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Isotopologue Profiling of *Legionella pneumophila*

ROLE OF SERINE AND GLUCOSE AS CARBON SUBSTRATES^{*[§]}

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Legionella pneumophila (Lp) is commonly found in freshwater habitats but is also the causative agent of Legionnaires' disease when infecting humans. Although various virulence factors have been reported, little is known about the nutrition and the metabolism of the bacterium. Here, we report the application of isotopologue profiling for analyzing the metabolism of *L. pneumophila*. Cultures of Lp were supplied with [U-¹³C₃]serine, [U-¹³C₆]glucose, or [1,2-¹³C₂]glucose. After growth, ¹³C enrichments and isotopologue patterns of protein-derived amino acids and poly-3-hydroxybutyrate were determined by mass spectrometry and/or NMR spectroscopy. The labeling patterns detected in the experiment with [U-¹³C₃]serine showed major carbon flux from serine to pyruvate and from pyruvate to acetyl-CoA, which serves as a precursor of poly-3-hydroxybutyrate or as a substrate of a complete citrate cycle with *Si* specificity of the citrate synthase. Minor carbon flux was observed between pyruvate and oxaloacetate/malate by carboxylation and decarboxylation, respectively. The apparent lack of label in Val, Ile, Leu, Pro, Phe, Met, Arg, and Tyr confirmed that *L. pneumophila* is auxotrophic for these amino acids. Experiments with [1,2-¹³C₂]glucose showed that the carbohydrate is also used as a substrate to feed the central metabolism. The specific labeling patterns due to [1,2-¹³C₂]glucose identified the Entner-Doudoroff pathway as the predominant route for glucose utilization. In line with these observations, a mutant lacking glucose-6-phosphate dehydrogenase (Δzwf) did not incorporate label from glucose at significant levels and was slowly outcompeted by the wild type strain in successive rounds of infection in *Acanthamoeba castellanii*, indicating the importance of this enzyme and of carbohydrate usage in general for the life cycle of Lp.

The Gram-negative bacterium *Legionella pneumophila* (Lp)⁵ can be found in freshwater habitats where it replicates within protozoa, mainly amoebae such as *Acanthamoeba castellanii*. Lp can also be transmitted to humans by contaminated aerosols. After entering the human lung, the pathogen is phagocytosed by alveolar macrophages wherein it is able to replicate, leading to Legionnaires' disease, an atypical pneumonia.

Lp survives within amoebae and macrophages because of its ability to establish a replication vacuole that is derived from the endoplasmic reticulum. Once within a vacuole, Lp differentiates into the replicative form. When nutrients become limiting, a regulatory cascade triggers the differentiation to a spore-like mature intracellular form (MIF), the so-called transmissive form, which metabolically seems to be nearly dormant (1–6). These forms exhibit a thickened cell wall and high amounts of cytoplasmic granules of poly-3-hydroxybutyrate (PHB), a general energy and carbon storage compound of bacteria (1, 7, 8). When released from spent hosts, these transmissive forms of Lp are able to persist for long periods in the environment. It has also been shown that Lp is able to differentiate into a "viable but nonculturable" status of greatly reduced metabolic activity after persistence in water (9). Notably, *Legionella* species can also grow in defined culture media under laboratory conditions (10–15). However, less is known about the routes of nutrient utilization during growth in culture media as well as during intracellular multiplication (16–20).

Legionella exhibits a strictly respiratory form of metabolism and does not grow anaerobically (18). The amino acids Arg, Ile, Leu, Val, Met, Ser, and Thr are reportedly essential for growth of Lp in culture (10–12, 14, 15, 21–23), whereas a partial requirement for Cys (or cystine) has also been observed (24). It is also well known that Lp uses amino acids as preferred energy and carbon sources (4, 10–12, 14, 21, 22). More specifically, Ser, Glu, Tyr, and/or Thr are efficiently used as carbon and energy sources *in vitro* (14), and Cys, Gln, Ser, and Arg support growth *in vivo* (19). From *in silico* analysis of the known Lp genome sequences it is proposed that Lp is auxotrophic for the amino acids Cys, Met, Arg, Thr, Val, Ile, and Leu (18, 25, 26). There-

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⁵ The abbreviations used are: Lp, *Legionella pneumophila*; PHB, poly-3-hydroxybutyrate; EMP, Embden-Meyerhof-Parnas; ED, Entner-Doudoroff; GC/MS, gas chromatography/mass spectrometry; PP, pentose phosphate; AYE, ACES-buffered yeast extract; ACES, 2-[(2-amino-2-oxoethyl)amino]ethanesulfonic acid; BCYE, buffered charcoal-yeast extract; CDM, chemically defined medium; CFU, colony forming unit(s); RT, reverse transcriptase; TBDMS, tert-butyldimethylsilyl; WT, wild type.

fore, it is not surprising that *Lp* harbors genes for ~12 classes of ABC (ATP-binding cassette) transporters and amino acid permeases, as well as various different amino peptidases and proteases. In addition, it has been shown that amino acid transporters of the host cell and of *Lp* are essential for intracellular replication; specifically, a neutral amino acid transporter of the host cell (SLC1A5 of MM6 monocyte cells) is necessary for *Lp* to replicate within this host (19). Furthermore, a bacterial Thr transporter (PhtA) is reported to be essential for replication of *Lp* in bone marrow-derived macrophages from mice (27).

In earlier studies, it was also suggested that *Lp* is able to use glucose as a carbon source (14, 15, 20), although the addition of glucose to the medium did not support *in vitro* growth (18, 20). As no active sugar transport could be demonstrated *in vitro* (14, 16, 18), it is generally believed that *Lp* does not utilize sugars as a carbon source but relies on gluconeogenesis (18). On the other hand, some of the various ABC-type transport systems might be involved in sugar uptake, because *Lp* also possesses putative systems for degradation of cellulose, chitin, starch, and glycogen (28). Recently, *Lp* has been shown to actively degrade cellulose (29). On the basis of this evidence it can be assumed that glucose is also catabolized, but the metabolic routes are still unknown.

The four sequenced genomes of *Lp* (*Lp* Philadelphia (26), *Lp* Paris, *Lp* Lens (25), and *Lp* Corby (30)) indicate the presence of the Embden-Meyerhof-Parnas (EMP) pathway as well as the Entner-Doudoroff (ED) pathway. However, *in vitro* enzyme assays did not detect activities for the ED pathway in strains Knoxville-1 and Philadelphia-1 (17, 20). Genes encoding the pentose phosphate (PP) pathway are also present, with the exception of 6-phosphogluconate dehydrogenase and the transaldolase (16, 18, 25, 26).

As the activity of pyruvate dehydrogenase was low in cell-free extracts (17, 20), it was hypothesized that the bulk of the acetyl-CoA entering the citrate cycle or used as precursor for the storage compound PHB is derived from fatty acid catabolism (18). Indeed, *Lp* possesses huge amounts of phospholipases exerting extracellular and cell-associated activities (31–33). However, the glyoxylate shunt appears to be absent in the *Lp* strains sequenced thus far.

One powerful method of studying metabolic pathways in growing microbes is based on incorporation experiments with stable isotope-labeled precursors (e.g. ^{13}C -labeled glucose) followed by the determination of the resulting isotopologue patterns in key metabolites, such as protein-derived amino acids or other storage compounds. Using stoichiometric models, isotopologue profiles can serve as constraints in metabolic flux calculations (^{13}C -based metabolic flux analysis) (34). Metabolic flux analysis is now well established for the analysis of metabolic flux in microorganisms growing under standardized conditions (reviewed in Refs. 34 and 35). These models typically rely on the use of minimal media with only one possible carbon source. It is therefore difficult to adapt metabolic flux analysis calculations to organisms with complex multiple carbon usage (*i.e.* when growing in complex media). However, because of the specificity of the detected isotopologue profiles, observation-driven analysis can also trace metabolic pathways in cases with unknown and/or multiple carbon usage (36, 37). In this study, the metab-

olism of *Lp* grown under culture conditions was analyzed for the first time by ^{13}C -labeled isotopologue (^{13}C -isotopologue) profiling using glucose or Ser as precursors.

EXPERIMENTAL PROCEDURES

Strains, Growth Conditions, and Media—*A. castellanii* ATCC30010 was cultured in PYG 712 medium (2% proteose-peptone, 0.1% yeast extract, 0.1 M glucose, 4 mM MgSO_4 , 0.4 M CaCl_2 , 0.1% sodium citrate dihydrate, 0.05 mM $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \times 6 \text{ H}_2\text{O}$, 2.5 mM NaH_2PO_4 , and 2.5 mM K_2HPO_4) at 20 °C. *Escherichia coli* DH5 α was used to clone recombinant plasmid DNA. Experiments were done with *L. pneumophila* Paris (CIP 107629 (25)). As described previously (28), *Lp* strains were cultured in AYE medium (ACES-buffered yeast extract broth: 10 g of ACES, 10 g of yeast extract, 0.4 g of L-cysteine, and 0.25 g of iron pyrophosphate in 1 liter (pH 6.8)) (see also [supplemental Table S1](#)) or on ACES-buffered charcoal-yeast extract (BCYE) agar plates at 37 °C. Alternatively, *Lp* Paris was cultivated in a chemically defined medium (CDM) (for details see [supplemental Table 1](#)) adapted from Ristroph *et al.* (12).

^{13}C Labeling Experiments—1 liter of growth medium (AYE or CDM) was supplemented with 2 g of [$\text{U-}^{13}\text{C}_6$]glucose, 2 g of [$1,2\text{-}^{13}\text{C}_2$]glucose, or 0.3 g of [$\text{U-}^{13}\text{C}_3$]Ser. 500 ml of the supplemented AYE medium was inoculated with 1 ml of an overnight culture of *Lp* Paris. For supplemented CDM the inoculum was 4 ml of an overnight culture grown in AYE medium. Incubation was carried out at 37 °C and 220 rpm, and the optical density at 600 nm (A_{600}) was determined at regular intervals. An A_{600} of 1.0 was determined as exponential growth, whereas an A_{600} of ~2.0 correlated with stationary growth. Cultures in AYE medium reached exponential growth after 16 h and stationary growth at 29 h. Cultures grown in CDM became stationary at 40 h. Before harvesting, a culture aliquot was plated on LB agar plates to rule out the possibility of contamination. The bacteria were killed with sodium azide at a final concentration of 10 mM and pelleted at $5500 \times g$ and 4 °C for 15 min. The pellet was washed twice with 200 ml of water and then once with 2 ml of water. The supernatant was discarded, and the bacterial pellet was autoclaved at 120 °C for 20 min.

Strain Construction—The *lpp0483* mutant strain of *Lp* Paris (Δzwf) was constructed as described previously (28, 38). In brief, the gene *lpp0483* (*zwf*) was inactivated by insertion of a kanamycin resistance (kan^R) cassette into the chromosomal gene. The chromosomal region containing the *lpp0483* gene was PCR-amplified with the primers *lpp0483_for* and *lpp0483_rev*, and the product (2639 bp) was cloned into the pGEM-T Easy vector (Promega). On this template, an inverse PCR was performed using the primers *lpp0483_inv_for* and *lpp0483_inv_BamHI_rev*, with the reverse primer bearing a BamHI restriction site. These primers amplified 4912 bp corresponding to the pGEM backbone and the flanking regions of *lpp0483*. The resulting PCR product was BamHI-digested and ligated to the kan^R cassette (1210 bp amplified via PCR from the plasmid pGEM-Kan R subcloned into a pGEM-T Easy vector) using primers containing BamHI restriction sites at the ends (*Kan_BamHI_for* and *Kan_BamHI_rev*). All primers are listed in Table 1. For chromosomal recombination, the construct (*i.e.* PCR fragment containing the kan^R cassette with flanking

regions of the gene of interest, ~900 bp upstream and ~250 bp downstream) was introduced into the *Lp* Paris strain by transformation. Three independent Δzwf mutant strains were generated, and two of them were used for intracellular replication assays.

Intracellular Multiplication in *A. castellanii*—For *in vivo* growth of *Lp* Paris and its derivatives in *A. castellanii*, we followed a protocol described previously (28). In brief, 3-day-old cultures of *A. castellanii* were washed in AC buffer (PYG 712 medium without proteose-peptone, glucose, and yeast extract) and adjusted to 5×10^5 cells. Stationary phase *Legionella* bacteria grown on BCYE agar were diluted in water and mixed with *A. castellanii* at a multiplicity of infection of 0.01. After invasion for 1 h at 37 °C, the *A. castellanii* layer was washed twice, defining the start point of the time course experiment. The number of colony forming units (CFU) of legionellae was determined by plating on BCYE agar. Each infection was carried out in duplicates and was done at least three times.

Intracellular Multiplication/Survival in *A. castellanii*—The intracellular multiplication was carried out as described above but without the washing step. After 3 days, *A. castellanii* cells were resuspended, 100- μ l aliquots were lysed, and serial dilutions were spread on BCYE agar to determine the number of CFU. To study the replication rates in repeating rounds of infection, the remaining solution was incubated at 37 °C for a further 3 days and diluted (1:1000). The number of CFU was determined by plating the remaining solution on BCYE agar. 1 ml of the remaining dilution was used to reinfect fresh amoeba cultures as described previously. Four rounds of infection were performed in total, and each infection was carried out in duplicates and done at least three times.

Intracellular Multiplication/Survival Assay in "Competition"—The infection procedure was similar to the assay described above, but equal amounts of bacteria of the wild type and the Δzwf mutant strain (kanamycin resistant) were used together to co-infect the *A. castellanii* cells. After 3 days, *A. castellanii* cells were resuspended, 100- μ l aliquots were lysed, and serial dilutions were spread on BCYE agar with and without kanamycin to determine the number of CFU. The remaining infection solution was incubated at 37 °C for a further 3 days and diluted (1:1000). The resulting solution was used to determine the number of CFU on BCYE with and without kanamycin and to reinfect fresh amoebae. Four rounds of infection were performed in total. To determine the number of wild type bacteria, the CFU on BCYE-kanamycin agar was subtracted from the CFU on BCYE plates without kanamycin. In the survival assay, the mixture of the first infection was incubated for a further 19 days. To follow the Δzwf and WT strain recovery capabilities, each sample was plated (100 μ l) on BCYE and/or BCYE-kanamycin plates. Each infection was carried out in duplicates and was done at least three times.

RT-PCR—Total RNA was extracted from bacteria grown in AYE medium to the appropriate growth phase, incubated with Dnase I, and then repurified. RT-PCR reactions were performed with a OneStep RT-PCR kit (Qiagen) using gene-specific primers. The RT reaction was carried out at 50 °C for 30 min with 0.5 μ g of total RNA. PCR amplification was performed with each primer at 0.6 μ M and each dNTP at 400 μ M in

TABLE 1
Primers used in this study

Oligonucleotide	Sequence
PCR	
lpp0483_for	TACATTGAGAAAAAGCGAAGCCAA
lpp0483_rev	TGCTGTTTAATAATCGCCTTTTCGA
lpp0483_inv_for	ACAGCCTTATAATATCTTTC
lpp0483_inv_BamHI_rev	CGGGATCCCGTTAATTTTGATAATCATC
Kan_BamHI_for	CGGGATCCCGCTATCTGGACAAGGGAAAAC
Kan_BamHI_rev	CGGGATCCCGGAAGAACTCCAGCATGAGAT
RT-PCR	
accC-For	GAACTTGGCATTTCAGACTGTTGC
accC-Rev	AGCATGTCCTTTCCCATCACC
edd-For	AGAGCAGCTTATCTGAATCAAATGG
edd-Rev	AAAACCTGCAAAACCCCTGA
fadD-For	AATGGAGTACCATGAAATTTGACG
fadD-Rev	CCAGAGGAGATATCCAGGTGTA
fumC-For	AACACGTGTAGAAACAGACAGCATG
fumC-Rev	AGGGGTTTCATGAGAAGCCAGA
pfp-For	CTGGTGGTGTGACCGCTGTAA
pfp-Rev	GGGACTTGGCCTATAGACCATT
pgl-edd-For	CTAGAACAGCTTTCATTCAGAG
pgl-edd-Rev	GAGTACGGTTGATGCGCTTATA
pykA-For	GCCAGTAAGGAACCTGAAATTTCTG
pykA-Rev	CTTCTGCTTCAATTGCTGTACGC
ppsA-For	ACTATAGATTGGACATCTTGGCA
ppsA-Rev	GGGTTTCCAACATTCACATCACT
rpiA-For	AAGAAGCTGGCAGCAATCAACAC
rpiA-Rev	CCAAGGCCATAGGTGTTGAGAA
sucA-For	TTGTCTGGAGGAAGTATGGCTTATG
sucA-Rev	TGAAGGGTTAAACGCCAAGC
zwf-pgl-For	GCCAAAGGAAGTCAACGCTTA
zwf-pgl-Rev	GAGAATCAATTGCGCTATTCACC

1 \times OneStep RT-PCR buffer containing 12.5 mM MgCl₂ and 2 μ l of OneStep RT-PCR enzyme mix. The total volume was 50 μ l. The cycling conditions were 94 °C for 1 min, 53–55 °C for 1 min, and 72 °C for 1 min for 25 to 35 cycles with a Thermocycler. The following gene-specific primer pairs were used: accC-For and accC-Rev; edd-For and edd-Rev; fadD-For and fadD-Rev; fumC-For and fumC-Rev; pfp-For and pfp-Rev; pykA-For and pykA-Rev; ppsA-For and ppsA-Rev; rpiA-For and rpiA-Rev; and sucA-For and sucA-Rev. All primers used for RT-PCR are listed in Table 1.

Protein Hydrolysis and Amino Acid Derivatization—Bacterial cell mass was suspended in 6 M hydrochloric acid and heated at 105 °C for 24 h under an inert atmosphere. The hydrolysate was placed on a cation exchange column of Dowex 50W \times 8 (H⁺ form, 200–400 mesh, 5 \times 10 mm) that was washed with water and developed with 2 M ammonium hydroxide. An aliquot of the eluate was dried under a stream of nitrogen, and the residue was dissolved in 50 μ l of water-free acetonitrile. A mixture of 50 μ l of *N*-(*tert*-butyldimethylsilyl)-*N*-methyl-trifluoroacetamide containing 1% *tert*-butyldimethylsilylchloride (Sigma) was added. The mixture was kept at 70 °C for 30 min. The resulting *N*-(*tert*-butyldimethylsilyl) (TBDMS)-amino acids were then analyzed by GC/MS.

Dichloromethane Extraction and Isolation of Amino Acids—The dried sample was heated under reflux with 10 ml of dichloromethane/100 mg of sample for 1 h. After filtration the filtrate was evaporated.

The filtered residue was dried and hydrolyzed with 6 M hydrochloric acid containing 0.5 mM thioglycolic acid. The mixture was boiled for 24 h under an inert atmosphere and then filtered. The solution was concentrated to a small volume under reduced pressure and lyophilized. The residue was dissolved in 8 ml of water. The solution was placed on top of a column of

Dowex 50W×8 (H⁺ form, 3 × 33 cm). The column was washed with 300 ml of water and then was developed with a linear gradient of 0–3 M hydrochloric acid (total volume, 2 liters). Fractions were collected, combined, evaporated to a small volume under reduced pressure, and lyophilized (36).

Mass Spectrometry—GC/MS analysis was performed on a GC-17A gas chromatograph and/or GC 2010 (Shimadzu, Duisburg, Germany) equipped with a fused silica capillary column (Equity TM-5; 30 m × 0.25 mm, 0.25 μm film thickness; SUPELCO, Bellefonte, PA) and a QP-5000 and/or GC-QP2010 plus mass selective detector (Shimadzu) working with electron impact ionization at 70 eV. An aliquot (1 μl) of a solution containing TBDMS amino acids was injected in a 1:10 split mode at an interface temperature of 260 °C and a helium inlet pressure of 70 kilopascals. The column was developed at 150 °C for 3 min and then with a temperature gradient of 10 °C/min to a final temperature of 280 °C that was held for 3 min. Data were collected using Class 5000 and/or GCMS Solution software (Shimadzu). Selected ion monitoring data were acquired using a 0.3-s sampling rate. Samples were analyzed at least three times. The theoretical isotope ratio and numerical deconvolution of the data were computed according to standard procedures: (i) determination of the “TBDMS derivate” spectrum of TBDMS-amino acid, (ii) determination of the mass isotopomer distribution of the labeled amino acid, and (iii) correction for incorporation of ¹³C from natural abundance into that amino acid (39).

NMR Spectroscopy—¹H and ¹³C NMR spectra were recorded at 25 °C using a DRX-500 spectrometer (Bruker Instruments, Karlsruhe, Germany) at transmitter frequencies of 500.1 and 125.6 MHz, respectively. Extracts with dichloromethane were dissolved in CDCl₃, and amino acids were measured in 0.1 M DCl. ¹³C enrichments were determined by quantitative NMR spectroscopy. For this purpose, ¹³C NMR spectra of the biolabeled specimens and of samples with natural ¹³C abundance (*i.e.* with 1.1% ¹³C abundance) were measured under the same experimental conditions. The ratios of the signal integrals of the labeled compounds and of the compounds at natural abundance were then calculated for each respective carbon atom. Absolute ¹³C abundances for certain carbon atoms (*i.e.* for carbon atoms with at least one attached hydrogen atom displaying a ¹H NMR signal in an uncrowded region of the spectrum) were determined from the ¹³C coupling satellites in the ¹H NMR spectra. The relative ¹³C abundances determined for all other positions were then referenced to this value, thus affording absolute ¹³C abundances for every single carbon atom. ¹³C-coupled satellites were integrated separately. The relative fractions of each respective satellite pair (corresponding to a given coupling pattern) in the total signal integral of a given carbon atom were calculated. These values were then referenced to the global ¹³C abundance affording concentrations of multiple ¹³C-labeled isotopologue groups (mol %).

RESULTS

Growth of *Lp* in Culture under Standardized Conditions—Cultures of *Lp* can be grown in various media although not in a medium that comprises only one possible carbon source. Typically, so-called AYE medium is used, which consists of yeast extract and ACES buffer and high amounts of iron ions and

TABLE 2

NMR data of ¹³C-labeled PHB obtained from incorporation experiments with *Lp*

Carbon	Chemical shift	¹³ C ¹³ C coupling constant ^a
	ppm	Hz
1	169.10	58.1 (2)
2	40.75	58.1 (1); 39.5 (3)
3	67.55	39.4 (2); 39.0 (4)
4	19.72	38.9 (3)

^a Coupling partners are indicated in parentheses.

TABLE 3

Abundances of carbon isotopologues of PHB obtained from incorporation experiments with *Lp*

<i>Lp</i> experiment	Isotopologue abundance		
	1,2- ¹³ C ₂	3,4- ¹³ C ₂	U- ¹³ C ₄
		mol %	
<i>Lp</i> WT (serine)	3.02	2.97	0.4
<i>Lp</i> WT (glucose)	5.17	5.25	0.98
<i>Lp</i> Δ <i>zwf</i> (glucose)	0.6	0.6	

cysteine. However, it is also possible to use a chemically defined medium consisting of ACES, 16 amino acids (not included are Ala, Gly, Asn, and Gln), ammonium chloride, and some inorganic salts (again including high amounts of iron ions; for details, see [supplemental Table S1](#)). To estimate the impact of the culture medium on the growth and metabolism of *Lp*, we cultivated the bacterium for 2 days at 37 °C in duplicates in 500 ml of AYE medium or CDM, each supplemented with 11 mM glucose. At timed intervals, the optical density at 600 nm (*A*₆₀₀) and the CFU were determined. *Lp* Paris had a generation time of 2.7 h in AYE medium and 4.4 h in CDM, and the final cell densities (optical density) were ~2.1 and 1.4, respectively (data not shown).

Serine Serves as a Major Carbon Substrate for *Lp*—In earlier studies, Ser was shown to support the growth of *Lp* (10, 14, 15), and high activity levels of Ser dehydratase converting Ser into pyruvate were detected in cell-free extracts of the bacterium (20). To analyze the metabolic fate of Ser in more detail, we added 3 mM [U-¹³C₃]Ser to the AYE medium (for details, see “Experimental Procedures”) and grew *Lp* for 29 h until the cells entered stationary phase (*A* = 2.1 at 600 nm). PHB and amino acids were extracted from the cells and analyzed by GC/MS and/or quantitative NMR spectrometry (for details, see “Experimental Procedures”).

NMR Analysis of the Dichloromethane Extract—The ¹³C NMR spectrum of the dichloromethane extract showed four intense signals due to PHB ([supplemental Fig. S1A](#); Table 2). Notably, the spectrum did not display signals of lipids or fatty acids at significant concentrations. All PHB signals showed pairs of satellites due to ¹³C¹³C couplings at high intensities. The quantitative signal analysis (Table 3) showed that ~3 mol % of PHB was multiply ¹³C-labeled and therefore derived from the supplied [U-¹³C₃]Ser or a multiply ¹³C-labeled downstream product. The coupling pattern (*i.e.* the size of the coupling constants for each carbon signal; Table 2) clearly showed that [1,2-¹³C₂]- and [3,4-¹³C₂]PHB were the predominant multiply ¹³C-labeled species. The isotopologue pattern can be explained by the well known mechanisms of PHB formation (7, 8) from [1,2-¹³C₂]acetyl-CoA and unlabeled acetyl-CoA (from the degrada-

tion of unlabeled components present in AYE medium) affording [1,2- $^{13}\text{C}_2$]acetoacetyl-CoA or [3,4- $^{13}\text{C}_2$]acetoacetyl-CoA (Fig. 1). Reduction yielded 3-[1,2- $^{13}\text{C}_2$]- or 3-[3,4- $^{13}\text{C}_2$]hydroxybutyryl-CoA, respectively, which was then condensed to PHB with the detected isotopologue composition. The minor U- $^{13}\text{C}_4$ -isotopologue in PHB can be explained by the statistical combination of two molecules of [1,2- $^{13}\text{C}_2$]acetyl-CoA.

GC/MS Analysis of Protein-derived Amino Acids—GC/MS analysis showed that 25% of the protein-derived Ser carried ^{13}C label (Fig. 2, gray bar; see numerical values in supplemental Table S3). Not unexpectedly, this finding documented that exogenous Ser was efficiently incorporated into *Lp* and was used for protein biosynthesis. The high ^{13}C enrichment values of other amino acids (3–12%; Ala > Glu > Gly = Asp; Fig. 2, gray bars) as well as the ^{13}C enrichment in PHB (3%) indicated that Ser was also shuffled into the central carbon metabolism of *Lp* where it was converted into pyruvate/Ala, oxaloacetate/Asp, and α -ketoglutarate/Glu, as well as into acetyl-CoA serving as a precursor of PHB. However, the ^{13}C enrichments of those metabolites were significantly lower (3–12%) than that of Ser (25%) suggesting (i) that higher fractions of Ala, Glu, Gly, and Asp were incorporated from the medium (*i.e.* in unlabeled form) as compared with Ser; and/or (ii) as expected, that Ser was not the unique carbon source for *Lp* under the experimental conditions and that, next to ^{13}C -labeled Ser, unlabeled carbon substrates were shuffled into the biosyn-

thetic pathways of Ala, Glu, Gly, and Asp. The lack of label in Ile, Leu, Val, and Tyr showed that these amino acids were derived from unlabeled amino acids present in the medium and/or were not synthesized *de novo*. Albeit with high standard deviations, a slight ^{13}C enrichment was detected in Pro, Thr, and Tyr. It remains open as to whether these amino acid were made *de novo* under the experimental conditions (16, 18).

The mass data also revealed the abundances of ^{13}C -labeled isotopologues comprising a given number of ^{13}C atoms (Fig. 2, patterned columns; see numerical values in supplemental Table S4). Ser and Ala were characterized by $^{13}\text{C}_3$ -isotopologues, whereas Asp and Glu were more complex mixtures of species comprising one, two, and three ^{13}C -labeled atoms (Fig. 2). Gly was a mixture of $^{13}\text{C}_1$ - and $^{13}\text{C}_2$ -isotopologues.

NMR Analysis of Protein-derived Amino Acids—To localize the label distribution at higher resolution, purified amino acids were also analyzed by quantitative NMR spectroscopy (36). Many of the ^{13}C NMR signals were multiplets caused by couplings between adjacent ^{13}C atoms in a given molecule. As examples, ^{13}C NMR signals of the C-3 of Asp and the C-2 of Ala are shown in supplemental Fig. S2. From the signal intensities of the satellites, the molar abundances of the underlying isotopologues were calculated and referenced to the values obtained by mass spectrometry. The coupling patterns of all labeled amino acids are shown graphically in Fig. 3 with bars connecting ^{13}C -labeled atoms in multiple ^{13}C -labeled isotopologues (see also supplemental Table S5).

The labeling patterns of Ser and Ala were characterized by a high abundance of the triple ^{13}C -labeled isotopologues and minor amounts of 1,2- $^{13}\text{C}_2$ -isotopologues. The triple ^{13}C -labeled isotopologues of Ser and Ala can be explained easily by the direct incorporation of exogenous [U- $^{13}\text{C}_3$]Ser into the protein-derived Ser and Ala fractions, respectively. The metabolic precursor for [U- $^{13}\text{C}_3$]Ala is [U- $^{13}\text{C}_3$]pyruvate. It appears safe to assume that the later compound is derived from [U- $^{13}\text{C}_3$]Ser by Ser dehydratase (Fig. 3, red arrow). The detection of the 1,2- $^{13}\text{C}_2$ -isotopologues lends support for the existence of Ser recycling via reactions of gluconeogenesis (Fig. 3). More specifically, the formation of phosphoenolpyruvate from [1,2- $^{13}\text{C}_2$]oxaloacetate (Fig. 3, green arrow) was conducive to the formation of [1,2- $^{13}\text{C}_2$]phosphoenolpyruvate and 3-[1,2- $^{13}\text{C}_2$]phosphoglycerate serving as the precursor of [1,2- $^{13}\text{C}_2$]Ser, which was then further converted into [1,2- $^{13}\text{C}_2$]pyruvate/Ala by Ser dehydratase.

The labeling profile of PHB can be taken as a reference for the labeling pattern of its precursor, acetyl-CoA. On this basis, the presence of [1,2- $^{13}\text{C}_2$]acetyl-CoA reflected its formation from [U- $^{13}\text{C}_3$]- and [1,2- $^{13}\text{C}_2$]pyruvate by pyruvate dehydrogenase. This isotopologue was then used for PHB synthesis but could also be shuffled into the citrate cycle, affording specific isotopo-

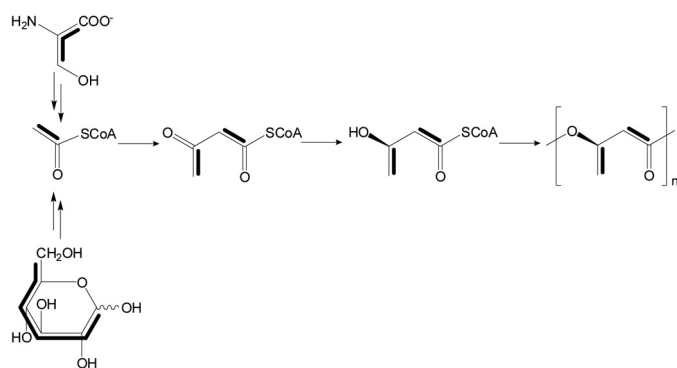


FIGURE 1. Formation of PHB in experiments with [U- $^{13}\text{C}_3$]Ser and [U- $^{13}\text{C}_6$]glucose. Multiple ^{13}C labeling is indicated by bars connecting ^{13}C -labeled atoms. The patterns of acetoacetyl-CoA and downstream products are overlays of isotopologues comprising two ^{13}C -labeled atoms in a single molecule.

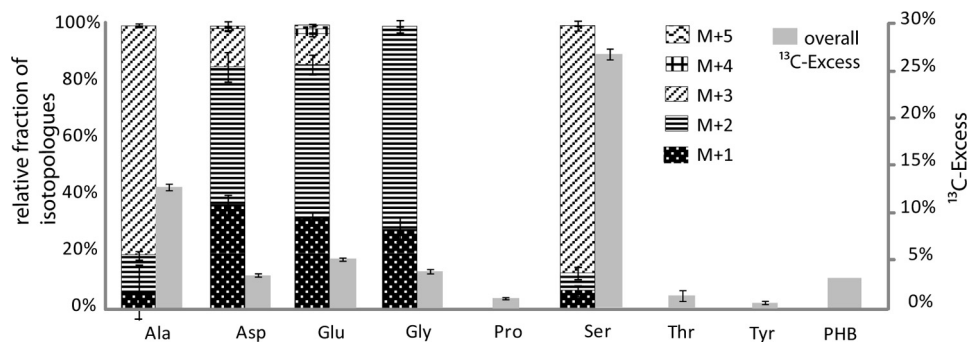


FIGURE 2. ^{13}C excess (gray columns; in mol % (right scale)) and isotopologue composition (patterned columns) of amino acids from the experiment with [U- $^{13}\text{C}_3$]Ser. The values represent means from three technical replicates, and the error bars indicate S.D. The patterned boxes indicate the relative contribution (%) of ^{13}C -isotopologues (M+1 to M+5 in different patterns) in the overall enrichments.

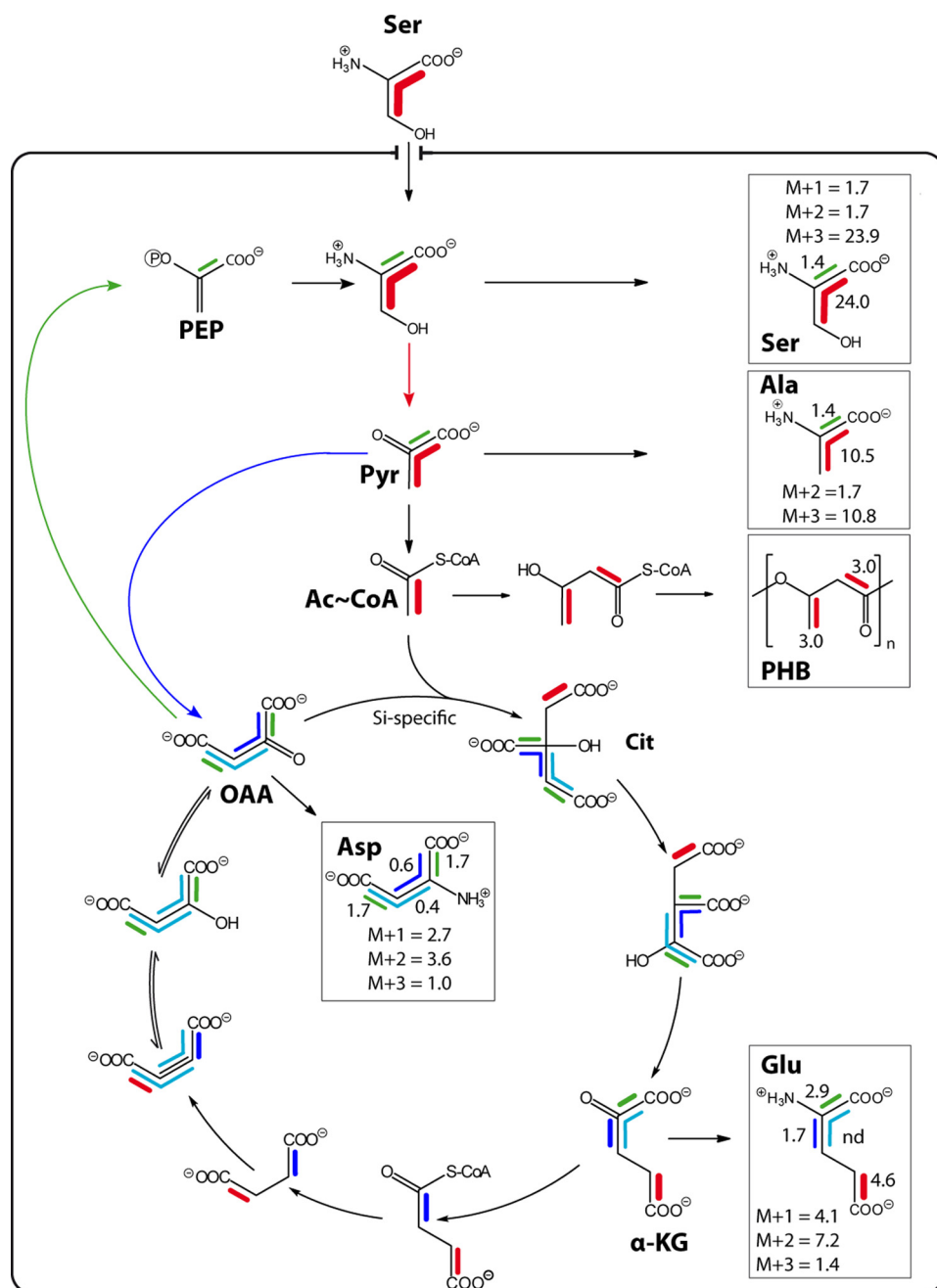


FIGURE 3. **Metabolic model for Ser utilization by *Lp Paris* grown in culture.** The scheme shows labeling patterns due to the incorporation of exogenous $[\text{U}-^{13}\text{C}_3]\text{Ser}$. Labeling patterns were detected in protein-derived amino acids and PHB (shown in boxes). Multiple ^{13}C -labeled isotopologues determined by NMR spectroscopy are indicated as bars connecting ^{13}C -labeled atoms in a given molecule. The numbers indicate the respective molar abundances. The molar abundances of isotopomer groups comprising one, two, or three ^{13}C -labeled atoms, as determined by mass spectrometry ($M+1$, $M+2$, and $M+3$, respectively), are also listed in the boxes. PEP, phosphoenolpyruvate; OAA, oxaloacetate; α -KG, α -ketoglutarate.

logue profiles in α -ketoglutarate and oxaloacetate, which serve as precursors for Glu and Asp, respectively (Fig. 3). Because of a *Si*-specific citrate synthase, label from $[1,2-^{13}\text{C}_2]\text{acetyl-CoA}$ (Fig. 3, red bar) was transferred to positions 4 and 5 of α -ketoglutarate and glutamate. Following the reactions of the citrate cycle, label from α - $[4,5-^{13}\text{C}_2]\text{ketoglutarate}$ afforded $[1,2-^{13}\text{C}_2]\text{succinate}$ and $[1,2-^{13}\text{C}_2]\text{fumarate}$ (Fig. 3, red bars). Because of the intrinsic symmetry of fumarate, malate synthase yielded a 0.5:0.5 mixture of $[1,2-^{13}\text{C}_2]$ - and $[3,4-^{13}\text{C}_2]\text{malate}$

from $[1,2-^{13}\text{C}_2]\text{fumarate}$. (Fig. 3, green bars). The same isotopologue mixture resulted in oxaloacetate and its amination product, aspartate (Fig. 3, green bars and $M+2$ values from the mass spectrum). The presence of triple ^{13}C -labeled isotopologues in Asp (Fig. 3, blue bars and $M+3$ values) also reflected the formation of oxaloacetate by carboxylation of $[\text{U}-^{13}\text{C}_3]\text{pyruvate}$ (Fig. 3, blue arrow). Primarily, this reaction yielded from $[\text{U}-^{13}\text{C}_3]\text{pyruvate}$ $[1,2,3-^{13}\text{C}_3]\text{oxaloacetate}$, which was then converted into $[1,2,3-^{13}\text{C}_3]\text{Asp}$. The fact that $[2,3,4-^{13}\text{C}_3]\text{oxaloacetate/Asp}$ also was observed at similar abundances as found for the $1,2,3-^{13}\text{C}_3$ -isotopologue can be explained by rapid equilibrium between oxaloacetate/malate/fumarate due to reversible malate dehydrogenase, malate synthase, and possibly also succinate dehydrogenase. Because of the symmetry of fumarate and succinate, the triple label was then randomized, affording the observed symmetric isotopologue distribution in oxaloacetate/Asp.

Labeled oxaloacetate also served as an acceptor of unlabeled acetyl-CoA. As shown in Fig. 3, label from $[3,4-^{13}\text{C}_2]\text{oxaloacetate}$ was transferred to positions 1 and 2 of α -ketoglutarate/Glu (green bar), and $[1,2-^{13}\text{C}_2]\text{oxaloacetate}$ yielded $[3-^{13}\text{C}_1]\text{Glu}$. Label from position 1 of oxaloacetate was lost as $^{13}\text{CO}_2$ during dehydrogenation of isocitrate. $[1,2,3-^{13}\text{C}_3]$ - and $[2,3,4-^{13}\text{C}_3]\text{oxaloacetate}$ gave rise to $2,3-^{13}\text{C}_2$ - and $1,2,3-^{13}\text{C}_3$ -labeled species in α -ketoglutarate/Glu, respectively. Notably, all of these isotopologues were detected in glutamate, although $[1,2,3-^{13}\text{C}_3]\text{Glu}$ could be identified only by mass spectrometry (as $M+3$ species).

Lp Is Able to Use Glucose as a Carbon Source—To obtain information about a potential usage of glucose, *Lp* was grown in AYE medium or CDM supplemented with 11 mM $[\text{U}-^{13}\text{C}_6]\text{glucose}$ and harvested at stationary phase. A sample work-up and analysis were done as described above. Fig. 4 indicates that the same set of amino acids labeled from ^{13}C Ser also acquired significant ^{13}C label ($>1\%$ ^{13}C enrichment; Ala $>$ Glu $>$ Asp $>$ Pro $>$ Ser) from $[\text{U}-^{13}\text{C}_6]\text{glucose}$ supplemented either to the AYE medium or to CDM. The percentage of

enrichment values of amino acids was remarkably similar, pointing to glucose metabolism irrespective of the culture medium used. In contrast to the small differences in the ^{13}C enrichments of amino acids, we noticed a rather large differ-

ence in the ^{13}C incorporation of $[\text{U-}^{13}\text{C}_6]\text{glucose}$ into PHB. More specifically, ^{13}C enrichment of PHB isolated from cells grown in AYE medium was ~ 3 -fold higher than the corresponding value in the experiments using CDM. The labeling patterns from the experiment with AYE medium are discussed in detail below.

Elevated Rate of Glucose Incorporation into PHB—The ^{13}C NMR signals of PHB detected in the dichloromethane extract of the labeled cells (supplemental Fig. S1B) displayed the same coupling pattern as observed previously for PHB labeled from $[\text{U-}^{13}\text{C}_3]\text{Ser}$. However, the intensities of the coupling satellites relative to the central signals were higher in the experiment with $[\text{U-}^{13}\text{C}_6]\text{glucose}$ (Table 3). The analysis of the signals corroborated the fact that $[1,2\text{-}^{13}\text{C}_2]$ - and $[3,4\text{-}^{13}\text{C}_2]\text{PHB}$ were again the dominant species, each with abundances of approximately 5%. As described above in detail, this isotopologue mixture can be explained by PHB biosynthesis from a mixture of $1,2\text{-}^{13}\text{C}_2$ -labeled and unlabeled acetyl-CoA. The minor $[\text{U-}^{13}\text{C}_4]\text{PHB}$ species was again assembled from two labeled molecules of acetyl-CoA. In summary, these data demonstrated that $[\text{U-}^{13}\text{C}_2]\text{acetyl-CoA}$ can be made from $[\text{U-}^{13}\text{C}_3]\text{Ser}$ or $[\text{U-}^{13}\text{C}_6]\text{glucose}$, indicating that Ser and glucose catabolism merge at a certain stage of the intermediary metabolism (*i.e.* prior to acetyl-CoA formation). During growth in AYE medium, glucose appeared to be used preferably as a PHB precursor. Under these conditions more than 6 mol % of PHB was derived from exogenous ^{13}C glucose (as compared with 0–5 mol % in amino acids).

Amino Acids—The ^{13}C incorporation into the bacterial amino acids as determined by GC/MS and NMR was 0.5–5% in the following order: Ala > Asp > Glu > Ser > Gly (Figs. 4 and 5). The same set of amino acids was also found to be labeled from $[\text{U-}^{13}\text{C}_3]\text{Ser}$, although at higher rates (3–12%). Generally, the relatively low incorporation rates of glucose into amino acids suggested that glucose did not serve as a major carbon source during the overall growth period of the experiment. Notably, the incorporation of glucose into pyruvate/alanine

(4.9%) was considerably lower than the rate into PHB (6.3%). This was in sharp contrast to the values of the experiment with $[\text{U-}^{13}\text{C}_3]\text{Ser}$ where the incorporation rate into PHB was lower (3.2%) than the respective value into pyruvate/alanine (12.2%). The discrepancy between the ^{13}C enrichments in pyruvate (serving as precursor for Ala) and acetyl-CoA (serving as precursor for PHB) immediately showed that these intermediates were not in isotopic equilibrium.

Ile, Leu, Phe, Tyr, His, Pro, and Val were also found to be unlabeled from $[\text{U-}^{13}\text{C}_6]\text{glucose}$. This confirms that *Lp* is auxotrophic for these amino acids as suggested. Surprisingly, some label appeared to be transferred from glucose to Ser,

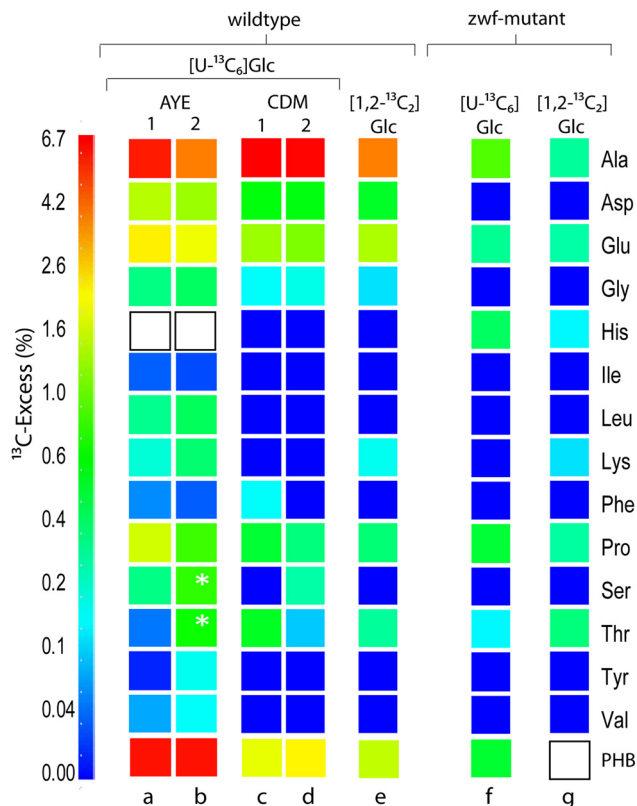


FIGURE 4. Overall ^{13}C excess (mol %) of labeled isotopologues in amino acids derived from protein after feeding of *L. pneumophila* Paris wild type and Δzwf mutant with 11 mM $[\text{U-}^{13}\text{C}_6]\text{glucose}$ and 11 mM $[1,2\text{-}^{13}\text{C}_2]\text{glucose}$ in different media. The color map indicates ^{13}C excess in quasi-logarithmic form to show even relatively small ^{13}C excess values. Each sample (different labeling experiments) was measured three times; the color for each amino acid correlates with the mean value of the three measurements. Asterisks indicate S.D. > 35%. PHB could not be measured in the experiment with $[1,2\text{-}^{13}\text{C}_2]\text{glucose}$ and the Δzwf mutant.

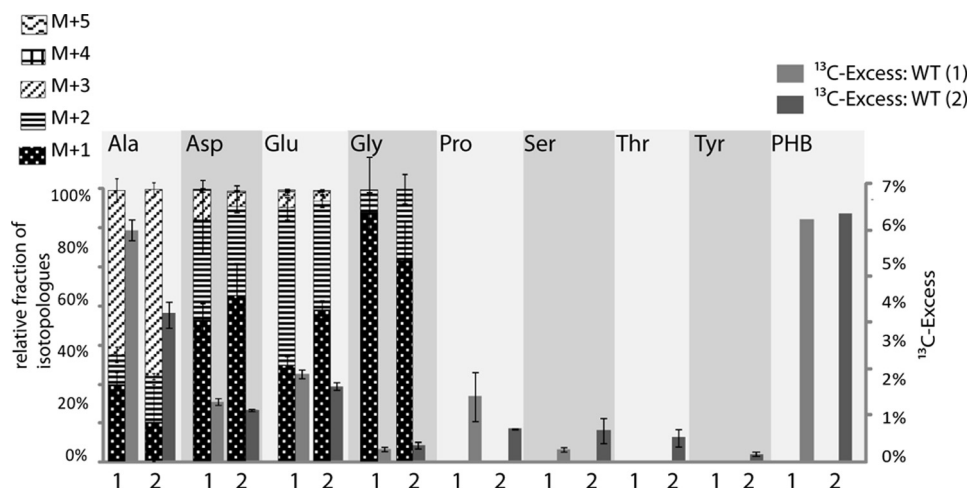


FIGURE 5. ^{13}C excess (gray columns; in mol % (right scale)) and isotopologue composition (patterned columns) of amino acids from experiments 1 and 2 with *L. pneumophila* wild type cultivated in AYE medium containing 11 mM $[\text{U-}^{13}\text{C}_6]\text{glucose}$. The values represent means from three measurements, and error bars indicate S.D. The patterned boxes indicate the relative contribution (%) (left scale) of ^{13}C -isotopologues (M+1 to M+5) in the overall enrichments.

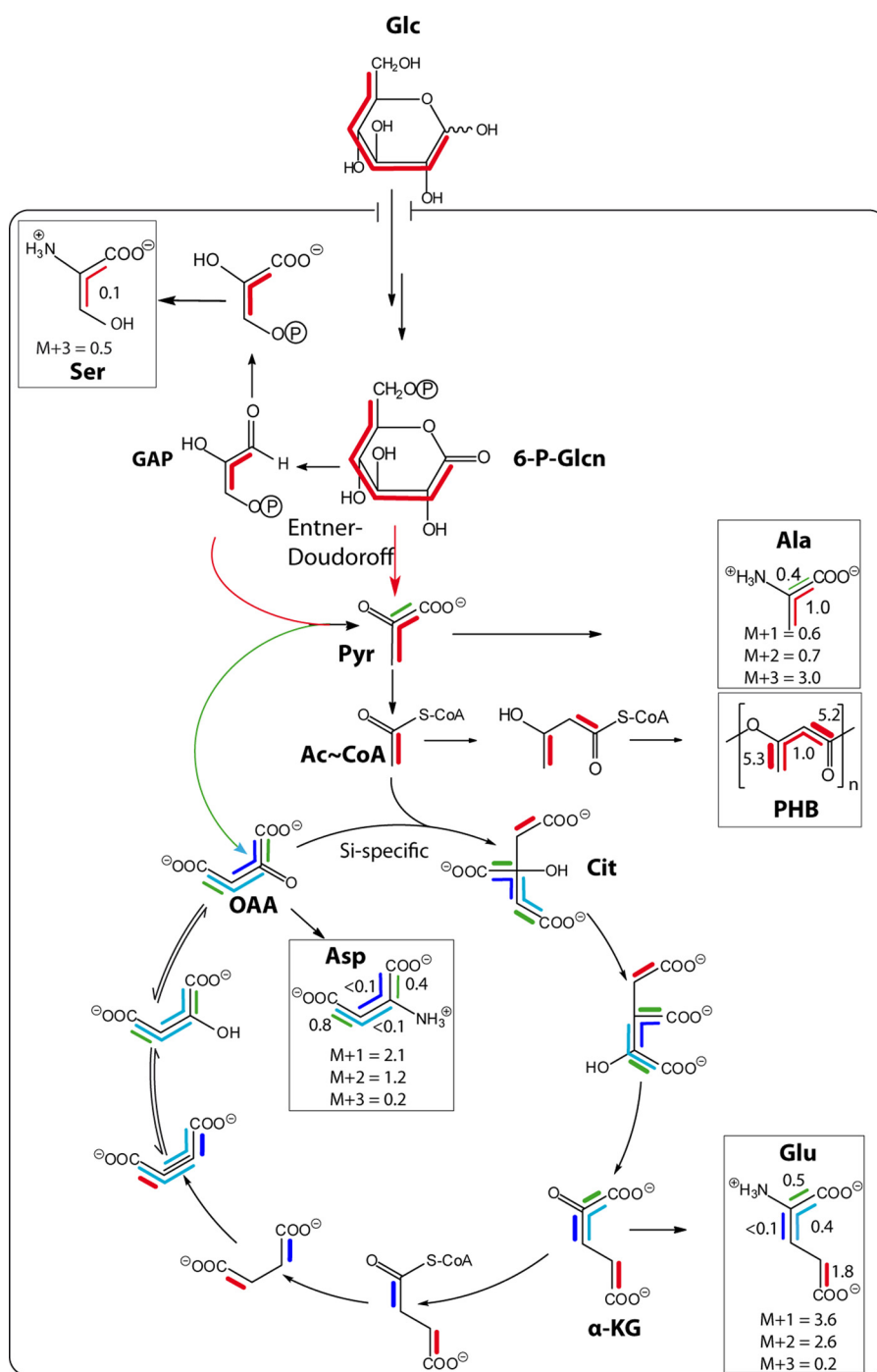


FIGURE 6. **Metabolic model for glucose utilization in *Lp* Paris grown in AYE medium supplemented with [U- $^{13}\text{C}_6$]glucose.** Labeling patterns were detected in protein-derived amino acids and PHB (shown in boxes). Multiple ^{13}C -labeled isotopologues determined by NMR spectroscopy are indicated as bars connecting ^{13}C -labeled atoms in a given molecule. The numbers indicate the respective molar abundances. The molar abundances of isotopomer groups comprising one, two, or three ^{13}C -labeled atoms as determined by mass spectrometry (M+1, M+2, and M+3, respectively) are also listed for comparison. GAP, glyceraldehyde-3-phosphate dehydrogenase; 6-P-Glc, 6-phosphogluconate; Pyr, pyruvate; OAA, oxaloacetate; α -KG, α -ketoglutarate.

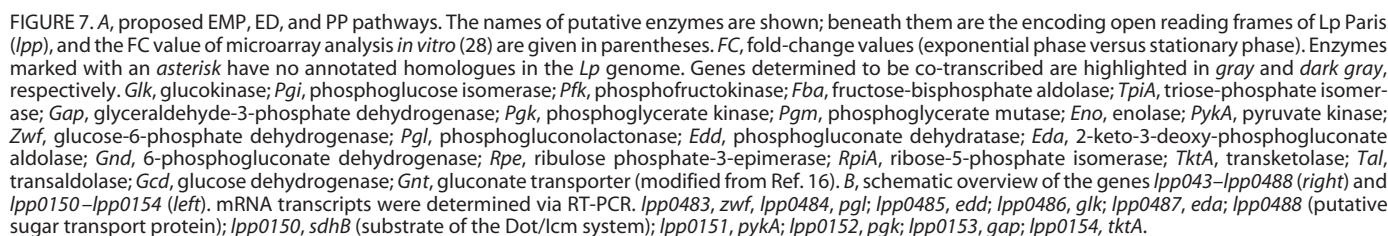
although exogenous Ser had been shown to be incorporated efficiently into proteins in the previous experiment. Moreover, a weak ^{13}C enrichment of Thr also could not be excluded on the basis of our experimental data.

The isotopologue distribution in each of the labeled amino acids was then determined by mass spectrometry and NMR

spectroscopy as described before. Ala was characterized by the U- $^{13}\text{C}_3$ -isotopologue. This isotopologue can be explained by glucose utilization via glycolysis, the PP pathway, and/or the ED pathway (Fig. 6). At lower abundances, Ser was also present as a U- $^{13}\text{C}_3$ -isotopologue, suggesting that 3-phosphoglycerate acquired at least some ^{13}C label by glycolysis. [1,2- $^{13}\text{C}_2$]Ala was observed as a minor isotopologue, suggesting that a small fraction of phosphoenolpyruvate/pyruvate was made from [1,2- $^{13}\text{C}_2$]oxaloacetate by decarboxylation (Fig. 6, green arrow). The labeling patterns in Asp and Glu supported carbon flux via the complete citrate cycle as already shown by the respective labeling profiles from [U- $^{13}\text{C}_3$]Ser. Thus, formation of oxaloacetate from pyruvate was again detected on the basis of $^{13}\text{C}_3$ -isotopologues in Asp and 1,2,3- $^{13}\text{C}_3$ - and 2,3- $^{13}\text{C}_2$ -isotopologues in Glu.

*Glucose Is Catabolized by the Entner-Doudoroff Pathway in *Lp**—To determine the glucose utilization pathway, we performed a labeling experiment with *Lp* Paris (*Lp*) in AYE medium supplemented with 11 mM [1,2- $^{13}\text{C}_2$]glucose. The ^{13}C enrichments and patterns in amino acids and PHB (Fig. 4, lane e) followed the same rules as described above. As shown in supplemental Fig. S3, label from [1,2- $^{13}\text{C}_2$]glucose was transferred at high rates to [1,2- $^{13}\text{C}_2$]Ala via the ED pathway but not to a 2,3- $^{13}\text{C}_2$ -labeled specimen representing a hypothetical product via glycolysis. Moreover, the apparent lack of label in Ser was also in line with the ED pathway, because glycolysis should have generated [2,3- $^{13}\text{C}_2$]Ser. The PP pathway was also excluded as a major pathway for utilizing glucose because no or only single labeled pyruvate/Ala (^{13}C at C-3) should

have occurred when [1,2- $^{13}\text{C}_2$]glucose was catabolized by this route. The low, single ^{13}C enrichment at C-1 of pyruvate/Ala rather reflected minor carbon flow from [^{13}C]oxaloacetate as already outlined above (supplemental Fig. S3). Pyruvate dehydrogenase should yield [1- $^{13}\text{C}_1$]acetyl-CoA from [1,2- $^{13}\text{C}_2$]pyruvate. [1- $^{13}\text{C}_1$]Acetyl-CoA was then conducive to the



The Δzwf mutant was then grown in AYE medium containing [U- $^{13}\text{C}_6$]- or [1,2- $^{13}\text{C}_2$]glucose as described above for the wild type strain. The Δzwf mutant strain showed strongly reduced incorporation rates (by an approximate factor of 10)

Hints for a Functional Role of Carbohydrate Utilization by *Lp*—To better understand the role of glucose metabolism in the life cycle of *Lp*, we ran infections of *A. castellanii*. The deletion of *zwf* in *Lp* did not significantly affect replication within the host (Fig. 8A). However, this was different when replication during successive rounds of infection was analyzed. After a first infection, which lasted 3 days, the mixture (comprising amoeba lysate and *Lp* WT or Δzwf mutant strain) was kept for 3 addi-

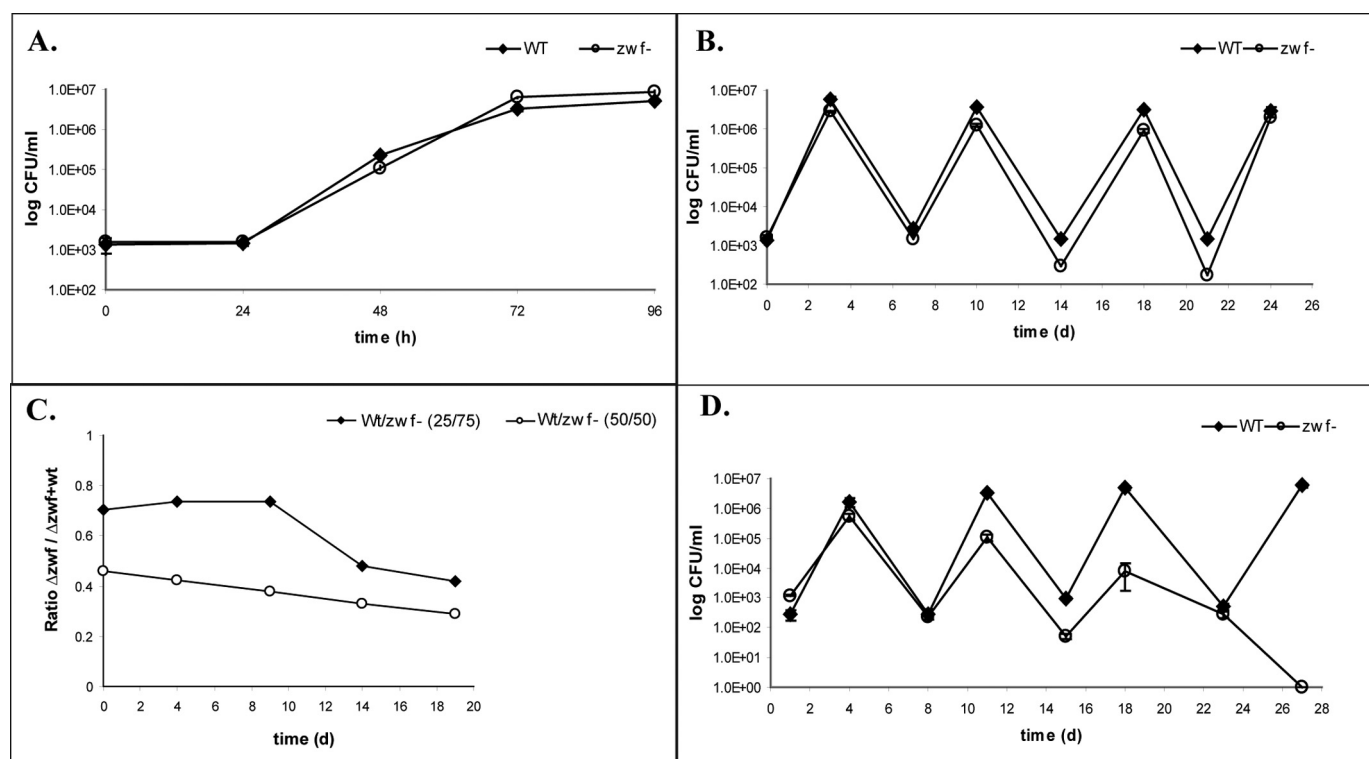


FIGURE 8. **Analysis of *L. pneumophila* Paris (WT) and Δzwf mutant strain (Δzwf) in co-cultures with *A. castellanii*.** Bacteria were used to infect monolayers of *A. castellanii* at a multiplicity of infection of 0.01 with (A) *Lp* Paris or the Δzwf mutant strain for 96 h (A); *Lp* Paris or Δzwf mutant strain for 3 days, resuspended and incubated for a further 3 days, diluted to $\sim 10^3$ bacteria/ml, and used to infect fresh amoebae (B). Four rounds of infection were performed. C, *L. pneumophila* Δzwf and WT strain survival over a 20-day period after co-infection of *A. castellanii* cells at two different ratios (circles, 50:50; diamonds, 75:25). D, infection was done as described in B, but *A. castellanii* cells were infected with both strains (WT and Δzwf) at the same time (in competition). At various time points postinoculation, bacteria were quantitated by plating aliquots on BCYE agar (see "Experimental Procedures"). Results are means \pm S.D. of duplicate samples and are representative of at least three independent experiments.

tional days. Then, we ran another infection using 1 ml of a 1:1000 dilution of the previous mixture in fresh infection buffer with fresh amoebae. To follow infection kinetics, each sample was plated on BCYE and/or BCYE kanamycin plates. Although there was only a minor difference during the second and third round of infection of amoebae (Fig. 8B), we noted that the viability of the Δzwf mutant strain dropped during the lag period. We then verified this observation by an experiment with a lag period of 20 days between the first and second rounds of co-infection and by evaluating the recovery percentage (ratios of $\Delta zwf / \Delta zwf + WT$) of both the mutant and the wild type strain (Fig. 8C). Then, we performed competition experiments with successive rounds of infection using the Δzwf mutant strain and the WT strain. Again, the mutant strain showed less fitness, and the observed effect accumulated with each additional round of infection until the mutant strain was outcompeted by the WT strain (Fig. 8D). Altogether, these results indicate an important role of the ED pathway (glucose-6-phosphate dehydrogenase) for the survival of *Lp* in the environment.

DISCUSSION

Lp survives within amoebae and macrophages because of its ability to establish a replication vacuole that is derived from the endoplasmic reticulum. Within this vacuole, *Lp* differentiates into the replicative form and multiplies. It was proposed that when nutrients become limiting, a regulatory cascade triggers the differentiation to a motile spore-like form. After the bacte-

ria are released from the host cells, these forms of *Lp* are well prepared to persist for long periods in the environment. It is known that *Legionella* exhibits a strictly respiratory form of metabolism and does not grow anaerobically (18). It is also current knowledge that *Lp* uses amino acids as primary energy and carbon sources (4, 10–12, 14) and that metabolic genes are expressed mainly in the exponential growth phase during *in vivo* growth within *A. castellanii* (28).

In this study, the metabolism of *Lp* was analyzed for the first time by comprehensive isotopologue profiling under culture conditions. Metabolic fluxes were estimated on the basis of the observed labeling profiles in amino acids and PHB. It had been reported previously that Ser is actively metabolized by legionellae and that Ser is necessary for the growth of *Lp* (10, 12, 14–16, 18, 19, 27). In the genome of strain Paris genes encoding for ~ 12 ABC transporters and amino acid permeases, e.g. a putative Ser transport protein (Lpp2269) and the putative amino acid transporters Lpp0026 and Lpp0357 are predicted. Indeed, using $[U-^{13}C_3]$ Ser as a supplement to AYE medium at a concentration of 3 mM, label was transferred to protein-derived $[^{13}C_3]$ Ser at $\sim 25\%$. Assuming that yeast extract present in AYE medium contributes unlabeled Ser at a similar concentration as the added ^{13}C -labeled Ser (see also supplemental Table S1), the incorporation of exogenous Ser into protein-derived Ser can be estimated as 50%. This high value supports the view that amino acid transporters, accepting Ser as a substrate, are active in *Lp*.

Albeit at lower levels of ^{13}C enrichment (0–13%), label from $[\text{U-}^{13}\text{C}_3]\text{Ser}$ was also distributed to other amino acids (Ala, Glu, Gly, and Asp) as well as to PHB. This lends evidence that Ser is catabolized to pyruvate by Ser dehydratase, known to be active in cell extracts of *Lp* (20). The enrichment values and isotopologue profiles of the storage compound, PHB, and amino acids derived from intermediates of the citrate cycle (*i.e.* Asp and Glu) also demonstrate that a carbon flux exists from pyruvate to acetyl-CoA serving as a precursor of PHB and a substrate of a complete citrate cycle with *Si* specificity of citrate synthase. Our data did not show any evidence for a functional glyoxylate bypass, corroborating data from genome sequence analysis (18, 25, 26).

The labeling profiles reflected only minor flux from pyruvate to oxaloacetate as well as from oxaloacetate to phosphoenolpyruvate/pyruvate by reactions of gluconeogenesis (including formation of $[\text{U-}^{13}\text{C}_2]\text{Ser}$ indicating that Ser can be made *de novo*). This is surprising because it was believed that the EMP pathway is used in the direction of gluconeogenesis and that Ser or pyruvate is required to maintain a pool of oxaloacetate (high activity of pyruvate carboxylase (20)). In addition, our results could not corroborate minor flux from pyruvate to acetyl-CoA, as suggested earlier on the basis of low activity for pyruvate dehydrogenase in cell lysates of *Lp* (20). However, at this time it is not possible to reach a conclusion on the importance of gluconeogenesis for *Lp*. Experiments are under way to investigate this question further.

The fact that many amino acids (Leu, Ile, Val, Phe, Tyr, Met, Arg, and His) were unlabeled corroborate that *Lp* is auxotrophic for many amino acids, as suggested by genome analysis (16, 18), and that amino acids (including Ser) can act as major carbon substrates for *Lp*. On the other hand, the detection of diluted label from exogenous $[\text{U-}^{13}\text{C}_3]\text{Ser}$ in metabolites derived from downstream central intermediates (*i.e.* acetyl-CoA, oxaloacetate, and α -ketoglutarate) lends support for the use of additional non-amino acid carbon substrates.

Inspired by this observation, we performed labeling experiments with 11 mM $[\text{U-}^{13}\text{C}_6]\text{-}$ or $[\text{1,2-}^{13}\text{C}_2]\text{glucose}$ as a supplement to AYE medium or CDM. In both experimental settings, glucose was incorporated into amino acids and PHB, affording ^{13}C enrichments up to 10% in pyruvate/Ala. Again, minor amounts of Ser were synthesized *de novo* in this experiment. Generally, the transfer of label from glucose to central metabolic intermediates providing precursors of the labeled amino acid is surprising, because it is believed that glucose or carbohydrates are not utilized by *Lp* (12, 15, 18, 20). Although the glycolytic pathway appeared to be complete (as suggested from the sequenced genomes), enzymatic assays indicated very low, if any, metabolic flux through this route (14, 16, 20). In addition, we confirmed that *Lp* does not exhibit a functional PP pathway, corroborated by the lack of orthologues of 6-phosphogluconate dehydrogenase and transaldolase within the recently sequenced *Lp* genomes. The ED pathway was also not thought to be active (16, 18). However, in our experiments, the labeling profiles of PHB and amino acids demonstrated carbon flux from glucose to pyruvate via the ED pathway and not via the EMP and/or the PP pathway. As further strong evidence, the Δzwf mutant of *Lp* Paris, impaired in the key reaction of the ED

pathway, was strongly reduced in its glucose utilization. However, the deletion of the *zwf* gene would also affect the catabolism of glucose via the PP pathway; but this seems to be a negligible concern, because our results (WT strain) demonstrated that the PP pathway was not used for glucose catabolism by *Lp*.

Further analysis of the putative *zwf* operon demonstrated that the genes *lpp0483* to *lpp0487* are transcribed as one mRNA unit. The putative glucose transport protein encoding gene *lpp0488* is transcribed separately as a monocistronic mRNA. It is also noteworthy that the glucokinase (*glk*, *lpp0486*) is located within the *zwf* operon encoding for genes of the ED pathway. The products of glucose degradation by the encoded enzymes of the *zwf* operon would be glyceraldehyde 3-phosphate and pyruvate, which could then enter into the lower part of glycolysis and the citrate cycle, respectively (see Fig. 7).

Further analysis of the Δzwf mutant strain demonstrated that the mutant strain was outcompeted by the wild type strain in a combined replication/survival assay with successive rounds of infection (Fig. 8D). In the first round of infection, the Δzwf mutant strain replicated as well as the wild type strain; however, during repeated infection cycles, the fitness of the Δzwf mutant strain was reduced in the presence of the wild type strain. Thus, the activity of glucose-6-phosphate dehydrogenase (ED pathway) and of glucose catabolism in general is important for full fitness of *Lp*.

In this context, it is important to note that *Lp* is also able to degrade cellulose (29), and we have identified a glucoamylase in *Lp* Paris the activity of which is responsible for starch and glycogen degradation of this strain.⁶ Moreover, it has been shown that a mutation in the chitinase gene (*chiA*) has a negative effect on the virulence of the mutant strain as compared with the isogenic wild type strain (40). Preliminary labeling experiments of *A. castellanii*, the host organism, were successful. Thus we now have an excellent basis for *in vivo* infection experiments using *Lp* and “prelabeled” *A. castellanii* cells to analyze the intracellular metabolism of the human pathogen *Lp*. This will provide further information for better understanding the mechanisms of intracellular pathogens and how *Lp* gets access to essential nutrients during intracellular parasitism of amoebae.

In summary, we were able to demonstrate that (i) Ser is used efficiently as a carbon source but is also synthesized *de novo* by *Legionella*, although Ser is absolutely required for *in vitro* and *in vivo* growth of *Lp*; (ii) glucose is metabolized mainly via the ED pathway, which is active during *in vitro* growth, and not via the EMP or PP pathway; (iii) carbon from glucose is incorporated preferably into the storage compound PHB; (iv) the citrate cycle is complete and active; (v) *Lp* is not able to synthesize Ile, Leu, Val, Phe, Met, Arg, and Tyr; and (vi) glucose metabolism via the ED pathway is necessary for full fitness of *Lp* during its life cycle.

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⁶ V. Herrmann, A. Eidner, K. Rydzewski, I. Blädel, M. Jules, C. Buchrieser, W. Eisenreich, and K. Heuner, submitted for publication.

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Supplemental Data

Table S1. Composition of the growth media for *L. pneumophila*.

Compound	CDM	AYE
	[mg/l]	
ACES (N-(2-acet- amido)-2-amino- ethanesulfonamid acid)	10,000	10,000
Alanine		560/440*
Arginine	350	260/140*
Asparagine		-/100*
Aspartate	510	530/160*
Cysteine	400	400 -/20*
Glutamate	600	940/660*
Glutamine		-/20*
Glycine		300/100*
Histidine	150	130/40*
Isoleucine	470	300/180*
Leucine	640	410/300*
Lysine	650	460/190*
Methionine	200	80/60*
Phenylalanine	350	260/200*
Proline	115	200/80*
Serine	650	160/130*
Threonine	330	160/110*
Tryptophan	100	-/50*
Tyrosine	400	120/80*
Valine	480	350/220*
NH ₄ Cl	315	
NaCl	50	10*
CaCl ₂	25	
KH ₂ PO ₄	1,180	
MgSO ₄	70	
Fe-pyrophosphate	250	250
Ca-ion		1.3*
Fe-ion		0.5*
Mg-ion		7.3*
K-ion		319.5*
Na-ion		49*
Cl-ion		38*
Sulfate		9*
Phosphate		327*

* calculated from the analysis of yeast extract (amino acids are listed as total amino acids including protein / free amino acids)

Table S2. ^{13}C -Excess (mol%) of amino acids from experiments with *L. pneumophila* wild-type and its mutant Δzwf fed with 11 mM $[\text{U-}^{13}\text{C}_6]\text{glucose}$ or 11 mM $[1,2\text{-}^{13}\text{C}_2]\text{glucose}$.

Legionella pneumophila Paris																		
wildtype														Δzwf				

Table S3. ^{13}C -Excess (mol%) of amino acids and PHB from experiments with *L. pneumophila* wild-type fed with 3 mM $[\text{U-}^{13}\text{C}_3]\text{Ser}$, cultivated in AYE medium.

<i>Legionella pneumophila</i> Paris	
	$[\text{U-}^{13}\text{C}_3]\text{Ser}$
Ala	12.18 % \pm 0.41%
Asp	3.28 % \pm 0.19%
Glu	4.95 % \pm 0.08%
Gly	3.68 % \pm 0.18%
Pro	0.83 % \pm 0.21%
Ser	25.62 % \pm 0.69%
Ile	0.06 % \pm 0.05%
Leu	0.05 % \pm 0.05%
Phe	0.14 % \pm 0.08%
Thr	1.16 % \pm 0.18%
Tyr	0.52 % \pm 0.06%
Val	0.15 % \pm 0.07%
PHB	3.39%

Table S4. ^{13}C -Isotopologue abundance in mol% of amino acids from experiments with *Legionella pneumophila* wild-type cultivated in AYE-medium fed with 3 mM $[\text{U-}^{13}\text{C}_3]\text{Ser}$. M represents the mass of the unlabelled amino acid under study. The number behind the mass represents the amount of ^{13}C -atoms. The data are mean values of three measurements.

<i>Legionella pneumophila</i> Paris wild-type									
$[\text{U-}^{13}\text{C}_3]\text{Ser}$									
Ala	M+0	86.74 %	±	1.30%	Phe	M+0	99.73 %	±	0.12%
	M+1	0.75 %	±	1.29%		M+1	0.00 %	±	0.00%
	M+2	1.75 %	±	0.18%		M+2	0.00 %	±	0.00%
	M+3	10.76 %	±	0.09%		M+3	0.12 %	±	0.05%
Asp	M+0	92.65 %	±	0.59%		M+4	0.02 %	±	0.01%
	M+1	2.72 %	±	0.26%		M+5	0.04 %	±	0.02%
	M+2	3.58 %	±	0.39%		M+6	0.03 %	±	0.03%
	M+3	0.98 %	±	0.05%		M+7	0.00 %	±	0.00%
	M+4	0.08 %	±	0.11%		M+8	0.03 %	±	0.04%
Glu	M+0	86.78 %	±	0.19%	Pro	M+9	0.03 %	±	0.05%
	M+1	4.15 %	±	0.33%		M+0	98.61 %	±	0.34%
	M+2	7.22 %	±	0.47%		M+1	0.03 %	±	0.05%
	M+3	1.42 %	±	0.10%		M+2	0.05 %	±	0.09%
	M+4	0.24 %	±	0.21%		M+3	1.21 %	±	0.18%
	M+5	0.19 %	±	0.07%		M+4	0.10 %	±	0.09%
Gly	M+0	95.71 %	±	0.27%	Ser	M+5	0.00 %	±	0.00%
	M+1	1.21 %	±	0.19%		M+0	72.67 %	±	1.01%
	M+2	3.08 %	±	0.09%		M+1	1.70 %	±	0.58%
Ile	M+0	99.90 %	±	0.10%	Thr	M+2	1.73 %	±	0.59%
	M+1	0.00 %	±	0.00%		M+3	23.90 %	±	0.51%
	M+2	0.04 %	±	0.06%		M+0	97.80 %	±	0.80%
	M+3	0.04 %	±	0.01%		M+1	0.51 %	±	0.89%
	M+4	0.02 %	±	0.01%		M+2	1.04 %	±	0.62%
	M+5	0.01 %	±	0.01%		M+3	0.50 %	±	0.47%
						M+4	0.13 %	±	0.03%

Table S4. (Continued)

Legionella pneumophila paris wildtype

[U- ¹³ C ₃]Ser									
Leu	M+0	99.92%	±	0.04%	Tyr	M+0	98.93%	±	0.37%
	M+1	0.03%	±	0.05%		M+1	0.00%	±	0.00%
	M+2	0.00%	±	0.00%		M+2	0.21%	±	0.37%
	M+3	0.00%	±	0.00%		M+3	0.29%	±	0.26%
	M+4	0.01%	±	0.01%		M+4	0.18%	±	0.16%
	M+5	0.03%	±	0.05%		M+5	0.05%	±	0.08%
						M+6	0.09%	±	0.10%
						M+7	0.16%	±	0.24%
						M+8	0.01%	±	0.01%
						M+9	0.08%	±	0.09%
				Val	M+0	99.50%	±	0.22%	
					M+1	0.29%	±	0.06%	
					M+2	0.19%	±	0.18%	
					M+3	0.00%	±	0.01%	
					M+4	0.02%	±	0.02%	
					M+5	0.00%	±	0.00%	

Table S5. ^{13}C -labelling of amino acids and PHB from *L. pneumophila* labelled with 3 mM $[\text{U-}^{13}\text{C}_3]\text{Ser}$, determined by NMR. The labelling pattern is given in terms of X-groups. This notation is based on digits for each C-atom. The first digit represents C-1, the second C-2, etc.; 1 signifies ^{13}C , 0 signifies ^{12}C , and X signify either ^{13}C or ^{12}C . The labelling status of X is totally undefined.

Amino acid	Pos	Chem. shift [ppm]	Coupling [Hz]	% $^{13}\text{C}^{13}\text{C}$	X-groups	mol%	GC/MS % $^{13}\text{C}/\text{C-atom}$
Ala	1	173.5		13.4	10X	1.8	13.3
			59.4	86.57	11X	11.5	
	2	49.4		10.67	010	1.4	
			59.3	10.19	110	1.4	
					011	0.0	
	3	15.8	59.3; 34.4	79.13	111	10.5	
				18.75	X01	2.5	
			34.3	81.25	X11	10.8	
Asp	1	171.7		52.8	10XX	2.3	4.4
			58.6	47.2	11XX	2.1	
	2	50		48.5	010X	2.1	
			57.6	39.5	110X	1.7	
			38.4	7.6	011X	0.3	
	3	34.3	57.6; 38	4	111X	0.2	
				45.5	X010	2.0	
			38.2	7.9	X110	0.3	
			55.7	38.5	X011	1.7	
			55.7; 37.5	8.2	X111	0.4	
	4	173.7		50.1	XX01	2.2	
			55.7	45.3	XX11	2.0	
Glu	1	—			10XXX		6.1
					11XXX		
	2	52.8		53.1	010XX	3.2	
			58.89	46.9	110XX	2.9	
					011XX		
	3	25.4		68.8	111XX		
					X010X	4.2	
			35.1	27.4	X011X, X110X	1.7	
			35.1; 35.1	3.8	X111X	0.2	
				20.4	XX010	1.2	
	4	29.9	55.2	68.2	XX011	4.2	
			36.3	2	XX110	0.1	
			55.2; 36.3	9.3	XX111	0.6	
	5	176.8		23.8	XXX01	1.5	
				76.2	XXX11	4.6	
Gly	1	170.6		34.49	10	1.7	4.8
			59	65.51	11	3.1	
	2	40.67		30.39	01	1.5	
			58.6	69.61	11	3.3	

Table S5. (Continued)

Amino acid	Pos	Chem. shift [ppm]	Coupling [Hz]	% ¹³ C ¹³ C	X-groups	mol%	GC/MS % ¹³ C/C-atom
Ser	1	170.9		6.5	10X	1.7	26.7
			58.3	93.5	11X	25.0	
	2	55.4		4.6	010	1.2	
			58.2	5.4	110	1.4	
				5.4	011	1.4	
			58.2; 36.9	89.9	111	24.0	
	3	59.9		10.8	X01	2.9	
			37.4	89.2	X11	23.8	
PHB	1	169.5		48.4	10XX	2.2	4.5
			58	51.6	11XX	2.3	
	2	41.2		30.3	010X	1.4	
			39.7	2.3	011X	0.1	
			58.1	59.3	110X	2.7	
			58.1; 40.1	8	111X	0.4	
	3	68		35.8	X010	1.6	
			39.3	57.4	X011	2.6	
					X110	0.0	
			39.3; 38.0	6.9	X111	0.3	
	4	20.15		34.2	XX01	1.5	
			38.8	65.8	XX11	3.0	

Table S6. ^{13}C -Isotopologue abundance in mol% of amino acids from experiments with *L. pneumophila* wild-type (Table S6a) and *L. pneumophila* mutant Δzwf (Table S6b), cultivated in different media (AYE-medium and CDM-medium) fed with $[\text{U-}^{13}\text{C}_6]\text{glucose}$ or $[1,2\text{-}^{13}\text{C}_2]\text{glucose}$. M represents the mass of the unlabelled amino acid under study. The number behind the mass represents the amount of ^{13}C -atoms. The data are mean values of three measurements.

Legionella pneumophila Paris wild-type																	
		11mM [U- ¹³ C ₆]Glc										11mM [1,2- ¹³ C ₂]Glc					
		AYE 1			AYE 2			CDM 1			CDM 2			AYE			
Ala	M+0	92.33	±	0.23	95.59	±	0.76	92.64	±	0.45	92.97	±	0.52	94.11	±	0.00	
	M+1	2.15	±	0.36	0.61	±	0.62	0.64	±	0.43	0.40	±	0.46	0.45	±	0.02	
	M+2	0.85	±	0.22	0.75	±	0.05	0.69	±	0.04	0.64	±	0.07	5.36	±	0.02	
	M+3	4.66	±	0.33	3.05	±	0.11	6.03	±	0.05	6.00	±	0.05	0.08	±	0.01	
Asp	M+0	96.30	±	0.14	96.41	±	0.27	99.07	±	0.10	99.04	±	0.05	97.95	±	0.06	
	M+1	1.92	±	0.23	2.19	±	0.44	0.00	±	0.00	0.04	±	0.08	1.99	±	0.07	
	M+2	1.36	±	0.45	1.20	±	0.09	0.50	±	0.11	0.54	±	0.11	0.00	±	0.00	
	M+3	0.40	±	0.14	0.19	±	0.09	0.42	±	0.02	0.38	±	0.06	0.06	±	0.02	
	M+4	0.01	±	0.02	0.01	±	0.01	0.01	±	0.01	0.00	±	0.00	0.00	±	0.00	
Glu	M+0	93.53	±	0.34	93.53	±	0.35	96.48	±	0.34	96.46	±	0.25	93.48	±	0.20	
	M+1	2.29	±	0.23	3.63	±	0.21	0.82	±	0.39	0.84	±	0.29	5.99	±	0.25	
	M+2	3.77	±	0.27	2.58	±	0.15	2.58	±	0.13	2.55	±	0.19	0.53	±	0.09	
	M+3	0.38	±	0.07	0.23	±	0.05	0.07	±	0.05	0.12	±	0.06	0.00	±	0.00	
	M+4	0.03	±	0.02	0.01	±	0.02	0.04	±	0.02	0.03	±	0.03	0.00	±	0.00	
	M+5	0.00	±	0.00	0.02	±	0.02	0.02	±	0.01	0.01	±	0.01	0.00	±	0.00	
Gly	M+0	99.43	±	0.05	99.41	±	0.10	99.74	±	0.18	99.68	±	0.09	99.75	±	0.03	
	M+1	0.52	±	0.04	0.43	±	0.08	0.22	±	0.16	0.30	±	0.10	0.23	±	0.01	
	M+2	0.05	±	0.07	0.15	±	0.03	0.04	±	0.03	0.02	±	0.01	0.02	±	0.02	
His	M+0							99.89	±	0.01	99.89	±	0.03	99.84	±	0.07	
	M+1							0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	
	M+2							0.00	±	0.00	0.01	±	0.01	0.00	±	0.00	
	M+3							0.02	±	0.02	0.06	±	0.02	0.00	±	0.00	
	M+4							0.00	±	0.00	0.01	±	0.01	0.06	±	0.08	
	M+5							0.06	±	0.03	0.03	±	0.01	0.04	±	0.02	
	M+6							0.02	±	0.02	0.01	±	0.02	0.05	±	0.02	
Ile	M+0	99.99	±	0.02	99.94	±	0.04	99.91	±	0.05	99.88	±	0.04	99.98	±	0.02	
	M+1	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	
	M+2	0.00	±	0.00	0.04	±	0.04	0.09	±	0.05	0.12	±	0.04	0.00	±	0.00	
	M+3	0.01	±	0.02	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	
	M+4	0.00	±	0.00	0.01	±	0.01	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	
	M+5	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.02	±	0.02	
Leu	M+0	99.33	±	1.16	99.04	±	0.29	100.00	±	0.00	99.99	±	0.00	99.98	±	0.00	
	M+1	0.00	±	0.00	0.13	±	0.23	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	
	M+2	0.58	±	1.00	0.75	±	0.08	0.00	±	0.00	0.00	±	0.00	0.01	±	0.01	
	M+3	0.09	±	0.16	0.03	±	0.03	0.00	±	0.00	0.00	±	0.00	0.01	±	0.01	
	M+4	0.00	±	0.00	0.05	±	0.01	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	
	M+5	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	

Table S6a. (Continued)

Legionella pneumophila Paris wild-type

		11mM [U- ¹³ C ₆]Glc										11mM [1,2- ¹³ C ₂]Glc				
		AYE 1			AYE 2			CDM 1			CDM 2			AYE		
Lys	M+0	0.00	±	0.00	0.00	±	0.00	99.98	±	0.04	99.99	±	0.02	99.64	±	0.04
	M+1	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
	M+2	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.14	±	0.03
	M+3	0.00	±	0.00	0.00	±	0.00	0.02	±	0.04	0.01	±	0.02	0.22	±	0.01
	M+4	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
	M+5	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
	M+6	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
Phe	M+0	99.84	±	0.12	99.91	±	0.06	98.97	±	0.24	98.89	±	0.40	99.50	±	0.27
	M+1	0.00	±	0.00	0.00	±	0.00	0.91	±	0.25	0.99	±	0.41	0.36	±	0.26
	M+2	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
	M+3	0.14	±	0.10	0.07	±	0.06	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
	M+4	0.01	±	0.02	0.00	±	0.00	0.12	±	0.01	0.12	±	0.00	0.13	±	0.01
	M+5	0.00	±	0.00	0.01	±	0.01	0.00	±	0.00	0.00	±	0.00	0.01	±	0.02
	M+6	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
	M+7	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
	M+8	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
	M+9	0.01	±	0.02	0.01	±	0.01	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
Pro	M+0	97.62	±	0.78	98.58	±	0.08	99.19	±	0.24	99.57	±	0.07	99.54	±	0.11
	M+1	0.00	±	0.00	0.15	±	0.13	0.26	±	0.23	0.00	±	0.00	0.00	±	0.00
	M+2	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
	M+3	1.22	±	0.33	1.17	±	0.09	0.13	±	0.06	0.12	±	0.09	0.18	±	0.02
	M+4	1.11	±	0.95	0.10	±	0.06	0.40	±	0.05	0.31	±	0.04	0.28	±	0.09
	M+5	0.05	±	0.09	0.00	±	0.00	0.02	±	0.04	0.00	±	0.00	0.00	±	0.00
Ser	M+0	99.42	±	0.21	99.08	±	0.40	99.83	±	0.06	99.75	±	0.09	99.94	±	0.01
	M+1	0.24	±	0.42	0.09	±	0.15	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
	M+2	0.33	±	0.25	0.30	±	0.41	0.00	±	0.01	0.00	±	0.00	0.00	±	0.00
	M+3	0.00	±	0.00	0.53	±	0.07	0.16	±	0.06	0.25	±	0.09	0.06	±	0.01
Thr	M+0	99.95	±	0.04	98.16	±	1.15	97.96	±	0.63	98.25	±	0.56	98.89	±	0.27
	M+1	0.00	±	0.00	1.13	±	1.39	2.04	±	0.63	1.75	±	0.56	0.86	±	0.25
	M+2	0.00	±	0.00	0.71	±	0.26	0.00	±	0.00	0.00	±	0.00	0.25	±	0.03
	M+3	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
	M+4	0.05	±	0.04	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
Tyr	M+0	99.96	±	0.04	99.54	±	0.23	99.70	±	0.18	99.82	±	0.13	99.58	±	0.20
	M+1	0.02	±	0.04	0.22	±	0.38	0.09	±	0.19	0.00	±	0.00	0.00	±	0.00
	M+2	0.00	±	0.00	0.00	±	0.00	0.12	±	0.11	0.08	±	0.13	0.04	±	0.06
	M+3	0.00	±	0.00	0.13	±	0.09	0.00	±	0.00	0.00	±	0.00	0.08	±	0.07
	M+4	0.01	±	0.02	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
	M+5	0.00	±	0.00	0.00	±	0.00	0.09	±	0.02	0.10	±	0.05	0.16	±	0.06
	M+6	0.00	±	0.00	0.02	±	0.03	0.00	±	0.00	0.00	±	0.00	0.05	±	0.06
	M+7	0.00	±	0.00	0.04	±	0.04	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
	M+8	0.00	±	0.00	0.01	±	0.02	0.00	±	0.00	0.00	±	0.00	0.05	±	0.02
	M+9	0.00	±	0.01	0.04	±	0.05	0.00	±	0.00	0.00	±	0.00	0.06	±	0.08
Val	M+0	99.83	±	0.20	99.42	±	0.59	99.94	±	0.08	100.00	±	0.00	99.99	±	0.01
	M+1	0.08	±	0.07	0.44	±	0.50	0.03	±	0.06	0.00	±	0.00	0.00	±	0.00
	M+2	0.00	±	0.00	0.13	±	0.09	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
	M+3	0.03	±	0.06	0.00	±	0.00	0.01	±	0.01	0.00	±	0.00	0.00	±	0.01
	M+4	0.05	±	0.08	0.01	±	0.01	0.01	±	0.01	0.00	±	0.00	0.01	±	0.01
	M+5	0.00	±	0.00	0.00	±	0.00	0.01	±	0.02	0.00	±	0.00	0.00	±	0.00

Table **S6b**. GC/MS data for *L. pneumophila* mutant Δzwf fed with [U- $^{13}\text{C}_6$]glucose and [1,2- $^{13}\text{C}_2$]glucose, cultivated in AYE medium.

<i>Legionella pneumophila</i> Paris Δzwf						
		11mM [U- $^{13}\text{C}_6$]Glc			11mM [1,2- $^{13}\text{C}_2$]Glc	
Ala	M+0	99.09	±	0.15	99.62	± 0.12 %
	M+1	0.11	±	0.11	0.04	± 2.19 %
	M+2	0.18	±	0.03	0.23	± 2.46 %
	M+3	0.63	±	0.04	0.11	± 0.97 %
Asp	M+0	99.91	±	0.04	99.99	± 5.82 %
	M+1	0.00	±	0.00	0.00	± 6.85 %
	M+2	0.00	±	0.00	0.00	± 0.00 %
	M+3	0.09	±	0.04	0.01	± 1.65 %
	M+4	0.00	±	0.00	0.00	± 0.00 %
Glu	M+0	99.30	±	0.49	99.27	± 20.47 %
	M+1	0.18	±	0.21	0.19	± 24.99 %
	M+2	0.52	±	0.35	0.53	± 9.25 %
	M+3	0.00	±	0.00	0.00	± 0.00 %
	M+4	0.00	±	0.00	0.00	± 0.00 %
	M+5	0.00	±	0.00	0.00	± 0.00 %
Gly	M+0	99.98	±	0.02	99.91	± 2.63 %
	M+1	0.01	±	0.02	0.09	± 1.02 %
	M+2	0.00	±	0.01	0.00	± 1.61 %
His	M+0	99.71	±	0.15	99.75	± 6.58 %
	M+1	0.00	±	0.00	0.00	± 0.00 %
	M+2	0.00	±	0.00	0.00	± 0.00 %
	M+3	0.04	±	0.04	0.11	± 0.26 %
	M+4	0.07	±	0.11	0.01	± 7.79 %
	M+5	0.17	±	0.04	0.10	± 2.29 %
	M+6	0.01	±	0.02	0.03	± 2.24 %
Ile	M+0	99.97	±	0.04	99.90	± 2.11 %
	M+1	0.00	±	0.00	0.00	± 0.00 %
	M+2	0.03	±	0.04	0.09	± 0.29 %
	M+3	0.00	±	0.00	0.00	± 0.00 %
	M+4	0.00	±	0.00	0.00	± 0.00 %
	M+5	0.00	±	0.00	0.00	± 2.01 %
Leu	M+0	100.00	±	0.00	99.95	± 0.23 %
	M+1	0.00	±	0.00	0.00	± 0.00 %
	M+2	0.00	±	0.00	0.02	± 0.98 %
	M+3	0.00	±	0.00	0.01	± 0.82 %
	M+4	0.00	±	0.00	0.00	± 0.11 %
	M+5	0.00	±	0.00	0.01	± 0.03 %
Lys	M+0	99.40	±	1.05	99.77	± 3.80 %
	M+1	0.00	±	0.00	0.00	± 0.00 %
	M+2	0.00	±	0.00	0.00	± 3.17 %
	M+3	0.20	±	0.28	0.23	± 1.33 %
	M+4	0.00	±	0.00	0.00	± 0.00 %
	M+5	0.00	±	0.00	0.00	± 0.00 %
	M+6	0.39	±	0.78	0.00	± 0.00 %

Table S6b. (Continued)

		<i>Legionella pneumophila</i> Paris Δzwf					
		11mM [U- ¹³ C ₆]Glc (2)			11mM [1,2- ¹³ C ₂]Glc		
Phe	M+0	99.20	±	0.46	99.75	±	0.27
	M+1	0.33	±	0.47	0.07	±	0.26
	M+2	0.00	±	0.00	0.00	±	0.00
	M+3	0.00	±	0.00	0.00	±	0.00
	M+4	0.27	±	0.13	0.15	±	0.01
	M+5	0.10	±	0.15	0.02	±	0.02
	M+6	0.00	±	0.00	0.00	±	0.00
	M+7	0.00	±	0.00	0.00	±	0.00
	M+8	0.05	±	0.10	0.00	±	0.00
	M+9	0.05	±	0.10	0.00	±	0.00
Pro	M+0	97.33	±	1.02	99.63	±	0.11
	M+1	0.00	±	0.00	0.00	±	0.00
	M+2	0.05	±	0.06	0.00	±	0.00
	M+3	0.14	±	0.11	0.15	±	0.02
	M+4	2.47	±	0.89	0.22	±	0.09
	M+5	0.01	±	0.02	0.00	±	0.00
Ser	M+0	99.94	±	0.04	99.97	±	0.01
	M+1	0.00	±	0.00	0.00	±	0.00
	M+2	0.00	±	0.00	0.00	±	0.00
	M+3	0.06	±	0.04	0.03	±	0.01
Thr	M+0	98.40	±	0.50	98.85	±	0.27
	M+1	1.29	±	0.82	1.03	±	0.25
	M+2	0.31	±	0.43	0.12	±	0.03
	M+3	0.00	±	0.00	0.00	±	0.00
	M+4	0.00	±	0.00	0.00	±	0.00
Tyr	M+0	99.58	±	0.34	99.78	±	0.20
	M+1	0.00	±	0.00	0.00	±	0.00
	M+2	0.03	±	0.06	0.00	±	0.06
	M+3	0.16	±	0.22	0.09	±	0.07
	M+4	0.00	±	0.00	0.00	±	0.00
	M+5	0.12	±	0.08	0.08	±	0.06
	M+6	0.11	±	0.22	0.00	±	0.06
	M+7	0.00	±	0.00	0.00	±	0.00
	M+8	0.00	±	0.00	0.01	±	0.02
	M+9	0.00	±	0.00	0.04	±	0.08
Val	M+0	99.96	±	0.03	99.98	±	0.01
	M+1	0.01	±	0.02	0.00	±	0.00
	M+2	0.00	±	0.00	0.00	±	0.00
	M+3	0.02	±	0.02	0.01	±	0.01
	M+4	0.00	±	0.00	0.01	±	0.01
	M+5	0.01	±	0.01	0.00	±	0.00

Supplemental Fig. S1. ^{13}C -NMR signals of PHB from dichloromethane extracts of *Lp* (A) wild-type growing in medium containing 0.3 g/l $[\text{U-}^{13}\text{C}_3]\text{Ser}$; (B) wild-type growing in medium containing 2 g/l $[\text{U-}^{13}\text{C}_6]\text{glucose}$; (C) Δzwf mutant growing in medium containing 2g/l $[\text{U-}^{13}\text{C}_6]\text{glucose}$; $^{13}\text{C}^{13}\text{C}$ -couplings are indicated. Structures indicate multiple ^{13}C -labelled PHB isotopologues as bars connecting ^{13}C atoms in a given molecule. The numbers indicate the respective molar abundances.

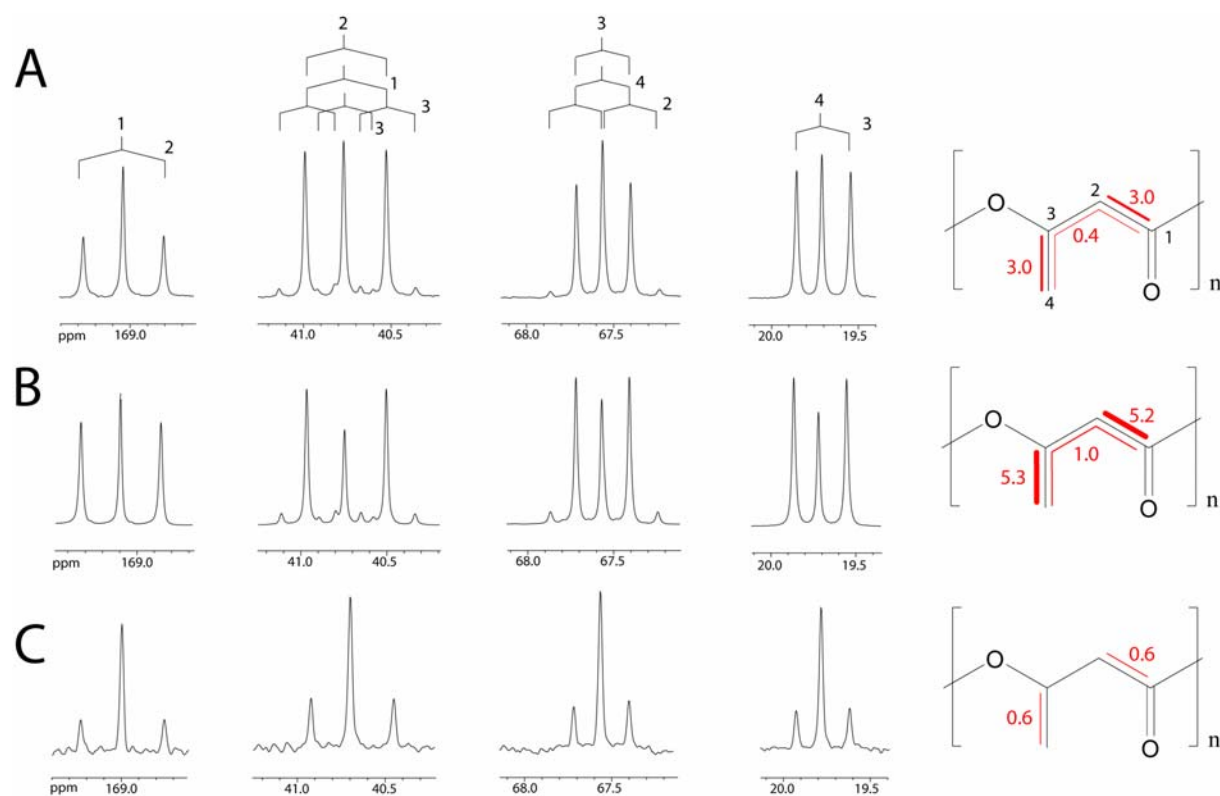


Fig S1

Supplemental Fig. S2. ^{13}C -NMR signals of C-3 of Asp (A) and C-2 of Ala (B). ^{13}C ^{13}C -couplings are indicated. Structures indicate multiple ^{13}C -labelled isotopologues as bars connecting ^{13}C atoms in a given molecule. The numbers indicate the respective molar abundances. While the signal of C-3 of Asp was characterized by an intense doublet (coupling constant, 55.3 Hz; coupling to C-4; indicated in red, Fig. 5A), the signal of C-2 of Ala was dominated by a double-doublet (coupling constants, 58.2 and 34.1 Hz, respectively; simultaneous coupling with C-1 and C-3; indicated in red, Supplemental Fig. 3). In case of Asp, the pattern showed the presence of a major isotopologue carrying ^{13}C at C-3 and C-4 (i.e. $[3,4\text{-}^{13}\text{C}_2]\text{Asp}$). As a minor isotopologue, $[2,3,4\text{-}^{13}\text{C}_3]\text{Asp}$ could be detected (indicated in green). Moreover, a minor coupling satellite (indicated in blue) reflected the presence of $[1,2,3\text{-}^{13}\text{C}_3]\text{Asp}$. In case of Ala, the signature was specific for the presence of two ^{13}C -neighbours, each of which coupled with ^{13}C -2 (i.e. $[1,2,3\text{-}^{13}\text{C}_3]\text{Ala}$). A minor satellite pair indicated the presence of $[1,2\text{-}^{13}\text{C}_2]\text{Ala}$.

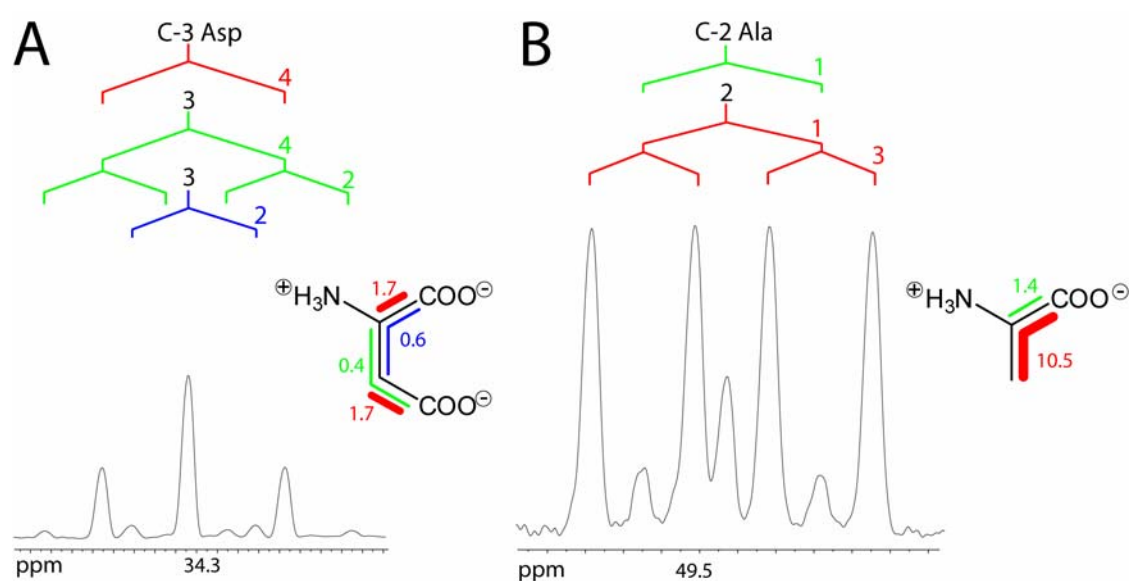


Fig. S2

Supplemental Fig. S3. Metabolic model for glucose utilization in *Lp* Paris grown in AYE medium supplemented with [1,2- $^{13}\text{C}_2$]glucose. Labelling patterns were detected in protein-derived amino acids and PHB shown in boxes. Multiple ^{13}C -labelled isotopologues determined by NMR spectroscopy are indicated as bars connecting ^{13}C atoms in a given molecule. The numbers indicate the respective molar abundances. The molar abundances of isotopomer groups comprising one, two or three ^{13}C -atoms, as determined by mass spectrometry (M+1, M+2, M+3, respectively) are also listed for comparison.

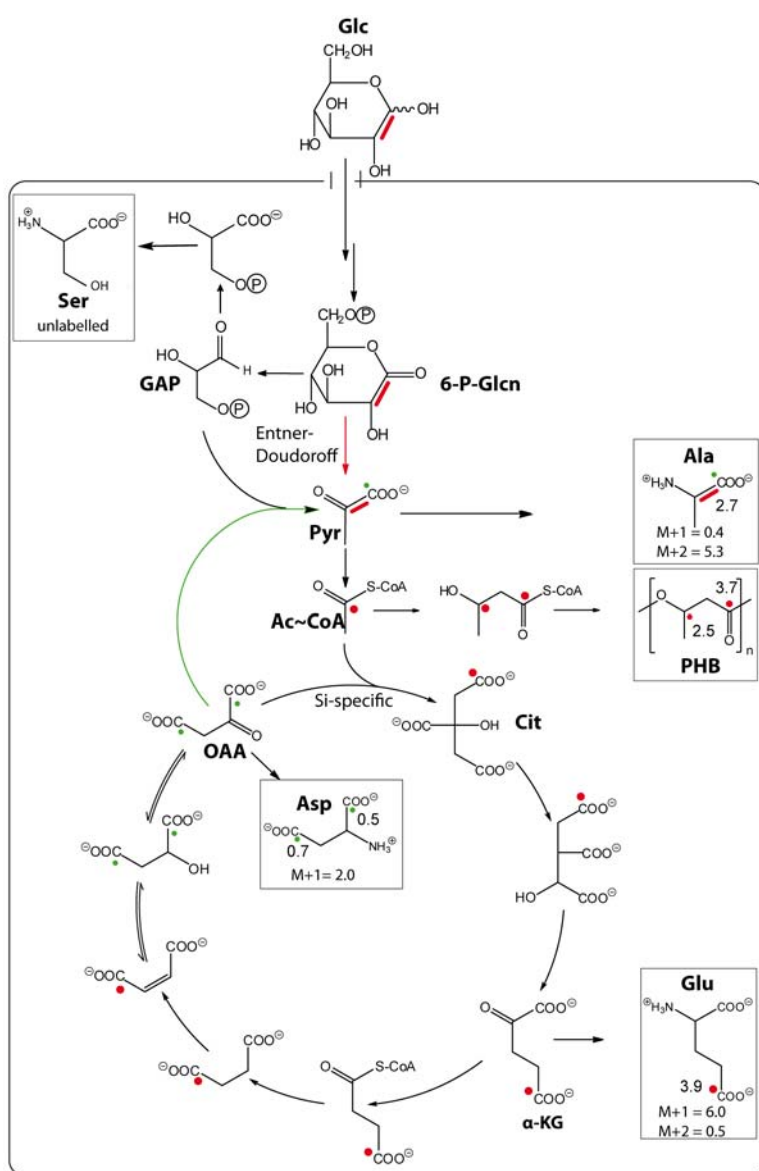


Fig. S3

Isotopologue Profiling of *Legionella pneumophila*: ROLE OF SERINE AND GLUCOSE AS CARBON SUBSTRATES

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