Legionella pneumophila CsrA regulates a metabolic switch from amino acid to glycerolipid metabolism

Ina Häuslein, Tobias Sahr, Pedro Escoll, Nadine Klausner, Wolfgang Eisenreich, Carmen Buchrieser

To cite this version:

Ina Häuslein, Tobias Sahr, Pedro Escoll, Nadine Klausner, Wolfgang Eisenreich, et al.. Legionella pneumophila CsrA regulates a metabolic switch from amino acid to glycerolipid metabolism. Open Biology, Royal Society, 2017, 7 (11), <10.1098/rsob.170149>. <pasteur-01684655>
Legionella pneumophila CsrA regulates a metabolic switch from amino acid to glycerolipid metabolism

Ina Häuslein\textsuperscript{1}, Tobias Sahr\textsuperscript{2,3}, Pedro Escoll\textsuperscript{2,3}, Nadine Klausner\textsuperscript{1}, Wolfgang Eisenreich\textsuperscript{1} and Carmen Buchrieser\textsuperscript{2,3}

\textsuperscript{1}Department of Chemistry, Biochemistry, Technische Universität München, Garching, Germany
\textsuperscript{2}Institut Pasteur, Biologie des Bactéries Intactocellulaires, Paris, France
\textsuperscript{3}CNRS UMR 3525, Paris, France

\textsuperscript{PE, 0000-0002-5933-094X; WE, 0000-0002-9832-8279; CB, 0000-0003-3477-9190}

1. Introduction

The Gram-negative, facultative intracellular pathogen Legionella pneumophila is widespread in natural and man-made aquatic systems, where it replicates within various free-living protozoan hosts like Acanthamoeba castellanii or Hartmannella vermiformis \cite{1,2}. However, L. pneumophila is also able to infect human alveolar macrophages when contaminated aerosols are inhaled by a susceptible human host, causing Legionnaires' disease, a severe life-threatening pneumonia \cite{3,4}. In both host systems, amoebae and macrophages, invasion occurs by phagocytosis followed by the establishment of an intracellular replication compartment, the Legionella-containing vacuole (LCV). The ability to replicate intracellularly is dependent on a functional Dot/Icm type IV secretion system (T4SS) that translocates over 300 effector proteins in the host cell, thereby triggering various processes such as the recruitment of vesicles derived
from the endoplasmatic reticulum or the direct manipulation of many host cell signalling pathways [5–7].

Although several different morphological forms have been characterized [8], the L. pneumophila life cycle can mainly be described as biphasic. It consists of a (i) replicative form where bacteria are rod shaped, non-flagellated and are able to replicate in the LCV, and (ii) a transmissive form where the bacteria are flagellated and virulence and transmission factors are expressed allowing the infection of new host cells [9]. This biphasic life cycle is crucial for the fitness of the pathogen and is linked to its metabolism. Indeed, when nutrients are abundant, as in a host cell, the presence of amino acids triggers, for example, the differentiation of L. pneumophila to a replicative form [10,11]. Replication of L. pneumophila and thereby nutrient scavenging leads to amino acid consumption that triggers the switch to the transmissive form [12–14] that is expressing the virulence and transmission factors necessary to leave the spent host cell and search for a new one.

A link between the biphasic life cycle and the metabolism is also reflected in the life-stage-specific usage of carbon nutrients [15–17]. Although this pathway is known to mainly use amino acids (e.g. serine) as carbon and energy source [18–20], the L. pneumophila genome analyses uncovered the presence of all enzymes of the glycolytic pathway and the Entner–Doudoroff (ED) pathway [21–23]. Indeed, their functionality in carbohydrate usage has been shown in transcriptome and proteome analyses, in in vivo experiments and by isotopologue profiling experiments, all confirming that L. pneumophila is able to metabolize glucose, whereby it predominantly uses the ED pathway [24,25]. In addition, L. pneumophila can use glycerol as a nutrient source. First hints came from early radio labelling experiments, in vitro studies as well as from transcriptome experiments that showed the upregulation of enzymes responsible for glycerol catabolism during intracellular growth in macrophages [20,26,27]. Recent in vitro isotopologue profiling studies of a L. pneumophila wt strain using [U-13C3]glycerol as a tracer demonstrated glycerol usage mainly in late growth phase where it serves as an additional substrate to feed the pentose phosphate pathway (PPP) and gluconeogenetic reactions [17]. Life-stage-specific substrate usage has also been demonstrated in labelling experiments with [U-13C3]serine and [U-13C6]glucose, highlighting that serine is more efficiently used in the replicative phase for energy generation via the tricarboxylic acid (TCA) cycle. Thus, the biphasic life cycle of L. pneumophila is represented by a switch from replicative to transmissive bacteria, a switch that is tightly linked to the metabolism and in particular to a life-cycle-specific substrate usage [15–17].

The metabolic changes occurring in the host cell during the intracellular replication of L. pneumophila are transmitted to regulatory systems and alternative sigma factors [28]. When nutrients are getting limited, the production of the stringent response messenger guanosine-3’,5’-bispyrophosphate (ppGpp) is induced, which in turn is activating among others the expression of RpoS and the two-component system LetA/LetS [10,29,30]. Consequently, the transcription of the three non-coding small RNAs RsmX, RsmY and RsmZ is induced, leading to the expression of transmissive traits and simultaneous repression of replicative traits due to binding to and thereby deactivating CsrA [31,32]. Thus CsrA is a key regulator of the switch from replicative to transmission competent bacteria [9,33–35]. CsrA prevents the translation of transmissive traits like motility, stress resistance or virulence during the exponential growth phase, while simultaneously activating replication [34–36]. The levels of csrA expression are furthermore regulated by the two-component system PmrA/PmrB that is essential for intracellular replication [31,37,38]. Another component of this regulatory network is the two-component system LqsTS/LqsR that is controlled by RpoS and LetA/LetS, and regulates quorum sensing in L. pneumophila [39,40].

Given the importance of CsrA in the life cycle switch between replicative and transmissive/virulent L. pneumophila and the fact that metabolic cues are regulating this biphasic life cycle, we investigated the role of the central regulator CsrA in the life-stage-specific carbon metabolism. In vitro 13C-isotopologue profiling and oxygen consumption experiments using the Seahorse Bioscience technology showed that the strong link between the Legionella life cycle and metabolism is reflected in different carbon source usage that is regulated by CsrA.

2. Results

2.1. Determination of L. pneumophila oxygen consumption reveals diminished energy generation by the csrA (csrA−) mutant strain

For a first evaluation of possible metabolic differences of the L. pneumophila csrA− strain (truncated mutant after Tyr48 [35]), aerobic bacterial respiration was analysed by measuring the oxygen consumption rate (OCR) of the exponentially grown wild-type (wt) and the csrA− strain in the presence of the substrates L-serine, L-alanine, L-glutamate, D-glucose, pyruvate, α-ketoglutarate, glycerol, butanoate or palmitate (figure 1). After poly-lysine coating of the L. pneumophila wt or csrA− strain on micro-plates, bacterial respiration was measured using an extracellular flux analyser (Seahorse Bioscience). When L-serine was added as the substrate a clear increase in respiration was observed, confirming that serine is an important source for energy in L. pneumophila during exponential growth [17,24,41]. The OCR was significantly downregulated in the csrA mutant when compared with the wt, suggesting a positive effect of CsrA on the utilization of serine with regard to bacterial respiration (figure 1a). Similar results were obtained when pyruvate, α-ketoglutarate L-alanine or palmitic acid were added as substrate (figure 1b–e). By contrast, no difference between the OCR of the wt and the csrA mutant strain was observed when D-glucose, L-glutamate or glycerol were provided (figure 1f,g,i), suggesting no usage of these substrates in bacterial respiration. Interestingly, the OCR after injection of palmitate and to a lesser extent also of butanoate indicated usage of fatty acids (FAs) for aerobic respiration and revealed a positive effect of CsrA, in particular on the usage of palmitate (figure 1e,h). In contrast, unsaturated FAs like arachidonic acid or oleic acid showed no or a significantly reduced OCR of the wt strain (electronic supplementary material, figure S1). Metabolism and carbon flux from fatty acid degradation was not known in L. pneumophila. Our data suggest that L. pneumophila is able to metabolize saturated FAs such as palmitate and butanoate, but probably not unsaturated FAs like oleate or arachidonate. To study these findings in more detail we performed
2.2. Serine uptake and metabolism are reduced in the csrA mutant strain

To investigate the role of CsrA in the carbon metabolism of serine, we performed labelling experiments with the wild-type and the csrA mutant grown in CE MDM medium supplemented with 6 mM [U-13C3]serine. Bacteria were harvested at E and PE growth phase and 13C overall enrichments were detected in the cell wall (figure 2; electronic supplementary material, figure c). In the wt strain 13C enrichments remained constant or decreased from E to PE phase (electronic supplementary material, table S1). Minor, but similar effects were also detectable during the PE phase, indicating that serine uptake and metabolism are generally downregulated in the csrA mutant. However, in both growth phases, the carbon flux from 13C-serine into gluconeogenetic reactions and into the PPP was strongly affected by this mutation, as 13C enrichments were mainly reduced in key metabolites related to the TCA cycle were only slightly changed, indicating smaller effects of CsrA on the carbon flux of serine into the TCA cycle (figure 2a).

In the csrA mutant (E phase) a slightly lower 13C incorporations were observed in Ala (besides Ser), but low ones in His or Man, which is in agreement with the previously proposed model of a bipartite metabolism of L. pneumophila wt, where Ser is mainly shuttled into the energy generating metabolic pathway of, for example the TCA via pyruvate [17]. Overall enrichment values increased slightly from E to PE phase in the wt and the csrA mutant in Ala, Asp, Glu, Lys, DAP and PHB. In contrast, in Gly, His and sugars 13C enrichments remained constant or decreased from E to PE phase (electronic supplementary material, table S1).

In the csrA mutant (E phase) a slightly lower 13C incorporation (p < 0.01) was detected in Asp, Glu, Gly, Lys and DAP, and a strongly reduced enrichment (p < 0.001) was seen in His as well as in Man, GlcN and Mur when compared with the wt strain (electronic supplementary material, table S1). Minor, but similar effects were also detectable during the PE phase, indicating that serine uptake and metabolism are generally downregulated in the csrA mutant. However, in both growth phases, the carbon flux from 13C-serine into gluconeogenetic reactions and into the PPP was strongly affected by this mutation, as 13C enrichments were mainly reduced in key metabolites of these pathways. In contrast, 13C enrichments in metabolites related to the TCA cycle were only slightly changed, indicating smaller effects of CsrA on the carbon flux of serine into the TCA cycle (figure 2a).
The relative fractions (%) of isotopologues containing one, two, three, ..., n $^{13}$C-atoms ($M+1$, $M+2$, $M+3$, ..., $M+n$) in the $^{13}$C-enriched samples were then determined from the intensities of the respective MS signals in comparison to the respective unlabelled compound (figure 2b and electronic supplementary material, table S2). Ala and Glu showed similar $^{13}$C distributions in the wt and the csrA mutant in Ala, Asp, Glu, Lys, DAP and PHB (figure 3; electronic supplementary material). In most metabolites, the enrichment values increased from E to PE growth phase in the wt but less or not at all in the csrA mutant. Only $^{13}$C enrichments in Man and GlcN remained constant or slightly decreased in the csrA mutant from E to PE phase (electronic supplementary material, table S3).

The wt and the csrA mutant showed similar enrichment values during E phase for the TCA cycle related amino acids Ala, Asp, Glu, Lys, DAP and PHB (figure 3; electronic supplementary material). In most metabolites, the enrichment values increased from E to PE growth phase in the wt but less or not at all in the csrA mutant. Only $^{13}$C enrichments in Man and GlcN remained constant or slightly decreased in the csrA mutant from E to PE phase (electronic supplementary material, table S3).

The relative fractions (%) of isotopologues containing one, two, three,..., n $^{13}$C-atoms ($M+1$, $M+2$, $M+3$, ..., $M+n$) in the $^{13}$C-enriched samples were then determined from the intensities of the respective MS signals in comparison to the respective unlabelled compound (figure 2b and electronic supplementary material, table S2). Ala and Glu showed similar $^{13}$C distributions in the wt and the csrA mutant in Ala, Asp, Glu, Lys, DAP and PHB (figure 3; electronic supplementary material). In most metabolites, the enrichment values increased from E to PE growth phase in the wt but less or not at all in the csrA mutant. Only $^{13}$C enrichments in Man and GlcN remained constant or slightly decreased in the csrA mutant from E to PE phase (electronic supplementary material, table S3).

The relative fractions (%) of isotopologues containing one, two, three,..., n $^{13}$C-atoms ($M+1$, $M+2$, $M+3$, ..., $M+n$) in the $^{13}$C-enriched samples were then determined from the intensities of the respective MS signals in comparison to the respective unlabelled compound (figure 2b and electronic supplementary material, table S2). Ala and Glu showed similar $^{13}$C distributions in the wt and the csrA mutant in Ala, Asp, Glu, Lys, DAP and PHB (figure 3; electronic supplementary material). In most metabolites, the enrichment values increased from E to PE growth phase in the wt but less or not at all in the csrA mutant. Only $^{13}$C enrichments in Man and GlcN remained constant or slightly decreased in the csrA mutant from E to PE phase (electronic supplementary material, table S3).
For numerical values, see electronic supplementary material, tables S4 and S8.

... deviations from six values (2/C2) the exponential (E) and post-exponential (PE) growth phase. 13C excess values (mol%) in protein-derived amino acids, diaminopimelic acid (DAP), polyhydroxy... L. pneumophila ... key metabolites from butyrate (PHB), mannose (Man), glucosamine (GlcN) and muramic acid (Mur) were determined by isotopologue profiling. Isotopologue distributions were determined for selected metabolites (Ala, Glu, His and Man). Shown are the relative fraction (in%) of isotopologues (M+1 to M+6). Error bars indicate standard deviations from six values (2 × biological replicates, 3 × technical GC/MS). Statistical significance is depicted as p-value (*p < 0.05, **p < 0.01 and ***p < 0.001). For numerical values, see electronic supplementary material, tables S4 and S8.

Figure 2. CsrA has regulatory impact on serine uptake and metabolism of L. pneumophila. (a) 13C excess (mol%) and (b) relative isotopologue distributions (%) in key metabolites from L. pneumophila wild-type and its csrA mutant grown in CE MDM supplemented with 6 mM [U-13C3]serine as tracer. Bacteria were harvested at the exponential (E) and post-exponential (PE) growth phase. 13C excess values (mol%) in protein-derived amino acids, diaminopimelic acid (DAP), polyhydroxybutyrate (PHB), mannose (Man), glucosamine (GlcN) and muramic acid (Mur) were determined by isotopologue profiling. Isotopologue distributions were determined for selected metabolites (Ala, Glu, His and Man). Shown are the relative fraction (in%) of isotopologues (M+1 to M+6). Error bars indicate standard deviations from six values (2 × biological replicates, 3 × technical GC/MS). Statistical significance is depicted as p-value (*p < 0.05, **p < 0.01 and ***p < 0.001). For numerical values, see electronic supplementary material, tables S4 and S8.

PHB (p-values <0.05). This was different in His, Man and GlcN where 13C incorporation was slightly higher or remained similar to the values of the wt (electronic supplementary material, table S3). Therefore, carbon flux from glucose into the TCA cycle seems to be predominantly affected by this mutation only during post-exponential growth. In contrast, gluconeogenetic reactions and carbon flux in the PPP seem to be already upregulated during E phase in the csrA mutant but not in the wt strain (figure 3a).

Similar results were obtained when analysing the isotopologue distributions, because the amounts of M+5 isotopologues in His and M+6 isotopologues in Man slightly increased in E and PE phase for the csrA mutant (figure 3b). The M+6 label in Man was derived from the direct conversion of fully labelled 13C-glucose into Man. In addition, M+6 isotopologues in Man can be formed via combination reactions of two fully labelled C3 precursors, which are previously built in glycolytic reactions or reactions of the ED pathway. M+5 label in His can be derived from fully labelled fructose 6-phosphate entering the PPP or again via combination reactions of two labelled precursors, which are recombined during gluconeogenetic reactions and/or in reactions of the PPP. Taken together, our results indicated, in contrast to what was observed for serine, a slightly higher carbon flux from glucose into the PPP and ED pathways as well as in the biosynthesis of sugars in the csrA mutant when compared with the wt strain (electronic supplementary material, table S4).

2.4. The glycerol metabolism is remarkably upregulated in the absence of CsrA

Early labelling experiments with [U-14C3]glycerol had suggested that glycerol is a potential nutrient for L. pneumophila [27]. Furthermore, transcriptome data showed that enzymes responsible for glycerol catabolism (lpp1369: glycerol kinase, lpp2257: glycerol 3-phosphate dehydrogenase) are upregulated during intracellular growth in macrophages [26]. Indeed, isotopologue profiling experiments of a wt strain demonstrated that L. pneumophila catabolizes this substrate, but exclusively at late stages of the developmental cycle, thereby mostly serving anabolic reactions in gluconeogenesis and the PPP [17].

To determine whether CsrA has a regulatory role on the metabolism of glycerol in L. pneumophila, we used labelling experiments with fully labelled 13C-glycerol. For comparisons, wild-type bacteria and a csrA− strain were grown in...
and (b) acids, diaminopimelic acid (DAP), polyhydroxybutyrate (PHB), mannose (Man), glucosamine (GlcN) and muramic acid (Mur) were determined by isotopologue profiling from six values (two biological and three technical GC/MS replicates). Statistical significance is given as p-value (*p < 0.05, **p < 0.01 and ***p < 0.001). For numerical values see electronic supplementary material, tables S3 and S4.

Figure 3. The carbon flux from glucose into the PPP and ED pathways as well as in the biosynthesis of sugars is higher in the csrA mutant. (a) 13C excess (mol%) and (b) relative isotopologue distributions (% in key metabolites from L. pneumophila wild-type and its csrA mutant grown in CE MDM supplemented with 11 mM [U-13C6]glucose as tracers. Bacteria were harvested at the exponential (E) and post-exponential (PE) growth phase. 13C excess values (mol%) in protein-derived amino acids, diaminopimelic acid (DAP), polyhydroxybutyrate (PHB), mannose (Man), glucosamine (GlcN) and muramic acid (Mur) were determined by isotopologue profiling. Isotopologue distributions were determined for selected metabolites (Ala, Glu, His and Man). Shown are the relative fractions (in%) of isotopologues (M+1 to M+6). For a better illustration, metabolites with 13C excess values lower than 30% are shown in the figure inset. Error bars indicate standard deviations calculated from six values (two biological and three technical GC/MS replicates). Statistical significance is given as p-value (*p < 0.05, **p < 0.01 and ***p < 0.001). For numerical values see electronic supplementary material, tables S3 and S4.

CE MDM medium supplemented with 50 mM [U-13C5]glycerol, and cells were harvested at E and PE phase. Similar to the results obtained with labelled glucose, highest 13C enrichments were detected in His, which is a marker for the PPP (see also table 1) and in the sugars Man, GlcN and Mur. Low but significant 13C enrichments were also found in Ala, Asp, Glu, Lys, DAP and PHB, which are metabolites related to a carbon flux directed towards the TCA cycle (figure 4; electronic supplementary material, figure S4). In both labelling experiments (wt and csrA mutant), 13C overall enrichments were increased from the E to PE phase in all metabolites except for His in the csrA- strain (electronic supplementary material, table S5).

Generally, 13C excess was higher in the csrA- strain during both growth phases for almost every metabolite, when compared with the wt strain. The csrA mutant showed significant enrichment already during E phase in metabolites that are related to the TCA cycle. Specifically, 13C enrichment of His was increased by a factor of more than 2 in the mutant, rising from 5.45% (wt) to 13.79% (csrA mutant; p < 0.001) in the E phase and from 6.40% (wt) to 13.99% (csrA mutant; p < 0.001) in PE phase. Similar results were obtained for the cell wall sugars. This indicated that glycerol, which is only favoured in later growth phases in L. pneumophila wt, is already taken up and metabolized very efficiently during E phase in the csrA mutant. Furthermore, the glycerol metabolism and probably also incorporation of this substrate are dramatically upregulated in the csrA- strain. This clearly demonstrates that CsrA has a negative, regulatory effect on the metabolism of glycerol in L. pneumophila, as the glycerol metabolism in general and predominantly the carbon flux into gluconeogenetic reactions and into the PPP seem to be affected (figure 4a).

This observation was also supported by the analyses of the isotopologue distributions in the respective metabolites (figure 4b; electronic supplementary material, table S6). For example, the amount of M+3 label in Ala increased in the csrA mutant. Since this isotopologue is directly derived from fully labelled glycerol, which is converted into pyruvate and subsequently used in Ala biosynthesis, this again suggested an upregulated incorporation and metabolism of glycerol in the absence of CsrA. Fully labelled pyruvate can then be used to build fully labelled acetyl-CoA, which is subsequently shuttled into the TCA cycle. Then, M+2 label is transferred to amino acids like Glu and Asp, which are
2.5. Differential substrate usage analyses indicates that CsrA activates the tricarboxylic acid cycle and represses glycerol metabolism in E phase

To better visualize the metabolic changes that depend on CsrA, we calculated the ratios of $^{13}$C enrichments in marker metabolites between the wt and the csrA mutant strain. His was chosen as a marker for PPP, because PRPP derived from the PPP is a precursor for His. Ala and Glu were chosen as indicators for the TCA cycle, because Ala is derived from pyruvate and Glu from $\alpha$-ketoglutarate, a direct intermediate of the TCA cycle (see also table 1). The two ratios $^{13}$C excess (mol%) His/Ala and $^{13}$C excess (mol%) His/Glu were calculated for the L. pneumophila wt and the csrA mutant strain at the E and PE growth phase, respectively. High values are indicating an intense carbon flux directed towards gluconeogenetic reactions and into the PPP, whereas low values are representing a high carbon flux of the respective substrate into the TCA cycle for energy generation. Ratios were calculated for labelling experiments with $[U-^{13}C_3]$serine, $[U-^{13}C_6]$glucose and $[U-^{13}C_3]$glycerol (figure 5).
Figure 5. Differential substrate usage of L. pneumophila wild-type and its csrA mutant. Shown are ratios of \(^{13}\)C excess in His (PPP) to Ala (pyruvate) or Glu (TCA cycle), respectively. Ratios were calculated for experiments with L. pneumophila wt and its csrA mutant grown with (a) \(^{13}\)C-serine, (b) \(^{13}\)C-glucose and (c) \(^{13}\)C-glycerol for E phase and PE phase respectively. Mean values were calculated from matrix calculations (resulting in 36 data points) for possible His/ Ala and His/Glu ratios with six datasets for His, Ala and Glu, respectively (two biological replicates; 2 x 3 technical replicates). The standard deviation was calculated from the resulting 36 His/Ala or His/Glu ratios, respectively. Statistical analysis was performed using two-tailed unpaired Student’s t-test for the analysis of differences between the wild-type and the csrA mutant at the E and PE growth phase. Statistical significance is given as p-value (*p < 0.05, **p < 0.01 and ***p < 0.001). For numerical values see electronic supplementary material, tables S7 and S8.

For the \(^{13}\)C-serine labelling experiments, these ratios were low in the wt and the mutant. Interestingly, the ratios were significantly reduced in the mutant, indicating that there is a higher carbon flux from serine into the TCA than into the PPP during both growth phases due to the csrA mutation (e.g. His/Ala: wt E: 0.64, csrA: E: 0.46; p < 0.001 and wt PE: 0.58, csrA: PE: 0.45; p < 0.001) (figure 5; electronic supplementary material, tables S7 and S8).

In contrast, experiments with \(^{13}\)C-glucose revealed high values for these ratios. This is in line with previous results obtained for a wt strain, which showed that glucose is predominantly shuttled into the PPP, the ED pathway and in glycolytic reactions [17]. In the csrA strain at both growth phases, these values were increased (e.g. His/Ala: wt E: 5.05, csrA: E: 5.70; p < 0.001 and wt PE: 4.36, csrA: PE: 5.95; p < 0.001), showing that CsrA has a regulatory impact on the carbon flux from glucose towards anabolic processes (figure 5).

Interestingly, the highest ratios were calculated for the experiments with \(^{13}\)C-glycerol, which indicates that carbon flow from this nutrient is mainly directed to the PPP in both the wt and the csrA mutant. Importantly, these values increased in E and PE phase dependent on CsrA. The incorporation and metabolism of glycerol is strongly upregulated in the absence of CsrA while the carbon flux is mostly restricted to reactions in the PPP (figure 5). Thus, CsrA clearly represses glycerol uptake and/or metabolism towards PPP in L. pneumophila wt, especially during early growth phases where high levels of this regulator are present in the bacteria.

2.6. Fatty acids are preferentially used for polyhydroxybutyrate biosynthesis dependent on the regulatory function of CsrA

The energy storage compound PHB that appears in granules predominantly in the transmissive growth phase is essential for long-term survival of L. pneumophila in the environment [42,43]. It is synthesized in numerous bacteria via (R)-3-hydroxybutanoyl-CoA, which is built from two acetyl-CoA molecules [16,44,45]. In a L. pneumophila wt strain it was shown that serine, but also glucose, is used to build acetyl-CoA, which is subsequently shuttled into the biosynthesis of PHB. Thereby, serine is partly used for PHB biosynthesis during replication, while glucose serves PHB biosynthesis at a later growth phase [16,17]. Here we analysed the putative role of CsrA as regulator of the time-dependent biosynthesis of this storage compound.

Analyses of the L. pneumophila csrA mutant had revealed significantly higher concentrations of this storage compound during E and stationary growth phase and has identified numerous enzymes involved in the biosynthesis of PHB as direct targets of CsrA [35]. However, labelling experiments with [U-\(^{13}\)C]serine, [U-\(^{13}\)C]glucose and [U-\(^{13}\)C]glycerol did not show significantly increased \(^{13}\)C incorporation into PHB in the csrA mutant (figure 2–4). Therefore, we hypothesized that another carbon source might serve for the biosynthesis of the higher amount of PHB in the csrA mutant.

Since all enzymes responsible for fatty acid degradation leading to acetyl-CoA, which can subsequently be used for the biosynthesis of PHB, are present in L. pneumophila [21–23], FAs could represent the carbon source for PHB biosynthesis. Indeed, when using fluorescently labelled palmitate (Bodipy FL C16) in vivo, we observed that L. pneumophila is able to take up and accumulate palmitate intracellularly. Furthermore, analyses by flow cytometry showed a slight but significant increase of palmitate accumulation in the csrA mutant when compared with the wt strain (figure 6a,b). This confirms our model that CsrA seems to have a negative regulatory effect on palmitate uptake and/or short-term utilization in oxidative phosphorylation (figure 1e). However, as judged by fluorescence microscopy, in both the wt and csrA strain, the FAs were stored in intracellular inclusion bodies similar to what is known for other bacteria such as Mycobacterium tuberculosis (figure 6c). Most interestingly, in M. tuberculosis, triacylglycerols released from the host cell are the main energy source, and the synthesis and accumulation of lipid droplets inside the bacteria is closely related to stress response, antibiotic resistance and dormancy [46].

To further analyse the impact of long-chain FAs, we performed for the first time also labelling experiments with [1,2,3,4-\(^{13}\)C]palmitic acid as \(^{13}\)C-carbon tracer. The L. pneumophila wt and the csrA mutant were grown in CE MDM medium with [1,2,3,4-\(^{13}\)C]palmitic acid and were harvested at E and PE phase. \(^{13}\)C enrichments and isotopologue profiles were determined in protein-derived amino acids, DAP and PHB as well as in lactate and stearic acid (figure 7; electronic supplementary material, table S8).
supplementary material, tables S9 and S10). High $^{13}$C enrichments were detected exclusively in the storage compound PHB. This demonstrates for the first time the effective usage of a fatty acid in \textit{L. pneumophila} and that acetyl-CoA derived from fatty acid degradation indeed serves predominantly for the biosynthesis of the storage compound PHB. Minor, but significant enrichments were also detected in Glu in the wt strain, indicating that minor amounts of labelled acetyl-CoA are also shuffled into the TCA cycle. $^{13}$C enrichments in PHB significantly increased in the \textit{csrA} (wt: $0.50\%$, \textit{csrA} mutant: $0.78\%$; $p < 0.001$) (electronic supplementary material, table S9). During E phase, also $^{13}$C carbon flux into the TCA cycle was upregulated in the \textit{csrA} strain in E phase (wt: $0.50\%$, \textit{csrA} mutant: $0.78\%$; $p < 0.001$) (electronic supplementary material, table S10). Additionally, a slightly increased carbon flux also occurred into the \textit{de novo} biosynthesis of stearic acid in the \textit{csrA} mutant during E phase ($p < 0.01$) (figure 7). In conclusion, CsrA is directly involved in the regulation of the growth phase-dependent biosynthesis of PHB by reducing carbon flux from FAs towards acetyl-CoA.

3. Discussion

The RNA-binding protein CsrA post-transcriptionally controls metabolism, motility and virulence in many bacteria [36,47]. \textit{Legionella pneumophila} CsrA is known to play a crucial role in the regulation of the bacterium’s biphasic life cycle, which is reflected in the expression of replicative traits during early stages of infection and virulence/transmissive traits during late stages of the infection [9]. However, among the over 500 CsrA targets of \textit{L. pneumophila} identified recently, many have functions in the carbon, amino acid and fatty acid metabolism as well as energy transfer and the biosynthesis of cofactors, vitamins and secondary metabolites [35].

Here we unveil, by using isotopologue profiling of a wt and a \textit{csrA} mutant strain, and the use of serine, glucose, glycerol or palmitic acids as $^{13}$C-carbon tracers, that CsrA has significant regulatory functions in many metabolic pathways of \textit{L. pneumophila}. We show that: (i) CsrA activates serine uptake and metabolism; (ii) CsrA influences the carbon flux through the glycolysis/ED pathways and gluconeogenesis; (iii) CsrA activates the flux towards the TCA; and (iv) CsrA has a negative, regulative effect on the metabolism of glycerol. Furthermore, by using for the first time $[1,2,3,4-^{13}$C$]_{4}$palmitic acid as a $^{13}$C-carbon tracer we revealed that carbon from palmitic acids are preferentially used for PHB biosynthesis dependent on the regulatory function of CsrA. Finally, the determination of the oxygen consumption rate of \textit{L. pneumophila} wt compared with the \textit{csrA} mutant when \textit{l}-serine, \textit{l}-alanine, pyruvate, \textit{α}-ketoglutarate or palmitic acid were used as carbon source, disclosed reduced energy generation by the \textit{csrA} mutant. This is in line with our recent results showing, for example, that genes coding for functions important for
serine incorporation (lpp2269) or metabolism (lpp0854) are targets of CsrA [35].

Previous studies had revealed a bipartite metabolic network with life-stage-specific usages of amino acids, carbohydrates and glycerol as major substrates. For example, serine is predominantly used in the E growth phase for energy generation in the TCA cycle [17]. Here, we show that CsrA is an important regulator of the bipartite metabolism in L. pneumophila. The carbon flux into the energy generating part of the bipartite metabolism (module two) that is derived from glucose was reduced in the csrA strain, whereas carbon flux into the upper part (module one) was not affected (figure 8). This is in line with previous proteome and transcriptome data in which CsrA positively affects several enzymes of the carbon metabolism via direct interaction, including the ED pathway and the second part of the glycolysis, whereas a glucose transporter was negatively affected by CsrA [35] (electronic supplementary material, figures S5 and S6). As a result, even though the overall uptake of glucose might be induced in the absence of CsrA, the attenuation of the ED pathway and the second part of the glycolysis leads to a reduced carbon flux directed towards module 2. Thereby, the operon coding for glyceraldehyde 3-phosphate, phosphoglycerate kinase, pyruvate kinase (lpp0151-lpp0154), three enzymes of the glycolytic cascade and the transketolase, an enzyme of the PPP, seems to play a key role in the regulation of carbon flux partitioning between module one and module 2. Indeed, we have shown previously that this operon is directly targeted by CsrA, inducing the transcription of the three glycolytic enzymes, whereas regulation of the transcription level of the transketolase is not under the control of CsrA [35]. Thus, our isotopologue analysis is in line with this finding as in the absence of CsrA, the incorporation of glucose is induced and glyceraldehyde 3-phosphate built from glucose is shuttled without hindrance into the PPP, because reactions of the transketolase are not affected whereas pyruvate biosynthesis from glyceraldehyde 3-phosphate is reduced. Apparently, the regulation of this operon by CsrA does not lead to a decreased carbon flux towards module 2.

Figure 7. Palmitic acid predominantly serves as carbon source for the PHB biosynthesis in the wt. (a) 13C excess (mol%) and (b) relative isotopologue distributions (%) in key metabolites from L. pneumophila wild-type and its csrA mutant grown in CE MDM supplemented with 0.8 mM [1,2,3,4-13C4]palmitic acid as tracers. Bacteria were harvested at the exponential (E) and post-exponential (PE) growth phase. 13C excess values (mol%) in protein-derived amino acids, dianopimelic acid (DAP), polyhydroxybutyrate (PHB), lactate (LACT) and stearic acid (STE) were determined by isotopologue profiling. Isotopologue distributions were determined for selected metabolites (Glu and PHB). Shown are the relative fractions (in%) of isotopologues (M+1 to M+6). For a better illustration, metabolites with 13C excess values of lower than 1% are shown in the figure inset. Error bars indicate standard deviations calculated from six values (two biological and three technical GC/MS replicates). Statistical significance is given as p-value (*p < 0.05, **p < 0.01 and ***p < 0.001). For numerical values, see electronic supplementary material, tables S9 and S10.
not only affect the formation of pyruvate via glyceraldehyde 3-phosphate, but also gluconeogenic reactions in the opposite direction to form glyceraldehyde 3-phosphate from pyruvate, because *L. pneumophila* also uses gluconeogenesis [17]. Therefore, besides the reduced uptake and use of serine in the csrA strain, the flux of $^{13}$C-serine into the upper part of the metabolism is lower in the wt strain compared with the csrA strain (right). Thickness of arrows indicates relative carbon flow intensities from the three different substrates, respectively, into the upper part of metabolism (module 1) in the csrA mutant, though serine metabolism is reduced in the csrA mutant. Also the carbon flux from serine into the upper part of metabolism is lower in the csrA strain. Metabolic flow of glucose is mainly restricted to module 1, though carbon flux also occurs into the energy generating part of metabolism in the csrA strain. In the csrA mutant carbon flux from glucose into the PPP was not affected, while glucose metabolism in the TCA cycle was reduced. The csrA mutation drastically changed glycerol metabolism in *L. pneumophila*. In the wt, glycerol metabolism is very low and almost exclusively restricted to the upper part of metabolism (module 1). In the *L. pneumophila* csrA-strain, glycerol metabolism is dramatically upregulated. Carbon flow also occurs predominantly into module 1, but carbon flow from glycerol into the TCA cycle has also been detectable. Framed and highlighted in yellow plus or minus signs are representing enzymes, which are upregulated [+] or downregulated [-] dependent on CsRA levels. In the *L. pneumophila* wt strain enzymes of the second part of the glycolysis are induced by Csra (lpp0151: pyruvate kinase, lpp0152: phosphoglycerate kinase, lpp0153: glyceraldehyde-3-phosphate dehydrogenase), whereas the glycerol kinase is negatively (lpp1369) affected. PRPP, phosphoribosylpyrophosphate; α-KG, α-ketoglutarate; Glu-6-P, glucose-6-phosphate; Fru-6-P, fructose-6-phosphate; 6-PG, 6-phosphogluconate; KDPG, 2-keto-3-deoxy-phosphogluconate; GAP, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; G3P, glyceraldehyde-3-phosphate; Pyr, pyruvate; PPP, pentose phosphate pathway; PHB, polyhydroxybutyrate; DAP, diaminopimelate; Man, mannose; ED pathway, Entner–Doudoroff pathway; TCA, tricarboxylic acid cycle.

Taken together, not only the developmental cycle is dependent on the Csra regulatory system, but also the life-stage-specific metabolism of *L. pneumophila*. Thereby, Csra is responsible for the upregulation of enzymes in the TCA cycle and the effective uptake and usage of serine. During later growth stages, this central regulator induces the metabolic flow from alternative carbon sources like glucose and more important glycerol, subsequently directing metabolism towards anabolic processes. Furthermore, Csra seems to be a key player in the regulatory network of the bipartite metabolism, predominantly via the regulation of the operon lpp0151-lpp0154, which comprises enzymes at the interface of the two metabolic modules (ED, PPP, gluconeogenesis = module 1; TCA cycle = module 2) (figure 8).

Using labelling experiments with [1,2,3,4-$^{13}$C$_4$]palmitic acid as tracer, we show for the first time the effective usage of a long-chain fatty acid by *L. pneumophila* as nutrient. It primarily serves for the biosynthesis of the storage compound PHB, which is predominantly built in later growth phases [42,43] (figure 9). This metabolic link between β-oxidation
and PHB biosynthesis has also been observed in other bacteria, such as *Pseudomonas putida* [48], and was hypothesized to be present in *L. pneumophila* [15]. The presence of many phospholipases in the genome of *L. pneumophila* might also hint at the utilization of FAs as nutrients. Some of them are virulence factors and are expressed at later growth phase further indicating a life stage specific regulation of fatty acid degradation [49]. Furthermore, short-chain FAs also trigger the developmental switch between the replicative and the transmissive growth phase of *L. pneumophila*, suggesting that fatty acid metabolism and virulence are directly connected [13]. Studies on cell-associated enzymes revealed lipolytic activities, whereby hydrolysis of eukaryotic membrane constituents could be an important virulence factor of *L. pneumophila* [50]. Indeed, the high phospholipase potential that is present in this pathogen might even act as a signal for an important intracellular pathogen might lyse the membrane of its host during major lipolytic activity in *L. pneumophila*, encoding the haemolytic phospholipase A (PlaB), which preferably is involved in the disease development [49]. The cell-associated enzymes revealed lipolytic activities, whereby hydrolysis of eukaryotic membrane constituents could be an important virulence factor of *L. pneumophila* [50]. Indeed, the high phospholipase potential that is present in this pathogen might even act as a signal for the coordination of fatty acid uptake, degradation and simultaneously on PHB biosynthesis during exponential growth.

In summary, palmitic acid predominantly serves as carbon source for PHB biosynthesis in the wt, probably during the later growth phase when amino acids are depleted and the degradation of host glycerolipid may emerge as a major source for carbon in *Legionella*. As a consequence of this metabolic switch, the carbon of glycerol is used to fill up the carbon and energy storage supplies. Both are crucial to survive in the extracellular environment or might even act as a signal for *L. pneumophila* to prepare the...
evasion of its host. CsrA seems to act as an organizer for the switch between the usages of the different carbon source during the life cycle. During replication, CsrA supports that serine and probably other amino acids are used as energy and carbon source for the TCA cycle (module 2), concomitantly repressing glycerol usage and the production of PHB. In later growth phases, this inhibitory effect is reduced due to binding of CsrA to the sRNAs RsmX,Y,Z, leading to an increased metabolism of glycerol, which is together with glucose now the main source for the PPP (module 1), and an enhanced carbon flux via fatty acid degradation into PHB biosynthesis. Thus CsrA not only regulates the switch from replicative to transmission competent bacteria, but it governs in parallel the regulation of the bipartite metabolism of \textit{L. pneumophila} and a metabolic switch from amino acid to carbohydrate/glycerolipid metabolism.

4. Material and methods

4.1. Bacteria, cells and growth conditions

In this study, the \textit{L. pneumophila} Paris wt strain and its isogenic csrA\textsuperscript{−} mutant strain \cite{35} were grown in ACES-buffered yeast extract broth or an ACES-buffered charcoal-yeast extract agar under aerobic conditions at 37°C. For labelling experiments, bacteria were grown at 37°C in a carbon enriched minimal defined media (CE MDM-all components, except Fe-pyrophosphate) are dissolved in 950 ml ddH\textsubscript{2}O. pH was adjusted to 6.5 using KOH. Then Fe-pyrophosphate was dissolved and filled up to 1 l) (electronic supplementary material, table S11), as reported previously \cite{17}. The csrA\textsuperscript{−} strain of \textit{L. pneumophila} Paris was constructed by inserting an apramycin-resistance cassette after the amino acid Tyr\textsuperscript{48} of the lpp0845 gene encoding the major CsrA in \textit{L. pneumophila} Paris \cite{35}.

4.2. Oxygen consumption experiments

\textit{Legionella pneumophila} Paris wt and csrA\textsuperscript{−} mutant were grown to exponential phase (OD\textsubscript{600} = 2–2.5) in BYE, at 37°C and 170 rpm in a light-protected and humidity-controlled incubator shaker. After centrifugation, bacteria were resuspended in PBS (pH 7.4) to a final concentration of OD\textsubscript{600} = 0.1 and 90 μl of the diluted cells were transferred to wells of the poly-D-lysine (PDL)-coated Microplate. To coat XF Cell Culture Microplate (Seahorse Bioscience), 15 μl of 1 mg ml\textsuperscript{-1} PDL in 100 mM Tris–HCl (pH 8.4) was added to each well, followed by overnight drying, and two ddH\textsubscript{2}O rinses. PDL treatment has been shown to not significantly alter respiration in \textit{Escherichia coli} \cite{52}. Cells were attached by 10 min centrifugation at 4000 rpm in a benchtop swinging bucket centrifuge and afterwards the volume in each well was raised to 175 μl PBS. Bacterial respiration, expressed as oxygen consumption rates (OCR), was quantified using an XF24 Extracellular Flux Analyzer (Seahorse Bioscience) according to the manufacturer’s instructions. Basal OCR was measured for approximately 30 min to acquire a baseline prior to the injection of compounds. The different substrates were solubilized in PBS and 25 μl were injected to the reaction chamber at final concentrations of L-serine, L-alanine and L-glutamate: 0.1 g 1\textsuperscript{-1}; D-glucose, glycerol, butanoate, α-ketoglutarate and pyruvate: 0.2 g 1\textsuperscript{-1}; palmitate, oleate and arachidonic acid: 0.1 mM.

4.3. Isotopologue labelling experiments of \textit{L. pneumophila}

\textsuperscript{13}C-labelled precursors used in this study ([U-\textsuperscript{13}C\textsubscript{3}]serine, [U-\textsuperscript{13}C\textsubscript{3}]glucose, [U-\textsuperscript{13}C\textsubscript{3}]glycerol and [1,2,3,4-\textsuperscript{13}C\textsubscript{4}]palmitic acid) were ordered from Sigma-Aldrich. For labelling experiments, \textit{L. pneumophila} wt and csrA\textsuperscript{−} strains were grown in CE MDM medium, whereas the amount of unlabelled serine, glucose and glycerol was displaced with 6 mM [U-\textsuperscript{13}C\textsubscript{3}]serine, 11 mM [U-\textsuperscript{13}C\textsubscript{3}]glucose and 50 mM [U-\textsuperscript{13}C\textsubscript{3}]glycerol, respectively. For labelling experiments with [1,2,3,4-\textsuperscript{13}C\textsubscript{4}]palmitic acid, bacteria were grown in CE MDM supplemented with additional 0.02% of labelled palmitic acid (0.8 mM). For these experiments, the respective \textit{L. pneumophila} strain was grown over night in 50 ml unlabelled CE MDM medium. Afterwards, the inoculum was suspended in 50 ml of CE MDM comprising the respective \textsuperscript{13}C-tracer and diluted to an OD\textsubscript{600} of 0.1. For every labelling experiment, bacteria were harvested at E and PE growth phase by centrifugation at 5000g for 5 min at 4°C. Cells were autoclaved for 30 min at 120°C, freeze-dried and stored at −80°C until further analysis.

4.4. Preparation of protein-derived amino acids, diaminopimelic acid and 3-hydroxybutyrate (derived from poly-hydroxybutyrate) for GC/MS analysis

For isotopologue profiling of protein-derived amino acid, DAP and PHB, 1 mg of the freeze-dried cell pellet was resolved in 0.5 ml of 6 N H\textsubscript{2}C. Sample was subsequently incubated at 105°C for 24 h, as described earlier \cite{24}. Next, the H\textsubscript{2}C was removed under a stream of nitrogen at 70°C and the remaining residue was subsequently resolved in 200 μl acetic acid. Sample was purified using a cation exchange column of Dowex 50Wx8 (H\textsuperscript{+} form, 200–400 mesh, 5 × 10 mm), which was previously washed with 1 ml of MeOH and 1 ml ddH\textsubscript{2}O. Column was evolved with 2 ml of ddH\textsubscript{2}O (eluate 1) and 1 ml of 4 M ammonium hydroxide (eluate 2). Both samples were dried under a stream of nitrogen at 70°C. The remaining residue of eluate 1 was used for further analysis of PHB, whereas the residue of eluate 2 comprises protein-derived amino acids and DAP.

For derivatization of 3-hydroxybutyrate, derived from hydrolysis of PHB with H\textsubscript{2}C, dried samples of eluate 1 were dissolved in 100 μl of N-methyl-N-(trimethylsilyl)-trifluoroacetamide (Sigma). Afterwards, samples were incubated at 60°C in a shaking incubator at 110 rpm overnight. The resulting TMS derivative (TMS) of 3-hydroxybutyrate was used in following GC/MS analysis.

For analysis of protein-derived amino acids and DAP, the remaining residue of eluate 2 was resolved in a mixture of 50 μl dry acetonitrile and 50 μl N-(tert-butyl(dimethyl)silyl)-N-methyl-trifluoroacetamide (Sigma) and incubated at 70°C for 30 min. The resulting TBDMS derivatives were used in following GC/MS analysis. Owing to degradation by acid hydrolysis, the amino acids tryptophan, arginine, methionine and cysteine could not be analysed. Furthermore, acid hydrolysis led to the conversion of glutamine and asparagine to glutamate and aspartate. Therefore, results for aspartate and glutamate correspond to asparagine/aspartate and glutamine/glutamate, respectively.
4.5. Preparation of lactate and stearate for GC/MS analysis

For isotopologue profiling of LACT and STE, 5 mg of the freeze-dried cell pellet was resolved in 1 ml of cold MeOH. Then 800 mg of glass beads (0.25–0.05 mm) were added and mechanical cell lysis and extraction occurred using a ribolyser system (Hybaid) for 3 × 20 s at 6.5 m s⁻¹. Afterwards, samples were centrifuged at 2300g for 10 min and the supernatant was dried under a stream of nitrogen. For analysis of LACT and STE, the remaining residue of the supernatant was resolved in a mixture of 50 μl dry acetonitrile and 50 μl N-(tert-butyldimethylsilyl)-N-methyl-trifluoroacetamide (Sigma) and incubated at 70 °C for 1 h. The resulting TBDMS derivatives were used for GC/MS analysis.

4.6. Preparation of mannose for GC/MS analysis

For isotopologue profiling of mannose, 5 mg of the freeze-dried sample was resolved in 0.5 ml methanolic HCl (3 N) and incubated overnight at 80 °C. After cooling down the sample, the supernatant was dried under a stream of nitrogen at 25 °C. Next, 1 ml acetone containing 20 μl concentrated H₂SO₄ was added and derivatization occurred at 25 °C for 1 h. Then 2 ml of saturated NaCl and 2 ml saturated Na₂CO₃ solution was added and extraction of this aqueous solution was done 2 × with 3 ml ethyl acetate. The combined organic phase was dried under a stream of nitrogen. In a second derivatization step, the dry residue was incubated overnight at 60 °C with 200 μl of a 1:1 mixture of dry ethyl acetate and acetic anhydride. For GC/MS analysis, the derivatization reagents were removed and the remaining residue was resolved in 100 μl anhydrous ethyl acetate. Resulting diisopropylidene/acetate derivatives were used for GC/MS analysis.

4.7. Preparation and derivatization of the cell wall sugars glucosamine (GlcN) and muramic acid (Mur) for GC/MS analysis

For isotopologue profiling of GlcN and Mur, 15 mg of the freeze-dried sample was resolved in 0.5 ml of 6 N HCl and incubated overnight at 105 °C. Following, solid components were removed by filtration and the filtrate was dried under a stream of nitrogen. One hundred microlitres of hexamethyldisilazane (HMDS) was added to the remaining residue and derivatization occurred for 3 h at 120 °C. Resulting TBDMS derivatives were used for further GC/MS analysis.

4.8. Gas chromatography/mass spectrometry: analysis and isotopologue profiling

GC/MS analysis was performed with a QP2010 Plus gas chromatograph/mass spectrometer (Shimadzu) equipped with a fused silica capillary column (Equity TM-5; 30 m × 0.25 mm, 0.25 μm film thickness; SUPELCO) and a quadrupole detector working with electron impact ionization at 70 eV. An aliquot (0.1 to 6 μl) of the derivatized samples were injected in 1:5 split mode at an interface temperature of 260 °C and a helium inlet pressure of 70 kPa. Selected ion monitoring was used with a sampling rate of 0.5 s and LaSolution software (Shimadzu) was used for data collection and analysis. All samples were measured three times for technical replicates. ¹³C excess values and isotopologue compositions were calculated as previously described [53]. This comprises (i) the detection of GC/MS spectra of unlabelled derivatized metabolites, (ii) determination of the absolute mass of isotopologue enrichments and distributions of labelled metabolites of the experiment, and (iii) correction of the absolute ¹³C incorporation by subtracting the heavy isotopologue contributions due to the natural abundances in the derivatized metabolites to calculate the isotopologue enrichments and distributions.

For amino acid and diaminopimelate analysis the column was first kept at 150 °C for 3 min after injection of the sample. Then the column was developed with a temperature gradient of 7 °C min⁻¹ to a final temperature of 280 °C, and this temperature was held for further 3 min. The amino acids alanine (6.7 min), glycine (7.0 min), valine (8.5 min), leucine (9.1 min), isoleucine (9.5 min), proline (10.1 min), serine (13.2 min), phenylalanine (14.5 min), aspartate (15.4 min), glutamate (16.8 min), lysine (18.1 min), histidine (20.4 min), tyrosine (21.0 min) and the cell wall component diaminopimelate (23.4 min) were detected and isotopologue calculations were performed with m/z [M-57]⁺ or m/z [M-85]⁺.

For analysis of LACT and STE, the column was kept at 100 °C for 2 min after sample injection. Following, the column was developed with a first gradient of 3 °C min⁻¹ until a final temperature of 234 °C. Subsequently, a second temperature gradient of 1 °C min⁻¹ until a final temperature of 237 °C, and a third temperature gradient of 3 °C min⁻¹ to a final temperature of 260 °C was performed. TBDMS derivatives of LACT (17.8 min) and STE (49.4 min) were detected. Isotopologue calculations were performed with m/z [M-57]⁺.

For diisopropylidene/acetate derivatives of the mannose, the column was kept at 150 °C for 3 min after sample injection. Then the column was developed with a first temperature gradient of 10 °C min⁻¹ to a final temperature of 220 °C, and a second temperature gradient of 50 °C min⁻¹ to a final temperature of 280 °C. The final temperature was held for a further 3 min. Isotopologue calculations were performed with a fragment, which still contains all six C-atoms of mannose (m/z [M-15]⁺).

For analysis of the cell wall components GlcN and Mur the column was first kept at 70 °C for 5 min. Following the column was developed with a temperature gradient of 5 °C min⁻¹ to a final temperature of 310 °C. The final temperature was kept for an additional minute. Isotopologue calculations of the respective TMS derivatives were performed with m/z [M-452]⁺ and m/z [M-434]⁺. Retention times and mass fragments of derivatized metabolites that were used for all isotopologue calculations are documented in electronic supplementary material, table S12.

4.9. Fluorescence-labelled palmitate quantification

Bodipy FL C16 (Thermo Fisher Scientific) was solubilized in DMSO at a concentration of 1 mM. Bacterial cultures of wt and csrA mutant were grown in BYE until reaching exponential phase (OD 2). Then 500 μl were centrifuged for 3 min at 5000g. Pellets were washed once in 1 ml PBS, resuspended in PBS and adjusted to OD 1. Bacterial cells were incubated with 5 μM of Bodipy FL C16 for 10 min at 37 °C. The cells
were pelleted and washed three times with 1 ml PBS before resuspended in 400 µl PBS. Fluorescence was analysed with a MACSQuant flow cytometer (Miltenyi Biotec). Images of fluorescent Bodipy FL C16-labelled bacteria were acquired using an EVOS FL Cell Imaging System (Thermo Fisher) using the GFP LED cube (ex.470/22, em.510/42) at 100× magnification.

Data accessibility. This article has no additional data.

Authors’ contributions. I.H., T.S., N.K. and P.E. carried out the culture and metabolic analyses of the L. pneumophila wt and mutant strains, conducted the data analysis, participated in the design of the study and drafted the manuscript. W.E. participated in the data analysis, writing of the manuscript and design of the study. C.B. conceived the study, participated in the design of the study and the writing of the manuscript. All authors gave final approval for publication.

Competing Interest. The authors declare that they have no competing interests.

Funding. Work in the C.B. laboratory is financed by the Institut Pasteur, the grant no. ANR-10-LABX-62-IBLEID, and Infect-Era project EUGENPATH (ANR-13-IFEC-0003-02) and the Pasteur-Weizmann consortium ‘The roles of non-coding RNAs in regulation of microbial life styles and virulence’. P.E. was supported by the Fondation pour la Recherche Médicale (FRM) grant N° DEQ20120523697. Work in the group of W.E. was supported by the Bundesministerium für Bildung und Forschung (BMBF) through ERA-NET Infect-Era in the context of the EUGENPATH network and the German Research Foundation (DFG; SPP1516, EI 384/11).

References

1. Rowbotham TJ. 1980 Preliminary report on the pathogenicity of Legionella pneumophila for freshwater and soil amoebae. J. Clin. Pathol. 33, 1179–1183. (doi:10.1136/jcp.33.12.1179)

2. Steinert M, Hentschel U, Hacker J. 2002 Legionella pneumophila: an aquatic microbe goes astray. FEMS Microbiol. Rev. 26, 149–162. (doi:10.1111/j.1574-6976.2002.tb00047.x)

3. Fraser DW et al. 1977 Legionnaires’ disease: description of an epidemic of pneumonia. N. Engl. J. Med. 297, 1189–1197. (doi:10.1056/NEJM197712012972201)

4. Horwitz MA, Silverstein SC. 1980 Legionnaires’ disease: a cosmopolitan pathogen? Lancet 2, 721–731. (doi:10.1046/j.1574-2958.1999.08260.x)

5. Hubber A, Roy CR. 2010 Modulation of host cell behaviour. Nat. Rev. Microbiol. 8, 261–283. (doi:10.1038/nrmicro2295)

6. Isberg RR, O’Connor TJ, Heidtman M. 2009 The developmental network of Legionella pneumophila. Annu. Rev. Cell Dev. Biol. 25, 261–283. (doi:10.1146/annurev.cellbio.092908.131722)

7. Isberg RR, O’Connor TJ, Heidtman M. 2009 The Legionella pneumophila replication vacuole: making a cosy niche inside host cells. Nat. Rev. Microbiol. 7, 13–24. (doi:10.1038/nrmicro1967)

8. Robertson P, Abdelhady H, Garduno RA. 2014 The many forms of a pleomorphic bacterial pathogen—the developmental network of Legionella pneumophila. Front. Microbiol. 5, 670. (doi:10.3389/fmicb.2014.00670)

9. Molofsky AB, Swanson MS. 2004 Differentiate to thrive: lessons from the Legionella pneumophila life cycle. Mol. Microbiol. 53, 29–40. (doi:10.1111/j.1365-2958.2004.04129.x)

10. Hammer BK, Swanson MS. 1999 Co-ordination of Legionella pneumophila virulence with entry into stationary phase by ppGpp. Mol. Microbiol. 33, 721–731. (doi:10.1046/j.1365-2958.1999.01519.x)

11. Sauer JD, Bachman MA, Swanson, MS. 2005 The phagosomal transporter A couples threonine acquisition to differentiation and replication of Legionella pneumophila in macrophages. Proc. Natl Acad. Sci. USA 102, 9924–9929. (doi:10.1073/pnas.0502767102)

12. Dalebroux ZD, Edwards RL, Swanson MS. 2009 Spot1 governs Legionella pneumophila differentiation in host macrophages. Mol. Microbiol. 71, 640–658. (doi:10.1111/j.1365-2958.2008.06555.x)

13. Edwards RL, Dalebroux ZD, Swanson MS. 2009 Legionella pneumophila couples fatty acid flux to microbial differentiation and virulence. Mol. Microbiol. 71, 1190–1204. (doi:10.1111/j.1365-2958.2008.06593.x)

14. Edwards RL, Jules M, Sahr T, Buchrieser C, Swanson MS. 2010 The Legionella pneumophila LetA/LetS two-component system exhibits rheostat-like behavior. Infect. Immun. 78, 2571–2583. (doi:10.1128/IAI.01107-09)

15. Eisenreich W, Heuner K. 2016 The life stage-specific pathometabolism of Legionella pneumophila. FEBS Lett. 590, 3868–3886. (doi:10.1002/1873-4638.12126)

16. Gillmaier N et al. 2016 Growth-related metabolism of the carbon storage poly-3-hydroxybutyrate in Legionella pneumophila. J. Biol. Chem. 291, 6471–6482. (doi:10.1074/jbc.M115.693481)

17. Haaszek I, Manske C, Goebel W, Eisenreich W, Hilbi H. 2016 Pathway analysis using 13C-glycerol and other carbon tracers reveals a bipartite metabolism of Legionella pneumophila. Mol. Microbiol. 100, 229–246. (doi:10.1111/mmi.13313)

18. Pine L, George JR, Reeves MW, Harrell WK. 1979 Carbon compounds as energy sources. J. Bacteriol. 138, 115–119.

19. Cazalet C et al. 2004 Evidence in the Legionella pneumophila genome for exploitation of host cell functions and high genome plasticity. Nat. Genet. 36, 1165–1173. (doi:10.1038/ng1447)

20. Chien M et al. 2004 The genomic sequence of the accidental pathogen Legionella pneumophila. Science 305, 1966–1968. (doi:10.1126/science.1099776)

21. Steinert M, Heuner K, Buchrieser C, Albert-Weissenberger C, Glöckner G. 2007 Legionella pathogenicity: genome structure, regulatory networks and the host cell response. Int. J. Med. Microbiol. 297, 577–587. (doi:10.1016/j.ijmm.2007.03.009)

22. Eybert E, Herrmann V, Jules M, Gillmaier N, Lautner M, Buchrieser C, Eisenreich W, Heuner K. 2010 Isotopologue profiling of Legionella pneumophila: role of serine and glucose as carbon substrates. J. Biol. Chem. 285, 22 232–22 243. (doi:10.1074/jbc.M110.128678)

23. Fonseca MV, Swanson MS. 2014 Nutrient salvaging and metabolism in Legionella pneumophila: dependence on the Entner-Doudoroff pathway and connection with intracellular bacterial growth. J. Bacteriol. 192, 2892–2899. (doi:10.1128/JB.01535-09)

24. Moore J, Harper TL, Chandra M, Clesceri LS, Plunkett G. 2001 Legionella pneumophila transcription during intracellular multiplication in human macrophages. Front. Microbiol. 2, 66. (doi:10.3389/fmicb.2011.00060)

25. Tesh MJ, Morse SA, Miller RD. 1983 Intermediary metabolism in Legionella pneumophila: utilization of amino acids and other compounds as energy sources. J. Bacteriol. 154, 1104–1109.

26. Fanosec MV, Swanson MS. 2014 Nutrient salvaging and metabolism by the intracellular pathogen Legionella pneumophila. Front. Cell. Infect. Microbiol. 4, 12. (doi:10.3389/fcimb.2014.00012)

27. Bachman MA, Swanson MS. 2001 RpoS co-operates with other factors to induce Legionella pneumophila virulence in the stationary phase. Mol. Microbiol. 40, 1201–1214. (doi:10.1046/j.1365-2958.2001.02465.x)

28. Dalebroux ZD, Yagi BF, Sahr T, Buchrieser C, Swanson MS. 2010 Distinct roles of ppGpp and DksA in Legionella pneumophila differentiation. Mol. Microbiol. 76, 201–219. (doi:10.1111/j.1365-2958.2010.07094.x)
31. Rasis M, Segal G. 2009 The LetA-RsmYZ-CsrA regulatory cascade, together with RpoS and PmrA, post-transcriptionally regulates stationary phase activation of Legionella pneumophila Icm/Dot effectors. Mol. Microbiol. 72, 995 – 1010. (doi:10.1111/j.1365-2958.2009.06705.x)

32. Sahr T, Brüggemann H, Jules M, Lomma M, Albert-Weissenberger C, Cazalet C, Buchrieser C. 2009 Two small ncRNAs jointly govern virulence and transmission in Legionella pneumophila. Mol. Microbiol. 72, 741 – 762. (doi:10.1111/j.1365-2958.2009.06677.x)

33. Forsbach-Birk V, McNealy T, Shi C, Lynch D, Marre R. 2004 Reduced expression of the global regulator CsrA in Legionella pneumophila affects virulence-associated regulators and growth in Acanthamoeba castellanii. Int. J. Med. Microbiol. 294, 15 – 25. (doi:10.1016/j.ijmm.2003.12.003)

34. Molofsky AB, Swanson MS. 2003 Legionella pneumophila CsrA is a pivotal repressor of transmission traits and activator of replication. Mol. Microbiol. 50, 445 – 461. (doi:10.1046/j.1365-2958.2003.03706.x)

35. Sahr T, Rusniok C, Impens F, Oliva G, Sismeiro O, Coppee JY, Buchrieser C. 2017 The Legionella pneumophila genome evolved to accommodate multiple regulatory mechanisms controlled by the CsrA-system. PLoS Genet. 13, e1006629. (doi:10.1371/journal.pgen.1006629)

36. Valkuskas CA, Potts AH, Babitzke P, Ahmer BM, Romero T. 2015 Regulation of bacterial virulence by Csr (Rom) systems. Microbiol. Mol. Biol. Rev. 79, 193 – 224. (doi:10.1128/MMBR.00052-14)

37. Al-Khodr S, Kalachikov S, Morozova I, Price CT, Abu Kwaik Y. 2009 The PmrA/PmrB two-component system of Legionella pneumophila is a global regulator required for intracellular replication within macrophages and protozoa. Infect. Immun. 77, 374 – 386. (doi:10.1128/IAI.00181-08)

38. Hovel-Miner G, Pampou S, Faucher SP, Clarke M, Morozova I, Morozow P, Russo JJ, Shuman HA, Kalachikov S. 2009 SigmaS controls multiple pathways associated with intracellular replication of Legionella pneumophila. J. Bacteriol. 191, 2461 – 2473. (doi:10.1128/JB.01578-08)

39. Tiaden A, Sping T, Carranza P, Brüggemann H, Riedel K, Eberl L, Buchrieser C, Hilbi H. 2008 Synergistic contribution of the Legionella pneumophila fps genes to pathogen-host interactions. J. Bacteriol. 190, 7532 – 7547. (doi:10.1128/JB.01002-08)

40. Tiaden A, Sping T, Weber SS, Brüggemann H, Bosshard R, Buchrieser C, Hilbi H. 2007 The Legionella pneumophila response regulator LqsR promotes host cell interactions as an element of the virulence regulatory network controlled by RpoS and LetA. Cell Microbiol. 9, 2903 – 2920. (doi:10.1111/j.1462-5822.2007.01005.x)

41. George JR, Pine L, Reeves MW, Harrell WK. 1980 Amino acid requirements of Legionella pneumophila. J. Clin. Microbiol. 11, 286 – 291.

42. Garduno RA, Garduno E, Hilzt M, Hoffman PS. 2002 Intracellular growth of Legionella pneumophila gives rise to a differentiated form dissimilar to stationary-phase forms. Infect. Immun. 70, 6273 – 6283. (doi:10.1128/IAI.70.11.6273-6283.2002)

43. James BW, Mauchline WS, Dennis PJ, Keevil CW, Wait R. 1999 Poly-3-hydroxybutyrate in Legionella pneumophila: an energy source for survival in low-nutrient environments. Appl. Environ. Microbiol. 65, 822 – 827.

44. Poirier Y, Erard N, MacDonald-Comber Petitet J. 2002 Synthesis of poly(3-hydroxyalkanoate) in Legionella pneumophila, an energy source for survival in low-nutrient environments. Appl. Environ. Microbiol. 65, 822 – 827.

45. Steinbächel A, Schlegel HG. 1991 Physiology and molecular genetics of poly(beta-hydroxy-alkanoic acid) synthesis in Alcaligenes eutrophus. Mol. Microbiol. 5, 535 – 542. (doi:10.1111/j.1365-2958.1991.tb00725.x)

46. Daniel J, Maamor H, Deb C, Sirakova TD, Kolattukudy PE. 2011 Mycobacterium tuberculosis uses host triacylglycerol to accumulate lipid droplets and acquires a dormancy-like phenotype in lipid-loaded macrophages. PLoS Pathog. 7, e1002093. (doi:10.1371/journal.ppat.1002093)

47. Van Assche E, Van Puyvelde S, Vanderleyden J, Steenackers HP. 2015 RNA-binding proteins involved in post-transcriptional regulation in bacteria. Front. Microbiol. 6, 141. (doi:10.3389/fmicb.2015.00141)

48. Huijbers GN, de Rijk TC, de Waard P, Eggink G. 1994 13C nuclear magnetic resonance studies of Pseudomonas putida fatty acid metabolic routes involved in poly(3-hydroxyalkanoate) synthesis. J. Bacteriol. 176, 1661 – 1666. (doi:10.1128/jb.176.6.1661-1666.1994)

49. Kuhle K, Flieger A. 2013 Legionella phospholipases implicated in virulence. Curr. Top. Microbiol. Immunol. 376, 175 – 209. (doi:10.1007/81_2013_348)

50. Bender J, Rydzewski K, Breiich M, Schunder E, Heuner K, Flieger A. 2009 Phospholipase PlaB of Legionella pneumophila represents a novel lipase family: protein residues essential for lipolytic activity, substrate specificity, and hemolysis. J. Biol. Chem. 284, 187 – 195. (doi:10.1074/jbc.M109.026021)

51. Brüggemann H et al. 2006 Virulence strategies for infecting phagocytes deduced from the in vivo transcriptional program of Legionella pneumophila. Cell Microbiol. 8, 1228 – 1240. (doi:10.1111/j.1462-5822.2006.00703.x)

52. Dwyer DJ et al. 2014 Antibiotics induce redox-related physiological alterations as part of their lethality. Proc. Natl Acad. Sci. USA 111, E2100 – E2109. (doi:10.1073/pnas.1401876111)

53. Eylett E, Schar J, Mertins S, Stoll R, Bacher A, Goebel W, Eisenreich W. 2008 Carbon metabolism of Listeria monocytogenes growing inside macrophages. Mol. Microbiol. 69, 1008 – 1017. (doi:10.1111/j.1365-2958.2008.06357.x)