***Listeria monocytogenes* TcyKLMN cystine/cysteine transporter facilitates glutathione synthesis and virulence gene expression**

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Abstract

Listeria monocytogenes (*Lm*) is a saprophyte and a human intracellular pathogen. Upon invasion into mammalian cells, it senses multiple metabolic and environmental signals that collectively trigger its transition to the pathogenic state. One of these signals is the tripeptide glutathione, which acts as an allosteric activator of *Lm*'s master virulence regulator, PrfA. While glutathione synthesis by *Lm* was shown to be critical for PrfA activation and virulence gene expression, it remains unclear how this tripeptide is synthesized under changing environments, especially in light of the observation that *Lm* is auxotrophic to one of its precursors, cysteine. Here, we show that the ABC transporter TcyKLMN is a cystine/cysteine importer that supplies cysteine for glutathione synthesis, hence mediating the induction of the virulence genes. Further, we demonstrate that this transporter is negatively regulated by three metabolic regulators: CodY, CymR and CysK, which sense and respond to changing concentrations of branched chain amino acids (BCAA) and cysteine. The data indicate that under low concentrations of BCAA, TcyKLMN is up-regulated, driving the production of glutathione by supplying cysteine, thereby facilitating PrfA activation. These findings provide molecular insight into the coupling of *Lm* metabolism and virulence, connecting BCAA sensing to cysteine uptake and glutathione biosynthesis, as a mechanism that controls virulence gene expression. This study exemplifies how bacterial pathogens sense their intracellular environment and exploit essential metabolites as effectors of virulence.

Importance

Bacterial pathogens sense the repertoire of metabolites in the mammalian niche and use this information to shift into a pathogenic state to accomplish successful infection. Glutathione is a virulence-activating signal that is synthesized by *L. monocytogenes* during infection of mammalian cells. In this study, we show that cysteine uptake via TcyKLMN drives glutathione synthesis and virulence gene expression. The data emphasize the intimate cross-regulation between metabolism and virulence in bacterial pathogens.

Introduction 57

Listeria monocytogenes (*Lm*) is a Gram-positive, facultative, intracellular pathogen 58
and the causative agent of listeriosis, a disease that can lead to severe clinical 59
manifestations in pregnant women, neonates and immunocompromised adults (1). *Lm* 60
is characterized by its intracellular lifestyle, but can also grow outside the host on soil 61
and vegetation, as well as on food products (2). In the mammalian host, *Lm* invades a 62
wide array of cells (phagocytic and non-phagocytic) by expressing specialized 63
proteins, named internalins, that facilitate its internalization (e.g., InlA and InlB) (3). 64
Upon internalization, the bacteria are initially found within a membrane-bound 65
vacuole, from which they escape into the host cell cytosol via the action of several 66
virulence factors - the pore-forming toxin Listeriolysin O (LLO, encoded by the *hly* 67
gene), and the two phospholipases PlcA and PlcB (4–6). In the host cell cytosol, the 68
bacteria utilize host-derived metabolites to support growth (7–10), and using ActA 69
protein hijack the host actin-polymerization machinery to move around the cell and 70
spread from cell to cell (11, 12). All of the above-mentioned virulence factors as well 71
as many others are positively regulated by PrfA, the master virulence regulator of *Lm* 72
(13, 14). 73

Multiple metabolic and physiological signals are responsible for the transition of *Lm* 74
from the saprophytic to the pathogenic state (15–20). Many of these signals converge 75
at PrfA, and directly or indirectly regulate its transcription, translation and activity. 76
One of these signals is low concentration of branched-chain amino acids (low 77
BCAA), a condition that is found in the intracellular niche. It was previously 78
demonstrated by our lab that *Lm* responds to low BCAA by upregulating the 79
expression of PrfA, which, in turn, activates the virulence genes (15). This response 80
was shown to depend on the global metabolic regulator CodY, which is also a sensor 81
of BCAA, as it directly binds isoleucine (15, 21, 22). Under high-BCAA conditions, 82
CodY was shown to bind isoleucine and in that form to repress the transcription of 83
many metabolic genes, including those involved in BCAA biosynthesis (21–25). 84
While this was considered the main mechanism of CodY regulation, we demonstrated 85
that in *Lm* CodY is also active under low BCAA conditions, i.e., in its isoleucine- 86
unbound form, where it up- and downregulates the transcription of many genes, 87
among them *prfA* (up-regulating its transcription), hence playing a role in the 88
induction of the virulence genes (15, 22). While these findings demonstrated that PrfA 89

expression is essentially linked to the availability of BCAA in the intracellular niche, 90
they further indicated that sensing of the metabolic host cell cytosol environment is 91
key to the regulation of *Lm* virulence. 92

93
Another metabolite that was recently shown to affect *Lm* virulence is glutathione. 94
Glutathione is a low-molecular-weight peptide thiol that is highly abundant in the host 95
cell cytosol in its reduced form (GSH), functioning as a redox buffer, antioxidant and 96
enzyme cofactor (26). Glutathione is also synthesized by some bacteria, mainly Gram- 97
negative and few Gram-positive (such as *Lm*), and plays a role in redox homeostasis 98
and bacterial survival under oxidative stress (26). A previous study demonstrated that 99
Lm's glutathione synthetase, GshF, plays a critical role in the induction of the 100
virulence genes during infection (17). GshF is a bifunctional enzyme that catalyzes 101
the two reactions that synthesize the tripeptide glutathione (i.e., L- γ -glutamyl-L- 102
cysteinylglycine) (27). It first ligates the γ -carboxyl group of L-glutamate to L- 103
cysteine, a reaction that is the rate-limiting step of glutathione biosynthesis, and then 104
condenses the product γ -glutamylcysteine with glycine. GSH itself was shown to 105
allosterically bind PrfA, and act as its activating cofactor (28, 29). More specifically, 106
the binding of glutathione to PrfA was shown to cause a conformational change that 107
primes its binding to DNA, as shown for other ligand-binding Crp/Fnr transcription 108
regulators (28, 29). 109

110
As indicated, cysteine is one of the building blocks of glutathione, and its rate-limiting 111
precursor, yet, it is not synthesized by *Lm*. *Lm* lacks the ability to reduce sulfate to 112
sulfide, which is required for cysteine biosynthesis, and hence has to import cysteine 113
from the environment (30). Notably, *Lm* is also auxotrophic to methionine (which also 114
has to be imported), and it cannot synthesize cysteine from methionine, as it lacks the 115
transsulfuration pathway that converts methionine to cysteine (9, 30). To date, two 116
transport systems have been shown to be involved in the acquisition of cysteine by 117
Lm. The first is the ABC-transporter Lmo0135-0137 (whose substrate binding protein 118
is CtaP), that was shown to support the uptake of free cysteine during growth in a 119
synthetic medium (31). The other system is the OppABCDF transporter, that was 120
shown to import oligopeptides, including cysteine-containing peptides, that were 121
shown to serve as a source of cysteine for glutathione synthesis and PrfA activation 122

(20). While these systems were reported to promote to *Lm* invasion and virulence 123
gene expression in mammalian cells, respectively, their contribution to *Lm* 124
intracellular growth was only partial (20, 31, 32), implying that additional systems are 125
involved in the acquisition of cysteine within the intracellular niche. 126
In this study, we report on TcyKLMN, another ABC-transporter that is directly 127
involved in cysteine uptake in *Lm*. We show that this transporter imports both cystine 128
and cysteine and plays a role in the activation of *Lm* virulence gene expression by 129
supplying cysteine for glutathione synthesis. Further, we demonstrate that this 130
transporter is regulated by CodY, CymR and CysK, three metabolic factors that act as 131
repressors under nutrient-rich conditions. The findings presented here establish that 132
cysteine import is key to the transition of *Lm* to the pathogenic state, and provide 133
another example of the coupling of metabolism and virulence in bacterial pathogens. 134
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136
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Results 138

Genes associated with cysteine uptake and metabolism modulate virulence gene expression 139 140

We previously performed a genetic screen of a mariner transposon mutant library in 141 search of genes that differentially regulate the virulence genes of *Lm* strain 10403S 142 under low BCAA. The screen identified multiple genes that were associated with 143 cysteine uptake and metabolism: *tcyN* (*LMRG_01497*), *ytmO* (*LMRG_01498*), 144 *LMRG_01492* and *cysK* (*LMRG_02645*) (**Figure 1A** and **S1A**) (33). Interestingly, the 145 first three genes mapped to the *ytmI* operon, which in *B. subtilis* was shown to encode 146 the cystine ABC-transporter, TcyJKLMN (**Figure 1A**, of note, in *Lm* this operon 147 lacks the *tcyJ* gene) (34). *tcyN* encodes the transporter's ATP-binding protein, 148 whereas *ytmO* and *LMRG_01492* encode a monooxygenase and an FMN reductase, 149 respectively, whose functions in cystine uptake/metabolism remain elusive. Apart 150 from this locus, the *cysK* gene was also identified, encoding an O-acetylserine thiol- 151 lyase (which is part of the cysteine synthase complex), which plays a role in cysteine 152 regulation and synthesis (**Figure 1A** and **S1B**). In *B. subtilis*, CysK was shown to be a 153 trigger enzyme that acts both as a metabolic enzyme and a transcription regulator, the 154 latter via interaction with CymR, a key transcription regulator in cysteine/methionine 155 metabolism (35). It was shown that CymR and CysK form a complex that negatively 156 regulates the *ytmI* operon by repressing the transcription of its activator YtlI (also 157 called AscR (36, 37), which is encoded upstream to the operon in the opposite 158 direction (a similar gene organization exists in *Lm*, **Figure 1A**) (38). Although *ytlI* 159 and *cymR* were not identified in our screen, we found homologues in the *Lm* genome 160 (*LMRG_01491* and *LMRG_01455*, respectively), suggesting that the *Lm ytmI* operon 161 is regulated similarly to that of *B. subtilis*. 162

To investigate the cysteine-associated genes and their role in the regulation of *Lm* 164 virulence, we first examined whether *Lm* strain 10403S is auxotrophic to cysteine and 165 methionine, as some variations have been reported for different *Lm* strains (30). As 166 shown in **Figure S2**, *Lm* strain 10403S is unable to grow in the absence of cysteine or 167 methionine, and cannot use either of them as a source for the other, hence had to be 168 supplied with both. We next confirmed the phenotypes of the mariner transposon 169 mutants, focusing on *cysK::Tn* and *tcyN::Tn*, using the same assay that was used in the 170 original screen. Virulence gene expression was monitored using a reporter system that 171

expresses the *luxABCDE* genes under the control of the PrfA-regulated *hly* promoter, which was cloned on the integrative plasmid pPL2 (pPL2-*Phly-lux*). Of note, wild type (WT) bacteria carrying this plasmid grown in a minimal defined medium containing low BCAA (low BCAA minimal medium, LBMM) display an enhanced luminescence profile, which represents the increased transcription of the *hly* gene [previously shown in (21)]. As mentioned, this upregulation of *hly* transcription in LBMM is completely dependent on CodY, as, under this condition, CodY activates the transcription of PrfA (15, 21). Examining the luminescence profiles of *cysK::Tn* and *tcyN::Tn* during growth in LBMM, we observed that the mutants differentially affect *Phly-lux* expression in comparison to WT bacteria. While the *cysK::Tn* mutant exhibited an enhanced luminescence profile, *tcyN::Tn* failed to show luminescence signals (**Figure 1 B-C**) (33). To validate these phenotypes, we generated clean deletion mutants of *cysK* and *tcyK*, the latter encoding the substrate binding protein (SBP) of TcyKLMN (*LMRG_01494*). SBPs of ABC transporters (specifically importers) are key factors that bind the transported substrate extracellularly and facilitate its import into the cell, hence determining the substrate specificity of the transporter (39). Since both *tcyK* and *tcyN* are essential components of TcyKLMN, we chose to delete *tcyK* instead of *tcyN*, to further investigate the transporter's substrate specificity. As shown in **Figure 1 D-E**, $\Delta cysK$ and $\Delta tcyK$ recapitulated the phenotypes of their corresponding transposon mutants. Of note, the mutants grew similarly to WT bacteria in LBMM and in the rich medium BHI (**Figures 1 D-E** and **Figure S3 A-B**). Since in *B. subtilis* CysK was shown to repress *tcyJKLMN* by forming a complex with CymR, we generated a $\Delta cymR$ mutant of *Lm* and analyzed its luminescence profile during growth in LBMM using the *Phly-lux* reporter system. The data indicated that $\Delta cymR$ behaves similarly to $\Delta cysK$, i.e., exhibits an enhanced luminescence profile, overall demonstrating that CysK and CymR negatively affect the transcription of *hly* (**Figure 1F** and **Figure S3C**). To confirm the effects of TcyK, CysK and CymR on the expression of the virulence genes, the transcription levels of *actA*, *plcA* and *hly* (three major virulence genes of *Lm*) were evaluated in $\Delta tcyK$, $\Delta cysK$ and $\Delta cymR$ mutants and compared to those in WT bacteria grown in LBMM, using qRT-PCR. As shown in **Figure 1G**, the transcription level of the virulence genes corroborated the luminescence data, demonstrating a low transcription level in $\Delta tcyK$ (~10-fold) and an enhanced transcription level in $\Delta cysK$ and $\Delta cymR$ (~15-fold) in comparison to WT bacteria. These phenotypes were further complemented by introducing a plasmid

containing a copy of *cysK* or *tcyK* to the corresponding mutants, demonstrating WT levels of *hly* transcription (Figure S4 and Figure S3 D-E). Taken together, these findings indicated that TcyKLMN, CysK and CymR play a role in the regulation of *Lm* virulence gene expression.

TcyKLMN is a cystine/cysteine transporter

The transport specificity of ABC transporters that functions as importers is dictated almost exclusively by the binding specificity of their cognate substrate binding proteins (SBP). Therefore, to determine the substrate specificity of *Lm* TcyKLMN, the SBP of the system, TcyK (containing only amino acids 38-286, i.e., without the membrane anchoring domain) was cloned, overexpressed in *E. coli* and purified to near homogeneity (Figure S5). We then used isothermal titration calorimetry (ITC) to measure the binding of different substrates to TcyK. We found that TcyK binds cystine (CSSC, the reduced form of cysteine) with a dissociation constant (K_D) of 13 μ M and L-cysteine with a K_D of 66.7 μ M (Figure 2A). We also tested TcyK for binding of additional amino acids, such as Gln, His, Glu, Thr and Ser, however, none of these were found to be recognized by TcyK (data not shown). To corroborate the ITC findings, we performed growth experiments of Δ *tcyK* and WT bacteria in minimal defined medium containing either cysteine or CSSC as a sole source. Since *Lm* is auxotrophic to cysteine, bacteria with reduced cysteine-import activity should display a growth disadvantage. Indeed, as shown in Figure 2B, when either CSSC or cysteine were added at limited concentrations (<0.5 mM), the growth of Δ *tcyK* was significantly attenuated relative to WT bacteria. These experiments suggest that *Lm* TcyKLMN imports both CSSC and cysteine.

TcyKLMN is negatively regulated by CodY, CysK and CymR

Since TcyKLMN was identified under low BCAA, we next investigated its expression level under this condition. To this end, we compared the transcription levels of *tcyK*, *tcyN* and *ytII* in WT bacteria grown in LBMM versus BHI. The results demonstrated that the transporter is highly expressed in LBMM (~100-fold in comparison to BHI, shown by the transcription level of *tcyK* and *tcyN*), and that the YtII activator gene is also up-regulated (~10-fold) (Figure 3B). Examining the *ytmI*-*ytII* intergenic region, we identified two putative CodY binding-sites upstream to the *ytII* gene (also identified in (22, 40)), suggesting that CodY indirectly regulates TcyKLMN via

regulation of the YtlI activator (**Figure 3A**). To test this hypothesis, we analyzed the transcription levels of *ytII* and *tcyK* in WT and $\Delta codY$ bacteria grown in BHI and LBMM, using qRT-PCR. The experiments confirmed that under nutrient-rich conditions (i.e., in BHI) CodY represses the *ytII* gene, and consequently *tcyK* (**Figure 3C**), and further demonstrated that this repression is relieved when BCAA are limited (i.e., in LBMM) (**Figure 3D**). We next investigated the role of CysK and CymR in the regulation of TcyKLMN (i.e., the *ytmI* operon). Of note, since *Lm* does not assimilate sulfate to sulfide, we assumed that CysK acts as a CymR co-repressor and not as an O-acetylserine thiol-lyase, a function that requires sulfide (**Figure S1B**). As mentioned, in *B. subtilis* it was shown that CysK and CymR form a complex that represses the TcyJKLMN transporter genes (35). Based on the *B. subtilis* CymR binding-motif (38), we identified two putative CymR binding-sites in the *ytmI*-*ytII* regulatory region of *Lm*: one upstream the *ytII* gene and the other upstream the *ytmI* operon (**Figure 3A**). To examine the potential regulation of this gene locus by CymR and CysK, we first analyzed the transcription levels of *ytII* and *tcyK* in $\Delta cymR$ and $\Delta cysK$ mutants in comparison to WT bacteria grown in BHI and LBMM. The data clearly demonstrated that CymR and CysK act as repressors, down-regulating the transcription of *ytII* and the *ytmI* operon under both BHI and LBMM conditions (**Figure 3 C-D**). Since in *B. subtilis* it was shown that CysK and CymR respond to the availability of cysteine, we repeated this experiment using a minimal defined medium containing low concentration of cysteine (LCMM, containing 0.08 mM cysteine). Interestingly, the data demonstrated that under these conditions, the repression of *ytII* by CymR and CysK was fully relieved, whereas *tcyK* (representative of the transporter genes) was still repressed by these proteins (~15-fold) (**Figure 3E**). These findings established that TcyKLMN is negatively regulated, both directly and indirectly, by at least three factors: CodY, CymR and CysK, which collectively respond to changes in BCAA and cysteine levels, and possibly to additional metabolites that affect CymR and CysK. Taken together, the data indicated that under nutrient-rich conditions, all three factors repress the *ytmI* operon, whereas under low BCAA conditions, the operon is up-regulated, albeit, not to its full capacity, as CymR and CysK still repress its transcription to some extent. These findings imply a complex regulation of TcyKLMN in response to different metabolic or environmental cues.

In light of these observations, we next examined whether the increased expression of the virulence genes in $\Delta cymR$ and $\Delta cysK$ is due to the increased expression of TcyKLMN. For this purpose, we generated double deletion mutants, lacking either *cysK* or *cymR* and *tcyK* ($\Delta cysK/\Delta tcyK$ and $\Delta cymR/\Delta tcyK$) and analyzed their *Phly-lux* luminescence profiles during growth in LBMM. Notably, both mutants failed to activate *Phly-lux* expression, similarly to $\Delta tcyK$, indicating that TcyKLMN itself plays a role in the activation of the virulence genes under low BCAA (**Figure 4** and **Figure S3 F-G**).

Cysteine import via TcyKLMN drives glutathione biosynthesis, which triggers the induction of the virulence genes

Having discovered that in LBMM, TcyKLMN is required for the induction of the virulence genes and not for bacterial growth (**Figure 1E**), we hypothesized that its role in cysteine import may directly feed into glutathione biosynthesis, which, in turn, stimulates the activity of PrfA. This would account for the failure of all the tested transporter mutants (*i.e.*, $\Delta tcyK$, *tcyN::Tn*, $\Delta cymR/\Delta tcyK$ and $\Delta cysK/\Delta tcyK$) to express the virulence genes in LBMM. To test this hypothesis, we first constructed a mutant deleted of the glutathione synthase gene ($\Delta gshF$) and examined its impact on the activation of virulence genes in LBMM using the *Phly-lux* reporter system. As shown in **Figure 5A**, $\Delta gshF$ displayed practically zero expression of the *lux* genes (similarly to $\Delta tcyK$), however this phenotype was fully restored to WT levels by the exogenous addition of GSH (20 mM). These observations indicated that glutathione biosynthesis is absolutely required for the activation of the virulence genes in LBMM. Similarly, the transcription of virulence genes in $\Delta tcyK$ was restored by the addition of GSH; $\Delta tcyK$ bacteria supplemented with 20mM of GSH exhibited a luminescence profile that was similar to that of WT bacteria, supporting the premise that TcyKLMN is involved in glutathione biosynthesis (**Figure 5B**). To directly examine this hypothesis, we measured the total concentration of glutathione (including reduced and oxidized forms, GSH + GSSG) in $\Delta tcyK$ bacteria in comparison to WT *Lm*, using $\Delta gshF$ as a control. Remarkably, the internal glutathione concentration of $\Delta tcyK$ was as low as that measured in $\Delta gshF$, below the detection level of the glutathione assay kit (**Figure 5C**), indicating that TcyKLMN plays a role in glutathione biosynthesis, most likely by supplying the rate-limiting precursor, cysteine.

In light of these findings, we next examined whether the enhanced expression of the virulence genes in $\Delta cysK$ and $\Delta cymR$ is a result of increased production of glutathione, as these strains highly express TcyKLMN, and hence presumably import more cysteine for glutathione synthesis. Notably, internal measurements of glutathione in $\Delta cysK$ and $\Delta cymR$ bacteria demonstrated a 2-fold increase in the amount of glutathione in comparison to WT bacteria, supporting the hypothesis that higher cysteine uptake can increase glutathione biosynthesis (Figure 5C). Moreover, these observations demonstrated that cysteine import by TcyKLMN feeds into glutathione biosynthesis and further confirmed that cysteine is indeed a rate-limiting substrate of GshF. To evaluate whether the enhanced virulence gene expression in $\Delta cysK$ and $\Delta cymR$ relies on glutathione biosynthesis, we combined these mutants with a deletion of *gshF* (generating the double deletion mutants: $\Delta cysK/\Delta gshF$ and $\Delta cymR/\Delta gshF$), and examined virulence gene expression in LBMM, using the *lux* reporter system. As shown in Figure 5D, these mutants failed to express the *lux* genes, confirming that glutathione biosynthesis is absolutely required for virulence gene expression. Of note, these mutants, as well as $\Delta gshF$, grew like WT bacteria both in BHI and LBMM, overall demonstrating that glutathione itself is not essential for growth under these conditions (Figure 5A, D and Figure S3 H-J). Finally, we examined whether CymR, CysK and CodY regulate the transcription of *gshF* in BHI and LBMM, and found that they do not (Figure S6), supporting the conclusion that they affect glutathione biosynthesis by regulating the import of cysteine via TcyKLMN. We also examined the possibility that TcyKLMN imports glutathione, and found that it does not, as evident from ITC experiments using TcyK, and growth experiments using glutathione as a source of cysteine (Figure S7 A-B). Of note, these experiments clearly demonstrated that *Lm* can use exogenous GSH as a sole source of cysteine (Figure S7B). Altogether, these findings demonstrated that when grown in LBMM, TcyKLMN is the main transport system that supplies cysteine for glutathione biosynthesis, which, in turn, drives the expression of the virulence genes via the activation of PrfA.

TcyKLMN contributes to the induction of virulence genes during infection of macrophage cells

Finally, given the identified role of TcyKLMN in glutathione biosynthesis, we sought to investigate its impact on *Lm* intracellular growth and virulence gene expression in macrophage cells. We first analyzed the transcription level of *tcyK*, *tcyN* and *ytII* in WT bacteria grown intracellularly in macrophage cells in comparison to bacteria grown in BHI. The data indicated a modest induction of TcyKLMN and its activator YtII within the intracellular niche (~3-fold) (**Figure 6A**). We next evaluated the expression of the virulence gene *plcA* in Δ *tcyK* and WT bacteria grown intracellularly in macrophage cells, using Δ *gshF* as a control. For this purpose, we used a fluorescence-based reporter system that we previously constructed, which relies on the expression of three consecutive *yfp* genes under the regulation of the *plcA* promoter (cloned in the integrative plasmid pPL2, pPL2-P*plcA*-3*yfp*) (33). Using this system, we observed a ~40% reduction in the expression of *plcA* in Δ *tcyK* in comparison to WT bacteria, whereas a more dramatic reduction of ~ 75% was observed in Δ *gshF* (**Figure 6B**). While these findings demonstrate that TcyKLMN contributes to the induction of the virulence genes during macrophage cell infection, they implied that it is not the sole supplier of cysteine within the intracellular niche, corroborating previous reports demonstrating that *Lm* also utilizes oligopeptides as a source of amino acids, including cysteine, which are imported by the Opp transporter (20, 41). We next examined the intracellular growth of Δ *tcyK* and Δ *gshF* in macrophage cells in comparison to WT bacteria. Notably, the data demonstrated a late growth defect for Δ *tcyK* that was apparent at 6 h post-infection (**Figure 6C**). Interestingly, a similar phenotype was reported for the Opp transporter deletion mutant (Δ *oppDF*), which also exhibited an intracellular growth defect at 6 to 7 h post-infection of macrophage cells (20). Evaluating Δ *gshF* intracellular growth in macrophage cells, we observed a more profound growth defect, starting at 3 h post-infection, indicating that glutathione biosynthesis by *Lm* is critical for intracellular growth, and hence has to be accompanied by cysteine uptake (**Figure 6D**). Taken together, the results presented here support the premise that *Lm* uses multiple transport systems to acquire cysteine in the intracellular niche, among them TcyKLMN.

Discussion

This study is a direct continuation of our previous reports demonstrating that low availability of BCAA is sensed by *Lm* as a signal to activate the expression of virulence genes. While we established that the metabolic regulator CodY is responsible for the sensing of this signal, we further determined that it directly enhances the transcription of PrfA, thereby promoting virulence gene expression. That said, at the time these studies were conducted, we did not know that glutathione is also involved, through its binding to PrfA and allosterically enhancing its activity (28, 42). Here, we show that the induction of the virulence genes under low BCAA conditions is entirely dependent on *Lm*'s *de novo* synthesis of glutathione, mediated by GshF. Further, we demonstrate that glutathione biosynthesis under this condition requires the active import of cysteine. As indicated, *Lm* is auxotrophic to cysteine and methionine and hence has to import these amino acids from the environment. To date, two transport systems have been linked to the acquisition of cysteine in *Lm* (as a free amino acid or in peptides), yet their role in supporting *Lm* intracellular growth was not robust, implying that other systems may be involved. Interestingly, studying the regulation of *Lm* virulence gene expression under low BCAA (i.e., in LBMM) led to the discovery of TcyKLMN as a cystine/cysteine importer that facilitates glutathione biosynthesis and PrfA activation. The results demonstrated that TcyKLMN is repressed under nutrient-rich conditions, and up-regulated when BCAA are limited. This repression was linked to CodY, which we found to repress the activator of the *ytmI* operon, YtII. As indicated, low BCAA is the same condition at which PrfA expression is up-regulated by CodY; therefore, it is possible that the parallel increase in cysteine uptake, and subsequent up-regulation of glutathione biosynthesis, may have evolved to cope with the heightened PrfA levels. Altogether, the findings presented here reveal an intriguing synchronization between PrfA expression and glutathione biosynthesis that is mediated by CodY, overall indicating that CodY regulation of *Lm* virulence is even more complex than previously considered.

An important observation made in this study was that glutathione levels are largely limited within the bacteria, and that the availability of cysteine determines its biosynthesis. In line with this conclusion, all the factors that were found to affect cysteine import, i.e., CodY, CymR, CysK and TcyKLMN, indirectly influenced the

activation of PrfA. In a way, these findings uncover a new pathway by which *Lm* 405
virulence can be regulated in the mammalian host responding to changes in cysteine 406
availability. CodY, CymR and CysK were also found to regulate the expression of 407
TcyKLMN, and hence to modulate the expression of the virulence genes. These 408
regulators still repressed TcyKLMN under conditions of low concentrations of BCAA 409
and cysteine, implying that other metabolic or environmental signals may be involved. 410
In *B. subtilis*, it was shown that the *ytmI* operon is differentially regulated under 411
disulfide stress and changes in sulfur availability, hence it is possible that the *Lm* 412
CymR and CysK respond to these conditions (36, 38). Moreover, since glutathione 413
plays a role in redox conditions, it is possible that CymR and CysK respond to these 414
conditions as well. In this respect, it was demonstrated in *Staphylococcus aureus* that 415
CymR senses oxidative stress via thiolation of its cysteine residue (Cys-25), and 416
hence the *YtmI* operon is up-regulated under oxidizing environments (43). Taken 417
together, these reports indicate that the *YtmI* operon is regulated by multiple 418
metabolic and environmental cues. Interestingly, the *ytmI* operon of *B. subtilis* is 419
independent of CodY regulation, as evident from RNA-seq experiments and genome- 420
wide analyses of CodY binding sites (44, 45). It is tempting to speculate that CodY 421
regulation of *Lm*'s TcyKLMN evolved to cope with the pathogenic lifestyle of this 422
bacterium, yet *Lm* and *B. subtilis* differ greatly in their sulfur, cysteine and methionine 423
metabolism, which can lead to differences in the regulation of these pathways. 424
Notwithstanding, this study revealed an intriguing link between CodY regulation, 425
cysteine import and glutathione biosynthesis in *Lm*, portraying additional mechanism 426
to control the expression of the virulence genes. 427

Here, we showed that the SBP of TcyKLMN (i.e., TcyK) specifically binds cysteine, 428
and to a better extent, CSSC, suggesting that it primes the import of these two related 429
substrates. CSSC is the oxidized form of cysteine, containing two cysteine molecules 430
that are linked via a disulfide bond, and hence is expected to be more prevalent in 431
oxidizing environments, such as within phagosomes. Outside the mammalian cells 432
(i.e., in the extracellular milieu), cysteine is considered to be abundant (produced and 433
secreted by the liver), yet it is quickly oxidized and imported into the cells via 434
specialized CSSC transporters, e.g. the CSSC/glutamate antiporter, cXT, which also 435
plays a role in glutathione synthesis (46–49). Interestingly, within the cells, 436
thioredoxin or glutathione are used to reduce the CSSC to cysteine, which is further 437
utilized in protein synthesis. Of note, mammalian cells can further biosynthesize 438

cysteine from methionine, using the transsulfuration pathway, or alternatively break
down glutathione to salvage cysteine (46). In respect to *Lm*'s intracellular lifestyle, it
is likely that it encounters CSSC within the phagosome/vacuole environment. It is
possible that TcyKLMN plays a role during cell invasion or during spread from cell to
cell (i.e., in the primary and secondary vacuoles, respectively), or when *Lm* switches
to persistent infection, i.e., when it resides in lysosome-like vacuoles for long periods
(50, 51). While we did not identify a phenotype for Δ tcyK upon mice infections (data
not shown), we observed a late intracellular growth defect during infection of bone
marrow-derived macrophage cells. These experiments further demonstrated a reduced
transcription level of *plcA* in Δ tcyK, supporting the premise that TcyKLMN
contributes to the activation of PrfA during *Lm* infection of mammalian cells. Since
CSSC and cysteine are relatively limited in the intracellular environment, it is not
surprising that *Lm* acquired multiple means to scavenge this essential amino acid. As
mentioned, *Lm* exploits peptides as a source of amino acids, and the Opp transporter
was shown to import cysteine-containing peptides as a source of cysteine for
glutathione biosynthesis (20, 41). The finding that Δ tcyK exhibits a 40% reduction in
plcA transcription, as compared to the 75% reduction in Δ gshF, supports the premise
that *Lm* exploits multiple systems to scavenge cysteine.

Reniere et al. suggested that *Lm* also exploits host glutathione (GSH) to activate PrfA
(17). However, this mode of PrfA activation appeared to be minor, as Δ gshF bacteria
hardly activated the virulence genes in the intracellular environment (17), overall
indicating that host-derived GSH cannot replace the glutathione produced by *Lm*. In
this regard, it remains an open question why *Lm* synthesizes GSH and uses it as an
intracellular signal, considering its high abundance in the intracellular niche, much
more than cysteine. Other bacterial pathogens, such as *Burkholderia pseudomallei* and
Francisella tularensis were shown to exploit host GSH as an activating signal of
virulence gene expression and as a source of cysteine, respectively (52, 53).
Intriguingly, *in vitro* experiments in minimal defined medium indicated that *Lm* is
capable of importing GSH and uses it both to activate PrfA, and as a source of
cysteine (**Figure 5 and S7B**) (54). While these findings imply that *Lm* encodes a
glutathione transporter, or, alternatively, uses a nonspecific system such as di/tri-
peptide transporters to import glutathione, it is not clear why it does not use them in
the intracellular niche. Despite its importance, a dedicated glutathione transporter was
not identified in Gram-positive bacteria. In Gram-negative bacteria the dipeptide

transporter DppBCDF was shown to import glutathione, using the SBP protein GbpA (55). It is of particular interest to decipher the structural and molecular mechanism by which glutathione is imported in Gram-positive bacteria, especially intracellular pathogens like *Lm*. Intriguingly, in the Gram-positive bacterium *Streptococcus mutans*, it was shown that the CSSC ABC-transporter, TcyBC (not found in *Lm*), imports both CSSC and glutathione using two distinct SPB proteins (55). The SBP that binds glutathione (GshT) was found to be encoded elsewhere on the bacterial chromosome, and not near the *tcyBC* genes. While this phenomenon of a shared permease is not new, the discovery that *S. mutans* holds a distinct SBP that primes glutathione import via another transporter was new, providing early insights into glutathione import in Gram-positive bacteria (56). Interestingly, we found that TcyK shares 30% amino-acid sequence identity with the *S. mutans* GshT, yet our data indicated that it does not bind glutathione.

In summary, this study demonstrated that cysteine import is critical for virulence gene expression in *Lm*. The data imply that multiple metabolic and environmental cues are involved in the regulation of cysteine import and hence affect glutathione biosynthesis, placing this function at the heart of *Lm* patho-metabolism. As different bacterial pathogens acquired different mechanisms to sense the mammalian niche, it is interesting to learn how they exploit host and bacterial metabolites as signals and effectors of virulence.

Materials and Methods	496
	497
Ethics statement	498
Experimental protocols were approved by the Tel Aviv University Animal Care and	499
Use Committee (01-15-052, 04-13-039) and were in accordance with the Israel	500
Welfare Law (1994) and the National Research Council guide (Guide for the Care and	501
Use of Laboratory Animals 2010).	502
Bacterial strains, plasmids and primers	503
<i>Listeria monocytogenes</i> (<i>Lm</i>) strain 10403S was used as the WT strain and as the	504
parental strain to generate allelic exchange mutant strains (Table S1). <i>E. coli</i> XL-1	505
blue strain (Stratagene) was used to generate vectors and <i>E. coli</i> SM-10 strain (57)	506
was used for plasmid conjugation to <i>Lm</i> . Plasmids and primers used in this study are	507
listed in Table S1 and Table S2 , respectively.	508
	509
Growth conditions	510
<i>Lm</i> bacteria were grown at 37°C, with agitation, in brain heart infusion (BHI), as a	511
nutrient-rich medium, or in minimal defined medium (MM). MM (phosphate buffer	512
48.2 mM KH ₂ PO ₄ and 1.12 M Na ₂ HPO ₄ , pH 7, 0.41 mg/ml magnesium sulfate, 10	513
mg/ml glucose, 100 µg/ml of each amino acid (methionine, arginine, histidine,	514
tryptophan, phenylalanine, cysteine, isoleucine, leucine and valine), 600 µg/ml	515
glutamine, 0.5 mg/ml biotin, 0.5 mg/ml riboflavin, 20 mg/ml ferric citrate, 1 mg/ml	516
para-aminobenzoic acid, 5 ng/ml lipoic acid, 2.5 mg/ml adenine, 1 mg/ml thiamine, 1	517
mg/ml pyridoxal, 1 mg/ml calcium pantothenate and 1 mg/ml nicotinamine) was	518
prepared as described previously (58). For analysis of auxotrophies, <i>Lm</i> was grown	519
with 0-2000 µg/ml of either cysteine or methionine or neither, as indicated. For	520
growth under low BCAA, MM was freshly made with 10-fold less isoleucine, leucine	521
and valine (resulting in a final concentration of 10 µg/ml) was named low-BCAA	522
minimal defined medium (LBMM). For growth under limited concentrations of	523
cysteine, MM was freshly made with 10-fold less cysteine (resulting in a final	524
concentration of 10 µg/ml). For growth in glutathione medium, 20 mM reduced	525
glutathione (GSH) were freshly added to LBMM. For growth with cysteine or with	526

either cystine (CSSC), reduced or oxidized glutathione (GSH or GSSG, respectively) 527
as a cysteine source, MM was prepared without cysteine and 0-2 mM of the respective 528
cysteine source was added to the medium. 529

Lux reporter assay

Overnight BHI cultures harboring a *Phly-lux* luciferase reporter system (pPL2-*Phly-luxABCDE*) (59) were washed 3 times with PBS, adjusted to OD₆₀₀ = 0.03 in fresh 532
LBMM or GSH medium, and grown in a 96-well plate. Luminescence and bacterial 533
growth (OD₆₀₀) were measured every 15 min after shaking for 1 min, using a Synergy 534
HT BioTek plate reader at 37°C for 12-48 h. 535

Bacterial RNA extraction and qRT-PCR

Bacteria grown in the indicated medium were harvested at mid-logarithmic growth 539
(OD₆₀₀ of ~0.3). Total RNA was extracted from bacteria using the *RNAsnap* method 540
(60). Briefly, bacterial pellets were washed with AE buffer (50 mM NaOAc pH 5.2, 541
10 mM EDTA) and then resuspended in 95% formamide, 18 mM EDTA, 1% 2- 542
mercaptoethanol and 0.025% SDS. Bacterial lysis was performed by vortexing 543
extracts with 100 µm zirconia beads (OPS Diagnostics), followed by incubation at 544
95°C. Nucleic acids were precipitated with ethanol and treated with Turbo-DNase 545
(Ambion), followed by standard phenol extraction. Total RNA (1 µg) was reverse- 546
transcribed to cDNA using qScript (Quanta). qRT-PCR was performed on 10 ng 547
cDNA using FastStart Universal SYBR Green Master (Roche) in a StepOne Plus real- 548
time PCR system (Applied Biosystems). The transcription level of each gene was 549
normalized to that of the reference gene *rpoD*. 550

Analysis of promoters and putative binding sites

Putative binding sites of transcriptional regulators were identified using the MAST 553
search tool (61), using the CymR motif of *Bacillus* (38) and CodY motif of Gram- 554
positive bacteria (62). Promoters (-10 and -35) were identified using BPROM (63). 555

Transcription start sites (TSS) were either previously identified (64) or manually predicted based on the promoter sequence.

Intracellular growth in macrophage cells

Bone marrow-derived macrophages (BMDM) were used for *Lm* infection experiments. The cells were isolated from 8 week-old female C57BL/6 mice (Envigo, Israel) and cultured in BMDM medium (Dulbecco's modified Eagle medium (DMEM) supplemented with 20% fetal bovine serum, sodium pyruvate (1 mM), L-glutamine (2 mM), β -mercaptoethanol (0.05 mM) and monocyte-colony stimulating factor (M-CSF, L929-conditioned medium), as described previously (65)). BMDM cells (2×10^6) were seeded in a 60 mm Petri dish, on glass coverslips, in 5 ml BMDM medium and incubated overnight (O.N.) in a 37 °C, 5% CO₂ forced-air incubator. *Lm* bacteria (8×10^6) grown O.N. at 30 °C without agitation, were used to infect BMDM cells (MOI of 1). Thirty minutes post-infection, macrophage monolayers were washed and fresh medium was added. Gentamicin was added 1 h post-infection to a final concentration of 5 μ g/ml in order to limit the growth of extracellular bacteria. At each time point, three coverslips were transferred to 2 ml sterile water to release the intracellular bacteria. Serial dilutions of the resulting lysate were plated on BHI agar plates and the CFUs were counted after 24 h incubation at 37 °C.

Intracellular *PplcA-3yfp* expression

WT and mutant strains expressing three consecutive YFP proteins under the regulation of the *plcA* gene promoter (cloned on the pPL2 integrative plasmid) were used to infect BMDM on 20 mm slides. Three hours post-infection, cells were fixed with 4% v/v paraformaldehyde-PBS and permeabilized with 0.05% v/v Triton X-100. DNA was stained with DAPI-containing Vectashield mounting medium (Vector laboratories inc.). Fluorescent images were captured using a Zeiss LSM 510-META confocal microscope.

Intracellular gene expression analysis

RNA was purified from WT *Lm* bacteria intracellularly grown in J774A.1 macrophage cells, as previously described for BMDM macrophages (66). Briefly, three 145 mm dishes were seeded with 2×10^7 cells that were then infected in parallel with 2×10^9 bacteria. Thirty minutes post-infection, cell monolayers were washed twice with PBS to remove unattached bacteria, and fresh medium was added. At 1 h post-infection (h.p.i.), gentamicin (50 μ g/ml) was added to limit extracellular bacterial growth. At 6 h.p.i., the macrophages were lysed with 20 mL cold water, and cell debris and nuclei were removed by centrifugation (800 xg, 3 min, 4°C). Released bacteria were quickly collected on 0.45 μ m filter membranes (Millipore) using a vacuum apparatus and snap-frozen in liquid nitrogen. Bacteria were recovered from the filters by vortexing the membranes in AE buffer (50 mM sodium acetate pH 5.2, 10 mM EDTA), and bacterial nucleic acids were extracted using the *RNAsnap* method (60), followed by ethanol precipitation. RNeasy Mini Kit DNase on column (QIAGEN) was used for DNase treatment.

Cloning, overexpression and purification of TcyK

Lm 10403S *tcyK*, encoding the TcyKLMN substrate binding protein, was synthesized and adjusted to the *E. coli* codon usage (Genescript). The gene was cloned into the pET-19b vector (Novagen) for expression with an N-terminal His-tag. His-tagged TcyK was overexpressed in *E. coli* BL21-Gold (DE3) (Stratagene) cultured in Terrific Broth (TB) and induced at mid-log phase by addition of 1 mM isopropyl b- D-1-thiogalactopyranoside (IPTG; 1.5 h, 37°C). Cells were harvested by centrifugation (13,600 $\times$ g, 20 min, 4°C) and the pellet was stored at -80°C until use. For purification, cells were homogenized in 50 mM Tris-HCl pH 8, 500 mM NaCl, complete EDTA-free protease inhibitor (Roche), 30 mg ml⁻¹ DNase (Worthington), and 1 mM MgCl₂. The cells were then ruptured by three passages through an EmulsiFlex-C3 homogenizer (Avestin), and the lysate was centrifuged at 34,500 $\times$ g for 30 min, at 4°C. The supernatant was loaded onto a nickel affinity column (HisTrap HP, GE Healthcare) on an AKTA Avant instrument. The protein was eluted using an imidazole gradient, and imidazole was removed from the sample by desalting (HiPrep 26/10, GE Healthcare). Protein purification was monitored by Coomassie staining of SDS-PAGE and size exclusion chromatography (Superdex 75 10/300 GL, GE Healthcare).

Isothermal titration calorimetry experiments

Calorimetric measurements were performed with the Microcal iTC200 System (GE Healthcare). Prior to measurements, purified TcyK was dialyzed against three exchanges of 50 mM Tris-HCl pH 8, 500 mM NaCl buffer. Stocks of cysteine, CSSC, GSSG, GSH or control amino acid (Gln, His, Glu, Thr and Ser) were prepared fresh in double-distilled water and diluted to a working concentration using the buffer from the last protein dialysis exchange. 1 mM Tris(2-carboxyethyl)phosphine (TCEP) was added to cysteine and GSH stocks as a reductant agent. Aliquots (2 μ L) of 500 μ M stocks were added by a rotating syringe to the reaction well containing 200 μ L of 50 μ M purified TcyK at 25°C. Data-fitting was performed with the Microcal analysis software.

Glutathione quantification

Total glutathione (GSH+GSSG) concentration in bacteria was measured as described previously (54). Briefly, bacteria were grown to mid-log phase in LBMM, resuspended in PBS containing 1 mM EDTA, pH 6.5, and lysed by vortexing with 100 μ m zirconia beads (OPS Diagnostics). Cold (4°C) lysates were deproteinated with an equal volume of metaphosphoric acid and quantified with a commercial kit (Cayman Chemical), according to the recommendations of the kit manufacturer.

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Figure Legends

Fig 1. The TcyKLMN transporter and the regulatory factors CysK and CymR play a role in the regulation of *Lm* virulence gene expression.

(A) A schematic representation of the *ytmI* operon and the genes identified in Friedman *et al.* 2017. Locations of transposon insertions are marked with a triangle. (B-F) Luminescence and OD 600nm measurements of bacteria grown in minimal defined media supplemented with low concentrations of branched chain amino acids (LBMM). Relative Luminescence Units (RLU) represent luminescence values normalized to the respective OD 600nm value. OD 600nm values represent growth. The data represent 3 biological replicates. Error bars indicate standard deviation. (G) qRT-PCR analysis of *actA*, *plcA* and *hly* transcription levels in indicated bacteria grown in LBMM. mRNA levels were normalized to *rpoD* mRNA and are represented as relative quantity (RQ), relative to mRNA level in WT bacteria. The data represent 3 biological replicates. Error bars indicate standard deviation. Asterisks represent P-values (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, n.s. = non- significant) calculated by Student's t-test. P-values represent a comparison to the BHI sample.

Fig 2. TcyKLMN is a cystine/cysteine transporter.

(A) Isothermal Titration Calorimetry (ITC) analysis, showing binding of TcyK to cystine (CSSC) or cysteine (Cys). Shown are the consecutive injections of 2 μ L aliquots from a 200 μ M Cys/CSSC solution into 200 μ L of 20 μ M TcyK. TCEP was used as a reducing agent when indicated. The upper panels show the calorimetric titration and the lower panels display the integrated injection heat derived from the titrations, for which the best-fit curve was used to calculate the K_D . The experiments were repeated independently 3 times, and the K_D value is presented as a mean $\pm$ standard deviation of these 3 experiments. (B) Growth of WT *Lm* and Δ *tcyK* bacteria grown in minimal defined media supplemented with different concentrations of cysteine (Cys) or cystine (CSSC) as a sole cysteine source. The data is presented as maximal OD 600nm values, corresponds to bacterial yield. The data represent 2 biological replicates. The experiment was repeated independently twice, with a total of 4 biological replicates. Error bars indicate standard deviation.

Fig 3. The TcyKLMN transporter is negatively regulated by CodY, CysK and CymR. (A) A schematic representation of the *ytmI-ytII* regulatory region. Putative CymR binding-sites were identified using the motif of *Bacillus*. Putative CodY binding sites were identified using the motif of gram-positive bacteria. Promoters (-10 and -35) were identified using BPRM. TSS denotes the transcription start sites, either identified by Wurtzel *et al.*, 2012, or predicted based on the promoter sequence. (B) qRT-PCR analysis of *tcyK*, *tcyN* and *ytII* transcription level in WT bacteria grown in rich media (BHI) and in LBMM. mRNA levels were normalized to *rpoD* mRNA and are represented as relative quantity (RQ), relative to mRNA level in WT bacteria grown in BHI. The data represent 3 biological replicates. Error bars indicate standard deviation. Asterisks represent P-values (* = P<0.05, ** = P<0.01, *** = P<0.001, n.s. = non- significant) calculated by Student's t-test. P-values represent a comparison to the BHI sample. (C-E) qRT-PCR analysis of *tcyK* and *ytII* transcription level in the indicated mutants grown in rich media (BHI) and minimal defined media supplemented with low concentration of either branched chain amino acids (LBMM) or cysteine (LCMM). mRNA levels were normalized to *rpoD* mRNA and are represented as relative quantity (RQ), relative to mRNA level in WT bacteria grown in the indicated medium. The data represent 3 biological replicates. Error bars indicate standard deviation. Asterisks represent P-values (* = P<0.05, ** = P<0.01, *** = P<0.001, n.s. = non- significant) calculated by Student's t-test. P-values represent a comparison to the respective WT sample, unless indicated otherwise.

Fig 4. Enhanced expression of virulence genes in Δ cymR and Δ cysK is TcyKLMN dependent. Luminescence of pPL2 *Phly-lux* reporter system (left) and growth (right) of Δ cysK/ Δ tcyK and Δ cymR/ Δ tcyK bacteria grown in minimal defined media supplemented with low concentration of branched chain amino acids (LBMM). Relative Luminescence Units (RLU) represent luminescence values normalized to the respective OD 600nm value. OD 600nm values represent growth. The data represent 3 biological replicates. Error bars indicate standard deviation.

Fig 5. The TcyKLMN transporter plays a role in glutathione biosynthesis. (A-B) Luminescence of pPL2 *Phly-lux* reporter system (left) and growth (right) of Δ gshF and Δ tcyK bacteria grown in minimal defined media with low concentration of branched chain amino acids (LBMM). 20 mM reduced glutathione was supplemented

to LBMM when indicated. Relative Luminescence Units (RLU) represent luminescence values normalized to the respective OD 600nm value. OD 600nm values represent growth. The data represent 3 biological replicates. Error bars indicate standard deviation. (C) Internal glutathione level during growth in LBMM in indicated bacteria. The data represent 3 biological replicates. Error bars indicate standard deviation. Asterisks represent P-values (***) = $P < 0.001$) calculated by Student's t-test. P-values represent a comparison to the WT sample. (D) Luminescence of pPL2 *Phly-lux* reporter system (left) and growth (right) of $\Delta cysK/\Delta gshF$ and $\Delta cymR/\Delta gshF$ bacteria grown in LBMM. Relative Luminescence Units (RLU) represent luminescence values normalized to the respective OD 600nm value. OD 600nm values represent growth. The data represent 3 biological replicates. Error bars indicate standard deviation.

Fig 6. The TcyKLMN transporter promotes *Lm* intracellular growth in macrophage cells. (A) qRT-PCR analysis of *tcyK*, *tcyN* and *ytII* transcription level in WT bacteria grown in rich media (BHI) or intracellularly in J774A.1 macrophage cell line (IC). mRNA levels were normalized to *rpoD* mRNA and are represented as relative quantity (RQ), relative to mRNA level in in the BHI sample. The data represent 3 biological replicates. Error bars indicate standard deviation. Asterisks represent P-values (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, n.s. = non- significant) calculated by Student's t-test. P-values represent a comparison to the BHI sample. (B) Intracellular expression of *plcA* as indicated by the fluorescence of YFP proteins expressed under the control of *plcA* promoter (pPL2-*PplcA*-3yfp) in WT *Lm* and indicated mutants, grown in bone marrow derived macrophages (BMDM). Fluorescence was measured 3 hours post infection using a florescence microscope. The data represent at least 3 biological replicates. Error bars indicate standard deviation. Asterisks represent P-values (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, n.s. = non- significant) calculated by Student's t-test. P-values represent a comparison to the WT sample. (C-D) Intracellular growth of $\Delta tcyK$ and $\Delta gshF$ mutants in BMDM cells in comparison to WT *Lm*. The experiment was repeated 3 times independently. Since we could not average the data, a representative result is shown here, and two additional biological repeats are shown in **Figure S8**. Error bars indicate standard deviation. h.p.i. = hours post infection.

Supplementary Figure Legends

Fig S1. Cysteine uptake and metabolic genes identified in a screen by Friedman

et al. (A) Phenotypes of transposon mutants as identified in Friedman *et al.*, 2017. WT bacteria and indicated transposon mutants were grown in minimal defined medium supplemented with low concentration of branched chain amino acids (LBMM). Relative Luminescence Units (RLU) represent luminescence values normalized to the respective OD 600nm value. (B) Schematic representation of cysteine transport via TcyKLMN and cysteine-glutathione biosynthesis. The genes identified in the genetic screen are marked in red. OAS=O-acetylserine.

Fig S2. *Lm* strain 10403S is auxotrophic to cysteine and methionine. (A-B)

Growth of WT *Lm* strain 10403S in minimal defined media supplemented with different concentrations of either cysteine or methionine. The data is presented as maximal OD 600nm values, corresponding to bacterial yield. The data represent 3 biological replicates. Error bars indicate standard deviation. (C) Growth of WT *Lm* in minimal defined media without cysteine supplemented either with 1000 µg/ml, 2000 µg/ml or no methionine. Growth of WT *Lm* in minimal defined media supplemented with standard concentrations of cysteine (100 µg/ml) is shown as a control. OD 600 nm values represent growth. The data represent 3 biological replicates. Error bars indicate standard deviation. (D) Growth of WT *Lm* in minimal defined medium without methionine, supplemented with either 1000 µg/ml, 2000 µg/ml or no cysteine. Growth of WT *Lm* in minimal defined media supplemented with standard concentrations of methionine (100 µg/ml) is shown as a control. OD 600nm values represent growth. The data represent 3 biological replicates. Error bars indicate standard deviation.

Fig S3. Growth of deletion mutants generated in this study in the rich medium

BHI. WT bacteria and indicated strains (A-J) were grown in BHI. OD 600nm values represent growth. The data represent 3 biological replicates. Error bars indicate standard deviation.

Fig S4. Complementation experiments of *ΔtcyK* and *ΔcysK* mutants. qRT-PCR analysis of *hly* transcription level in WT *Lm*, *ΔtcyK* and *ΔcysK* mutants and their respective complementation strains harboring a copy of *tcyK* or *cysK* gene under the *tet* promoter on pPL2 plasmid, grown in LBMM. mRNA levels were normalized to *rpoD* mRNA and are represented as relative quantity (RQ), relative to mRNA level in WT bacteria. The data represent at least 2 biological replicates. Error bars indicate standard deviation. Asterisks represent P-values (* = P<0.05, ** = P<0.01, *** = P<0.001, n.s. = non- significant) calculated by Student's t-test. P-values represent a comparison to the WT sample unless indicated otherwise.

Fig S5. Purification of a short variant of TcyK. (A) Coomassie staining of SDS-PAGE of Ni-NTA affinity purification of a His-tagged short TcyK (amino acids 38-286). Lane 1: molecular weight marker (in kDa), Lane 2: total protein extract, Lane 3: column-unbound fraction, Lane 4: wash with 60 mM of imidazole, Lanes 5-11: fractions eluted using a linear gradient of 60 to 250 mM imidazole. (B) Analysis of the purified protein by size exclusion chromatography. 80 μl of a 0.8 mg/ml protein solution were injected on a Superdex 75 10/300 GL column (GE Healthcare).

Fig S6. *gshF* is not transcriptionally regulated by CymR, CysK and CodY. qRT-PCR analysis of *gshF* transcription level in the indicated mutants grown in rich media (BHI) and minimal defined media supplemented with low concentration of either branched chain amino acids (LBMM). mRNA levels were normalized to *rpoD* mRNA and are represented as relative quantity (RQ), relative to mRNA level in WT bacteria grown in the indicated medium. The data represent at least 2 biological replicates. Error bars indicate standard deviation Asterisks represent P-values (* = P<0.05, ** = P<0.01, *** = P<0.001, n.s. = non- significant) calculated by Student's t-test. P-values represent a comparison to the respective WT sample.

Fig S7. The TcyKLMN transporter does not transport glutathione. (A) Isothermal Titration Calorimetry (ITC) analysis, showing no binding of TcyK to reduced glutathione (GSH). Shown are the consecutive injections of 2 μL aliquots from a 200 μM GSH solution into 200 μL of 20 μM TcyK. TCEP was used as a reducing agent. The upper panels show the calorimetric titration and the lower panels display the

integrated injection heat derived from the titrations. The experiment was repeated 818
independently 3 times. **(B)** Growth of WT *Lm* and *AtcyK* bacteria in minimal defined 819
media supplemented with different concentrations of reduced glutathione (GSH) as a 820
sole source of cysteine. The data is presented as maximal OD600 values, 821
corresponding to bacterial yield. The data represent 2 biological replicates. Error bars 822
indicate standard deviation. 823

Fig S8. Intracellular growth of *AtcyK* and *AgshF* mutants in BMDM cells in 825
comparison to WT *Lm*. The experiment was repeated independently 3 times, two 826
biological repeats for each mutant are shown here, supplementary to **Figure 6 C-D.** 827

References	840
	841
1. Swaminathan B, Gerner-Smidt P. 2007. The epidemiology of human listeriosis. <i>Microbes Infect</i> 9:1236–43.	842 843
2. Freitag NE, Port GC, Miner MD. 2009. <i>Listeria monocytogenes</i> — from saprophyte to intracellular pathogen. <i>Nat Rev Microbiol</i> 72:623–628.	844 845
3. Lecuit M, Cossart P, Pizarro-cerda J. 2003. Invasion of mammalian cells by <i>Listeria monocytogenes</i> : functional mimicry to subvert cellular functions. <i>Trends Cell Biol</i> 13:23–31.	846 847 848
4. Cossart P, Vicente MF, Mengaud J, Baquero F, Perezdiaz JC, Berche P. 1989. Listeriolysin-O Is Essential for Virulence of <i>Listeria-Monocytogenes</i> - Direct Evidence Obtained By Gene Complementation. <i>Infect Immun</i> 57:3629–3636.	849 850 851
5. Smith G a., Marquis H, Jones S, Johnston NC, Portnoy D a., Goldfine H. 1995. The two distinct phospholipases C of <i>Listeria monocytogenes</i> have overlapping roles in escape from a vacuole and cell-to-cell spread. <i>Infect Immun</i> 63:4231–4237.	852 853 854
6. Marquis H, Doshi V, Portnoy DA. 1995. The broad-range phospholipase C and a metalloprotease mediate listeriolysin O-independent escape of <i>Listeria monocytogenes</i> from a primary vacuole in human epithelial cells. <i>Infect Immun</i> 63:4531–4534.	855 856 857
7. Grubmüller S, Schauer K, Goebel W, Fuchs TM, Eisenreich W. 2014. Analysis of carbon substrates used by <i>Listeria monocytogenes</i> during growth in J774A.1 macrophages suggests a bipartite intracellular metabolism. <i>Front Cell Infect Microbiol</i> 4:156.	858 859 860 861
8. Chico-Calero I, Suárez M, González-Zorn B, Scortti M, Slaghuis J, Goebel W, Vázquez-Boland JA. 2002. Hpt, a bacterial homolog of the microsomal glucose- 6-phosphate translocase, mediates rapid intracellular proliferation in <i>Listeria</i> . <i>Proc Natl Acad Sci</i> 99:431–436.	862 863 864 865
9. Sauer J-D, Herskovits AA, O’Riordan MXD. 2019. Metabolism of the Gram-Positive Bacterial Pathogen <i>Listeria monocytogenes</i> . <i>Microbiol Spectr</i> 7.	866 867
10. Keeney K, Colosi L, Weber W, O’Riordan M. 2009. Generation of Branched-Chain Fatty Acids through Lipoate-Dependent Metabolism Facilitates Intracellular Growth of <i>Listeria monocytogenes</i> . <i>J Bacteriol</i> 191:2187–2196.	868 869 870
11. Tilney LG, Portnoy DA. 1989. Actin Filaments and the Growth, Movement, and Spread of the Intacellular Bacterial Parsite, <i>Listeria monocytogenes</i> . <i>JCell Biol</i> 109:1597–1608.	871 872 873
12. Kocks C, Gouin E, Tabouret M, Berche P, Ohayon H, Cossart P. 1992. L. monocytogenes-induced actin assembly requires the actA gene product, a surface protein. <i>Cell</i> 68:521–531.	874 875 876
13. de las Heras A, Cain RJ, Bielecka MK, Vázquez-Boland J a. 2011. Regulation of <i>Listeria</i> virulence: PrfA master and commander. <i>Curr Opin Microbiol</i> 14:118–27.	877 878
14. Radoshevich L, Cossart P. 2017. <i>Listeria monocytogenes</i> : towards a complete picture of its physiology and pathogenesis. <i>Nat Rev Microbiol</i> 2017 161 16:32–46.	879 880
15. Lobel L, Sigal N, Borovok I, Belitsky BR, Sonenshein AL, Herskovits AA. 2015. The	881

	metabolic regulator CodY links <i>Listeria monocytogenes</i> metabolism to virulence by directly activating the virulence regulatory gene <i>prfA</i> . <i>Mol Microbiol</i> 95:624–644.	882 883
16.	Haber A, Friedman S, Lobel L, Burg-Golani T, Sigal N, Rose J, Livnat-Levanon N, Lewinson O, Herskovits AA. 2017. L-glutamine Induces Expression of <i>Listeria monocytogenes</i> Virulence Genes. <i>PLOS Pathog</i> 13:e1006161.	884 885 886
17.	Reniere ML, Whiteley AT, Hamilton KL, John SM, Lauer P, Brennan RG, Portnoy DA. 2015. Glutathione activates virulence gene expression of an intracellular pathogen. <i>Nature</i> 517:170–173.	887 888 889
18.	Johansson J, Mandin P, Renzoni a, Chiaruttini C, Springer M, Cossart P. 2002. An RNA thermosensor controls expression of virulence genes in <i>Listeria monocytogenes</i> . <i>Cell</i> 110:551–561.	890 891 892
19.	Ripio MT, Brehm K, Lara M, Suarez M, Vazquez-Boland JA. 1997. Glucose-1-phosphate utilization by <i>Listeria monocytogenes</i> is PrfA dependent and coordinately expressed with virulence factors. <i>J Bacteriol</i> 197/11/26. 179:7174–7180.	893 894 895
20.	Kryptou E, Scortti M, Grundström C, Oelker M, Luisi BF, Sauer-Eriksson AE, Vázquez-Boland J. 2019. Control of Bacterial Virulence through the Peptide Signature of the Habitat. <i>Cell Rep</i> 26:1815-1827.e5.	896 897 898
21.	Lobel L, Sigal N, Borovok I, Ruppin E, Herskovits AA. 2012. Integrative genomic analysis identifies isoleucine and CodY as regulators of <i>Listeria monocytogenes</i> virulence. <i>PLoS Genet</i> 8:e1002887.	899 900 901
22.	Lobel L, Herskovits A a. 2016. Regulatory Activities of CodY Controlling Metabolism , Motility and Virulence in <i>Listeria monocytogenes</i> . <i>PLOS Genet</i> 12:1–27.	902 903 904
23.	Levdikov VM, Blagova E, Joseph P, Sonenshein AL, Wilkinson AJ. 2006. The structure of CodY, a GTP- and isoleucine-responsive regulator of stationary phase and virulence in gram-positive bacteria. <i>J Biol Chem</i> 281:11366–73.	905 906 907
24.	Sonenshein AL. 2005. CodY, a global regulator of stationary phase and virulence in Gram-positive bacteria. <i>Curr Opin Microbiol</i> 8:203–7.	908 909
25.	Brenner M, Lobel L, Borovok I, Sigal N, Herskovits AA. 2018. Controlled branched-chain amino acids auxotrophy in <i>Listeria monocytogenes</i> allows isoleucine to serve as a host signal and virulence effector. <i>PLoS Genet</i> 14:1–21.	910 911 912
26.	Aoyama K, Nakaki T. 2015. Glutathione in Cellular Redox Homeostasis: Association with the Excitatory Amino Acid Carrier 1 (EAAC1). <i>Mol</i> 2015, Vol 20, Pages 8742–8758 20:8742–8758.	913 914 915
27.	Gopal S, Borovok I, Ofer A, Yanku M, Cohen G, Goebel W, Kreft J, Aharonowitz Y. 2005. A Multidomain Fusion Protein in <i>Listeria monocytogenes</i> Catalyzes the Two Primary Activities for Glutathione Biosynthesis. <i>J Bacteriol</i> 187:3839–3847.	916 917 918
28.	Hall M, Grundström C, Begum A, Lindberg MJ, Sauer UH, Almqvist F, Johansson J, Sauer-Eriksson AE. 2016. Structural basis for glutathione-mediated activation of the virulence regulatory protein PrfA in <i>Listeria</i> . <i>Proc Natl Acad Sci U S A</i> 6:201614028.	919 920 921
29.	Wang Y, Feng H, Zhu Y, Gao P. 2017. Structural insights into glutathione-mediated activation of the master regulator PrfA in <i>Listeria monocytogenes</i> . <i>Protein Cell</i> 8:308–312.	922 923 924

30.	Tsai HN, Hodgson DA. 2003. Development of a Synthetic Minimal Medium for <i>Listeria monocytogenes</i> . <i>Appl Environ Microbiol</i> 69:6943–6945.	925 926
31.	Xayarath B, Marquis H, Port GC, Freitag NE. 2009. <i>Listeria monocytogenes</i> CtaP is a multifunctional cysteine transport-associated protein required for bacterial pathogenesis. <i>Mol Microbiol</i> 74:956–973.	927 928 929
32.	Schauer K, Geginat G, Liang C, Goebel W, Dandekar T, Fuchs TM. 2010. Deciphering the intracellular metabolism of <i>Listeria monocytogenes</i> by mutant screening and modelling. <i>BMC Genomics</i> 11:573.	930 931 932
33.	Friedman S, Linsky M, Lobel L, Rabinovich L, Sigal N, Herskovits AA. 2017. Metabolic genetic screens reveal multidimensional regulation of virulence gene expression in <i>Listeria monocytogenes</i> , and an aminopeptidase that is critical for PrfA protein activation. <i>Infect Immun</i> IAI.00027-17.	933 934 935 936
34.	Burguière P, Auger S, Hullo M, Danchin A, Martin-verstraete I, Burguie P. 2004. Three Different Systems Participate in l-Cystine Uptake in <i>Bacillus subtilis</i> Three Different Systems Participate in L -Cystine Uptake in <i>Bacillus subtilis</i> . <i>J Bacteriol</i> 186:4875–4884.	937 938 939 940
35.	Tanous C, Soutourina O, Raynal B, Hullo MF, Mervelet P, Gilles AM, Noirot P, Danchin A, England P, Martin-Verstraete I. 2008. The CymR regulator in complex with the enzyme CysK controls cysteine metabolism in <i>Bacillus subtilis</i> . <i>J Biol Chem</i> 283:35551–35560.	941 942 943 944
36.	Burguière P, Fert J, Guillouard I, Danchin A, Martin-verstraete I, Burguie P, Auger S. 2005. Regulation of the <i>Bacillus subtilis</i> ytmI Operon, Involved in Sulfur Metabolism. <i>J Bacteriol</i> 187:6019–6030.	945 946 947
37.	CM C, A D, P M, A S. 2014. Paralogous metabolism: S-alkyl-cysteine degradation in <i>Bacillus subtilis</i> . <i>Environ Microbiol</i> 16:101–117.	948 949
38.	Even S, Burguière P, Auger S, Danchin A, Martin-verstraete I, Burguie P, Soutourina O. 2006. Global Control of Cysteine Metabolism by CymR in <i>Bacillus subtilis</i> . <i>J Bacteriol</i> 188:2184–2197.	950 951 952
39.	Rees DC, Johnson E, Lewinson O. 2009. ABC transporters: The power to change. <i>Nat Rev Mol Cell Biol</i> 10:218–227.	953 954
40.	Biswas R, Sonenshein AL, Belitsky BR. 2020. Role of GlnR in Controlling Expression of Nitrogen Metabolism Genes in <i>Listeria monocytogenes</i> . <i>J Bacteriol</i> 202:209–229.	955 956 957
41.	Marquis H, Bouwer HGA, Hinrichs DJ, Portnoy DA. 1993. Intracytoplasmic growth and virulence of <i>Listeria monocytogenes</i> auxotrophic mutants. <i>Infect Immun</i> 61:3756–3760.	958 959 960
42.	Reniere ML, Whiteley AT, Portnoy DA. 2016. An In Vivo Selection Identifies <i>Listeria monocytogenes</i> Genes Required to Sense the Intracellular Environment and Activate Virulence Factor Expression. <i>PLoS Pathog</i> 12:1–27.	961 962 963
43.	Ji Q, Zhang L, Sun F, Deng X, Liang H, Bae T, He C. 2012. <i>Staphylococcus aureus</i> CymR is a new thiol-based oxidation-sensing regulator of stress resistance and oxidative response. <i>J Biol Chem</i> 287:21102–21109.	964 965 966
44.	Virginie M, Nakaura Y, P SR, Yamaguchi H, Losick R, Fujita Y, Sonenshein AL. 2003. Additional targets of the <i>Bacillus subtilis</i> global regulator CodY identified by	967 968

	chromatin immunoprecipitation and genome-wide transcript analysis. <i>J Bacteriol</i> 185:1911–1922.	969 970
45.	Brinsmade SR, Alexander EL, Livny J, Stettner AI, Segrè D, Rhee KY, Sonenshein AL. 2014. Hierarchical expression of genes controlled by the <i>Bacillus subtilis</i> global regulatory protein CodY. <i>Proc Natl Acad Sci</i> 111:8227–8232.	971 972 973
46.	Combs JA, DeNicola GM. 2019. The Non-Essential Amino Acid Cysteine Becomes Essential for Tumor Proliferation and Survival. <i>Cancers (Basel)</i> 11.	974 975
47.	Sato H, Tamba M, Ishii T, Bannai S. 1999. Cloning and Expression of a Plasma Membrane Cystine/Glutamate Exchange Transporter Composed of Two Distinct Proteins *. <i>J Biol Chem</i> 274:11455–11458.	976 977 978
48.	Kang M, Eichhorn CD, Feigon J. 2014. Structural determinants for ligand capture by a class II preQ1 riboswitch. <i>Proc Natl Acad Sci U S A</i> 111:E663-71.	979 980
49.	Shih AY, Erb H, Sun X, Toda S, Kalivas PW, Murphy TH. 2006. Cystine/Glutamate Exchange Modulates Glutathione Supply for Neuroprotection from Oxidative Stress and Cell Proliferation. <i>J Neurosci</i> 26:10514–10523.	981 982 983
50.	Kortebi M, 1 M, Mitchell G, Choux CP, Prevost M-C, Cossart P, Bierne H. 2017. <i>Listeria monocytogenes</i> switches from dissemination to persistence by adopting a vacuolar lifestyle in epithelial cells https://doi.org/10.1371/journal.ppat.1006734 .	984 985 986
51.	Birmingham CL, Canadien V, Kaniuk NA, Steinberg BE, Higgins DE, Brumell JH. 2008. Listeriolysin O allows <i>Listeria monocytogenes</i> replication in macrophage vacuoles. <i>Nat</i> 2008 4517176 451:350–354.	987 988 989
52.	Wong J, Chen Y, Gan YH. 2015. Host cytosolic glutathione sensing by a membrane histidine kinase activates the type VI secretion system in an intracellular bacterium. <i>Cell Host Microbe</i> 18:38–48.	990 991 992
53.	Alkhuder K, Meibom KL, Dubail I, Dupuis M, Charbit A. 2009. Glutathione provides a source of cysteine essential for intracellular multiplication of <i>Francisella tularensis</i> . <i>PLoS Pathog</i> 5.	993 994 995
54.	Portman JL, Dubinsky SB, Peterson BN, Whiteley AT, Portnoy DA. 2017. Activation of the <i>Listeria monocytogenes</i> Virulence Program by a Reducing Environment. <i>MBio</i> 8:1–13.	996 997 998
55.	Vergauwen B, Elegheert J, Dansercoer A, Devreese B, Savvides SN. 2010. Glutathione import in <i>Haemophilus influenzae</i> Rd is primed by the periplasmic heme-binding protein HbpA. <i>Proc Natl Acad Sci</i> 107:13270–13275.	999 1000 1001
56.	Vergauwen B, Verstraete K, Senadheera DB, Dansercoer A, Cvitkovitch DG, Guédon E, Savvides SN. 2013. Molecular and structural basis of glutathione import in Gram-positive bacteria via GshT and the cystine ABC importer TcyBC of <i>Streptococcus mutans</i> . <i>Mol Microbiol</i> 89:288–303.	1002 1003 1004 1005
57.	Simon R, Prierer U, Pühler A. 1983. A Broad Host Range Mobilization System for In Vivo Genetic Engineering: Transposon Mutagenesis in Gram Negative Bacteria. <i>Nat Biotechnol</i> 1:784–791.	1006 1007 1008
58.	Phan-thanh L, Gormon T. 1997. A chemically defined minimal medium for the optimal culture of <i>Listeria</i> . <i>Int J Food Microbiol</i> 35:91–95.	1009 1010
59.	Bron P a, Monk IR, Corr SC, Hill C, Gahan CGM, Corr C. 2006. Novel Luciferase	1011

	Reporter System for In Vitro and Organ-Specific Monitoring of Differential Gene Expression in <i>Listeria monocytogenes</i> . <i>Appl Environ Microbiol</i> 72:2876–2884.	1012 1013
60.	Stead MB, Agrawal A, Bowden KE, Nasir R, Mohanty BK, Meagher RB, Kushner SR. 2012. RNAsnap TM : a rapid, quantitative and inexpensive, method for isolating total RNA from bacteria. <i>Nucleic Acids Res</i> 40:e156.	1014 1015 1016
61.	Bailey TL, Boden M, Buske FA, Frith M, Grant CE, Clementi L, Ren J, Li WW, Noble WS. 2009. MEME Suite: Tools for motif discovery and searching. <i>Nucleic Acids Res</i> 37:202–208.	1017 1018 1019
62.	Levdikov VM, Blagova E, Young VL, Belitsky BR, Lebedev A, Sonenshein AL, Wilkinson AJ. 2017. Structure of the branched-chain Amino Acid and GTP-sensing global regulator, CodY, from <i>Bacillus subtilis</i> . <i>J Biol Chem</i> 292:2714–2728.	1020 1021 1022
63.	Solovyev V, Salamov A. 2010. Automatic Annotation of Bacterial Community Sequences and Application To Infections Diagnostic. <i>Metagenomics its Appl Agric Biomed Environ Stud</i> 61–78.	1023 1024 1025
64.	Wurtzel O, Sesto N, Mellin JR, Karunker I, Edelheit S, Bécavin C, Archambaud C, Cossart P, Sorek R. 2012. Comparative transcriptomics of pathogenic and non-pathogenic <i>Listeria</i> species. <i>Mol Syst Biol</i> 8:583.	1026 1027 1028
65.	Crimmins GT, Herskovits AA, Rehder K, Sivick KE, Lauer P, Dubensky TW, Portnoy D a. 2008. <i>Listeria monocytogenes</i> multidrug resistance transporters activate a cytosolic surveillance pathway of innate immunity. <i>Proc Natl Acad Sci U S A</i> 105:10191–6.	1029 1030 1031 1032
66.	Sigal N, Pasechnek A, Herskovits AA. 2016. RNA purification from intracellularly grown <i>Listeria monocytogenes</i> in macrophage cells. <i>J Vis Exp</i> 2016:1–7.	1033 1034 1035

Fig 1. The TcyKLMN transporter and the regulatory factors CysK and CymR play a role in the regulation of *Lm* virulence gene expression. See full legend in the next page.

Fig 1. The TcyKLMN transporter and the regulatory factors CysK and CymR play a role in the regulation of *Lm* virulence gene expression. **(A)** A schematic representation of the *ytmI* operon and the genes identified in Friedman *et al.* 2017. Locations of transposon insertions are marked with a triangle. **(B-F)** Luminescence and OD 600nm measurements of bacteria grown in minimal defined media supplemented with low concentrations of branched chain amino acids (LBMM). Relative Luminescence Units (RLU) represent luminescence values normalized to the respective OD 600nm value. OD 600nm values represent growth. The data represent 3 biological replicates. Error bars indicate standard deviation. **(G)** qRT-PCR analysis of *actA*, *plcA* and *hly* transcription levels in indicated bacteria grown in LBMM. mRNA levels were normalized to *rpoD* mRNA and are represented as relative quantity (RQ), relative to mRNA level in WT bacteria. The data represent 3 biological replicates. Error bars indicate standard deviation. Asterisks represent P-values (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, n.s. = non-significant) calculated by Student's t-test. P-values represent a comparison to the BHI sample.

Fig 2. TcyKLMN is a cystine/cysteine transporter. (A) Isothermal Titration Calorimetry (ITC) analysis, showing binding of TcyK to cystine (CSSC) or cysteine (Cys). Shown are the consecutive injections of 2 μL aliquots from a 200 μM Cys/CSSC solution into 200 μL of 20 μM TcyK. TCEP was used as a reducing agent when indicated. The upper panels show the calorimetric titration and the lower panels display the integrated injection heat derived from the titrations, for which the best-fit curve was used to calculate the K_D . The experiments were repeated independently 3 times, and the K_D value is presented as a mean $\pm$ standard deviation of these 3 experiments. **(B)** Growth of WT *Lm* and $\Delta tcyK$ bacteria grown in minimal defined media supplemented with different concentrations of cysteine (Cys) or cystine (CSSC) as a sole cysteine source. The data is presented as maximal OD 600nm values, corresponds to bacterial yield. The data represent 2 biological replicates. The experiment was repeated independently twice, with a total of 4 biological replicates. Error bars indicate standard deviation.

Fig 3. The TcyKLMN transporter is negatively regulated by CodY, CysK and CymR. See full legend in the next page.

Fig 3. The TcyKLMN transporter is negatively regulated by CodY, CysK and CymR. (A) A schematic representation of the *ytml-ytlI* regulatory region. Putative CymR binding-sites were identified using the motif of *Bacillus*. Putative CodY binding sites were identified using the motif of gram-positive bacteria. Promoters (-10 and -35) were identified using BPPROM. TSS denotes the transcription start sites, either identified by Wurtzel *et al.*, 2012, or predicted based on the promoter sequence. **(B)** qRT-PCR analysis of *tcyK*, *tcyN* and *ytlI* transcription level in WT bacteria grown in rich media (BHI) and in LBMM. mRNA levels were normalized to *rpoD* mRNA and are represented as relative quantity (RQ), relative to mRNA level in WT bacteria grown in BHI. The data represent 3 biological replicates. Error bars indicate standard deviation. Asterisks represent P-values (* = P<0.05, ** = P<0.01, *** = P<0.001, n.s. = non- significant) calculated by Student's t-test. P-values represent a comparison to the BHI sample. **(C-E)** qRT-PCR analysis of *tcyK* and *ytlI* transcription level in the indicated mutants grown in rich media (BHI) and minimal defined media supplemented with low concentration of either branched chain amino acids (LBMM) or cysteine (LCMM). mRNA levels were normalized to *rpoD* mRNA and are represented as relative quantity (RQ), relative to mRNA level in WT bacteria grown in the indicated medium. The data represent 3 biological replicates. Error bars indicate standard deviation. Asterisks represent P-values (* = P<0.05, ** = P<0.01, *** = P<0.001, n.s. = non- significant) calculated by Student's t-test. P-values represent a comparison to the respective WT sample, unless indicated otherwise.

Fig 4. Enhanced expression of virulence genes in $\Delta cymR$ and $\Delta cysK$ is TcyKLMN dependent. Luminescence of pPL2 *Phly-lux* reporter system (left) and growth (right) of $\Delta cysK/\Delta tscyK$ and $\Delta cymR/\Delta tscyK$ bacteria grown in minimal defined media supplemented with low concentration of branched chain amino acids (LBMM). Relative Luminescence Units (RLU) represent luminescence values normalized to the respective OD 600nm value. OD 600nm values represent growth. The data represent 3 biological replicates. Error bars indicate standard deviation.

Fig 5. The TcyKLMN transporter plays a role in glutathione synthesis. See full legend in the next page.

Fig 5. The TcyKLMN transporter plays a role in glutathione biosynthesis. (A-B) Luminescence of pPL2 *Phly-lux* reporter system (left) and growth (right) of $\Delta gshF$ and $\Delta tcyK$ bacteria grown in minimal defined media with low concentration of branched chain amino acids (LBMM). 20 mM reduced glutathione was supplemented to LBMM when indicated. Relative Luminescence Units (RLU) represent luminescence values normalized to the respective OD 600nm value. OD 600nm values represent growth. The data represent 3 biological replicates. Error bars indicate standard deviation. **(C)** Internal glutathione level during growth in LBMM in indicated bacteria. The data represent 3 biological replicates. Error bars indicate standard deviation. Asterisks represent P-values (***) = $P < 0.001$) calculated by Student's t-test. P-values represent a comparison to the WT sample. **(D)** Luminescence of pPL2 *Phly-lux* reporter system (left) and growth (right) of $\Delta cysK/\Delta gshF$ and $\Delta cymR/\Delta gshF$ bacteria grown in LBMM. Relative Luminescence Units (RLU) represent luminescence values normalized to the respective OD 600nm value. OD 600nm values represent growth. The data represent 3 biological replicates. Error bars indicate standard deviation.

Fig 6. The TcyKLMN transporter promotes *Lm* intracellular growth in macrophage cells. **(A)** qRT-PCR analysis of *tcyK*, *tcyN* and *ytII* transcription level in WT bacteria grown in rich media (BHI) or intracellularly in J774A.1 macrophage cell line (IC). mRNA levels were normalized to *rpoD* mRNA and are represented as relative quantity (RQ), relative to mRNA level in in the BHI sample. The data represent 3 biological replicates. Error bars indicate standard deviation. Asterisks represent P-values (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, n.s. = non- significant) calculated by Student's t-test. P-values represent a comparison to the BHI sample. **(B)** Intracellular expression of *plcA* as indicated by the fluorescence of YFP proteins expressed under the control of *plcA* promoter (pPL2-*PplcA*-3yfp) in WT *Lm* and indicated mutants, grown in bone marrow derived macrophages (BMDM). Fluorescence was measured 3 hours post infection using a florescence microscope. The data represent at least 3 biological replicates. Error bars indicate standard deviation. Asterisks represent P-values (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, n.s. = non- significant) calculated by Student's t-test. P-values represent a comparison to the WT sample. **(C-D)** Intracellular growth of $\Delta tcyK$ and $\Delta gshF$ mutants in BMDM cells in comparison to WT *Lm*. The experiment was repeated 3 times independently. Since we could not average the data, a representative result is shown here, and two additional biological repeats are shown in **Figure S8**. Error bars indicate standard deviation. h.p.i. = hours post infection.

A

B

Fig S1. Cysteine uptake and metabolic genes identified in a screen by Friedman *et al.* (A) Phenotypes of transposon mutants as identified in Friedman *et al.*, 2017. WT bacteria and indicated transposon mutants were grown in minimal defined medium supplemented with low concentration of branched chain amino acids (LBMM). Relative Luminescence Units (RLU) represent luminescence values normalized to the respective OD 600nm value. (B) Schematic representation of cysteine transport via TcyKLMN and cysteine-glutathione biosynthesis. The genes identified in the genetic screen are marked in red. OAS=O-acetylserine.

Fig S2. *Lm* strain 10403S is auxotrophic to cysteine and methionine. (A-B) Growth of WT *Lm* strain 10403S in minimal defined media supplemented with different concentrations of either cysteine or methionine. The data is presented as maximal OD 600nm values, corresponding to bacterial yield. The data represent 3 biological replicates. Error bars indicate standard deviation. (C) Growth of WT *Lm* in minimal defined media without cysteine supplemented either with 1000 $\mu\text{g/ml}$, 2000 $\mu\text{g/ml}$ or no methionine. Growth of WT *Lm* in minimal defined media supplemented with standard concentrations of cysteine (100 $\mu\text{g/ml}$) is shown as a control. OD 600 nm values represent growth. The data represent 3 biological replicates. Error bars indicate standard deviation. (D) Growth of WT *Lm* in minimal defined medium without methionine, supplemented with either 1000 $\mu\text{g/ml}$, 2000 $\mu\text{g/ml}$ or no cysteine. Growth of WT *Lm* in minimal defined media supplemented with standard concentrations of methionine (100 $\mu\text{g/ml}$) is shown as a control. OD 600nm values represent growth. The data represent 3 biological replicates. Error bars indicate standard deviation.

Fig S3. Growth of deletion mutants generated in this study in the rich medium BHI. WT bacteria and indicated strains (A-J) were grown in BHI. OD 600nm values represent growth. The data represent 3 biological replicates. Error bars indicate standard deviation.

Fig S4. Complementation experiments of $\Delta tcyK$ and $\Delta cysK$ mutants. qRT-PCR analysis of *hly* transcription level in WT *Lm*, $\Delta tcyK$ and $\Delta cysK$ mutants and their respective complementation strains harboring a copy of *tcyK* or *cysK* gene under the *tet* promoter on pPL2 plasmid, grown in LBMM. mRNA levels were normalized to *rpoD* mRNA and are represented as relative quantity (RQ), relative to mRNA level in WT bacteria. The data represent at least 2 biological replicates. Error bars indicate standard deviation. Asterisks represent P-values (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, n.s. = non-significant) calculated by Student's t-test. P-values represent a comparison to the WT sample unless indicated otherwise.

Fig S5. Purification of a short variant of TcyK. (A) Coomassie staining of SDS-PAGE of Ni-NTA affinity purification of a his-tagged short TcyK (amino acids 38-286). Lane 1: molecular weight marker (in kDa), Lane 2: total protein extract, Lane 3: column-unbound fraction, Lane 4: wash with 60 mM of imidazole, Lanes 5-11: fractions eluted using a linear gradient of 60 to 250 mM imidazole. (B) Analysis of the purified protein by size exclusion chromatography. 80 μ l of a 0.8 mg/ml protein solution were injected on a Superdex 75 10/300 GL column (GE Healthcare).

Fig S6. *gshF* is not transcriptionally regulated by CymR, CysK and CodY. qRT-PCR analysis of *gshF* transcription level in the indicated mutants grown in rich media (BHI) and minimal defined media supplemented with low concentration of either branched chain amino acids (LBMM). mRNA levels were normalized to *rpoD* mRNA and are represented as relative quantity (RQ), relative to mRNA level in WT bacteria grown in the indicated medium. The data represent at least 2 biological replicates. Error bars indicate standard deviation Asterisks represent P-values (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, n.s. = non- significant) calculated by Student's t-test. P-values represent a comparison to the respective WT sample.

Fig S7. The TcyKLMN transporter does not transport glutathione. (A) Isothermal Titration Calorimetry (ITC) analysis, showing no binding of TcyK to reduced glutathione (GSH). Shown are the consecutive injections of 2 μ L aliquots from a 200 μ M GSH solution into 200 μ L of 20 μ M TcyK. TCEP was used as a reducing agent. The upper panels show the calorimetric titration and the lower panels display the integrated injection heat derived from the titrations. The experiment was repeated independently 3 times. **(B)** Growth of WT *Lm* and $\Delta tcyK$ bacteria in minimal defined media supplemented with different concentrations of reduced glutathione (GSH) as a sole source of cysteine. The data is presented as maximal OD₆₀₀ values, corresponding to bacterial yield. The data represent 2 biological replicates. Error bars indicate standard deviation.

Fig S8. Intracellular growth of $\Delta tcyK$ and $\Delta gshF$ mutants in BMDM cells in comparison to WT *Lm*. The experiment was repeated independently 3 times, two biological repeats for each mutant are shown here, supplementary to **Figure 6 C-D**.