is impaired in motility and cell adhesion. Swarming of *L. monocytogenes* WT and *ΔcodY* bacteria, as well a *codY* complemented strain on BHI **A.** and LBMM **B.** soft agar plates. *ΔflaA* strain was used as a negative control. Results are representative of at least 3 biological repeats. Bar charts represent the diameters of bacterial growth zones, based on 3 independent biological repeats. **C.** An attachment assay of WT, *ΔcodY* and *codY* complemented *L. monocytogenes* strains to Caco2 epithelial cells. *ΔflaA* is used as a negative control. The results represent at least 3 biological repeats Error bars represent standard error of the mean (SE). Asterisk (*) represents p-value < 0.05. Student's t-test was used for statistical analysis.

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([Fig 6B](#)). Introducing an ectopic copy of the *codY* gene to the Δ*codY* mutant (Δ*codY+-pLIV2-codY*) restored bacterial motility on both media to WT levels ([Fig 6A and 6B](#)). Since the flagella plays a critical role in *L. monocytogenes* attachment to mammalian cells [49, 50], we further examined the ability of the Δ*codY* mutant to attach to Caco2 epithelial cells. An attachment assay was performed with WT bacteria, Δ*codY*, Δ*codY+pLIV2-codY* and a Δ*flaA* mutant as a control. Indeed we observed a reduced attachment of Δ*codY* bacteria to Caco2 cells, a defect that was rescued in a Δ*codY* complemented strain (Δ*codY+pLIV2-codY*) ([Fig 6C](#)). Overall, these findings support a more central role for CodY in *L. monocytogenes* pathogenesis, beyond activation of virulence genes *per se*, a role that encompasses regulation of metabolic and motility genes, functions important for successful mammalian infection.

Discussion

In this study, we applied a genome-wide approach to identify CodY's regulon, target genes and regulatory functions in *L. monocytogenes*. Unlike previous studies of CodY, we examined both rich and minimal growth conditions, in attempt to explore further the possibility that CodY retains activity also when BCAAs, its primary ligands, are in limiting amounts. In contrast to the current dogma that CodY functions only in the presence of its ligand BCAAs, our results clearly demonstrate that CodY functions under both conditions, rich and minimal, and that under each condition it can serve as a repressor and as an activator of genes, establishing for the first time a global regulatory role for CodY under low levels of BCAAs ([Fig 7](#)). Furthermore, this study reveals a broader role for CodY in *L. monocytogenes* physiology, and particularly in regulation of virulence, as novel CodY direct target genes were discovered that are known to contribute to *L. monocytogenes* infection of mammalian cells (discussed below). Notably, this study not only builds on previous knowledge of how CodY serves to monitor and fine tune bacterial metabolism, but also expands our understanding of CodY's spectrum of activities and impact on other central bacterial processes. For the first time, CodY is established as an integrator of bacterial motility, stress related and virulence functions and metabolic adaptations.

The two systems level analyses that we employed to delineate the role of CodY in *L. monocytogenes* growth under rich and minimal conditions were RNA-Seq and ChIP-Seq. The RNA–Seq analysis revealed that CodY affects the expression of hundreds of genes, establishing this transcription factor as a central regulator of *L. monocytogenes*. The ChIP-Seq analysis showed
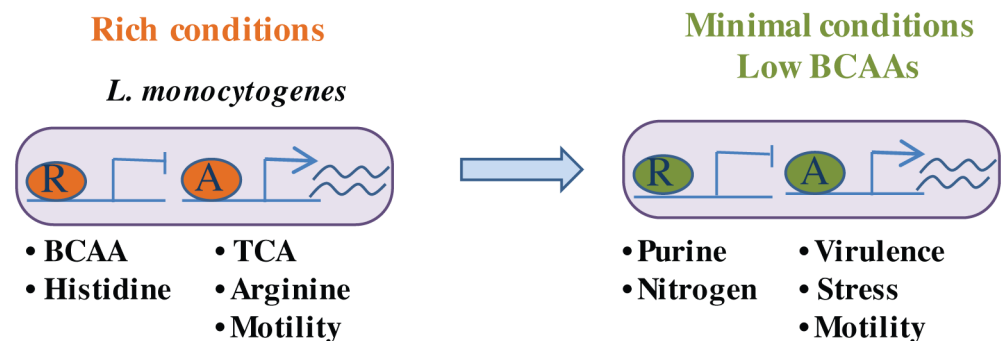

Fig 7. CodY serves as both a repressor and as an activator of genes under rich and minimal growth conditions. Unlike previous studies describing a function for CodY only under rich conditions, here we show that CodY exhibit a global regulatory role as a repressor and as an activator of genes under both rich and minimal conditions, the latter limiting for BCAAs. The coordinated regulation of metabolic, stress and virulence genes by CodY, most likely allows *L. monocytogenes* bacteria to swiftly switch from being a saprophyte to virulent bacteria. The color-coded oval represents CodY protein under the different conditions bound to regulatory regions of genes. R represents repressor and A activator.

that CodY directly regulates 33% of its regulon, under both rich and minimal growth conditions. In total, more genes were regulated by CodY (directly and indirectly) under rich nutrient conditions (*i.e.*, in BHI), with the majority repressed, essentially validating CodY's global role as a repressor of metabolic genes. Nevertheless, a third of the CodY genes regulated under this condition were found to be activated by CodY, many of them related to the TCA cycle (discussed below), demonstrating CodY's ability to serve as an activator as well.

As previously reported in other bacteria, we found that CodY represses amino acid biosynthesis (mainly BCAAs and histidine), purine, riboflavin and certain carbon and nitrogen metabolism genes under rich nutrient conditions [37–42, 48]. However, we found that CodY activates critical enzymes of the TCA cycle, including glutamate/glutamine derivatives and the arginine biosynthesis pathway, in contrast to what was shown in *B. subtilis* and *L. lactis*, where CodY was found to repress these genes [37, 41, 51]. This intriguing discrepancy suggests *L. monocytogenes* may have evolved distinct metabolic network/fluxes to fit its unique lifestyle. Specifically, our data predict that under rich nutrient conditions CodY directs metabolic flux from pyruvate to the TCA cycle through pyruvate carboxylase (PycA), which generates oxaloacetate, while blocking pyruvate flux to the BCAAs biosynthesis pathway through direct repression of the pyruvate oxidase gene, *poxB* and the *ilv* operon (both shown to be directly repressed by CodY) (Fig 8). This model is based on our findings that CodY upregulates most of the downstream TCA cycle genes (encoding four consecutive enzymes converting oxaloacetate to 2-oxoglutarate). Since the TCA cycle of *L. monocytogenes* is missing the enzyme that converts 2-oxoglutarate to succinate (2-oxoglutarate dehydrogenase) [52, 53], this step may be bypassed by glutamate synthase converting 2-oxoglutarate together with glutamine to two molecules of glutamate, which are then further converted to GABA by glutamate decarboxylase (GadG), and to succinate by the GABA shunt [54]. A support for this metabolic bypass is provided by the observation that the gene encoding glutamate decarboxylase (*gadG*) was also found to be directly activated by CodY, while the enzyme that reverts glutamate to 2-oxoglutarate, namely glutamate dehydrogenase (encoded by *gdhA*), was found to be directly repressed by CodY under these conditions (Fig 8). Moreover, further down this pathway, we found that CodY activates expression of fumarate reductase, which converts succinate to fumarate, in agreement with the premise that CodY positively regulates the TCA cycle and the glutamine/glutamate-GABA bypass (Fig 8). Interestingly, since *L. monocytogenes* is also missing the enzyme malate dehydrogenase, which converts malate to oxaloacetate [52, 53], conversion of fumarate to malate is a dead end reaction. In this regard, our observation that CodY activates expression of arginine biosynthesis genes during growth in BHI may be explained as a way to consume fumarate through a reversed arginine pathway, generating carbamoyl phosphate that can further feed to nitrogen or pyrimidine metabolism (Fig 8). In general, this alternative metabolic flux may generate energy and essential precursors to other metabolic pathways to support rapid growth of *L. monocytogenes* in rich medium. In contrast, during growth in LBMM CodY does not up regulate the expression of the TCA cycle or the arginine metabolism genes and most likely diverts the flux of pyruvate to the generation of BCAAs (Fig 8). This model may explain the observation that the bacteria grow more slowly in LBMM than in rich medium (S1 Fig).

Recently, the second messenger molecule c-di-AMP was shown to be involved in regulation of the TCA cycle and was reported to be essential specifically during growth in rich medium conditions, but not during growth in minimal medium [55]. Interestingly c-di-AMP was found to negatively regulate the pyruvate carboxylase, PycA, (via an allosteric binding) thus controlling the conversion of pyruvate to oxaloacetate [56]. Therefore, under conditions where c-di-AMP is absent (simulated by a diadenylate cyclase mutant, *ΔdacA*) and it is expected that PycA activity is enhanced, it follows that the TCA cycle is accelerated, resulting in generation and accumulation of intermediates and byproducts that may be toxic to the bacteria. We propose that while
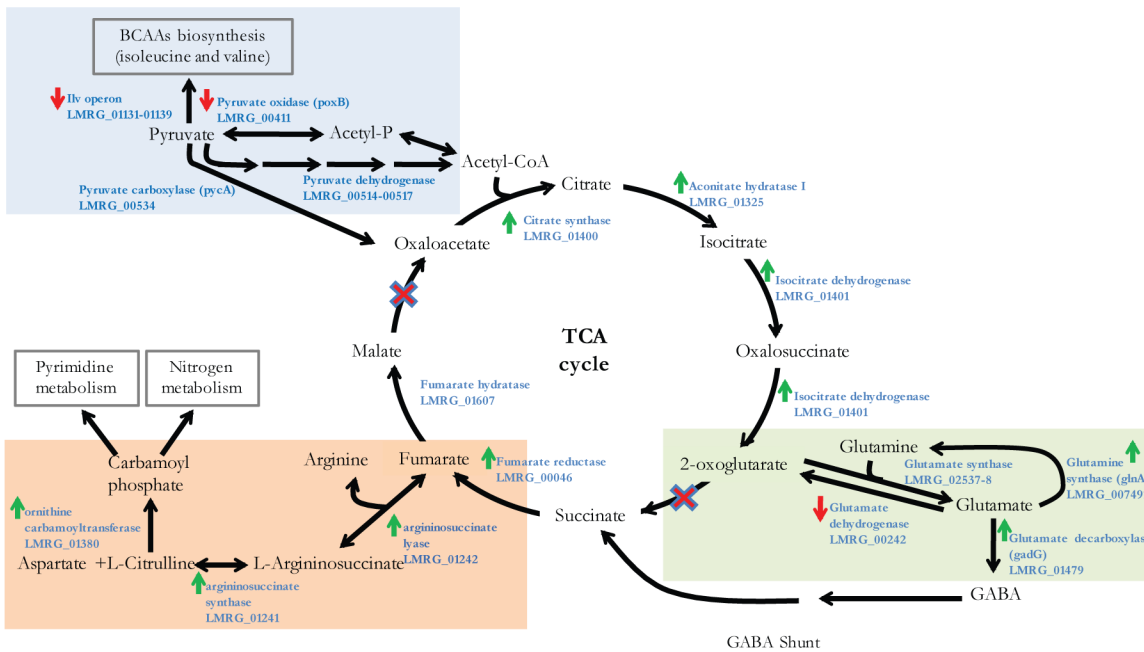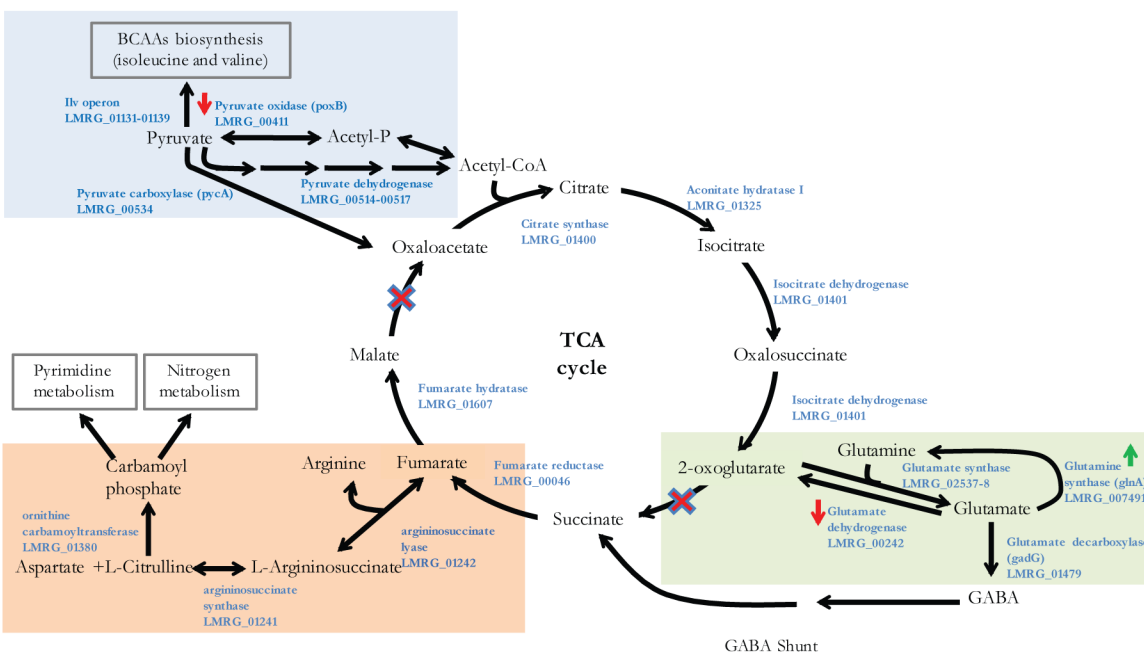
Fig 8. A Model for CodY regulation of *L. monocytogenes* central metabolism. Illustration of central metabolic pathways that are regulated by CodY in *L. monocytogenes* in BHI **A.** and LBMM **B.** In light blue—pyruvate metabolism, light green—nitrogen metabolism, light orange—arginine metabolism, in the middle the TCA cycle. Green arrows represent enzymes that are induced by CodY, while red arrows represent enzymes repressed by CodY, based on the RNA-Seq analysis.

c-di-AMP may play a role in regulation of the TCA cycle under rich conditions (i.e., by preventing a high flux of pyruvate to the TCA cycle), in minimal medium this function may be dispensable, since part of the pyruvate flux is directed by CodY to the BCAAs biosynthesis pathway. A corollary of this hypothesis is that inactivation of CodY, which in turn reduces flux through the

TCA cycle, may alleviate the toxic phenotype of the *ΔdacA* mutant under rich nutrients conditions. Interestingly, a recent study reported an indirect but intriguing link between DacA and CodY, whereby a mutation in the *codY* gene rescued the virulence defect of a *ΔrelA* mutant, which in turn rescued the growth defect of *ΔdacA* [55]. While more research should be done to explore these phenotypes, it is clear that c-di-AMP and CodY play important roles in shaping *L. monocytogenes* core metabolism and by doing so affect virulence. Notably, the former is a known listerial immunostimulatory ligand, which is recognized by the innate immune system during infection, and thus exerts additional phenotypes within the host [57–59].

Another novel metabolic relationship identified in this study is the direct activation of GlnR expression by CodY. GlnR is a conserved transcription regulator of nitrogen metabolism genes (such as those involved in glutamine, glutamate and ammonium metabolism) [60]. Although both CodY and GlnR were previously reported to independently repress nitrogen related genes (*e.g.*, *gdhA*) [39, 48], a direct relationship was not previously documented. The activation of *glnR* by CodY may underlie CodY's observed robust regulation of nitrogen metabolism genes.

In addition to metabolic regulation, this study identified a role for CodY in regulation of stress responses. Specifically, under rich nutrient conditions we found that CodY represses, directly and indirectly, the stress induced protease HslUV, the chaperon GroEL-GroES, the osmoprotectant transport system OpuCA and the stress responsive alternative sigma factor, SigB (σ^B). The latter is shown here for the first time to be a direct target of CodY, indicating a hierarchical regulation of stress related genes down-stream to CodY. Notably, σ^B is a critical sigma factor of *L. monocytogenes*, playing a major role in regulation of stress related and virulence genes during mammalian infection [61, 62]. Sigma B itself is directly involved in PrfA regulation, as it binds the *prfA* promoter and facilitates transcription during infection [61, 63, 64]. Our novel finding that CodY regulates *sigB* raises the possibility that during mammalian infection, conditions in which BCAAs are limited, CodY may promote *prfA* transcription by at least two mechanisms: directly via binding to the *prfA* gene and indirectly by relieving *sigB* repression. In this regard, we identified also several virulence related genes to be indirectly repressed by CodY, *e.g.*, *inlA* and *inlB*, which mediate bacterial internalization into mammalian cells [3, 4]. These genes were shown previously to be positively regulated by σ^B [65] and thus may be repressed in BHI as a result of *sigB* repression by CodY. Similarly, the bile salt hydrolase (*bsh*) and the osmoprotectant system *opuCA* are indirectly repressed by CodY during growth in BHI and are both known to be positively regulated by σ^B [61, 65]. More generally, these findings reveal tight regulatory cross-talk between three central factors, σ^B, PrfA and CodY that together coordinate *L. monocytogenes* adaptation to the mammalian niche with regards to stress, virulence and metabolism, respectively.

One of the big surprises of this study was the extent of CodY regulation under conditions when BCAAs are limiting. We found that CodY regulates (activates and represses) genes involved in metabolism, motility and virulence. Cluster IV is of particular interest, comprising genes activated by CodY in LBMM, as this cluster includes most of *L. monocytogenes* major virulence factors in addition to some metabolic genes. CodY regulation of Cluster IV strengthens the premise that CodY attunes virulence functions and metabolic requirements to better adapt the bacterium to the intracellular niche. Notably, three novel CodY direct targets were identified within this cluster in addition to the *prfA* gene: the *actA* gene; a cysteine transporter gene; and a PTS system operon (S3 Fig). We confirmed CodY binding to the *actA* regulatory region by ChIP-RT-qPCR and EMSA assays. Nevertheless, it remains somewhat intriguing why CodY directly regulates both *actA* and *prfA* genes, as *actA* is already under the direct regulation of PrfA. One possible explanation is that by regulating *actA* CodY is serving as a direct regulatory link between metabolism and motility (in this case intracellular motility), in addition to general regulation of virulence. A role for CodY in *L. monocytogenes* motility is further highlighted by

our findings that CodY directly activates flagellar and chemotaxis genes (*i.e.*, extracellular motility) under both growth conditions. Taken together, these observations suggest that CodY plays a major role in coordinating sensing of nutrients with bacterial movement under diverse conditions and niches. CodY regulation of motility was also documented in *B. cereus*, where it was shown that CodY positively regulates motility genes and that a Δ*codY* strain is less motile [40]. Similarly, we showed in the present study that an *L. monocytogenes codY* mutant is impaired in motility and attachment to mammalian cells.

Above all, the present study establishes that CodY regulation is more complex than classically considered. Previous studies depicted CodY as a transcriptional regulator that represses gene expression either by binding to promoter regions to interfere with RNA polymerase binding or by binding to internal sites leading to transcriptional roadblocks [45, 66, 67]. In light of our new data, it appears that CodY could function in all possible states and forms, for example under high and low BCAAs levels, as a repressor and as an activator (under both conditions), with the ability to bind multiple binding sites with different affinities around the genome; such complexity will make future study of CodY highly interesting yet challenging. It is most likely that *in vivo* the different binding sites are subject to genome wide binding competition, which is dependent on the concentrations of the different CodY forms (for example bound or unbound to a specific ligand). The data also suggests that additional factors are involved in mediating CodY binding to the different sites, as many strong sites that are bound under rich conditions (e.g., upstream to the *ilv* operon) do not appear to be bound in minimal conditions, although BCAAs are still present, while other binding sites are bound under both conditions. A model of cooperative binding was suggested before for CodY to explain such phenomena [45], where under a given condition CodY may cooperatively bind DNA in high affinity, but if conditions are changed even a bit (*e.g.*, a slight drop in BCAA levels), binding is completely lost. Under this scenario, other binding sites with lower affinities might now be accessible for CodY binding and thus better compete. This model may explain why we were able to detect many direct binding sites that are specific to LBMM. In addition, it is most likely that CodY does not work alone and that other transcription regulators and factors influence its activity, as was observed in the case of the histidine and the arginine operons. Such factors can affect CodY's DNA accessibility, binding affinity or conformation and thus modulate gene expression [28, 68, 69]. Notably, GTP is another known ligand of CodY that was shown in other bacteria to affect CodY activity [25, 28]. It is possible that GTP modulates CodY activity also in *L. monocytogenes*, and that CodY responds to varying concentrations of GTP in a similar manner it responds to BCAAs. Under this scenario, the relative concentrations of the two ligands under changing environments may determine CodY activities and binding affinities, a hypothesis that awaits further investigation. Of note, we did not observe differences in the transcription of the three *relA* paralogs of *L. monocytogenes* (*relA*, *relP* and *relQ*) in the RNA-seq data, genes that their products are known to affect intracellular levels of GTP.

As in other genome wide binding studies, we observed many CodY binding regions that do not appear to be associated with transcriptional regulation. This phenomenon is generally explained by one of the following scenarios: non-specific protein binding that influences DNA topology; an active mechanism that serves to titer the regulator itself; or redundancy with other co-regulators, as was recently shown for CodY and ScoC interactive regulation, where CodY deletion alone did not result in changes in gene expression of the BCAAs permase, *braB* [69–75]. This notwithstanding, the observation that many of the identified binding regions do not contain a CodY putative binding box as identified in other bacteria [72, 76, 77], suggests that CodY binding sites in *L. monocytogenes* are more diverged than in *B. subitlis* and *L. lactis* and/ or that additional CodY recognition sites might exist. Unfortunately, the resolution of our ChIP-Seq data did not allow us to identify such new sites, though new techniques may lead in

the future to identification of such sites. Overall, this study expands our understanding of CodY functions in *L. monocytogenes*, and we expect these insights to impact the study of CodY in other pathogenic and non-pathogenic bacteria.

Materials and Methods

Bacterial strains and media

L. monocytogenes 10403S [78] was used as the wild type strain. Brain heart infusion (BHI, Merck) was used as a rich medium, while high BCAAs minimal medium (HBMM) was used as defined medium as in [79] with 100 μg ml⁻¹ of BCAAs [isoleucine, leucine and valine] and low BCAAs minimal medium (LBMM) was made with 10 μg ml⁻¹ for each BCAA, which is 76 μM for leucine and isoleucine and 85 μM for valine. Bacteria were grown at 37°C with agitation in BHI, HBMM or LBMM. Bacterial strains used in this study are listed in S5 Table.

RNA extraction, RNA-Seq sample preparation and real time PCR analysis

Bacteria (WT and Δ*codY* strains) were grown to mid-exponential phase in BHI, HBMM or LBMM at 37°C (OD600 = 0.35). RNA was extracted using the RNAsnap method [80]. For RNA-Seq samples, DNase I (Qiagen) treatment was performed on Qiagen RNAeasy columns. For RT-PCR analysis, DNase I (Fermentas) treatment was followed by phenol-chloroform extraction. For RNA-Seq samples, the RNA integrity number (RIN) was evaluated using a TapeStation instrument (Agilent Technologies) and then rRNA was depleted using the Ribo-Zero kit (Epicentere). RNA-Seq libraries were prepared using the TruSeq RNA sample Prep kit (Illumina) and sequenced (50 nt per read) by HiSeq 2500 instrument (Illumina) at the Technion Genome Center (Haifa, Israel). For RT-qPCR analysis, 1 μg of total RNA was reverse transcribed using QScript reverse transcription kit (Quatna). 16 ng of cDNA were used for RT-qPCR analysis with FastStart Universal Green Master Mix (Roche) using a StepOnePlus instrument (Applied Biosystems). The transcription level of each gene of interest was normalized to that of the *rpoD* mRNA. For the ChIP-RT-PCR analysis, each target gene was first normalized by the levels of *rpoD* and *bglA* DNA in each sample (normalization to multiple control genes is recommended and done using StepOne software) and then the ChIP sample was normalized to its no-ChIP sample using the StepOne V2.3 software. First, ΔCt for each sample is calculated as ΔCt = Average Ct—Normalization Factor (NF), while Normalization Factor is the mean of the selected endogenous controls (single or multiple genes), which is used to normalize the Ct value of each sample. Next, Fold enrichment is calculated as RQ = $2^{(-\Delta Ct\ (ChIP\ sample))} / 2^{(-\Delta Ct\ (no\text{-}ChIP\ sample))}$ RT-qPCR primers are described in S5 Table.

Bacterial ChIP

Bacterial ChIP analysis was performed as described in [17, 81]. Bacteria were grown in 50 ml of BHI, HBMM or LBMM at 37°C with shaking at 250 rpm to O.D. of ~0.35. 1% formaldehyde was added to the cultures, which were then incubated at room temperature with shaking at 100 rpm for 20 min. 0.5 M Glycine was added to quench excess formaldehyde by shaking for 5 min at room temperature at 100 rpm. Afterwards, the samples were centrifuged at 4000 rpm (2600 g) for 10 min at 4°C, washed twice with cold TBS (50 mM Tris-HCl pH 7.5, 150 mM NaCl) and kept at -80°C. Cross-linked samples were resuspended in 0.2 ml of lysis buffer (10 mM Tris pH 8, 20% sucrose, 50 mM NaCl, 10 mM EDTA and 10 mg ml⁻¹ of lysozyme) and incubated for 30 min at 37°C, and then 0.8 ml of IP-buffer (50 mM HEPES-KOH pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X100, 0.1% sodium deoxycholate and 0.1% SDS) supplemented

with 1 mM PMSF was added. The samples were lysed using sonication (6 rounds of 30 s) and were centrifuged 10 min at 14000 rpm (18000 g) at 4°C and the supernatants were transferred to new 1.5ml tubes. Sheered DNA was analyzed by electrophoresis to observe bands between 200–500 bps. 0.8 ml of sonicated sample was used for immuno-precipitation by adding 20 µl of magnetic A/G beads (Millipore, Cat. 16–663) and 5 µl of anti-his tag antibody (Abcam, Cat. 18184). Samples were then incubated with rotation overnight at 4°C to allow immuno-binding. Beads were collected using magnetic stands and the supernatant was transferred to a new tube to be used as control DNA. Beads were washed twice with 0.5 ml of IP-buffer, once with 0.5 ml of IP-buffer supplemented with 500 mM NaCl, once with 1 ml of washing buffer (10 mM Tris pH 8, 250 mM LiCl, 1 mM EDTA, 0.5% IP-40 and 0.5% sodium deoxycholate) and once with TE buffer (50 mM Tris pH 7.5 and 10 mM EDTA). Finally, the samples were resuspended in 0.1 ml of elution buffer (50 mM Tris pH 7.5, 10 mM EDTA and 1% SDS) and incubated 10 min at 65°C. Then the beads were removed by magnetic stands and the supernatants were transferred to a new tube. 80 µl of TE buffer and 2.5 µl of RNase A (8 mg ml^{-1}) were added and incubated for 1.5 h at 42°C, followed by 2 h incubation at 42°C with 20 µl of Proteinase K (Fermentas). Then, the samples were incubated overnight at 65°C and the next day the DNA was isolated using Qiagen DNA concentration and cleanup kit. For ChIP-Seq analysis, 50 ng of ChIP and control DNA were used to prepare ChIP DNA libraries using the TruSeq ChIP sample Prep kit and sequenced (50 nt per read) on HiSeq 2500 instrument (Illumina) at the Technion Genome Center (Haifa, Israel).

RNA-Seq anaylsis

On average ~10 million reads were obtained per cDNA library in fastq file, providing a 168-fold genomic coverage (data was deposited in GEO, accession number—GSE76159). The quality of the reads was evaluated using FastQC (Babraham Bioinformatics) and if needed, trimming of reads was performed using the fastX tool kit (http://hannonlab.cshl.edu/fastx_toolkit/). Mapping of reads followed by upper quartile normalization by gene expression and differential expression analysis by the negative binomial distribution as the statistical model was performed using Rockhopper V1.3 with default parameters [82]. For curated clustering of differentially expressed genes 3 criteria were used: minimal value of 10 normalized counts in at least one of the samples, minimal fold change between the WT and *ΔcodY* samples of at least 1.8 in either the BHI or LBMM media, and a significant q-value (<0.05) of the negative binomial analysis performed by Rockhopper V1.3. Hierarchical clustering of the differentially expressed genes was performed by the average linkage method using Cluster V3.0 (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm), and a heatmap was generated using TreeView V3 (http://jtreeview.sourceforge.net).

ChIP-Seq analysis

fastq files were obtained and processed as mentioned above (data was deposited in GEO, accession number—GSE76821). Reads were mapped to *L. monocytogenes* genome using Bowtie2 running with default parameters [83]. Peak calling for ChIP-Seq analysis was performed using MACS V1.4.2 with default parameters [84] and with SeqMonk. CodY motif search based on *L. lactis* and *B. subtilis* CodY motifs was performed using MAST [47].

Functional enrichment analysis

Differentially expressed genes from each cluster of the RNA-Seq analysis were used as input for the MIPS server [44, 85] for functional enrichment analysis.

Purification of CodY-6His

L. monocytogenes CodY-6His was expressed in *E. coli* strain BL-21 from the pET28 expression plasmid. 10 ml of overnight bacterial culture were diluted in 0.5 L of LB medium supplemented with 30 µg ml^{-1} of kanamycin. Bacteria were grown till O.D.$_{600}$ = 0.3, and then induced with 1 mM IPTG for 4 h. The bacteria were harvested by centrifugation (2600 g, 10 min), washed in 50 ml of cold buffer A (0.3 M NaCl, 50 mM NaH$_2$PO$_4$, pH 8) and resuspended in 15 ml of buffer A supplemented with 10 mM imidazole and 1 mM PMSF. Bacteria were lysed by an ultra high-pressure homogenizer (Stansted Fluid Power) at 12000 psi. Cell debris were removed by centrifugation at 16,000 g for 20 min and the lysate was incubated with 1ml of Ni-NTA beads (Sigma) for 1 h at 4°C with tilting. The Ni-NTA beads were then loaded on a column and washed with 10 ml buffer A supplemented with 10 mM imidazole. The protein was eluted by 250 mM imidazole in buffer A and dialyzed overnight against 100 ml of buffer A. Protein concentration was determined using a Nanodrop 1000 (Thermo) spectrophotometer. 0.5 µg of purified protein were separated on SDS-PAGE gel followed by Commassie staining to test for the purity of the protein.

Electrophoretic mobility shift assay (EMSA)

For EMSA probes of CodY target genes the upstream intergenic region up to ~100 bps into the coding sequence of the target gene was amplified using PCR and labeled using Roche DIG Gel Shift kit. Purified CodY-6His was incubated with 4 ng of DIG-labeled target DNA in binding buffer (20 mM Tris-Cl pH 8, 50 mM KCl, 2 mM MgCl$_2$, 0.5 mM EDTA, 1mM DTT, 0.05% NP-40, 5% glycerol, 25 µg ml^{-1} salmon sperm DNA) for 15 min at room temperature. The samples were then loaded onto a pre-run 8% native acrylamide gel (running buffer composed of: 35 mM HEPES, 43 mM imidazole buffer, pH 7.4) and separated for 1.5 h at 200 V. Detection of DIG labeled probes was performed using Roche DIG detection kit and visualized using Super RX-N FUJIFILM. For calculations of average apparent K_D values, the ratio between the free DNA probe and total DNA probe at each CodY concentration (i.e., at each lane) was quantitated by densitometry analysis using ImageJ software for each EMSA gel [86]. Regression analysis was performed for each gel and the apparent K_D value was calculated. Average apparent K_D values depicted in the manuscript are based on 2–3 regression analyses of each probe. To demonstrate the reproducibility of the EMSA gels, averaged quantifications of 2–3 biological repeats of each probe were fit using exponential least-squares regression analysis (shown in S4 Fig). The apparent K_D values were determined as the concentration at which 50% of the DNA probes were unbound, as deduced the average from the regression analyses. List of primers used for the amplification of target DNA sequences is found in S5 Table.

Motility assay

BHI and LBMM soft agar plates were prepared (0.3% agar) with 1 mM IPTG. 1 µl from the overnight bacterial cultures in BHI was spotted on the soft agar plates and grown for 48 h at 30°C. For the analysis of swarming capability, diameters of the bacterial growth zones were measured using a standard ruler. Pictures were taken using Olympus camera.

Attachment assay

L. monocytogenes strains were grown overnight in 3 ml BHI cultures at 30°C without shaking. CaCo2 cells were cultured overnight in 6-well plates in CaCo2 medium (MEM with 20% FBS, 1 mM sodium pyruvate, 2 mM L-glutamine and 5ml MEM non-essential amino acids X100 solution) supplemented with penicillin and streptomycin in 37°C incubator with 5% CO$_2$. The next day, the cells were washed twice with PBS and replenished with fresh CaCo2 medium without

antibiotics. The bacteria were washed twice with PBS and approximately 1.6×10^7 *L. monocytogenes* bacteria were used to infect 2×10^6 CacCo2 cells. Thirty minutes post-infection, the cells were washed 6 times with PBS and lysed with 1 ml cold water. Serial dilutions were plated on BHI plates and colony-forming units (CFUs) were counted after overnight incubation at 37°C.

Supporting Information

S1 Fig. Growth curves of WT and *ΔcodY L. monocytogenes bacteria* in BHI and LBMM media. Optical density measurements of WT *L. monocytogenes* and *ΔcodY* bacteria during growth in BHI (**A**) and LBMM (**B**). Results are average of 3 independent experiments. Error bars represent standard deviation.
(TIF)

S2 Fig. Biological repeats of RNA-Seq transcriptome analysis of WT and *ΔcodY L. monocytogenes* bacteria grown in BHI and LBMM media. Correlations between biological repeats of WT (**A**) and *ΔcodY* (**B**) *L. monocytogenes* transcriptome analyses in BHI medium. Correlations between biological repeats of WT (**C**) and *ΔcodY* (**D**) *L. monocytogenes* transcriptome analyses in LBMM medium. Each point in the graphs represents a gene and each axis represents a biological repeat. R^2 represents linear regression correlation values. Of note, *ΔcodY* 3 LBMM sample was found to have more then twice of its reads aligning to non-coding regions compared to all other RNA-Seq samples. Furthermore, its correlation with the two other *ΔcodY* LBMM samples was significantly low. Therefore, this sample was omitted from the analysis.
(TIF)

S3 Fig. Schematic representation of the regulatory regions, which were selected for EMSA analysis. Schematic representation of the regulatory regions of *hisZ* (**A**), *rbsV* (**B**), *glpF* (**C**), *argG* (**D**), *gadC* (**E**), *feoA* (**F**), *actA* (**G**), *gdhA* (**H**), *poxB* (**I**), *glnR* (**J**) and *fliN* (**K**) genes, which were selected for EMSA analysis. The regulatory region of each gene is presented. CodY motifs are highlighted.
(PDF)

S4 Fig. Densitometry analyses of EMSA gels. An averaged regression analysis is shown for each probe. Analysis is based on densitometry measurements of the ratio between the free DNA probe and the total DNA probe at each CodY concentration (i.e., each lane) using ImageJ software [86]. Quantifications of 2–3 biological repeats were fitted via exponential least-squares regression analysis. The average apparent K_D values depicted in the manuscript are based on 2–3 independent regression analyses made for each probe and are not derived from the averaged graphs presented here. Error bars represent standard error of the mean.
(TIF)

S1 Table. RNA-Seq dataset. This file contains transcriptomic data analyzed by the Rockhopper software. The first sheet (ALL) represents the expression of all Listeria monocytogenes 10403S annotated genes and ncRNA, in addition to novel ncRNA and ORFs detected by Rockhopper (V 1.3), in WT and *codY* mutant *L. monocytogenes* strains in BHI and LBMM media. Each of the following sheets represent a cluster of genes differentially regulated between WT and the *codY* mutant in either or both types of media. In order to be defined as a differentially expressed gene, 3 criteria should be satisfied: 1. Have a q-vaule of <0.05. q-value represents the p-value after correcting for multiple testing by the FDR method. 2. Have a fold change of >1.8 between the WT and *codY* mutant in either one of the media. 3. Have a normalized expression value of >10 in at least one condition, in which the > 1.8 fold change difference is observed.
(XLSX)

S2 Table. Functional enrichment analysis by clusters. This table contains functional enrichment analysis of the clusters of the differentially expressed genes. Each sheet represents a different cluster of CodY regulated genes. The functional enrichment analysis was performed using the MIPS server ([44] Mewes et al, MIPS: analysis and annotation of proteins from whole genomes, Nucleic acids research, 2004). Results are shown for the RNA-seq differentially expressed genes.
(XLSX)

S3 Table. ChIP-Seq dataset. This table contains CodY binding regions, identified by the ChIP-seq analysis. There are two sheets—for the BHI and LBMM growth conditions. Fold change and FDR values were calculated by the MACS [[84] Zhang, Y et al, Model-based analysis of ChIP-Seq (MACS). Genome biology. 2008] algorithm. The "exp" column represents the existence of CodY regulation on the genes associated with the peaks. The data in the "CodY box" column indicates the existence of a CodY consensus sequence binding box in the peak region identified in the ChIP-seq analysis. CodY boxes were identified with statistical significance using MAST [Bailey TL and Gribskov M, 1998 [47]]. The input consensus sequence to MAST was based on previous CodY box analysis [den Hengst et al, 2005 [37], and Belitsky and Sonenshein, 2008 [87]]. The consensus generation was made using the MEME algorithm [Bailey TL and Elkan C, 1994 [46]]. Only sites with E value < 10 and P value < 0.05 are shown.
(XLSX)

S4 Table. Direct CodY regulon. This Table contains *L. monocytogenes* CodY direct regulon divided into distinct clusters of regulation. The data in the "transcriptional unit" column represent the first gene of an operon. The data in the "CodY box" column indicates the existence of a CodY consensus sequence binding box in either the peak region identified in the ChIP-seq analysis or in the regulatory region of the transcriptional unit. CodY boxes were identified with statistical significance using MAST [Bailey TL and Gribskov M, 1998 [47]]. The input consensus sequence to MAST was based on previous CodY box analysis [den Hengst et al, 2005 [37], and Belitsky and Sonenshien, 2008 [87]]. The consensus generation was made using the MEME algorithm [Bailey TL and Elkan C, 1994 [46]]. Only sites with E value < 10 and P value < 0.05 are shown. Validated direct target genes of CodY are marked in bold.
(XLSX)

S5 Table. List of strains and primers used in this study. This table contains the list of bacterial strains and primers used in this study.
(XLSX)

Acknowledgments

Author Contributions

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

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