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The metabolic regulator CodY links *L. monocytogenes* metabolism to virulence by directly activating the virulence regulatory gene, *prfA*

Lior Lobel[1], **Nadejda Sigal**[1], **Ilya Borovok**[1], **Boris R. Belitsky**[2], **Abraham L. Sonenshein**[2], and **Anat A. Herskovits**[1,*]

[1] The Department of Molecular Microbiology and Biotechnology, The George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv, 69978, Israel.

[2] The Department of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, MA 02111.

Summary

Metabolic adaptations are critical to the ability of bacterial pathogens to grow within host cells and are normally preceded by sensing of host-specific metabolic signals, which in turn can influence the pathogen's virulence state. Previously, we reported that the intracellular bacterial pathogen *Listeria monocytogenes* responds to low availability of branched-chain amino acids (BCAA) within mammalian cells by up-regulating both BCAA biosynthesis and virulence genes. The induction of virulence genes required the BCAA-responsive transcription regulator, CodY, but the molecular mechanism governing this mode of regulation was unclear. In this report, we demonstrate that CodY directly binds the coding sequence of the *L. monocytogenes* master virulence activator gene, *prfA*, 15 nt downstream of its start codon, and that this binding results in up-regulation of *prfA* transcription specifically under low concentrations of BCAA. Mutating this site abolished CodY binding and reduced *prfA* transcription in macrophages, and attenuated bacterial virulence in mice. Notably, the mutated binding site did not alter *prfA* transcription or PrfA activity under other conditions that are known to activate PrfA, such as during growth in the presence of glucose-1-phosphate. This study highlights the tight crosstalk between *L. monocytogenes* metabolism and virulence' while revealing novel features of CodY-mediated regulation.

Introduction

Microorganisms respond to different physical and chemical signals, such as temperature, salinity and nutrient availability, in order to adapt to diverse ecological niches. These signals mainly serve to regulate the metabolic status of the organism, but can also trigger induction of a niche-specific set of genes that further optimize the organism's growth. While observations of such phenomena in pathogenic bacteria were made years ago, the molecular

*To whom correspondence should be addressed: anathe@post.tau.ac.il Department of Molecular Microbiology and Biotechnology, The George S. Wise Life Sciences Faculty, Tel Aviv University, Tel Aviv, Israel, 69978 Phone: 972-3-640-7502 Fax: 972-3-640-9407.

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mechanisms that link the sensing of environmental signals to regulation of specific genes during infection are just beginning to be deciphered. Within the host, pathogens sense nutritional and physical signals that lead to the induction of genes specifically expressed in the host (Abu Kwaik & Bumann, 2013, Brown *et al.*, 2008). These genes promote the pathogen's ability to invade and manipulate the host, as well to adapt to the host metabolic environment (Fuchs *et al.*, 2012, Bretl *et al.*, 2011). For example, *Legionella pneumophila* senses changes in threonine concentrations within host cells and switches from the replicative to the transmissive phase (Sauer *et al.*, 2005). *Vibrio cholerae* senses and responds to reduced oxygen tension by up-regulation of virulence genes via a thiol-based sensor domain located within its virulence transcription regulator (Liu *et al.*, 2011). Other examples include the response of *Salmonella typhimurium* to high iron concentrations, leading to enhanced adherence to and invasion of enterocytes (Kortman *et al.*, 2012), and induction by *Listeria monocytogenes* of virulence genes in response to glucose-1-phosphate, a sugar that is mainly available in mammalian cells (Ripio *et al.*, 1997a). Though there are many examples of signaling-dependent gene expression in host-pathogen interactions, the mechanisms by which metabolic signals are sensed and transduced to regulatory cascades that co-regulate metabolism and virulence remain largely unknown.

L. monocytogenes, is a Gram-positive, foodborne intracellular pathogen and the causative agent of listeriosis in animals and humans (Swaminathan & Gerner-Smidt, 2007). *L. monocytogenes* invades host cells either passively via phagocytosis or actively by expressing surface proteins that induce bacterial internalization (Cossart, 2011). Once inside the cell, *L. monocytogenes* is initially contained in a vacuole from which it rapidly escapes by expressing the pore-forming cytolysin listeriolysin O (LLO), two additional phospholipases, PlcA and PlcB, and components of the Com system (Rabinovich *et al.*, 2012, Mengaud *et al.*, 1987, Portnoy *et al.*, 1988, Geoffroy *et al.*, 1991, Leimeister-Wachter *et al.*, 1991, Goebel *et al.*, 1988). In the host cell cytosol, *L. monocytogenes* replicates and gains mobility via polymerization of host actin filaments, enabling the bacteria to spread from cell to cell without being exposed to the extracellular environment (Tilney & Portnoy, 1989). Remarkably, most of the *L. monocytogenes* virulence factors are positively regulated by the master activator of virulence, PrfA (de las Heras *et al.*, 2011). Regulation of the *prfA* gene is highly complex and multilayered. *prfA* is transcribed from three distinct promoters: two proximal promoters, P1 and P2, located upstream of the *prfA* translation initiation codon and one distal promoter, P3, located upstream of the *plcA* gene and from which expression results in a bicistronic *plcA-prfA* transcript (Freitag & Portnoy, 1994). Transcription from the P1 and P2 promoters requires the sigma factors SigA and SigA or SigB, respectively, while SigA-dependent transcription from P3 is dependent on PrfA itself (Rauch *et al.*, 2005, Freitag & Portnoy, 1994, Lobel *et al.*, 2012). In addition, *prfA* is negatively regulated by the *trans*-acting small RNAs, SreA and SreB, both of which are synthesized as S-adenosylmethionine-dependent riboswitches under rich nutritional conditions, and that block PrfA synthesis by pairing with the 5’ untranslated region (UTR) of *prfA* mRNA (Loh *et al.*, 2009). Moreover, the *prfA* 5’-UTR contains a thermosensory structure that allows *prfA* mRNA translation at 37°C, but blocks translation at 30°C by sequestering the ribosome binding site (Johansson *et al.*, 2002). Lastly, the 5’ coding region stabilizes *prfA* mRNA and thus increases the efficiency of its translation (Loh *et al.*, 2012). In addition to its

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transcriptional regulation, PrfA is also regulated post-translationally. When grown in rich laboratory media *L. monocytogenes* expresses PrfA but it remains inactive (Renzoni *et al.*, 1997). Upon invasion of host cells, however, PrfA becomes active, leading to a rapid activation of virulence genes (Scortti *et al.*, 2007). Since PrfA is a member of the cyclic-AMP receptor protein (Crp) family of transcriptional regulators, of which many require the binding of a cofactor for full activity, it has been postulated that upon invasion into host cells a specific cofactor binds PrfA and activates it. While the identity of this putative cofactor remains unknown, mutations were identified within PrfA that render it constitutively active (PrfA* mutations) (Ripio *et al.*, 1997a, Miner*et al.*, 2008, Ripio *et al.*, 1997b, Shetron-Rama *et al.*, 2003, Vega *et al.*, 2004, Wong & Freitag, 2004). Together these observations suggest that conformational changes in PrfA regulate its activity (Freitag *et al.*, 2009).

As for metabolic signals, PrfA is known to be regulated by sugar availability. It was shown that sugars transported by the PEP-dependent phosphotransferase system (PTS), such as glucose and cellobiose, repress PrfA activity, while the presence of non-PTS host-derived sugars, such as glucose-1-phosphate and glycerol, trigger *prfA* transcription and PrfA activity. (Mertins *et al.*, 2006, Joseph *et al.*, 2008, Milenbachs *et al.*, 1997). Notably, the activation of PrfA in rich media conditions was shown to require activated charcoal in the medium, though the reason for this effect has not been clearly determined (Ripio *et al.*, 1996). Iron availability also seems to play a role in *prfA* regulation, though the mechanism is not clear (Conte *et al.*, 1996, Böckmann*et al.*, 1996). We have recently discovered that branched-chain amino acids (BCAA) [isoleucine, leucine and valine] also serve as a metabolic signal for induction of *prfA* and its downstream regulated genes (Lobel *et al.*, 2012). Limiting BCAA availability activates virulence, a response that requires a sensor of BCAA, the transcription factor called CodY, a known global regulator of metabolic genes in low G+C Gram-positive bacteria (Sonenshein, 2005). CodY, which has been studied most thoroughly in *Bacillus subtilis*, responds to intracellular availability of BCAA (and GTP) via direct binding to these metabolites (Levdikov *et al.*, 2006, Shivers & Sonenshein, 2004). Multiple reports have demonstrated that interaction with isoleucine allows CodY to bind to the promoters of hundreds of genes (Belitsky & Sonenshein, 2013, Preis *et al.*, 2009, Shivers *et al.*, 2006, Molle *et al.*, 2003, Sonenshein, 2007). In its isoleucine-bound state, CodY acts as a repressor of many metabolic pathways, including the BCAA biosynthesis pathway (encoded in part by the *ilv* operon), and as a positive regulator of other genes (Shivers *et al.*, 2006, Molle *et al.*, 2003, Preis *et al.*, 2009). Upon intracellular depletion of BCAA, CodY dissociates from these promoters thus altering their transcription (Sonenshein, 2007). Interestingly, some genus/species-specific differences in the functionality of this regulator were reported. Whereas the activity of CodY limits virulence of *Staphylococcus aureus* (Montgomery *et al.*, 2012, Rivera *et al.*, 2012) and *Clostridium difficile* (Dineen *et al.*, 2010), CodY enhances the expression of virulence genes in *L. monocytogenes, Bacillus anthracis* and several other human bacterial pathogens, though the molecular mechanism that mediates this enhancement has not been deciphered (Kreth *et al.*, 2011, Chateau *et al.*, 2011, van Schaik *et al.*, 2009, Hendriksen *et al.*, 2008, Flores *et al.*, 2013, Li *et al.*, 2013, Lobel *et al.*, 2012).

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In this report, we demonstrate that CodY directly activates expression of the *prfA* gene by binding to a sequence located unexpectedly within the 5' coding region. Using electrophoretic mobility shift (EMSA) and chromatin immuno-precipitation (ChIP) assays, we identified a CodY-binding site, located ~ 15-bp downstream of the *prfA* start codon, that is necessary for *prfA* expression under low BCAA concentrations. Mutating this site abolished CodY binding and resulted in lower transcription of *prfA* and PrfA-dependent virulence genes under low BCAA conditions. Moreover, both a *codY* mutant strain and a mutant altered in the *prfA* CodY-binding site were attenuated for virulence in both cultured macrophages and mice. Overall, this study defines a direct regulatory link between CodY and PrfA, and highlights the tight interaction between *in vivo* metabolism (*i.e.,* during infection) and virulence in *L. monocytogenes*.

Results

CodY is required for the induction of virulence genes in response to low concentrations of BCAA, but not in response to glucose 1-phosphate

As mentioned above, two major metabolic signals are currently known to trigger robust expression of *L. monocytogenes* virulence genes and thus switch the bacteria to the virulent state: one is the availability of phosphorylated hexoses (*e.g.*, glucose-1-phosphate), and the second is the low availability of BCAA. Both conditions are found in the cytosol of mammalian cells (Chico-Calero *et al.*, 2002, Ripio *et al.*, 1997a, Lobel *et al.*, 2012) and our previous work demonstrated the involvement of CodY in the response to low BCAA (Lobel *et al.*, 2012). To explore the possibility that the two metabolic signals use the same regulatory pathway, we first examined whether CodY is also required for the induction of virulence genes in bacteria growing in glucose-1-phosphate containing media. To this end, we used a *lux* reporter system to measure the expression of the *hly* gene (encoding LLO toxin) during growth in a defined minimal medium (MM) (Phan-Thanh & Gormon, 1997) containing relatively low or high concentrations of BCAA (~80 μM and ~800 μM, respectively, of each BCAA; named "low-BCAA MM" or "high-BCAA MM") and in LB-MOPS activated charcoal medium supplemented with glucose-1-phosphate or glucose as a control (25 mM of each, "LB-MOPS-G1P" or "LB-MOPS-Glu", respectively). To follow PrfA-dependent *hly* transcription, an integrative plasmid containing the *lux* operon under the control of the *hly* promoter (pPL2-P_{hly}*lux*) (Bron *et al.*, 2006, Lobel *et al.*, 2012) was introduced by conjugation into the wild-type (WT) *L. monocytogenes* strain 10403S and its *codY* mutant derivative. Transconjugants were subjected to growth in the different media and parallel measurements of luminescence and optical density (OD_{600}) were taken. As shown in Figure 1, *hly* was highly induced under low-BCAA MM and LB-MOPS-G1P conditions (~100-fold and ~1000-fold, respectively) and was not induced in the control media, high-BCAA MM and LB-MOPS-Glu. Notably, CodY was absolutely necessary for *hly* transcription during growth in low-BCAA MM, but was largely dispensable during growth in LB-MOPS-G1P medium (Figure 1 A-B). In the latter condition a biphasic luminescence profile was reproducibly observed. A *codY* complemented strain constitutively expressing *codY* from the integrative plasmid pPL2-P_{hly}*lux*-P-*codY* exhibited WT levels of *hly* transcription under low-BCAA MM conditions, strengthening the premise that CodY indeed mediates *hly* induction in response to low availability of BCAA (Figure

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1C). Together, the results indicate that the two metabolic signals (glucose 1-phosphate and BCAA) exploit distinct regulatory pathways that trigger *hly* transcription, and that CodY is primarily responsible for the induction under low concentrations of BCAA.

CodY preferentially binds the regulatory region of the *prfA* gene under low concentrations of BCAA

Since we have previously shown that induction of the *hly* promoter under conditions of limiting BCAA is not only CodY-dependent but also PrfA-dependent, and that *prfA* transcription is reduced in a *codY* mutant, we reasoned that the regulatory effect of CodY on *hly* and additional virulence genes is mediated via PrfA itself. We have demonstrated that CodY specifically activates the transcription of *prfA* from its two proximal promoters P1 and P2, though we did not determine whether this regulation occurs directly or indirectly via another factor (Lobel *et al.*, 2012). To investigate if CodY directly regulates *prfA* by binding to its regulatory region, we performed electrophoretic mobility shift assays (EMSA), in which increasing amounts of purified recombinant 6His-tagged CodY (CodY-His_6) were incubated with a *prfA* promoter probe containing the complete *plcA*-*prfA* intergenic region plus 100-bp of the *prfA* coding sequence (Figure 2A). For CodY purification, a *codY* gene with six histidine codons appended to the 3' end was cloned in the pET28 vector and expressed as described in the Materials and Methods section. The EMSA assay was performed under two conditions, with and without BCAA (10 mM of each), and included two control DNA probes: one containing the *ilvD* promoter region of the *ilv* operon known to be directly regulated by CodY (Molle *et al.*, 2003) and the second containing the promoter of the *codVWXY* operon (encoding the *codY* gene), as in *Lactococus lactis* the *codY* gene was shown to be auto-regulated (den Hengst *et al.*, 2005). In line with our expectations, the EMSA assays demonstrated that CodY binds to all three probes, albeit with different affinities. CodY binding to the *prfA* probe exhibited a K_D of 236 ± 49 nM, whereas its binding to the *ilvD* and *codV* probes exhibited a much lower K_D of 3.1 ± 1.5 nM and 51.74 ± 1.68 nM, respectively, under conditions containing 10 mM of BCAA (Figure 2 B-D) (average K_D values were calculated based on 3 regression analyses of 3 independent EMSA gel experiments, Figure S1). As expected, a significant decrease in CodY binding to the *ilvD* and the *codV* control probes was observed in the absence of BCAA (K_D = 48 ± 20.7 nM and 96 ± 11.4 nM, respectively), though CodY binding to the *prfA* probe under these conditions remained largely unchanged (K_D = 232 ± 66 nM) (Figure 2 and S1). We also performed EMSA assays with specific unlabeled competitor probes to verify the specificity of CodY binding (Figure S2). While these *in vitro* experiments have demonstrated binding of CodY to the regulatory region of the *prfA* gene (though with lower affinity in comparison to the control probes), they did not demonstrate a role for BCAA in this binding as insinuated by the *in vivo* experiments. To better examine this question we set up to assess CodY ability to bind the *prfA* regulatory region under *in vivo* conditions, i.e., during bacterial growth in rich or low BCAA-containing media. For this purpose we performed a chromatin immunoprecipitation assay in combination with real-time, quantitative PCR (ChIP-RT-qPCR) to analyze the binding of His-tagged CodY to regulatory DNA sequences *in vivo* (Waldminghaus & Skarstad, 2010). First, a derivative of strain 10403S was constructed that has a sixhistidine tag at the 3'-end of the chromosomal *codY* gene (*L.m. codY-6his* strain). The functionality of the His-tagged CodY was similar to that of the native

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CodY, as shown by its ability to support bacterial growth in brain heart infusion (BHI) medium and to repress *ilvD* transcription in this rich medium (Figure S3). The *L.m. codY-6his* strain was then grown in the two rich media, BHI and LB-MOPS-G1P, and in low-BCAA MM. During mid-exponential growth phase, bacteria were treated with formaldehyde to covalently crosslink proteins to DNA. Bacteria were then lysed, CodY-His_6 was immunoprecipitated, and CodY-bound DNA was isolated and quantitated by RT-qPCR using specific primers for the regulatory regions of *prfA*, *ilvD*, *codV* and *LMRG_01206* as a negative control. Using this method, the association of CodY with *prfA, ilvD*, *codV* and *LMRG_01206* promoter regions was evaluated, this time under relevant *in vivo* conditions. Notably, we found an enriched association of CodY with the *prfA* regulatory region during growth in low-BCAA MM in comparison to BHI or LB-MOPS-G1P (both contain excess amounts of BCAA), whereas the *ilvD* and *codV* promoter-containing regions were more highly associated with CodY during growth in the rich media than in low-BCAA MM (Figure 3 A-D). Notably, the results demonstrate the opposite effect of BCAA on CodY binding to the different promoters and support the hypothesis that, under conditions of limiting BCAA *in vivo*, CodY binds the *prfA* regulatory region to enhance its expression. Intrigued by the observation that the EMSA experiment failed to detect the effect of BCAA on CodY binding to the *prfA* probe, we hypothesized that this could be due in part to the lack of natural competitor binding sites within this *in vitro* system. *In vivo* CodY binds multiple promoters/binding sites with different affinities and it is most likely that the dynamics of CodY binding to weak binding sites, such as the one of *prfA*, is greatly influenced by the competition between the different binding sites in each given condition (Belitsky & Sonenshein, 2013). To address this question we developed a different *in vitro* system that is based on a CodY pull-down competition-binding assay. In this set up, both *ilvD* and *prfA* probes were incubated with purified His-tagged CodY and competed for its binding under different BCAA concentrations. Briefly, purified CodY-His_6 was incubated with the *prfA* probe and the *ilvD* probe under increasing concentrations of BCAA (0, 1 and 10 mM). Following incubation, CodY was precipitated using cobalt Talon beads and the amounts of *prfA* and *ilvD* probes bound to CodY were quantitated by RT-qPCR using specific primers. The results of this experiment indicated an opposite trend of CodY binding to the different probes. While binding of CodY to the *ilvD* probe was enhanced when BCAA concentrations were increased, the *prfA* probe competed more effectively for CodY binding when BCAA concentrations were reduced (Figure 3E). These *in vitro* results are in accordance with the *in vivo* data showing that CodY binds *prfA* to a greater extent when BCAA are limited and support the premise that gene regulation by CodY is influenced by its competitive binding to multiple sites with different affinities under varying BCAA concentrations (Belitsky & Sonenshein, 2013). In line with this model, we next examined whether reducing the affinity of CodY to BCAA (by mutating its BCAA binding site) will favor binding of CodY to P_{prfA} in comparison to P_{ilvD}. Since the affinity of CodY to its different binding sites greatly depends on BCAA concentration, such an experiment could further demonstrate the differential effect of BCAA on CodY binding. To this end, we selectively mutated the BCAA-binding site within CodY, and assessed whether such a mutant of CodY could still activate *prfA*. The BCAA-binding site of CodY was characterized structurally in *B. subtilis* and was shown to be highly conserved (Levdikov *et al.*, 2006). Moreover, mutations made within this site reduced to varying extents the ability

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of CodY to respond to isoleucine and thus to repress metabolic genes (Villapakkam *et al.*, 2009). Here we chose to change arginine-61 to alanine (R61A), as this residue is part of the BCAA-binding pocket and was shown to contribute to the isoleucine response (Levdikov *et al.*, 2006, Villapakkam *et al.*, 2009). The *CodY-R61A* strain together with WT *L.m.* and *codY* bacteria were subjected to growth in the rich medium BHI and in low-BCAA MM and the transcription levels of the *ilvD* and *prfA* genes were analyzed using RT-qPCR. The *CodY-R61A* bacteria grown in BHI accumulated *ilvD* transcripts at a high level' similar to that seen in *codY* bacteria, whereas WT bacteria exhibited much lower expression of this gene (Figure 3F). This result suggested that the *CodY-R61A* mutant had lost BCAA-dependent repressing activity to a significant extent. On the contrary, a different picture was seen in low-BCAA MM where the *CodY-R61A* mutant was still able to activate the *prfA* gene' similarly to WT bacteria, whereas the *codY* mutant was not (Figure 3G). These results imply that in high BCAA conditions CodY is preferentially bound to some binding sites (e.g., *ilvD*) and when BCAA levels drop CodY is still able to bind to other sites (e.g., *prfA*). These observations accord with the data showing that CodY's affinity to the *ilvD* probe is 80-fold higher than to *prfA* probe when BCAA are present, but only 5-fold higher when BCAA are absent (Figure 2). Altogether, these experiments indicated a direct *in vitro* and *in vivo* association between CodY and the regulatory region of the *prfA* gene under conditions that are consistent with those that support CodY-dependent activation of *prfA* transcription (Figure 1 and (Lobel *et al.*, 2012)).

CodY binds a core sequence of AATAT within the coding region of the *prfA* gene

Next, we aimed to determine the exact location at which CodY binds the *prfA* regulatory region (as designated in the *prfA* probe, Figure 2A). To this end, a set of new probes was synthesized to include each of the two halves and four quarters of the full-length *prfA* probe (Figure 4A) and subjected to further EMSA analysis. As shown in Figure 4, CodY demonstrated specific binding to the downstream half of the full-length *prfA* probe (K_D = 216 ± 25 nM, Figure 4B and Figure S1), and primarily to its 3' end, as indicated by the exclusive binding of CodY to the fourth quarter (K_D = 229 ±14.5, Figure 4C and Figure S1). Interestingly, the fourth quarter probe includes the first 100-bp of the *prfA* coding sequence. These results suggested that CodY might have an intragenic binding site within the *prfA* coding region and not, as usually found with transcription activators, upstream of the transcription start point. Interestingly, close examination of this 100-bp sequence revealed that it contains two overlapping 15-bp motifs similar to the CodY box consensus in both *L. lactis* and *B. subtilis* (each with 4- and 5-bp mismatches) (Belitsky & Sonenshein, 2013, den Hengst *et al.*, 2005), that were located 15-45 bp downstream of the *prfA* gene ATG start codon (Figure 4D).

To delineate the physiological relevance of the identified putative CodY-binding site(s) within the *prfA* gene we first performed a mutational analysis of this region to identify nucleotides critical for CodY binding. A series of double-stranded probes (~50 or 70-bp) containing the putative CodY-binding sites bearing mutations in different nucleotides, as well as the WT putative binding sites and CodY-binding sites found in *B. subtilis* and *L. lactis*, were synthesized and assessed for CodY binding under *in vitro* conditions (Figure 5A and Table S2). CodY binding to the different probes was evaluated using the same

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competition-binding assay described above, this time using the full-length *prfA* probe (Figure 2A) and each of the competitor probes. Purified CodY-His_6 was incubated with the full-length *prfA* probe and with each of the short mutated probes in a ratio of 1:10, respectively. Following incubation, CodY was precipitated and the amount of the full-length *prfA* probe bound to CodY was quantitated by RT-qPCR using primers specific only to this *prfA* probe (Table S2). In this assay, mutated probes that preserved CodY binding effectively competed with the full-length *prfA* probe and thus reduced its amount bound to CodY, whereas probes that failed to compete did not alter CodY binding to the *prfA* probe. First, the binding of CodY to the full-length *prfA* probe was validated in comparison to a probe containing a random sequence, and, as expected, no competition was observed (the level of the full-length *prfA* probe bound to CodY was similar to that of the control sample containing the *prfA* probe alone) (Figure 5B). Conversely, probes containing the consensus *L. lactis* CodY-binding motif (den Hengst *et al.*, 2005) or a high affinity *B. subtilis* CodY-binding motif (Belitsky & Sonenshein, 2013) competed efficiently for CodY binding. This latter result was also observed when a probe containing the *L. monocytogenes* WT *prfA* putative CodY-binding sites was used (Figure 5B). Next, the ability of the mutated probes to compete with the full-length *prfA* probe for CodY binding was measured. Notably, we found a 5-nt sequence (AATAT, overlapping the two putative CodY box motifs) to be the most critical for CodY binding, since changing this sequence to GGCGC (as in the probe mut6) completely abolished the ability to compete for CodY binding (Figure 5 A-B). Specific mutations made within these 5 nucleotides only partially affected CodY binding (probes mut7 to mut13), suggesting that together these 5-nt represent a critical sequence that is necessary for the interaction with CodY (Figure 5B). Interestingly, increasing the G-C content of another sequence downstream of the AATAT site (mut4 probe), which overlapped one of the CodY boxes, had no effect on CodY binding, suggesting that CodY might bind upstream of this putative site. Next, an EMSA analysis comparing CodY binding to the WT and to the mut6 binding sites (WT probe and mut6 probe, respectively, in the frame of the 2nd half of the *prfA* probe) confirmed that CodY binds to the mut6 probe with a much lower affinity, demonstrating a K_D of > 800 nM in comparison to a K_D of 216 ± 25.5 nM with the WT probe (Figure 5C, and Figure S1). Furthermore, performing EMSA assays using the mut6 *prfA* probe as a competitor to the WT *prfA* probe showed that the mut6 probe does not compete with the WT *prfA* probe for CodY binding (Figure S2). To further examine the importance of the AATAT sequence, CodY binding *in vivo* was assessed after the mut6 mutation was introduced into the chromosomal *prfA* gene by allelic exchange (*L.m. mut6-prfA* mutant). The mutation was introduced into the background of the *L.m. codY-6his* strain to allow ChIP analysis. The ability of CodY to bind the WT or the mutated binding site sequence within the chromosomal *prfA* gene was evaluated *in vivo* during bacterial growth in low-BCAA MM using ChIP-RT-qPCR analysis as described above. The results of this experiment clearly demonstrated that the AATAT sequence is important for CodY binding *in vivo*, as the *L.m. mut6-prfA* mutant DNA was not enriched in the CodY-bound fraction whereas the WT sequence was enriched 3.5-fold compared to the control genes (Figure 5D). Taken together, these results establish the existence of a CodY-binding site(s) within the 5'-end of the *prfA* gene and define the AATAT nucleotides as critical for CodY binding *in vitro* and *in vivo*.

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CodY positively regulates PrfA via binding to its identified site within the *prfA*-coding region

Having identified the sequence critical for CodY to bind to the *prfA* gene, we investigated the biological importance of this interaction in the regulation of *L. monocytogenes* virulence. Ideally, we wanted to employ the *L.m. mut6-prfA* mutant for this purpose. However, as this mutation results in two amino acid substitutions in the PrfA protein (K10R and Y11R), we first questioned whether these mutations impair PrfA structure and activity. To this end, we compared the growth of WT and *L.m. mut6-prfA* strains in LB-MOPS-G1P medium, which is known to require an active PrfA protein and is independent of CodY (Figure 1B). Of note, PrfA is required to activate the expression of the glucose-1-phosphate transporter, UhpT, which facilitates the uptake and utilization of this sugar (Chico-Calero *et al.*, 2002). As shown in Figure 6A, both strains grew similarly in the LB-MOPS-G1P medium, whereas a *prfA* mutant was unable to grow. Since during growth in this medium *L. monocytogenes* expresses and secretes several virulence factors in a PrfA-dependent manner, we next analyzed and compared the activity of two such factors, LLO and PlcA, in the supernatants of WT and *L.m. mut6-prfA* cultures. The activity of LLO was measured by a red blood cell hemolysis assay, whereas the activity of PlcA was measured by a PI-PLC specific assay, with *prfA, hly* and *plcA* mutants used as control strains (Rabinovich *et al.*, 2012). The WT and *L.m. mut6-prfA* strains exhibited similar levels of LLO and PlcA activities in their supernatants, suggesting that both strains express comparable levels of active PrfA proteins in LB-MOPS-G1P medium (Figure 6 B-C). Overall, the results indicate that the *prfA*-mut6 mutation does not interfere with PrfA protein activity. Another support for this premise came from a 3D-structural comparative analysis between the solved PrfA structure and the predicted structure of the mut6-PrfA protein (based on homology modeling), which demonstrated no change in the structure of the two proteins, including critical domains, such as the DNA binding and dimerization domains (Figure S4).

Next, the effect of the mutated CodY-binding site on the regulation of *prfA* and other virulence genes was tested. The pPL2-P_{hly}*lux* reporter plasmid was introduced into the WT, *codY* and *L.m. mut6-prfA* strains to monitor *hly* transcription during growth in low-BCAA MM and LB-MOPS-G1P media. As shown in Figure 7, CodY and the CodY-binding site were both necessary for the induction of *hly* under conditions of limiting concentrations of BCAA, whereas they were largely dispensable during growth in LB-MOPS-G1P (Figure 7 A-B). The biphasic/delayed luminescence pattern observed with the *codY* mutant during growth in LB-MOPS-G1P (also Figure 1B) was not evident in the *L.m. mut6-prfA* mutant, suggesting that a *codY* mutation causes this phenotype by a mechanism independent of the AATAT sequence (Figure 7B). Further analysis of the transcription levels of *hly, actA* and, most importantly, *prfA* during bacterial growth confirmed that both CodY and its binding site in *prfA* are necessary to activate the transcription of *prfA* and the virulence genes in low BCAA medium, but not during growth in LB-MOPS-G1P medium (Figure 7 C-D). Based on these data we conclude that CodY specifically regulates the transcription of *prfA* and its downstream virulence genes via direct binding to the identified site located within the *prfA* gene.

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Both CodY and its binding site within the *prfA* gene are important to promote virulence in macrophage cells and in mice

Lastly, the roles of CodY and its binding site were evaluated during infection of macrophage cells and mice. First, the intracellular growth ability of the WT, *codY*, *L.m. mut6-prfA* and *L.m. CodY R61A* strains was monitored in bone marrow-derived (BMD) macrophages. Notably, both *codY* and *L.m. mut6-prfA* mutants exhibited a similar intracellular growth defect in comparison to WT bacteria and to bacteria harboring the *codY R61A* mutation (Figure 8A). These results further indicated that while both CodY and its DNA binding site are important for intracellular growth, efficient binding of BCAA may not be required. Transcription analysis of *prfA* gene during intracellular growth of *codY* and *L.m. mut6-prfA* mutants confirmed that both CodY and its binding site play a role in its activation during infection (Figure 8B). Next, the fitness of WT, *codY* and *L.m. mut6-prfA* mutant strains was evaluated in young C57BL/6 female mice. Mice were injected intravenously with $4{\times}10^4$ of WT, *codY, L.m. CodY R61A* or *L.m. mut6-prfA* bacteria (5 mice in each group) and bacterial counts in the spleens and livers of the infected mice were analyzed at 72 hours post-infection. As shown in Figure 8C, both *codY* and *L.m. mut6-prfA* mutants colonized the livers and spleens of the infected mice to a lesser extent than did WT and *L.m, CodY R61A* bacteria, exhibiting 5-10 fold decreases in colony-forming units (CFUs) recovered from both organs. These results clearly indicate that both CodY and its binding site within the *prfA* gene contribute to *L. monocytogenes* virulence.

Discussion

Bacterial pathogens have co-evolved physical and metabolic adaptations in order to invade and grow successfully within mammalian cells, but how and at what level these seemingly different adaptations are intertwined has remained elusive. This report, together with our previous studies, reveals an interesting case in which host cell metabolism plays an important role in activation of bacterial virulence. We have previously shown that upon *L. monocytogenes* invasion of macrophage cells, certain pathways (e.g., those for biosynthesis of purines, histidine, arginine and BCAA) are activated in the bacteria in order to cope with the low availability of nutrients within the mammalian cell's cytosol (Lobel *et al.*, 2012). Among these pathways we found that the BCAA biosynthesis appears to be the main metabolic pathway interlinked with *L. monocytogenes* virulence. Low concentrations of BCAA were found to trigger enhanced induction of PrfA and the virulence genes it controls *in vitro*, suggesting that *L. monocytogenes* might recognize BCAA deficiency as a signature of the mammalian nutritional environment (Lobel *et al.*, 2012).

In the present study we discovered a direct link between the BCAA-sensing regulator CodY and the expression of PrfA. We found that CodY binds within the 5’ end of the *prfA* coding sequence *in vitro* and that binding in bacterial cells occurs preferentially under conditions of limiting concentrations of BCAA. A five-bp sequence within the CodY-binding site proved to be necessary for CodY-dependent activation of *prfA* transcription, implying that binding of CodY to that site is important for virulence gene expression *in vitro* and *in vivo*, and for colonization of liver and spleen. Furthermore, we found that activation of *prfA* transcription by CodY is less dependent on BCAA binding than is CodY-dependent repression of *ilvD*. In

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summary, these data demonstrate that CodY plays a direct role in *prfA* activation and affects virulence specifically when BCAA are in limiting amounts. As discussed in more detail below, this is the first report indicating that CodY activity as a regulatory protein correlates with a drop in BCAA concentration *in vivo* and that CodY can activate transcription by binding within a coding sequence.

While in most cases analyzed to date CodY has been shown to function as a repressor whose activity is increased when BCAA availability is high, there are several examples of positive regulation by CodY, again when BCAA are available (Shivers & Sonenshein, 2004). Many transcription factors (the classic example is AraC) are known to function as both activators and repressors (van Hijum *et al.*, 2009) and, in some cases, to demonstrate both activities at the same target gene when the regulator is in its effector-bound or effector-unbound states (Schleif, 2010). Other regulatory proteins bind to different targets in the effector-bound (*holo*) (Wunsche *et al.*) and – unbound (*apo*) states (Balderas-Martinez *et al.*, 2013). For example, Lrp (a regulatory protein in Gram-negative bacteria that controls both metabolic and virulence genes) and the Fur regulators have been shown to be active in both their *holo* and *apo* forms (Carpenter *et al.*, 2009, Butcher *et al.*, 2012, Baek *et al.*, 2011, Newman & Lin, 1995, van der Woude *et al.*, 1995, Cho *et al.*, 2008). In fact, Lrp was shown to repress genes in its *holo* form, while activating others in the *apo* form (Chen *et al.*, 2005). Such examples raise the possibility that CodY may also have more complex modes of action than presently known and may be more versatile than anticipated. Since binding of BCAA to CodY induces a significant conformational change that appears to separate the C-terminal winged helix-turn-helix motifs of the dimeric protein (Levdikov *et al.*, 2006, Levdikov *et al.*, 2009), thereby facilitating binding to DNA, we did not expect that limiting the availability of BCAA would enable CodY to bind DNA. Nevertheless, the observation that a CodY protein that is mutated within its BCAA-binding site loses its ability to repress *ilvD* but retains ability to activate *prfA* raises the possibility that CodY might possess an additional functional conformation when BCAA are limited.

A second unexpected conclusion of this study is that CodY activates *prfA* transcription by binding to a site within the coding region. Gene activation typically occurs via binding of transcription factors to sequences upstream of the RNA polymerase binding site, enabling the factors to interact directly with RNA polymerase without blocking the progress of the transcription machinery (van Hijum *et al.*, 2009). Nevertheless, intragenic binding sites can also play a role in activation of transcription, as opposed to a more conventional role in repression (Munson & Scott, 2000, Qi & Hulett, 1998, Munson *et al.*, 2001, Mitra *et al.*, 2005, Ouyang *et al.*, 2011, Liu *et al.*, 1998, Gal-Mor *et al.*, 2011, Shi *et al.*, 2004, Feng *et al.*, 2003). For example, *B. subtilis* PhoP, which regulates the response to phosphate starvation, activates genes by binding to sites internal to their coding sequences thereby enhancing full promoter activity (Liu *et al.*, 1998). In *E. coli*, the AraC-family Rns virulence activator promotes its own transcription by binding to two intragenic sites (Munson & Scott, 2000). Binding to these internal sites leads to a stronger interaction of RNA polymerase with the promoter region and the formation of an open transcription initiation complex. ChIP-Seq analyses of other bacterial global regulators have demonstrated that the binding of transcription activators is not restricted to upstream sequences but can involve binding

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within coding regions (Wang *et al.*, 2013, Park *et al.*, 2013, Butcher *et al.*, 2012, Butcher *et al.*, 2011, Cho *et al.*, 2008, Munson & Scott, 2000, Martin & Rosner, 2001). Interestingly, a recent genome-wide analysis of CodY-binding sites in *B. subtilis* revealed that 47% of the total sites identified were within coding regions. Some of these sites have been shown to mediate negative regulation (Belitsky & Sonenshein, 2013); though others may be involved in gene activation. It is important to note, however, that although the studies reported here support the idea that binding of CodY within a coding sequence can lead to gene activation, the exact molecular mechanism underlying this positive regulation remains to be delineated.

In this regard, it is interesting that the 5'-UTR of *prfA* mRNA is highly dynamic, responding to different metabolic and environmental cues. The RNA thermosensing structure was shown to overlap with a region that interacts with two non-coding RNAs that are themselves SAM-responsive riboswitches (Loh *et al.*, 2009). One could imagine that CodY binding to a site just downstream of the 5'-UTR region might slow down the RNA polymerase leading to stabilization of upstream RNA structures (of the 5'-transcribed UTR) that facilitate continuation of transcription rather than termination. In such a case, the relatively weak binding of CodY to the intragenic site may still allow the RNA polymerase to proceed with elongation of transcription. In that way, CodY binding to *prfA* might be strong enough to permit interaction with RNA polymerase or the 5'-UTR, but not so strong as to interfere with transcription elongation. Notably, similar mechanisms were proposed for PhoP and other regulators and were suggested to represent a new mode of regulation by activators (Liu *et al.*, 1998). Alternative mechanisms are equally possible, such as those involving DNA-looping or cooperative interaction with additional regulators, as already shown for CodY and CcpA (Wunsche *et al.*, 2012, Shivers *et al.*, 2006), and/or adjacent binding sites (van Hijum *et al.*, 2009).

The ability of CodY to bind to the relatively weak *prfA* site under conditions of limiting BCAA raises important mechanistic questions. Binding of CodY to many *B. subtilis* sites is highly cooperative (Belitsky & Sonenshein, 2013). If the same is true of *L. monocytogenes* CodY binding sites, but the *prfA* site demonstrates non-cooperative binding, the *prfA* site would compete well with other sites at low BCAA levels, i.e., when the concentration of ligand-bound CodY is below the threshold for cooperativity. At concentrations above the threshold, binding to *prfA* would be relatively poor, i.e., cooperative binding sites would compete much more effectively for CodY, explaining why *prfA* expression would be activated by CodY only at limiting BCAA concentrations. Our data demonstrating that the *prfA* site competes better for CodY binding when BCAA are limited are in line with this premise.

Overall the data presented here support a model whereby *L. monocytogenes* exploits a simple yet elegant coupling mechanism to sense a host-specific metabolic cue and induce virulence genes. Namely, a global regulator that functions predominantly as a repressor (when bound to BCAA) is recruited upon a shift in conditions (*i.e.,* isoleucine limitation) to function as a *prfA* activator. Using this mechanism, *L. monocytogenes* can respond to a drop in BCAA availability (as happens upon invasion of host cells) by releasing repression of many metabolic pathways, including BCAA biosynthesis, that facilitate growth and survival. In addition, ligand-limited CodY appears to have the ability to activate transcription of *prfA*,

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leading to induction of the major *Listeria* virulence genes. Interestingly, if Listeria had evolved to use CodY in what seems to be the conventional way (i.e., as a BCAA-dependent repressor of *prfA*), invasion of the host would still lead to PrfA synthesis when BCAA are limiting, but strong repression by CodY in high BCAA could interfere with other, CodY-independent mechanisms of *prfA* regulation, such as in response to G-1-P. Importantly, this study highlights the regulation of PrfA by BCAA and CodY, though it represents only one signaling pathway out of many that have been shown to affect PrfA expression. How these distinct pathways are integrated into the overall scheme of PrfA regulation *in vivo* is not completely clear and will no doubt be the topic of future studies.

Experimental Procedures

Ethics Statement

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 used as the wild type strain (WT) and served as the parental strain to generate allelic exchange mutant strains (Table S1). *E. coli* XL-1 Blue (Stratagene®) was used for vector propagation and *E. coli* strain SM-10 was used for transfer of plasmids to *L. monocytogenes* by conjugation. *L. monocytogenes* strains were grown in brain heart infusion (BHI, Merck®) rich medium, or in LB-MOPS medium that was pre-incubated with 0.2% activated charcoal and supplemented with either 25 mM of glucose (Glu) or glucose-1-phosphate (G1P) (Ripio *et al.*, 1997a) or in minimal defined medium (MM) (Phan-Thanh & Gormon, 1997) at 37°C with agitation. MM contains 100 μg ml^{-1} of each of the BCAAs, which are 760 μM for leucine and isoleucine and 850 μM for valine. For growth under limiting concentrations of nutrients, MM was freshly made with 10-fold less of the BCAA [isoleucine, leucine and valine] (10 μg ml^{-1} for each amino acid, which is 76 μM for leucine and isoleucine and 85 μM for valine.) and termed low-BCAA MM. Lists of bacterial strains and plasmids used in this study are provided in Table S1.

In vitro growth of *L. monocytogenes* in different laboratory media

Bacteria from overnight cultures grown in BHI medium were adjusted to $O.D._{600}$ 0.03 in fresh BHI medium or low/high -BCAA MM (10 μg and 100 μg of each BCAA per ml, respectively) or LB-MOPS-G1P or Glu (25mM) activated charcoal medium and grown in a Synergy HT Biotek® plate reader at 37°C for 24 h. O.D. measurements were made every 15 min. For luminescence assays, *L. monocytogenes* strains harboring the P_{hly}-luciferase reporter system (pPL2-P_{hly}*lux*) were used and luminescence measurements, presented as relative luminescence units (RLU, relative to the blank medium luminescence), were taken every 15 min.

Generation of *L.m. codY-6his*, *L.m. mut6-prfA* and *L.m. CodY R61A* strains

Upstream and downstream regions of the *codY* and *prfA* genes were amplified using Phusion DNA polymerase (NEB®) and specific primers containing the 6His-tag or the mut6

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mutation. The PCR products were cloned in pKSV7oriT vector (Smith & Youngman, 1992) (A list of all primers used in this study is provided in Table S2). Cloned plasmids were sequenced and conjugated to *L. monocytogenes* using *E. coli* SM-10 strain (Simon R, 1983). *L. monocytogenes* conjugants were then grown at 41°C for two days on BHI with chloramphenicol to promote plasmid integration into the bacterial chromosome by homologous recombination. For plasmid curing, bacteria were passaged several times in fresh BHI without chloramphenicol at 30°C to allow plasmid excision via the generation of an allelic exchange. Bacteria were then seeded on BHI plates and chloramphenicol sensitive colonies picked for validation of allelic exchange by PCR, enzyme restriction analysis and sequencing.

Purification of CodY-His$_6$

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 then harvested by centrifugation (4000 rpm, 10 min), washed in 50 ml of cold Buffer A (0.3M NaCl, 50 mM NaH2PO4, pH 8) and resuspended in 15 ml of buffer A 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 was removed by centrifugation at 16,000 g for 20 min and the lysate was incubated with 1ml of Ni-NTA beads (Sigma ®) for 1h at 4°C with tilting. The Ni-NTA beads were then loaded on a column and washed with 10 ml wash Buffer A supplemented with 10 mM imidazole. The protein was eluted by 250 mM imidazole in Buffer A and dialyzed against 100 ml of Buffer A. Protein concentration was determined using a Nanodrop 1000 (Thermo®) spectrophotometer. A small sample was separated on SDS-PAGE gel followed by Commassie staining to test for the purity of the protein. For DNA footprinting analysis a slightly different protocol for CodY purification was used. *L. monocytogenes* CodY was purified as described previously for *B. subtilis* CodY (Belitsky & Sonenshein, 2008) using pET24b plasmid (pET24b-*codY*) (Bennett *et al.*, 2007) and *codY* expression was induced using 0.2 mM IPTG.

Electrophoretic Mobility Shift Assay

Purified CodY-6His was incubated with 5 ng of 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 and separated for 1.5 h at 200 V. The DNA was transferred to a positively charged nylon membrane (Pall®) by overnight electrophoresis (15V) in TBE buffer. Following DNA strand separation in 0.4 M NaOH, crosslinking was performed by UV exposure. The membrane was probed with a radiolabeled DNA generated using NEBlot® kit (New England BioLabs) for 16 h, and then washed 3 times with SSC X 2 + 0.2% SDS, SSC X 0.2 + 0.2% SDS and SSC X 2. Finally the membrane was visualized by a radioactive sensitive phosphor-screen (Sigma®), emitting light on a light sensitive film (FUJI®). EMSA assay used for competition experiments was performed as detailed above, with the exception of labeling the DNA probes with DIG using Roche DIG Gel Shift kit®, adding 10X of competitor unlabeled DNA probe. Detection of

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labeled DNA was done using Roche DIG detection kit®. For calculations of average K_D values, the fraction of the free DNA probe at each CodY concentration was quantitated by densitometric analysis using ImageJ software for each EMSA gel (Schneider *et al.*, 2012). Regression analysis was performed for each gel and the Kd value was calculated. Average Kd values were 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 figure S2). The Kd values were determined as the concentration in which 50% of the DNA probes were unbound as deduced from the regression analysis. List of primers used for the amplification of target DNA sequences/probes is found in Table S2.

ChIP RT-qPCR analysis

The chromatin immunoprecipitation (ChIP) protocol was adapted from ref (Waldminghaus & Skarstad, 2010). Briefly, *L. monocytogenes codY-6His* strain was grown in BHI or low-BCAA MM to $O.D._{600}$ 0.3 or in LB-MOPS-G1P media to $O.D._{600}$ 0.6. Then 1.5% of formaldehyde was added for 20 min at room temperature, followed by quenching with 0.5 M glycine for 5 min. The cells were harvested by centrifugation, and washed twice with cold TBS. At this point, the cells were frozen in liquid N_2 and kept in -80°C. Following thawing, the cells were resuspended in 200 μl of resuspension buffer (20% sucrose, 10 mM TRIS pH 8, 50 mM NaCl, 10 mM EDTA with freshly added 10 mg ml^{-1} lysozyme and 2 U of Mutanolysin, Sigma). The samples were incubated at 37°C for 30 min and then 800 μl of IP buffer (50 mM HEPES-KOH pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X 100, 0.1 % sodium deoxycholate and 0.1 % SDS) and 1 mM of PMSF were added. The samples were then sonicated (6 rounds of 30 sec) and cell debris was removed by centrifugation at 14,000 g for 10 min at 4°C. The lysate was then transferred to a new 1.7 ml tube and 20 μl of 50% slurry protein A/G (Sigma) and 5 μl of anti-6His antibody (ABCAM® ab-18184) were added and incubated overnight with slow rotation at 4°C. Beads were collected by centrifugation (1 min, 3500 g) and the supernatant, which contains unbound DNA, was transferred to a new 1.7 ml tube to serve as control DNA for further analysis. The beads were washed twice with 500 μl cold IP buffer, once with 500 μl of cold high salt IP buffer (0.5 M NaCl), once with 500 μl cold wash buffer (10mM Tris pH 8.0, 250 mM LiCl, 1 mM EDTA, 0.5% NP-40, 0.5% sodium deoxycholate) and once with 500 μl of cold TE pH 8 buffer. Finally the beads were resuspended in 100 μl of elution buffer (50 mM Tris pH 8, 10 mM EDTA and 1% SDS) and incubated at 65°C for 10 min. Beads were removed by 1 min centrifugation. 100 μl of ChIP DNA or control DNA were supplemented with 80 μl of TE buffer with 2.5 μl RNase (8 mg ml^{-1}, Fermentas®) and incubated at 42°C for 1.5 h. DNA was released by incubation with 20 μl of proteinase K (Fermentas®) for 2 h at 42°C, following 16 h at 65°C. DNA was purified using QIAGEN MinElute kit® and eluted in 10 μl water. RT-qPCR analysis was performed on 1–10 ng of ChIP and control DNA using a Step One Plus Real Time PCR system (Applied Biosystems®). DNA levels were normalized to two house keeping genes: *bglA* and *rpoD* using Step One™ software. The enrichment value of each ChIP sample was calculated based on its cognate DNA control sample.

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In vitro CodY pull-down competition binding assay

The assay is based on a DNA pull down protocol that was modified from (Dineen *et al.*, 2010). For the screen of CodY binding site mutants (figure 6)' 300 nM purified CodY-His_6 were incubated with 7nM of *prfA* probe in 250 μl pull down buffer (20 mM TRIS pH 8, 50 mM sodium glutamate, 5% glycerol, 10mM $MgCl_2$, 0.05% NP-40, 25 μg ml^{-1} *E. coli* tRNA) with or without ~400nM of competitor DNA probe for 25 min at room temperature. For the *prfA* and *ilvD* probes competition assay (figure 5D), 200 nM purified CodY-His_6 were incubated with 30 nM *ilvD* probe and 300 nM *prfA* probe in 250 μl pull down buffer supplemented with 0, 1 or 10 mM of BCAA for 25 min at room temperature. Following incubation, 50 μl of TALON® cobalt affinity beads (pre-washed with the same buffer) were added for 20 min with an occasional tilting. Beads were washed 6 times in pull down buffer (if BCAA were present in the binding reaction, the same concentration was included in these washes), centrifuged at 1000 g for 2 min at each wash and finally resuspended in 100 μl of 10 mM TRIS, pH 8 buffer. DNA was released by boiling for 10 min followed by incubation with 1 μl of Proteinase K (Fermentas®) for 1 h at 65°C. Proteinase K was inactivated by boiling for 10 min and beads were removed by centrifugation. The resulting supernatant was diluted 1:10 in DNase free water and analyzed by RT-qPCR analysis using a Step One Plus Real Time PCR system (Applied Biosystems®) with primers specific to the indicated probes.

Analysis of LLO and PlcA activity

L. monocytogenes bacteria were grown in LB-MOPS-G1P medium at 37°C overnight (12 h), and supernatants were separated by centrifugation. The hemolytic activity assay was performed as described previously (Glomski *et al.*, 2002): bacterial supernatants were treated with 5 mM dithiothreitol (DTT), serially diluted in PBS, and incubated with 0.5% sheep red blood cell suspension (NovaMed®); hemolysis was measured by following the change in absorbance at 540 nm. The PI-PLC activity assay was adapted from Geoffroy et al. (Geoffroy *et al.*, 1991): 1 ml of sodium-cholate (58 mM), CaCl2 (10 mM), and 0.036 g phosphatidyl-inositol (P6636; Sigma®) were mixed with 7 ml NaCl (0.15M). One hundred μl of the assay solution was then mixed with 100 μl of bacterial supernatants and incubated in a plate reader at 37°C for 10 h, following turbidity assessment at 510 nm.

Bacterial RNA purification

RNA was harvested from bacteria grown to mid-log phase ($O.D._{600}$ = 0.35) in low-BCAA MM. Precultures were grown in MM overnight prior to the experiments. In LB-MOPS-G1P experiments, RNA was harvested from bacteria at post log-phase ($O.D._{600}$ ~ 0.7). RNA was extracted by a standard phenol-chloroform extraction protocol including a DNase I treatment. RNA from bacteria growing inside macrophages at 2 hours post infection was harvested as described previously (Rabinovich *et al.*, 2012). Briefly, bacteria were harvested by filtration and the filters were frozen rapidly in liquid nitrogen. Later, bacteria were released from filters by washing and bacterial RNA was isolated using phenol-chloroform extraction. Bacterial RNA was further amplified using MessageAmpTM II Bacteria Prokaryotic RNA Kit (Ambion®).

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Real time quantitative PCR analysis of gene expression

One microgram (1μg) of RNA was reverse transcribed to cDNA using the QScript reverse transcription kit (Roche®). RT-qPCR was performed on 16 ng of cDNA using SYBER Green (Roche®) in a Step-one Plus real time PCR system (Applied Biosystems®). The transcription level of each gene of interest was normalized to that of a reference gene: *bglA* in the intracellular experiments and *rpoD* mRNA in the low-BCAA MM and LB-MOPS-G-1-P medium experiments. Statistical analysis was performed using the StepOne™ V2.3 software. RT-qPCR primers are described in Table S2.

Intracellular growth of *L. monocytogenes*

Bone marrow-derived macrophages (BMDM) used for infection experiments were isolated from 6-8 week old female C57/BL6 mice (Harlan laboratories) as described previously (Celada *et al.*, 1984). BMDM were cultured in DMEM-based media supplemented with 20% fetal bovine serum, sodium pyruvate (1 mM), L-glutamine (2 mM), β-Mercaptoethanol (0.05 mM), and M-CSF (L929-conditioned medium). Approximately 8×10^6 *L. monocytogenes* bacteria were used to infect 2×10^6 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 medium added. At 1-hour post infection (h.p.i.) gentamicin (50 μg ml^{-1}) 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 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.

In vivo mice infections

L. monocytogenes bacteria were grown in BHI medium at 30°C overnight. Bacterial cultures were washed twice in Ringer's lactate solution and counted (~ 2×10^9 bacteria per ml). C57BL/6 (6-8 weeks old) female mice (Harlan Laboratories, Ltd, Israel) were infected via tail vein injections with 4×10^4 bacteria in 200 μl of PBS. Animals were observed daily for any signs of illnesses and were euthanized 72 hours post-infection. Spleens and livers were harvested and homogenized in 0.2% saponin, and the numbers of viable bacteria in each organ were determined by plating serial dilutions of homogenates onto BHI agar plates. The experiment involved 5 mice in each group and was repeated twice, yielding similar results.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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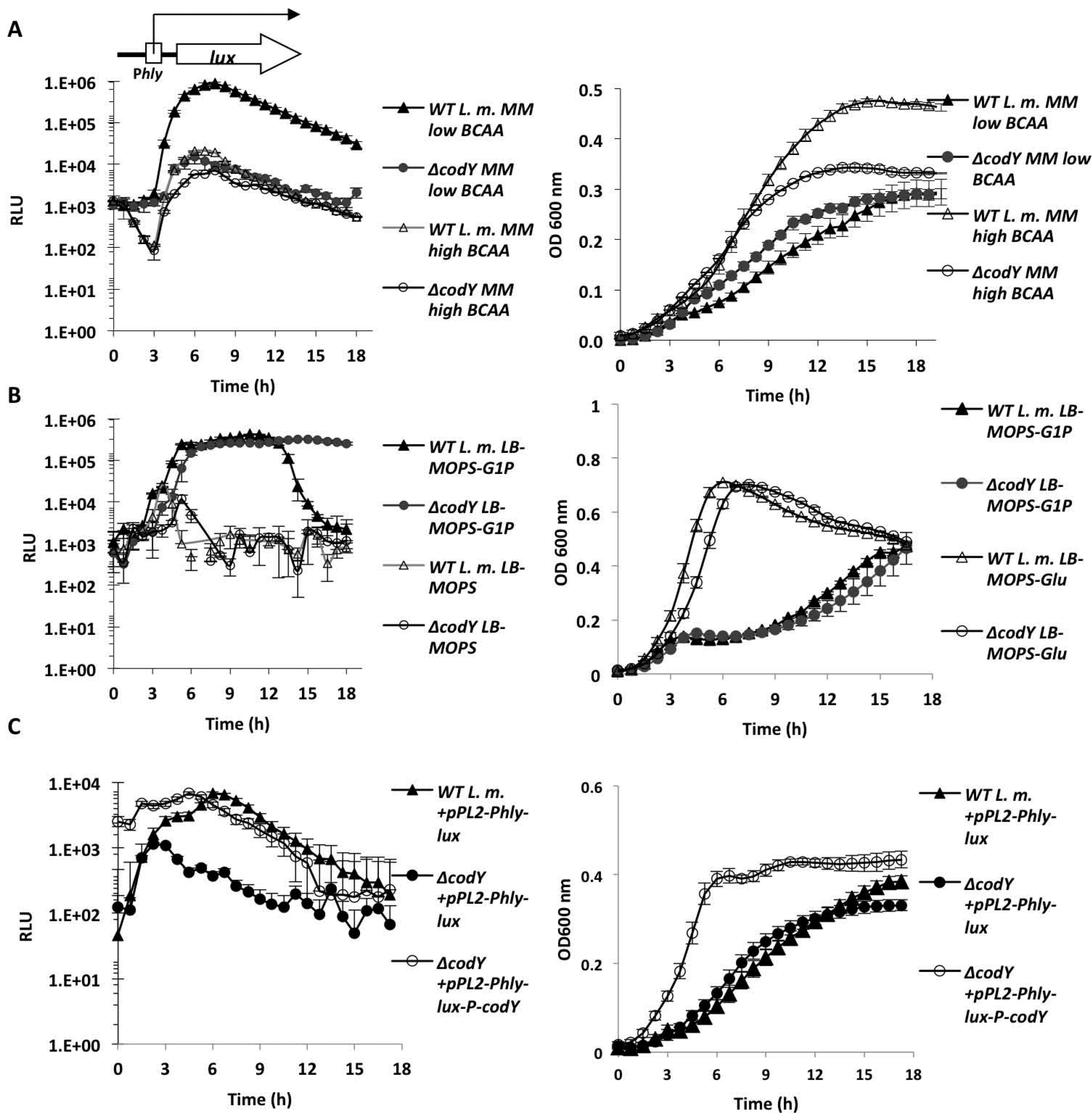

Figure 1. Induction of *hly* gene under low BCAA conditions is CodY-dependent, while its induction by glucose-1-phosphate is CodY-independent

A. Luminescence (left panels) and optical density (right panels) measurements of WT *L. monocytogenes* and *codY* bacteria harboring the pPL2-P$_{hly}$*lux* plasmid indicating P$_{hly}$ promoter activity during growth in low-BCAA MM and high-BCAA MM. **B.** Luminescence (RLU) and optical density measurements of WT *L. monocytogenes* and *codY* bacteria harboring the pPL2-P$_{hly}$*lux* plasmid during growth in LB-MOPS-G1P and LB-MOPS-Glu media. **C.** Complementation of *codY* strain. A copy of the *codY* gene under the regulation

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of the SPAC/*lacOid* promoter (taken from pLIV2 plasmid) was introduced into the *codY* strain on the integrative plasmid pPL2 together with the *hly*-*lux* reporter system (pPL2-P_{hly}*lux*-P-*codY*). Both *codY* and its complemented strain were grown in low-BCAA MM and the P_{hly} promoter activity was measured. Results are averages of at least 4 independent experiments, representing 3 biological replicates in each experiment. Error bars represent standard deviation.

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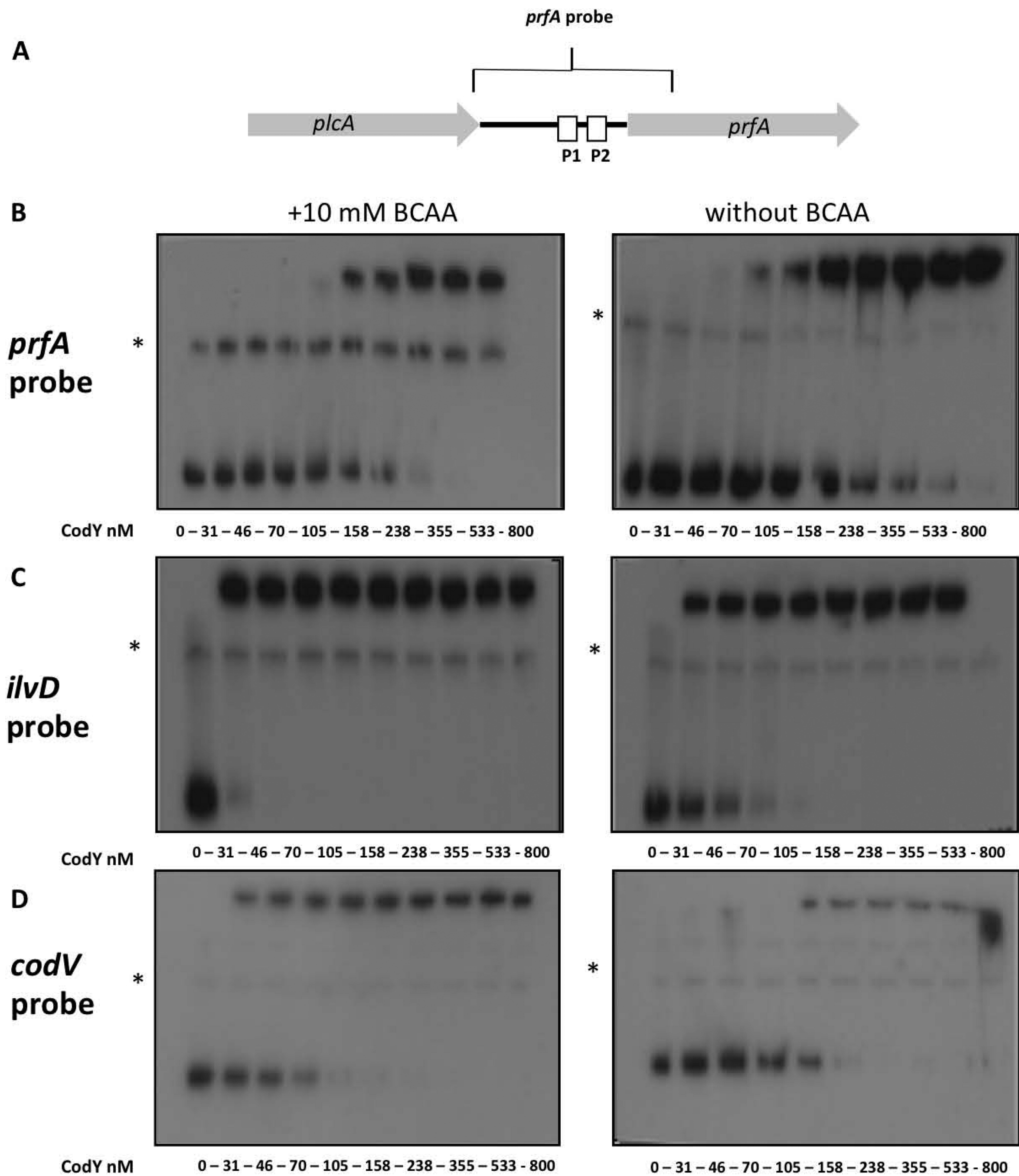

Figure 2. CodY binds the regulatory regions of *prfA* gene, *ilv* operon and the *cod* operon

A. Schematic representation of the full-length *prfA* probe (367-bp) encoding *prfA* intergenic regulatory promoter region and the first 100 nucleotides of *prfA* coding sequence. **B.** Electrophoretic mobility shift assay (EMSA) of CodY binding to *prfA* probe with (left panel) and without (right panel) 10 mM of BCAA (isoleucine, leucine and valine). **C.** EMSA analysis of CodY binding to the *ilvD* probe with and without 10 mM of BCAA. **D.** EMSA analysis of CodY binding to the *codV* probe with and without 10 mM of BCAA. Primers used for amplification of DNA probes are described in Table S2. Results are

representative of at least 3 independent biological repeats. Non-specific bands are marked with asterisks (*).

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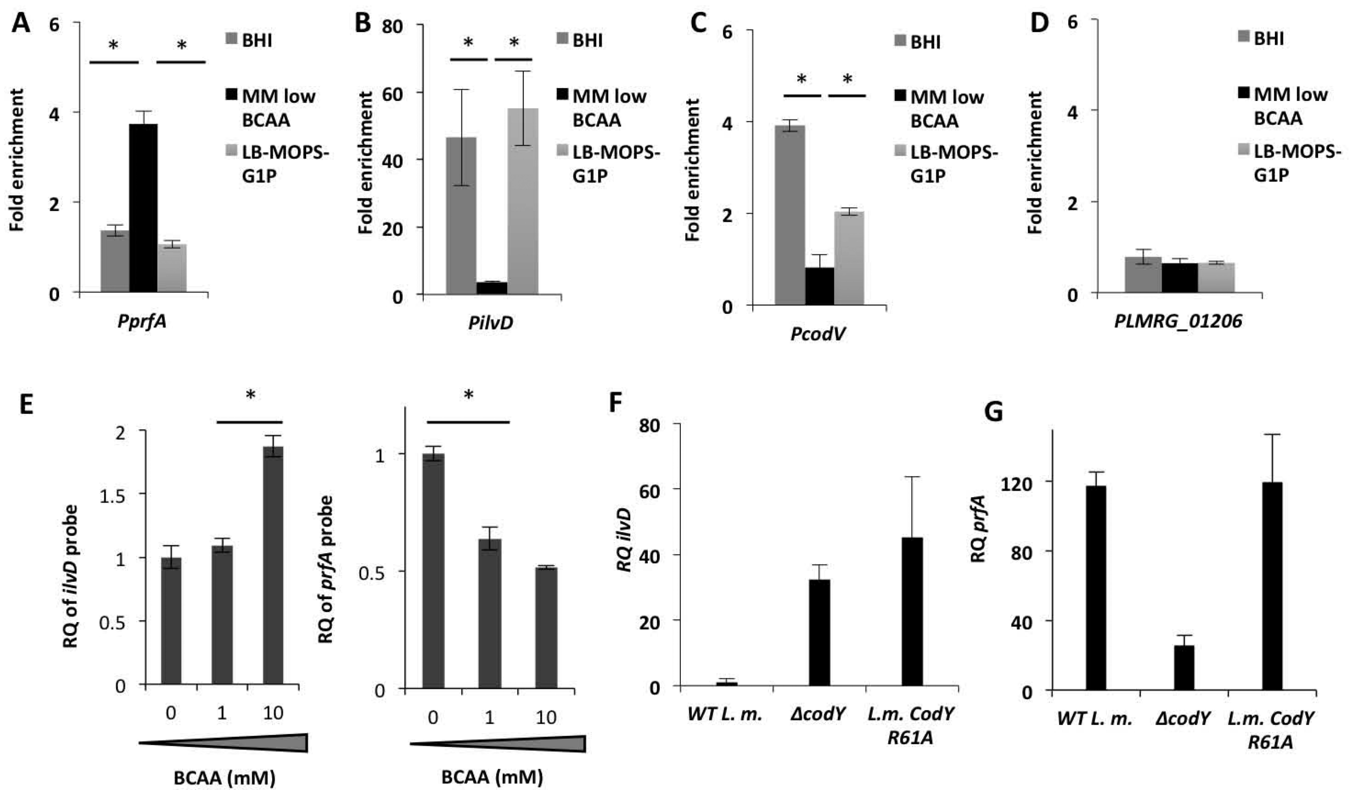

Figure 3. CodY preferentially binds the regulatory region of the *prfA* gene under limiting concentrations of BCAA

In vivo ChIP-RT-qPCR analysis of 6His-tagged CodY binding to regulatory region of **A.** *prfA* gene (*PprfA*), **B.** *ilvD* promoter (*PilvD*), **C.** *codV* promoter (*PcodV*) and **D**. *LMRG_01206* promoter (*PLMRG_01206*) as a negative control, during bacterial growth in BHI, low-BCAA MM and LB-MOPS G1P media. Fold enrichment of CodY association with each one of the tested sequences was normalized to the control sequences of *bglA* and *rpoD* and to its no-ChIP control. Results are average of 4 independent biological repeats. Error bars represent standard deviation (SD), * represents P value < 0.05. **E**. *In vitro* CodY pull-down competition binding assay. Both *ilvD* and full-length *prfA* probes were mixed to compete for 6His-tagged CodY binding at increasing concentrations of BCAA. Following incubation CodY was precipitated using cobalt beads and the amounts of *prfA* and *ilvD* probes bound to CodY were quantitated by RT-qPCR using specific primers. **F.** RT-qPCR analysis of *ilvD* transcription levels in WT *L.m.*, *codY* and *L.m. CodY R61A* bacteria grown to mid-log in BHI rich medium. **G.** RT-qPCR analysis of *prfA* transcription levels in WT *L. m.*, *codY* and *L.m. CodY R61A* bacteria grown to mid-log in low-BCAA MM. The RQ values are normalized to *ilvD* or *prfA* expression in WT bacteria grown in BHI. Results are average of 3 independent biological repeats. Error bars represent 95% confidence interval, * represents P value < 0.05.

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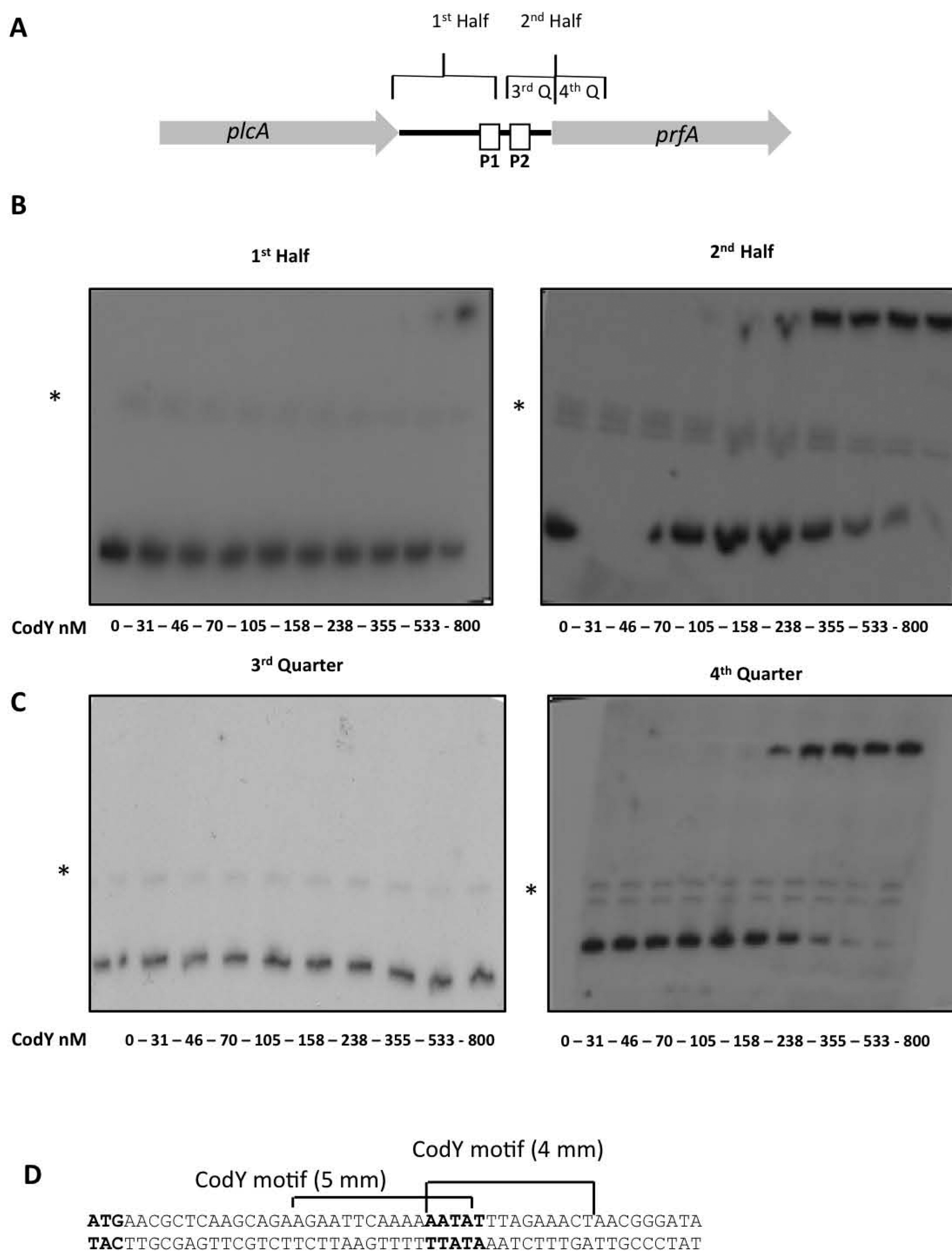

Figure 4. Mapping CodY binding site to the 5' -coding sequence of *prfA* gene using EMSA

A. Schematic representation of the *prfA* probe and its two halves and four quarters segments. **B.** EMSA analysis of CodY binding to the two halves of the *prfA* probe. **C.** EMSA analysis of CodY binding to the 3rd and 4th quarters of *prfA* probe. The results are representative of at least 3 independent biological repeats. Experiments were performed without BCAA. Non-specific bands are marked with asterisks (*). **D.** Sequence of the putative CodY boxes identified in the 4th quarter of the *prfA* probe.

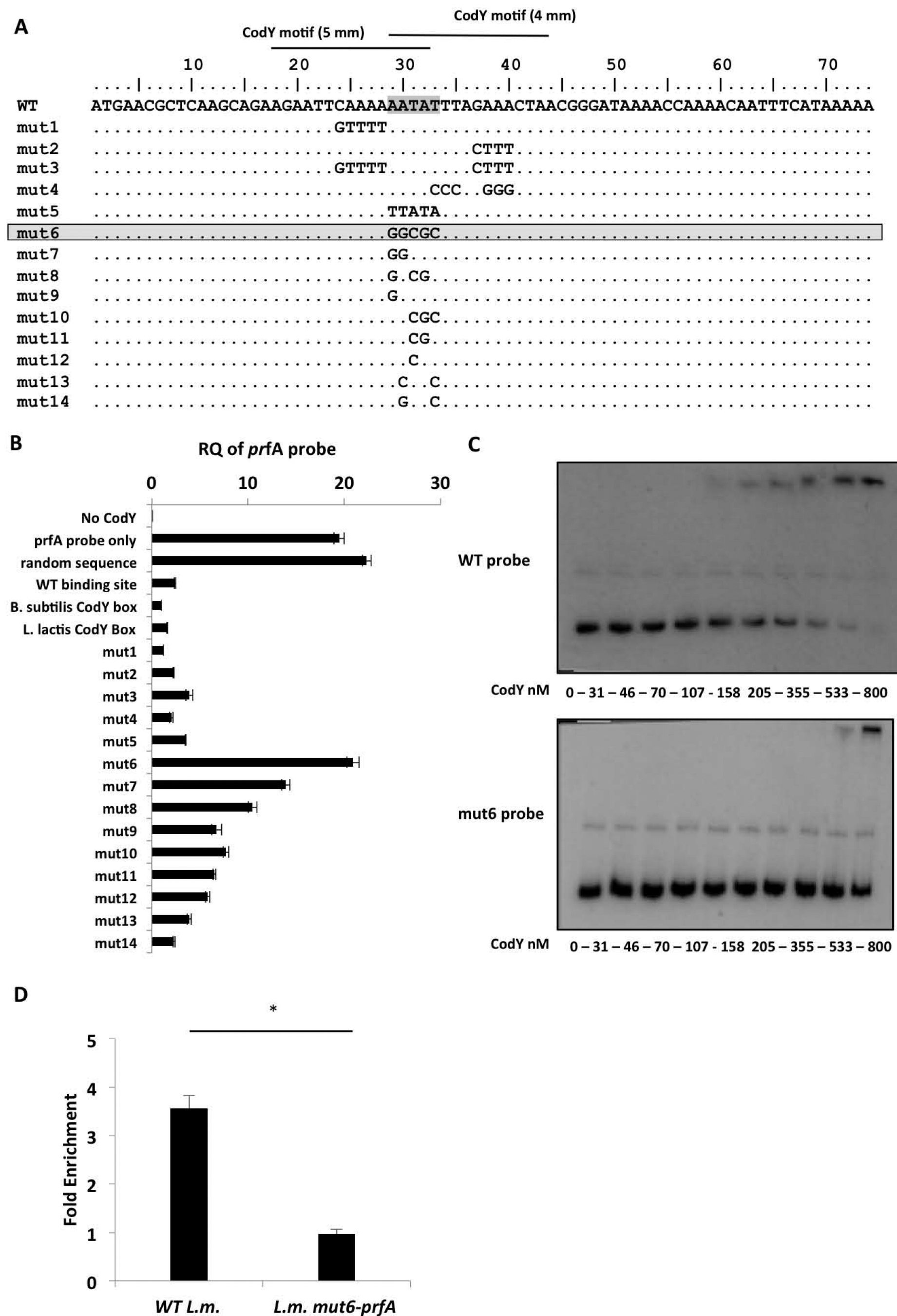

Figure 5. Mutational analysis of the CodY-binding region identifies an AATAT sequence that is critical for CodY binding

A. Representation of the 74-bp probes synthesized to contain different mutations within the putative CodY-binding region (mut-1 to -14). Critical nucleotides for CodY binding are highlighted. **B.** A CodY pull-down competition binding assay between the 367-bp *prfA* probe and each one of the 74-bp competitor probes. The assay is based on a DNA pull down assay using a 6His-tagged CodY protein. Analysis of CodY binding to the *prfA* probe was done using RT-qPCR with primers specific to the full-length prfA probe. Controls include a

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sample without CodY (No-CodY), a sample without a competitor (*prfA* probe only), a sample with random sequence probe as competitor and probes containing the WT *prfA* CodY-binding site, *B. subtilis* or *L. lactis* CodY-boxes as competitors. Results are average of 3 independent biological repeats. Error bars represent standard error of the mean. **C.** EMSA analysis of CodY binding to *prfA* 2nd half probe (WT probe) and to *prfA* 2nd half probe containing the mut6 mutation (mut6 probe). Results are representative of 3 independent biological repeats. **D.** ChIP-RT-qPCR analysis of CodY binding to WT and *L.m. mut6-prfA prfA* gene during growth in low-BCAA MM. Results are average of at least 3 independent biological repeats. Error bars represent standard deviation (SD), * represents P value < 0.05.

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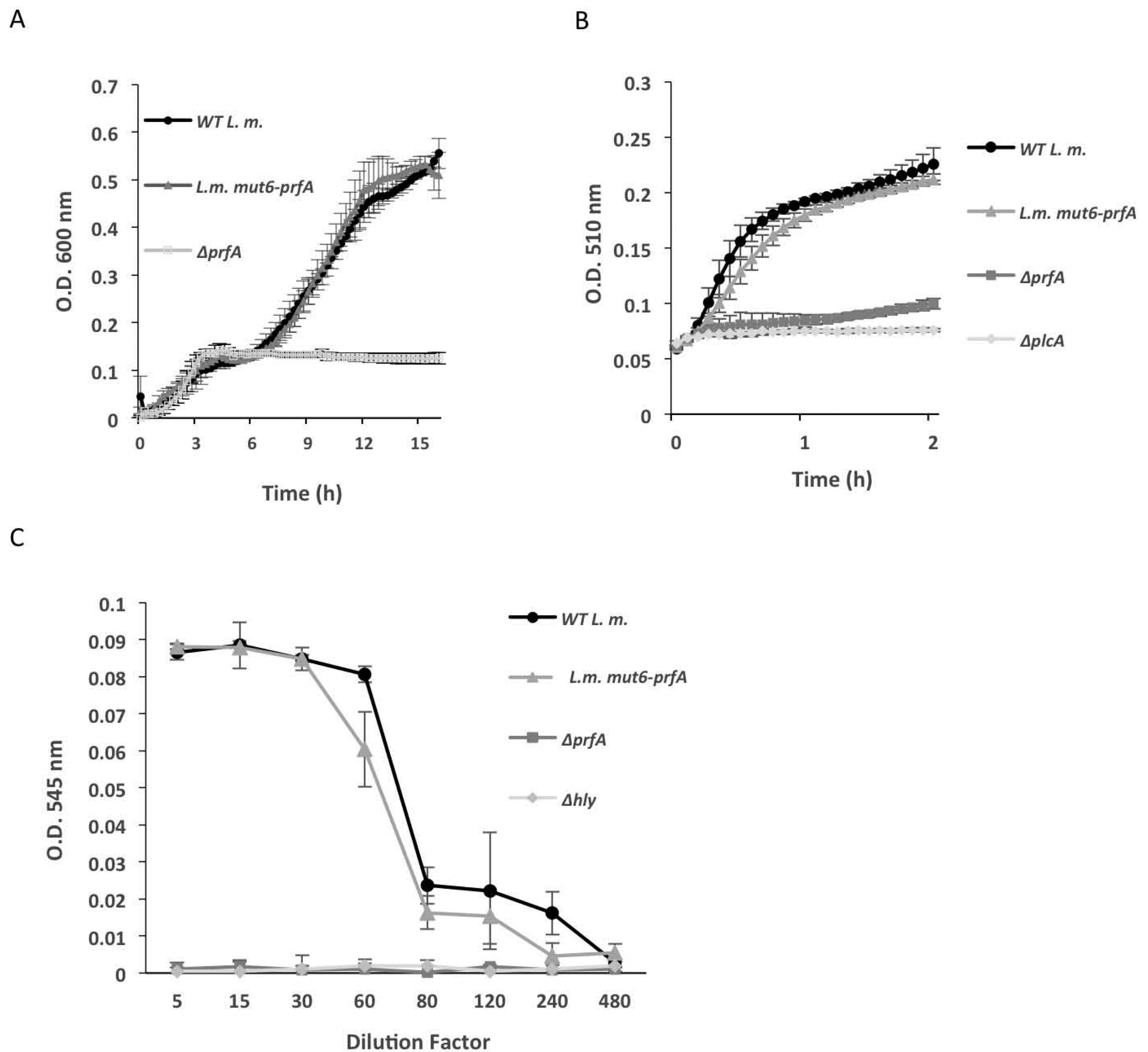

Figure 6. *mut6-prfA* is not impaired in PrfA activity

A. Growth of WT *L. m.*, *L.m. mut6-prfA* and *prfA* bacteria in LB-MOPS-G1P medium. **B.** PlcA activity in the supernatants of WT *L.m.*, *L.m. mut6-prfA*, *prfA* and *plcA* mutants measured using a PI-PLC phospholipase activity assay. Bacteria were grown in LB-MOPS-G1P medium and supernatants were harvested at mid-exponential growth. Results are average of 3 independent biological repeats. Error bars represent standard error of the mean. **C.** Analysis of LLO activity in the supernatants of WT *L.m.*, *L.m. mut6-prfA*, *prfA* and *hly* mutants using hemolysis assay in red blood cells. Bacteria were grown in LB-MOPS-G1P medium and supernatants were harvested at mid-exponential growth.

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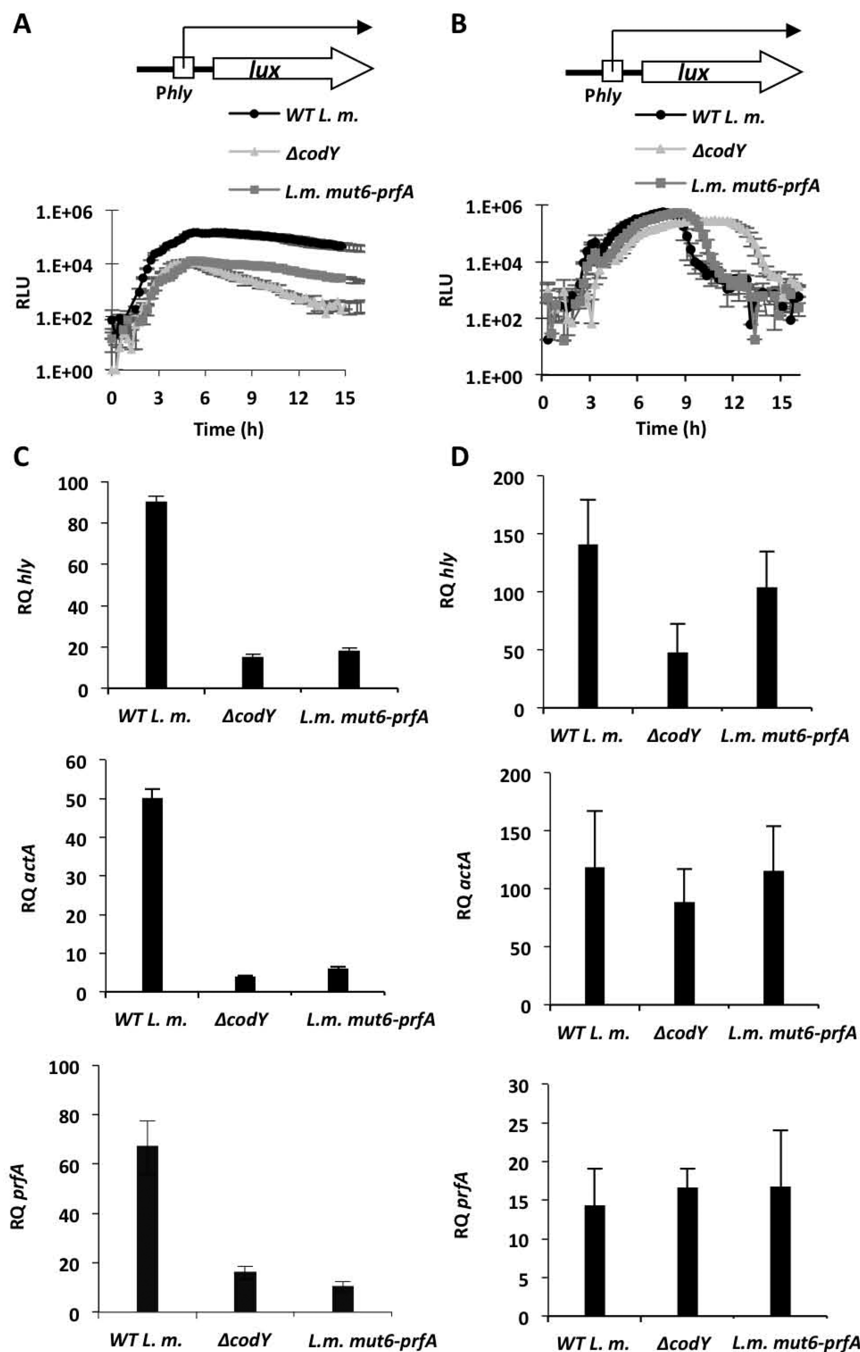

Figure 7. CodY and its *prfA*-intragenic binding site are important for *prfA* activation at low concentrations of BCAA

A. Luminescence of WT *L. m.*, *codY* and *L.m. mut6-prfA* bacteria harboring the integrative reporter plasmid pPL2-P_{hly}*lux* during growth in low-BCAA MM. **B.** Luminescence of WT *L. m.*, *codY* and *L.m. mut6-prfA* bacteria harboring the integrative reporter plasmid pPL2-P_{hly}*lux* during growth in LB-MOPS-G1P medium. Error bars represent standard deviation. **C.** RT-qPCR analysis of *hly*, *actA* and *prfA* genes in WT *L. m.*, *codY* and *L.m. mut6-prfA* bacteria grown in low-BCAA MM. **D.** RT-qPCR analysis of *hly*, *actA* and *prfA* genes in WT

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L. m., *codY* and *L.m. mut6-prfA* bacteria grown in LB-MOPS-G1P medium. The results represent 3 independent biological repeats. Transcription levels are represented as relative quantity (RQ), relative to the transcription levels during growth in BHI. Error bars represent 95% confidence interval.

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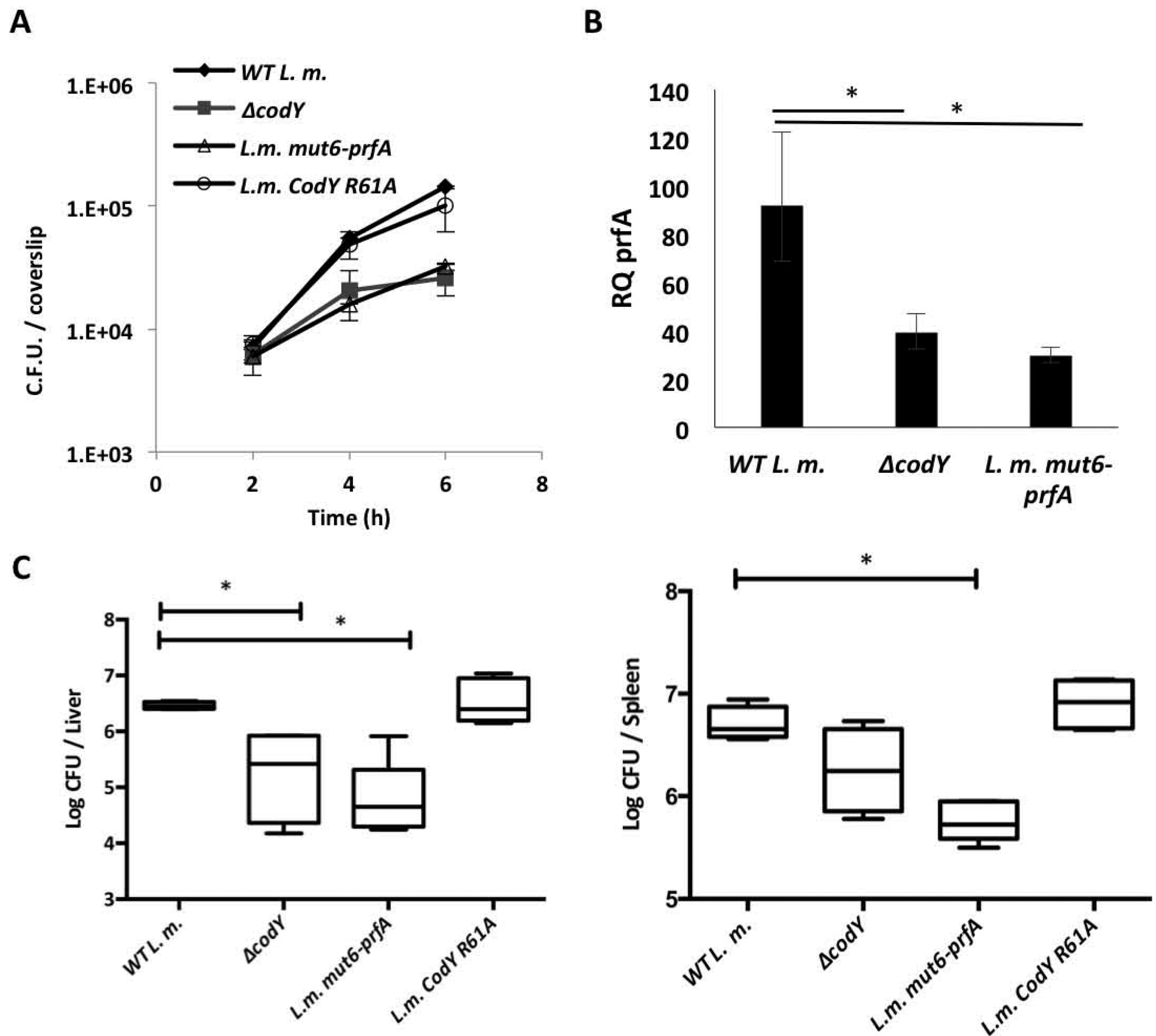

Figure 8. CodY and its *prfA*-intragenic binding site play a major role in *L. monocytogenes* virulence

A. Intracellular growth of WT *L. m.*, *codY*, *L.m. mut6-prfA* and *L.m. CodY R61A* bacteria in BMD macrophages. Results are representative of 3 independent biological repeats. Error bars represent the standard error of the mean. **B.** RT-qPCR analysis of *prfA* during intracellular growth of WT *L. m.*, *codY* and *L.m. mut6-prfA* bacteria in bone marrow derived (BMD) macrophages. Results are average of 3 independent biological repeats. Transcription levels are represented as relative quantity (RQ), relative to levels in WT *L.m* bacteria grown to mid-log in BHI rich medium. Error bars represent 95% confidence interval, * represents P value < 0.05. **C**. Intravenous infection of C57BL/6 mice with WT *L. m.*, *codY, L.m. mut6-prfA* and *L. m. CodY R61A* bacteria. Bacterial colony forming units (CFUs) were numerated at 72 h.p.i. from livers and spleens taken from 10 infected mice for

each strain. The results are mean of 2 independent experiments in which 5 mice were infected in each group. Mann-Whitney tests were performed for statistical significance (* P value < 0.05).

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