Amino Acid Uptake and Metabolism of Legionella pneumophila Hosted by Acanthamoeba castellanii

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Legionella pneumophila survives and replicates within a Legionella-containing vacuole (LCV) of amoebae and macrophages. Less is known about the carbon metabolism of the bacteria within the LCV. We have now analyzed the transfer and usage of amino acids from the natural host organism Acanthamoeba castellanii to Legionella pneumophila under in vivo (LCV) conditions. For this purpose, A. castellanii was 13C-labeled by incubation in buffer containing [U-13C6]glucose. Subsequently, these 13C-prelabeled amoebae were infected with L. pneumophila wild type or some mutants defective in putative key enzymes or regulators of carbon metabolism. 13C-Isotopologue profiling using 13C-prelabeled amoebae reflects key metabolic features of intracellular Legionella.

Background: Legionella replicates within vacuoles of host cells, but less is known about its metabolism during intracellular growth.

Results: Isotopologue profiles of metabolites from Legionella grown in 13C-prelabeled amoebae provide fingerprints of intracellular metabolism.

Conclusion: Host-derived amino acids are efficiently used for bacterial protein biosynthesis.

Significance: Isotopologue profiling using 13C-prelabeled amoebae reflects key metabolic features of intracellular Legionella.

The Gram-negative bacterium Legionella pneumophila is an environmental aquatic pathogen that replicates within the cells of primitive host organisms such as Acanthamoeba castellanii, Hartmannella sp., Naegleria sp., or Dictyostelium discoideum (1–3). Nevertheless, Legionella is an opportunistic pathogen and inhalation of contaminated aerosols causes Legionnaires’ disease, a severe pneumonia (4). Once in the lung macrophages, Legionella uses the same mechanisms required for replication as in amoebae (5). The bacteria inhibit the phagolysosomal pathway and build up a specialized compartment called Legionella-containing vacuole (LCV) by secretion of dot/icm effector proteins. Inside the LCV, Legionella starts to replicate, and when nutrition becomes limiting, the bacteria switch to the transmissive form, which finally ends up in the so called mature intracellular form. The mature intracellular form exhibits a spore-like shape with a thick cell wall and high amounts of the cytoplasmatic energy and carbon storage poly-3-hydroxybutyrate (PHB) (6–9).

In the replicative phase, the bacteria are metabolically highly active and resistant to sodium ions, a trait genetically related to avirulence (10). The maximum of metabolic activity is reached from mid to late exponential growth phase (11). In the transmissive phase, Legionella becomes metabolically dormant, sensitive against sodium ions, osmotically resistant, flagellated (motile), and cytotoxic. Subsequently, the virulent bacteria reach the host cytosol and are released from the host cell (12–15). Transcriptional analyses reflect the biphasic life style of Legionella because half of the genes predicted in the genome showed a shift in gene regulation during the life cycle of the bacterium (16). During the replicative phase in amoebae, genes encoding proteins for the uptake and catabolism of peptides (amino peptidases and proteinases) are up-regulated, especially for the metabolism of Ser, Thr, Gly, Tyr, Ala, and His. Addition-
Isotopologue Profiling of Intracellular L. pneumophila

Under in vitro conditions, glucose was shown to also feed the central metabolism of L. pneumophila (11, 23, 24). The Entner-Doudoroff pathway appeared as the main route of glucose metabolism and turned out to be relevant for intracellular growth (23, 37), but the genome of L. pneumophila also contains genes of an active glycolysis and pentose phosphate pathway (PPP) (23, 38, 39). Specifically, feeding of L. pneumophila with stable isotope (13C)-labeled amino acids or glucose followed by determination of the resulting isotopologue pattern of key metabolites (e.g. amino acids and PHB) allowed identification of the rates and the pathways of serine and glucose usage under extracellular in vitro conditions (23). In the meantime, protocols have been established that enable isotopologue profiling for bacteria replicating under intracellular conditions, e.g. in the LCV of U937 macrophages or amoebae as host organisms (40).

Following these protocols, we have now used 13C-prelabeled A. castellanii to investigate the nutrient usage and general carbon pathways of L. pneumophila Paris wild type and some of its mutant strains defective in putative key enzymes or regulators of carbon metabolism. With our study, the direct incorporation and usage of amino acids derived from the host is demonstrated for all strains under study of L. pneumophila growing in the LCV of A. castellanii.

EXPERIMENTAL PROCEDURES

Strains, Growth Conditions, Media, and Buffer—Escherichia coli DH5α, serving as host for amplification of recombinant plasmid DNA, was grown in LB broth or on LB agar (41, 42). L. pneumophila Paris wild type was used in this study (32). The following mutant strains were used for the in vivo infection assays: L. pneumophila Paris Δketo (lpp1788, acetyl-CoA acetyltransferase, β-ketothiolase) (this study; see below for details), Δzwf (lpp0483, glucose-6-phosphate-dehydrogenase) (23), ΔrpoN (σ54 factor) (43), and ΔfleQ (σ24 activator protein, master regulator of flagellar regulation) (43).

L. pneumophila was grown in N-(2-acetoamido)-2-aminoethanesulfonic acid-buffered yeast extract (AYE) broth consisting of 10 g of N-(2-acetoamido)-2-aminoethanesulfonic acid, 10 g of yeast extract, 0.4 g of l-Cys, and 0.25 g of ferric pyrophosphate/liter (adjusted to pH 6.8 with 3 M KOH and sterile filtered) at 37 °C with agitation at 250 rpm (44) or on buffered charcoal-yeast extract (BCYE) agar for 3 days at 37 °C. Bacterial growth in broth was monitored by determining the A600 with a Thermo Scientific GENESYS 10 Bio spectrophotometer (VWR, Darmstadt, Germany). When appropriate, media were supplemented with antibiotics at final concentrations suitable for L. pneumophila or E. coli as follows: kanamycin at 8 or 40 μg/ml, respectively, and ampicillin at 100 μg/ml for E. coli. For cultivation of L. pneumophila on agar plates, kanamycin was used in the final concentration of 12.5 μg/ml. A. castellanii ATCC 30010 was cultured in PYG 712 medium (2% proteose peptone, 0.1% yeast extract, 0.1 μl glucose, 4 mM MgSO4 × 7H2O, 0.4 mM CaCl2 × 2H2O, 0.1% sodium citrate dihydrate, 0.05 mM Fe(NH4)2(SO4)2 × 6H2O, 2.5 mM NaH2PO4, and 2.5 mM KH2PO4) at 20 °C. The Actinamaboea (Ac) buffer was PYG 712 medium without peptone, yeast extract, and glucose. We controlled the used strain of A. castellanii for the absence of bacterial parasites or endosymbions by PCR using prokaryotic 16 S rDNA specific primers (data not shown).

Intracellular Replication/Survival Assay in A. castellanii—The intracellular multiplication/survival assay with and without competition was carried out as described previously (23). DNA Techniques and Sequence Analysis—Genomic and plasmid DNAs were prepared according to standard protocols and the manufacturer’s instructions (45). PCR was carried out using a TRIO-Thermoblock (Biotrema, Göttingen, Germany) and Taq DNA polymerase (Qiagen). Foreign DNA was introduced into E. coli by electroporation with a gene pulser (Bio-Rad) according to manufacturer’s specifications at 1.7 kV, 100 μF, and 25 mF. Both strands of plasmid DNA were sequenced with infrared, dye-labeled primers by using an automated DNA sequencer (LI-COR-DNA 4000; MWG-Biotech, Ebersberg, Germany). Primers were obtained from Eurofins MWG Operon (Ebersberg, Germany). The XbaI restriction enzyme was from New England Biolabs (Frankfurt, Germany).

Gene Cloning and L. pneumophila Paris Δketo Knock-out Mutant Construction—The knock-out mutants of gene lpp1788, annotated as a β-ketothiolase (EC 2.3.1.16), was con-

ally, the genes encoding enzymes of the Entner-Doudoroff pathway and the glucoamylase GamA were found to be induced during replication in A. castellanii, indicating the usage of carbohydrate sources from amoebae (16). Indeed, GamA is responsible for glycogen- and starch-degrading activities of L. pneumophila and is expressed during replication in A. castellanii (17). The Legionella biphasic life cycle can be modeled in broth where the exponential phase corresponds to the replication of the bacteria in the LCV (13). Earlier studies indicated that Legionella uses amino acids as energy and carbon sources (11, 13, 18–23). Under these in vitro conditions, Arg, Ile, Leu, Met, Thr, Ser, Val, and Cys were shown to be essential for L. pneumophila (11, 18–25). Ser, Thr, Tyr, and particularly Glu were suggested as preferred carbon and energy sources (11, 25). Indeed, external Cys, Glu, Ser, and Met, but also Val, Glu, Tyr, Arg, and Thr stimulated intracellular replication in the LCV of human macrophages (26, 27). More recently, it was shown that the concentration of free amino acids in the cytosol of A. castellanii was increased because of infection with L. pneumophila activating the amoebal proteasome by the bacterial AnkB protein (28, 29). Thus, the Legionella ankB null mutant had a severe defect for replication in Acanthamoeba, which was rescued by supplementation with various amino acids, pyruvate, or citrate. All of these results provide strong evidence for the requirement of host-derived amino acids for replication of Legionella in the LCV (26–31). Indeed, some amino acids (Ile, Leu, Phe, Tyr, Val, Arg, and Thr stimulated intracellular replication in the LCV of host cells is still obscure (for a recent review, see Ref. 36).

Under in vitro conditions, glucose was shown to also feed the central metabolism of L. pneumophila activating the amoebal proteasome by the bacterial AnkB protein (28, 29). Thus, the Legionella ankB null mutant had a severe defect for replication in Acanthamoeba, which was rescued by supplementation with various amino acids, pyruvate, or citrate. All of these results provide strong evidence for the requirement of host-derived amino acids for replication of Legionella in the LCV (26–31). Indeed, some amino acids (Ile, Leu, Phe, Tyr, Val, Arg, and Thr stimulated intracellular replication in the LCV of host cells is still obscure (for a recent review, see Ref. 36).

In the meantime, protocols have been established that enable isotopologue profiling for bacteria replicating under intracellular conditions, e.g. in the LCV of U937 macrophages or amoebae as host organisms (40).

Following these protocols, we have now used 13C-prelabeled A. castellanii to investigate the nutrient usage and general carbon pathways of L. pneumophila Paris wild type and some of its mutant strains defective in putative key enzymes or regulators of carbon metabolism. With our study, the direct incorporation and usage of amino acids derived from the host is demonstrated for all strains under study of L. pneumophila growing in the LCV of A. castellanii.
constructed as described before (16, 46). In brief, lpp1788 (keto) was inactivated by insertion of a kanamycin resistance (kanR) cassette into the chromosomal gene. The chromosomal region containing the lpp1788 gene and flanking regions (1149 and 1296 bp) were PCR-amplified with the primers Keto-fwd and Keto-rev. The product was cloned into the pGEM-T Easy vector (Promega) resulting in pIB-Keto1. On this template, an inverse PCR was performed using the primers Keto-inv-R and Keto-inv-U3 introducing an XbaI restriction site. The PCR product contained the pGEM-Teasy backbone and the chromosomal flanking regions of lpp1788. It was religated (pIB-Keto3) and XbaI-digested. A kanR cassette with XbaI restriction sites was cloned into pIB3 resulting in pIB-Keto4. For chromosomal recombination, the construct (i.e. PCR fragment containing the kanR cassette with flanking regions of the gene of interest) was amplified per PCR with the primers Keto-fwd and Keto-rev. Natural transformation of L. pneumophila Paris was done as described before with modification (47). In brief, 5 ml of an exponentially grown overnight culture was harvested by centrifugation at 1500 × g for 10 min, and 3 ml of the supernatant was removed. The remaining culture was resuspended, transferred to a plastic tube, and incubated with the PCR product for 3 days at 30 °C without agitation. Subsequently, bacteria were grown on antibiotic selective media for 4 more days at 37 °C and 5% CO₂. Screening for mutants obtained by homologous recombination was performed by PCR with kanR legiossion-ellae. Three independent Δketo mutant strains were generated and confirmed by PCR analysis with primer pairs binding outside of the recombinant site (Keto-outsideF/R) in combination with Keto-rev/fwd and KmU/R. All primers are listed in Table 1.

| Primer | 5′–3′ sequence | Reference |
|--------|-----------------|-----------|
| Keto-fwd | ACTGGTACACCACTATGACATGTTACG | This study |
| Keto-rev | TATCGCGGATGCGATAGGAATATGCAAGT | This study |
| Keto-inv-U3 | TATCGCGGATGCGATAGGAATATGCAAGT | This study |
| Keto-inv-R2 | GAGCTTACAGACGGCTTATACCAACGAC | This study |
| Keto-outsideF | CAGAGATAGCACTAGCTTC | This study |
| Keto-outsideR | ACTTCGACAGATAGCACTCAGA | This study |
| KmR-XbalU | TGAGCTGACAGATAGCACTCAGA | This study |
| KmR-XbalR | GCCACTGTCTGAACGACTCTCTCG | This study |

**TABLE 1**

Primer used in this study to generate L. pneumophila Δketo

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**Isotopologue Profiling of Intracellular L. pneumophila**

Isotopologue Profiling of L. pneumophila from 13C6-Glucose—L. pneumophila was cultivated in 12-cell culture flasks (175 cm²). A volume of 50 ml of PYG medium was inoculated (1:10) and incubated for 3 days at room temperature. The adhered amoebae were washed two times by gently shaking three times until GC-MS analysis. A. castellanii—A. castellanii was cultivated in 12-cell culture flasks (175 cm²). A volume of 50 ml of PYG medium was inoculated (1:10) and incubated for 3 days at room temperature. The adhered amoebae were washed two times by gently shaking the flask containing 10 ml of Ac buffer. For prelabeling, the amoebae were incubated in Ac buffer supplemented with 11 mM [U-13C₆]glucose for 24 h at 37 °C. To remove external [U-13C₆]glucose, the amoebae were washed two times with Ac buffer, and the flasks were incubated in 50 ml of fresh Ac buffer for 2 h at 37 °C. L. pneumophila, grown on a BCYE agar plate for 3 days, was diluted in Ac buffer. The suspension was adjusted to an A₆₀₀ of 1, and 1 ml/flask was used for infection (about 10⁹ cells/ml, resulting in an MOI of 100). After 1 h at 37 °C, all extracellular bacteria were removed by washing two times with 10 ml Ac buffer. Subsequently, the infected amoebae were incubated for further 22 h at 37 °C. The infection status was checked by light microscopy, and cells were harvested in late exponential phase of the bacteria just before lysis of the host cell. At this time point, an aliquot was plated out on LB agar plates, and the plates were incubated for 24 h to exclude the possibility of contaminations. Cells were transferred to 50-ml tubes and killed by addition of sodium azide at a final concentration of 10 mM. The amoebae were frozen for at least 2 h at −80 °C and thawed in a 37 °C water bath and vortexed for 20 s. Successful lysis was monitored microscopically, and intactness of the bacteria was checked via plating the suspension on BCYE agar plates without sodium azide treatment before and after the lysis step. Differential centrifugation allowed separation of unlysed amoebae and high density cellular components (fraction 1, F1), L. pneumophila (fraction 2, F2), and the cytosolic proteins of A. castellanii (fraction 3, F3) as follows. The suspension was centrifuged at 600 × g for 4 °C for 15 min. The pellet contained F1 and was washed three times with Ac buffer. The supernatant was transferred to a new tube and centrifuged at 3600 × g and 4 °C for 15 min. The resulting pellet (F2) was washed two times in distilled water, and the supernatant was filtrated through a 0.22-μm pore filter to exclude contaminating bacteria. The supernatant was precipitated by addition of 100% TCA to a final concentration of 10% and incubated on ice for 30 min or overnight at 4 °C. The denatured proteins (F3) were spun down at 4600 × g and 4 °C for 30 min. All three pellets were autoclaved at 120 °C for 20 min and stored at −20 °C until Western blot fractionation control and work-up for GC-MS analysis. For Western blot fractionation control, the pellets generated from 12 flasks were solved in 600 μl of distilled water, 10 μl were used for SDS-PAGE, and the remaining solution was pelletted again for storage until GC-MS analysis.

**SDS-PAGE and Immunoblotting Control Experiments**—SDS-PAGE and Western blotting were done as described elsewhere (49, 50). Pellets F1–F3 from fractionation of four flasks with A. castellanii alone and four flasks with A. castellanii/ L. pneumophila Paris were solved in 200 μl of distilled water. For SDS-PAGE, 10 μl of each fraction was resuspended with 4 ml of Roti-Load denaturating sample buffer, boiled for 10 min, and loaded on a 12% SDS-polyacrylamide gel. Unfractionated A. castellanii (10 μl) from one flask in 50 μl of double distilled H₂O and L. pneumophila Paris (10 μl of L. pneumophila Paris,
A. castellanii was served as control (see Fig. 1A). To specify whether the contamination of the Legionella fraction (F2) was below 10%, which is indispensable to analyze samples in GC-MS, the noninfected Acanthamoeba cytosol (F3) was diluted in distilled water to imitate 0.5 and 5% contamination in a final volume of 10 μl (see Fig. 1B). A. castellanii derived proteins were detected with polyclonal (1:200) (51) or a monoclonal antibody (α-actin MAB1501 from Millipore; 1:1000). Legionella was detected using MONOFLUO anti-Legionella staining reagent from the Legionella IFA test kit (32.514; Bio-Rad), which contained a monoclonal antibody against the outer membrane protein Momp (1:100). The secondary HRP-linked antibodies were goat α-rabbit IgG (H+L) for detection of A. castellanii and goat α-mouse IgG (H+L) for detection of actin or Momp (Dianova, Hamburg, Germany) in a concentration of 1:5000. The blots were incubated with commercially available Pierce ECL Western blotting substrate ECL detection solution (Thermo Fisher Scientific) for 1 min and exposed to film. A Western blot analysis of the fractions was done before each GC-MS analysis to exclude possible cross-contaminations of the fractions.

Acid Hydrolysis and Derivatization for GC-MS Analysis—Pellet F2 or F3, respectively, was analyzed as described earlier (23). The resulting N-(tert-butyldimethylsilyl) amino acids and trimethylsilyl-3-hydroxybutyrate were then subjected to GC-MS analysis.

Mass Spectrometry—GC-MS analysis was performed with a GCMS-QP 2010 Plus (Shimadzu, Duisburg, Germany) as described earlier (23). Data were collected using the GCMS solution version 2 software (Shimadzu). Samples were analyzed at least three times. The theoretical isotope ratio and numerical deconvolution were computed according to standard procedures: (i) determination of the mass spectra of N-(tert-butyldimethylsilyl) amino acids and trimethylsilyl-3-hydroxybutyrate, respectively, (ii) determination of the mass isotopomer distribution of the labeled compounds, and (iii) correction for incorporation of 13C from natural abundance (23, 52–54). Arg, Cys, Trp, and Met were not detected by using this method (54). Furthermore, acidic treatment used for protein hydrolysis converted Asn and Gln into Asp and Glu. The labeling data given for Asp and Glu therefore represent Asn/Asp and Gln/Glu averages, respectively.

RESULTS
Optimization of the 13C Labeling and Infection Assay Using A. castellanii as Host

The amoeba A. castellanii is a natural host of Legionella in aquatic reservoirs. To analyze the transfer and usage of substrates from the host to intracellular L. pneumophila, we labeled A. castellanii with the stable 13C-isotope by using [U-13C6]glucose as a supplement to the growth buffer. To receive high yields of 13C-labeled amino acids in proteins of A. castellanii, we tested two different approaches. On the one hand, we grew A. castellanii for 3 days in PYG medium supplemented with 11 mM [U-13C6]glucose. Then the cells were split. This procedure was performed three times. On the other hand, A. castellanii cells were grown in PYG for 3 days, the medium was exchanged with Ac-buffer containing 11 mM [U-13C6]glucose, and the cells were further incubated for 24 h. Then the cells were harvested and protein-derived amino acids from both experiments were analyzed by GC-MS. 13C-Excess values (mol %) are given in Table 2 (first and second columns). Because the second method afforded higher 13C enrichments in amino acids, this setting was used to label A. castellanii in the experiments described below.

These prelabeled amoebae were then infected with L. pneumophila at a MOI of 100. After co-incubation for 2 h, remaining extracellular bacteria were removed by repeated exchange of the infection buffer. After 22 h of incubation in Ac buffer (without glucose), the amoebae were round shaped, and the LCV was filled with bacteria. Only 6–8% of the A. castellanii cells harbored motile bacteria, indicating that the majority of the infected hosts were not yet lysed at the time of harvest.

Control of the Fractionation Protocol

The infected amoebae were harvested and lysed, and subsequently, the amoebal cytosol (F3) was separated from the still intact bacteria (F2) by differential centrifugation followed by sterile filtration (see “Experimental Procedures” and Ref. 40). The first centrifugation step (“low speed”) resulted in fraction 1 (F1), which contained a mixture of high density compartments of the amoebal cytosol, amoebal ghosts, and unlysed amoebae with bacteria. Centrifugation of the supernatant (“high speed”) resulted in pelleted bacteria (F2) and a further supernatant. This supernatant was sterile filtered to ensure the absence of bacteria, and then the dissolved proteins were precipitated from this fraction (resulting in F3).

To assess cross-contamination rates of the fractions, we performed Western blot analysis using a polyclonal α-A. castellanii antibody (α-Ac), an A. castellanii specific monoclonal α-actin antibody and a L. pneumophila specific monoclonal α-major outer membrane protein antibody (α-Momp). For a further control, the complete fractionation protocol was also performed with uninfected A. castellanii cells. An Acanthamoeba-specific protein pattern was detected in F1 and F3 using α-Ac antiserum, no matter whether the amoebae were infected or not. In the L. pneumophila fraction F2, only a minor contamination (caused by A. castellanii-specific proteins) was visible (Fig. 1, A and C). A cross-reaction of Legionella proteins was observed with the α-A. castellanii antiserum, but not with the actin antibody (Fig. 1A, F2 and Lp).

To verify the sensitivity of both α-Acanthamoeba antisera, we diluted the noninfected Acanthamoeba fraction F3 in H2O.

| TABLE 2 |
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| 13C excess values of selected amino acids from different fractions |
| ni, not infected; i, infected (mean value of wild type, Δ[ΔS][ΔN]Ac and Δ[ΔS][ΔN]AcH2O, and ΔΔS); Ac, A. castellanii lysates; Ac-b, A. castellanii buffer; F2 and 3, fractions 2 and 3 reflecting amino acids from L. pneumophila and A. castellanii, respectively. |
| | 9 d, PYG | 24 h, Ac-b | 24 h Ac-b + 22 h | Mean value |
| | F3-i | F2-i | F3-i/F2-i/F3-i |
| Ala | 6.7 | 17.1 | 9.8 | 10.1 | 8.8 | 87 |
| Glu | 4.3 | 7.5 | 7.0 | 6.8 | 4.1 | 60 |
| Asp | 2.8 | 5.2 | 4.6 | 4.5 | 2.5 | 57 |
| Gly | 0.8 | 3.8 | 1.9 | 1.8 | 1.7 | 96 |
| Pro | 2.0 | 4.9 | 4.3 | 3.3 | 3.6 | 109 |
| Phe | 1.3 | 4.9 | 4.6 | 3.9 | 4.2 | 106 |
| Ser | 1.6 | 5.3 | 3.0 | 3.2 | 3.0 | 94 |

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We could determine that even a dilution of 0.5% of F3 was detectable in our Western blot by the $\alpha$-A. castellanii and the $\alpha$-actin antibody (Fig. 1B). At a concentration higher than 5%, the reaction reached its saturation point (Fig. 1D). The $\alpha$-Momp antibody clearly detected Legionella in the fractions F1 and F2, but not in F3 (Fig. 1C, $\alpha$-Momp). Because there was nearly no Acanthamoeba-specific signal detectable in fraction F2, the contamination of the Legionella fraction F2 seems to be below 0.5% and therefore applicable for isotopologue analysis.

In a further control experiment, we plated the bacteria before and after the amoeba lysis step and found no reduction in CFU, indicating that the bacteria were not lysed during this procedure. Altogether, we could therefore exclude a significant contamination of the sterile filtered Acanthamoeba cytosol fraction (F3) by Legionella-derived proteins (F2) and vice versa.

Metabolism and Amino Acid biosynthesis of A. castellanii

To determine the transfer of carbon substrates, e.g. amino acids from A. castellanii to the intracellular bacteria, by comparative isotopologue profiling, it was crucial to know the isotopic signatures of amino acids in the prelabeled host cells. Therefore, noninfected amoebae were labeled with [U-13C6]glucose following the same settings as used to prelabel amoebae for the infection experiments. Thus, A. castellanii were incubated for 24 h in Ac buffer containing 11 mM [U-13C6]glucose. The cells were then hydrolyzed under acidic conditions, and the resulting amino acids were analyzed by GC-MS. Of 14 detected amino acids, nine amino acids acquired significant 13C label from [U-13C6]glucose indicating de novo biosynthesis of these amino acids, albeit at different rates (Fig. 2A and supplemental Table S1). The 13C excess values decreased from Ala (17%) > Glu (7.5%) > Asp (5%) = Phe (5%) = Pro (5%) = Ser (5%) > Gly (4%) > Tyr (3%) > Thr (1%), whereas de novo synthesis from glucose was not detectable for His, Ile, Leu, Lys, and Val. When the amoebae were incubated for further 22 h in medium without [U-13C6]glucose (i.e. following the protocol of the infection assay), 13C excess of Ala, Ser, and Gly was found reduced by a factor of about 2, whereas the values of the other labeled amino acids (Glu, Asp, Phe, Tyr, and Thr) remained nearly constant (Table 2, third column). This shows that only Ala, Ser, and Gly were subjected to some metabolic turnover during the 22-h interval of the infection assay. The isotopologue compositions of the amoebal amino acids were important fingerprints to analyze the usage of these amino acids by the intracellular bacteria (see below), and therefore, these profiles are now described in some detail (see also Fig. 3A and supplemental Table S2a).

Alanine—The labeling pattern of Ala was characterized by a high abundance of the triple 13C-labeled isotopologue (M + 3). This species can be explained by uptake of [U-13C6]glucose by the amoebae, its conversion into the 6-phosphate, and degra-
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Amino Acid and PHB Metabolism of Intracellular L. pneumophila

The metabolism of intracellular Legionella was investigated with L. pneumophila Paris and its isogenic \( \Delta \)feQ, \( \Delta \)rpoN, \( \Delta \)zwf, \( \Delta \)keto, \( \Delta \)rfp, \( \Delta \)zwf, and \( \Delta \)rpoN. The bacteria were reisolated (fraction 2, F2) after infection of prelabeled A. castellanii. Overall \( ^{13}C \) excess (mol %) of labeled isotopologues amino acids and PHB measured by GC-MS analysis. The color map indicates \( ^{13}C \) excess in quasilogarithmic form to show even relatively small \( ^{13}C \) excess values. Each sample (two different labeling experiments) was measured three times; the color for each amino acid correlates with the mean value of the three measurements.

dation to \([U-^{13}C_6]\)glucose by pyruvate via glycolysis or the PPP. Amination of \([U-^{13}C_3]\)pyruvate then yields the detected \( ^{13}C_3 \)-Ala. The observed minor fraction of the \( ^{13}C_2 \)-labeled isotope could lead to \( ^{13}C_2 \)pyruvate by the malic enzyme. Alternatively, the M + 2 isotope of Ala could also reflect the action of transketolases during the PPP. The \( ^{13}C_1 \) isotope of Ala could be produced via gluconeogenesis that may be up-regulated during incubation in the low nutrition buffer for 22 h. Specifically, \( ^{13}C_1 \)OAA, which was generated from \( ^{13}C_2 \)acetyl-CoA entering the TCA could be transformed to \( ^{13}C_3 \)PEP and \( ^{13}C_1 \)pyruvate.

Aspartate—The high amount of \( ^{13}C_2 \)-labeled Asp (M + 2) is explained via \( ^{13}C_2 \)acetyl-CoA entering the TCA cycle. Thereby generated \( ^{13}C_2 \)OAA is transaminated to \( ^{13}C_2 \)Asp. The small amount of \( ^{13}C_3 \)Asp is formed by PEP carboxylase, which carboxylates \( ^{13}C_3 \)P to \( ^{13}C_3 \)OAA. During incubation in low nutrition infection buffer, the anaplerotic reaction of the malic enzyme could lead to a single \( ^{13}C \)-isotopologue via \( ^{13}C_2 \)acetyl-CoA entering the TCA.

Glutamate—The \( ^{13}C_2 \) isotopologue can be again explained via \( ^{13}C_2 \)acetyl-CoA entering the TCA. Isotopologues comprising more than two \( ^{13}C \) atoms reflect multiple cycling in the TCA using \( ^{13}C_2 \)OAA or \( ^{13}C_3 \)OAA combining with labeled or unlabeled acetyl-CoA, respectively. For example, the M + 4 labeling can be explained by combining \( ^{13}C_2 \)acetyl-CoA and \( ^{13}C_2 \)acetyl-CoA.

Proline—The precursor for the de novo synthesis of Pro is \( \alpha \)-ketoglutarate, and therefore the isotopologue composition reflects that of Glu.

Serine—The predominant M + 1 labeling could indicate the action of Ser-hydroxymethyltransferase using unlabeled Gly and \( ^{13}C_1 \)-labeled methylene-tetrahydrofolate as substrates. The \( ^{13}C_1 \)-isotopologue was not abundant, which excludes major flux from \([U-^{13}C_6]\)glucose via \( ^{13}C_3 \)-labeled glycolytic intermediates into Ser.

Glycine—Gly showed M + 1 and M + 2 isotopologues at similar relative amounts. This pattern can be explained by the interconversion of Ser and Gly, but the enrichment of the M + 2 fraction suggests an alternative route for Gly biosynthesis from a \( ^{13}C_2 \)-labeled source.

Phenylalanine and Tyrosine—Phe and Tyr were synthesized by the shikimate pathway from PEP and erythrose 4-phosphate (E4P) via the intermediate chorismate. This is reflected by the isotopologue patterns of Phe and Tyr which are characterized by M + 3 and M + 4 isotopologues, respectively. The M + 3 pattern is formed when \( ^{13}C_3 \)PEP and unmarked E4P enter the pathway, whereas M + 4 labeling occurs when \( ^{13}C_2 \)E4P, derived from \( ^{13}C_6 \)fructose 6-phosphate, is condensed with unlabeled PEP. Thus, the data demonstrate an active shikimate pathway in the eukaryotic amoeba, but also efficient flux via the transketolase reaction in the PPP to provide the E4P unit. Generally, infection with L. pneumophila wild type or its isogenic \( \Delta \)zwf, \( \Delta \)keto, \( \Delta \)rfp, \( \Delta \)zwf, or \( \Delta \)rpoN did not significantly change the labeling patterns of amino acids from the host, as described above (Figs. 2A and 3A and supplemental Tables S1 and S2).

Amino Acid and PHB Metabolism of Intracellular L. pneumophila

The metabolism of intracellular Legionella was investigated with L. pneumophila Paris and its isogenic \( \Delta \)feQ, \( \Delta \)rpoN, \( \Delta \)zwf, \( \Delta \)keto, \( \Delta \)rfp, \( \Delta \)zwf, and \( \Delta \)rpoN.
and Δlpp1788 (Δketo) mutant strains (43, 55). *L. pneumophila* Paris was selected because various microarray data of *in vitro* and *in vivo* (*A. castellanii*) grown bacteria were already published for this strain (16, 32, 43). The mutant strains were selected because the deleted genes refer to putative key regulators or enzymes of carbon metabolism. Thus, FleQ and RpoN are regulatory proteins of the flagellar regulon (43, 56), which were also shown to be involved in the expression of amino acid transport proteins and genes of the His and Arg biosynthesis pathways (43). Furthermore, ΔfleQ and ΔrpoN mutant strains of *L. pneumophila* were able to replicate in a general infection assay within *A. castellanii*, but both mutants showed a reduced fitness in competition to the wild type strain (55). The Δzwf gene encodes the first enzyme of the Entner-Doudoroff pathway involved in glucose metabolism and was also shown to exhibit a reduced fitness in competition with the wild type strain (23).

In the Δketo strain, the gene encoding a β-ketothiolase (*lpp1788*) was inactivated (this study). This β-ketothiolase was proposed to be involved in fatty acid degradation and PHB biosynthesis/degradation. It was not yet known whether this gene influences the intracellular replication of *L. pneumophila*. Therefore, we analyzed the Δketo strain in the intracellular replication/survival assay with and without competition. We could demonstrate that the mutant behaved similar as the wild type strain in these assays (Fig. 4). Thus, all *L. pneumophila* strains analyzed in this study grew similar in the general infection assay. With the exception of the Δketo, ΔrpoN, and ΔfleQ strains, all other mutant strains investigated in this study showed a reduced fitness when growing in competition with the wild type strain (Fig. 4) (55).

Because the impact of the Δketo, ΔrpoN, and ΔfleQ mutations on the metabolic network of *L. pneumophila* was not known at all, we first analyzed these strains under *in vitro* conditions by isotopologue profiling using [U-13C3]serine or [U-13C6]glucose as supplements to the growth medium (23). In the experiment with [U-13C3]serine, the labeling patterns of amino acids from the Δketo, Δzwf, ΔfleQ, and ΔrpoN strains were apparently identical with the respective patterns of amino acids from *in vitro* experiments with the wild type strain (Fig. 5A and sup-
Isotopologue Profiling of Intracellular L. pneumophila

In the next step, we determined and compared the isotopologue profiles of amino acids in the amoebal and bacterial protein fractions (F3 and F2, respectively) from the in vivo infection experiments using $^{13}$C-prelabeled amoebae as hosts. Generally, the $^{13}$C isotopologue patterns of amino acids from the analyzed L. pneumophila strains (F2 fractions, wild type, and mutants) resembled the respective patterns in amino acids from A. castellanii (F3 fractions) from the same experiment (Figs. 2 and 3). Thus, unlabeled amino acids in the host were found unlabeled also in the bacteria, whereas labeled host amino acids were $^{13}$C-enriched in the protein fraction of L. pneumophila. Both the molar abundances and the isotopomer compositions of the labeled amino acids from amoebae or bacteria were similar if not identical, respectively, with the exception of Ala, Asp, and Glu. To better visualize these differences, we calculated the ratios of $^{13}$C excess (mol %, mean value of all experiments) in amino acids from bacterial and amoebal proteins (F2/F3). As shown in Table 2, these ratios were 0.57 (Asp), 0.60 (Glu), 0.87 (Ala), 0.95 (Ser, Gly), 1.06 (Phe), and 1.09 (Pro). Thus, in particular the Legionella-derived amino acids Asp and Glu showed a significant lower $^{13}$C excess value than in A. castellanii (Fig. 6). However, not only the $^{13}$C excess values were different. Whereas the relative amounts of M + 1 in Asp and Glu from F2 and F3 were similar, the M + 2 and M + 3 fractions were significantly lower in the bacterial fraction F2 (Fig. 3 and supplemental Tables S1 and S2). Together with our careful fractionation control by Western blotting, the differences in Asp and Glu labeling confirmed that the detected similar labeling patterns of other amino acids from the bacterial and the host cell fraction were not due to cross-contaminations. However, more importantly, the decrease in $^{13}$C incorporation and the changes in the isotopologue profiles indicate that these amino acids were not only taken from the host and ready-made incorporated into bacterial protein, but that especially Asp and Glu were also made de novo by the intracellular bacteria from unlabeled compounds and metabolic intermediates provided from the host cell. The increase in the relative amounts of M + 1 isotopologues probably reflects contributions of $^{13}$C1-labeled precursors from the TCA of L. pneumophila. It can be concluded that either these intermediates or related metabolites are transferred from the host cell or that the labeled host amino acids were catabolized by L. pneumophila providing $^{13}$C1-labeled precursors for Asp and Glu biosynthesis. Another interesting finding is that for the amino acids Phe and Pro, which in vitro were rarely synthesized using [U-$^{13}$C$_6$]glucose or [U-$^{13}$C$_6$]glucose as substrates (Fig. 5), in vivo the $^{13}$C excess values were significantly higher in both fractions (Fig. 2). Again, this indicated the usage of host cell made Phe and Pro in the synthesis of bacterial proteins under in vivo conditions.

Whereas the $^{13}$C excess values of Phe and Pro seemed to be slightly reduced in the $\Delta$keto strain but increased in the $\Delta$leQ and $\Delta$rhoN mutants (Fig. 2), the profiles of the other bacterial amino acids were almost unchanged in comparison with the respective patterns of the wild type strain. With the $\Delta$zwf strain, we could not detect any differences in the $^{13}$C profiles notably in sharp contrast to the earlier in vitro results (23).
The $^{13}$C excess values of PHB (measured as a derivate of 3-hydroxybutyrate from the acidic hydrolysate of F2) were only about 1 mol % with a dominating M + 1 and M + 2 pattern (ratio 1:1) in the wild type, Δzwf, and ΔrpoN strains (supplemental Tables S1 and S2). Nevertheless, these enrichments reflected synthesis of bacterial PHB from host-derived $^{13}$C-labeled substrates, obviously via $^{13}$C$_2$- and $^{13}$C$_1$-acetyl-CoA. The $^{13}$C enrichments of PHB in the Δketo and ΔfleQ strain slightly differed and were ~0.7 and 1.5%, respectively (supplemental Table S1).

**DISCUSSION**

Metabolism of *A. castellanii—Acanthamoeba* is a chemoheterotrophic organism and metabolizes glucose mainly via glycolysis and the PPP. Pyruvate can be converted into acetyl-CoA, which enters the TCA cycle. Using these pathways, the carbon backbone of many amino acids generated by *Acanthamoeba* are provided (57, 58). Indeed, isotopologue profiling performed in this study showed that label from exogenous $[^{13}$C$_6$]glucose is transferred to Ala, Ser, Glu, Asp, Pro, Gly, Phe, Tyr, and Thr, whose precursors stem from degradation products of glucose via glycolysis, the PPP, and the TCA pathway, respectively. In the non-nutrient Ac buffer, $[^{13}$C$_6$]glucose was more efficiently used by *A. castellanii* as a precursor for amino acids than in the nutrient-rich PYG medium. A further incubation of $^{13}$C-labeled *A. castellanii* in unlabeled Ac buffer (i.e. without any glucose) caused a decrease in $^{13}$C-excess (Table 2, first through third columns) predominantly in Ala, Gly, and Ser, probably because of a further de novo synthesis of these amino acids from the nonlabeled citrate present in the Ac buffer (1 g/liter). We could not detect de novo synthesis of Ile, Leu, Val, His, and Lys from $[^{13}$C$_6$]glucose in *A. castellanii*. Obviously, the amoeba is auxotrophic for these amino acids. Indeed, it was already shown earlier that *Acanthamoeba* minimal medium needs to be supplemented with Ile, Leu, Val, Arg, and Met, as long as glucose is present as a single carbon source (59). If acetate is the carbon source, then also Gly was needed for growth in minimal medium. Notably, we could not detect $^{13}$C labeled Phe and Tyr in the experiment with [U-$^{13}$C$_6$]glucose. To our knowledge, this is the first direct experimental demonstration of de novo synthesis of aromatic amino acids in *A. castellanii*. In accordance, it has been observed earlier that some pathogenic protozoa including *A. castellanii* show reduced growth by the presence of inhibitors of the shikimate pathway (62–64). Interestingly, *Naegleria gruberi*, another natural host for *Legionella*, is negative for the shikimate pathway as shown by genome sequence analysis (65). On the basis of the similar labeling profiles in experiments with uninfected or infected *A. castellanii*, the amino acid and core metabolism (providing precursors of the nonessential amino acids) of *Acanthamoeba* was not significantly modulated by intracellular (i.e. in the LCV) replicating *Legionella*. This is in contrast to earlier observations with *Chlamydia pneumoniae*, *Listeria monocytogenes*, or *Salmonella enterica* serovar typhi.
murium, which modulated carbon fluxes of host metabolism, in particular fluxes via glycolysis and glutaminolysis of primary cells (66, 67).

Metabolism of L. pneumophila in the LCV of 13C-Prelabeled A. castellanii—To determine the uptake and usage of host-derived amino acids, 13C-Prelabeled A. castellanii were infected by L. pneumophila wild type strain or mutant strains and incubated for 22 h in medium without 13C tracers. This setting determines that the majority of LCVs is not lysed and L. pneumophila at the end of the nonmotile replicative phase with high metabolic activity (14).

In our infection experiments, we estimated the growth phase of L. pneumophila by determining the motility of the bacteria within the amoeba at the time of harvest, indicating that not more than 8% of the analyzed bacteria had entered the transmissive phase. It is known that L. pneumophila becomes flagellated when entering the transmissive phase (12, 15), and it has been published recently that this happens in vivo when the bacteria enter the cytosol (14).

Initial MS analysis revealed a very similar pattern of labeled amino acids from intracellularly grown L. pneumophila strains and their host cells. Therefore, we analyzed in considerable

### TABLE 3

13C excess values of selected amino acids and PHB of Lp Paris strains in vitro

|            | Wild type | ΔfeQ | ΔrpoN | Δketo |
|------------|-----------|------|-------|-------|
|            | [U-13C3]Ser | [U-13C3]Glc | [U-13C3]Ser | [U-13C3]Glc | [U-13C3]Ser | [U-13C3]Glc | [U-13C3]Ser | [U-13C3]Glc |
| Ala        | 7.3       | 4.9  | 6.8   | 9.2   | 7.7       | 11.2  | 6.9   | 8.6   |
| Glu        | 3.5       | 2.1  | 2.8   | 4.0   | 3.2       | 5.6   | 2.7   | 4.7   |
| Asp        | 1.7       | 1.4  | 1.3   | 2.3   | 1.6       | 2.8   | 1.4   | 2.8   |
| Gly        | 2.6       | 0.3  | 2.0   | 0.2   | 2.2       | 0.3   | 1.7   | 0.1   |
| PHB        | 2.1       | 6.3  | 2.0   | 9.0   | 1.7       | 10.1  | 0.6   | 1.6   |

### FIGURE 6

Overall 13C excess (mol %) of labeled amino acids of A. castellanii (red) or L. pneumophila (green) measured by GC-MS analysis. The results of A. castellanii (uninfected) and A. castellanii infected with L. pneumophila wild type (Lp WT) or the isogenic mutant strains Lp Δketo, Δzwf, ΔfeQ, and ΔrpoN are shown.
detail whether these results were due to severe cross-contamination of the analyzed fractions. The Legionella-specific α-Momp antibody clearly detected Legionella in fractions F1 and F2, as expected, but not in the A. castellanii fraction F3. In addition, no PHB could be detected in fraction F3. Furthermore, there was no specific signal visible in F2 using the Acanthamoeba-specific α-actin antiserum. The purity of the fractions was further confirmed by the finding that three L. pneumophila-derived amino acids (Ala, Asp, and Glu) showed significantly different 13C excess values than the respective A. castellanii-derived amino acids (Fig. 6).

For a discussion of the observed labeling patterns, the reader is referred to Fig. 7. In prelabeled A. castellanii cells, we detected significant 13C enrichments and specific isotopologue profiles (Fig. 7, asterisk) in Ala, Asp, Glu, Ser, Phe, Tyr, Pro, Gly, and Thr. On the other hand, Val, Leu, Ile, Lys, and His did not acquire any 13C label. These labeling patterns and fingerprints provided the basis to study the transfer of amino acids as nutrients for intracellular Legionella. The detection of the same unlabeled and labeled amino acids with their specific isotopologue fingerprints in amino acids from the bacterial fraction indicated that the whole set of amino acids were transferred...
from the host cytosol to the LCV, taken up by \textit{L. pneumophila} and subsequently directly used for protein biosynthesis. This close correlation of the labeling patterns in amino acids from \textit{A. castellanii} and its intracellular \textit{L. pneumophila} underlines the hypothesis of a synchronized evolution of amino acid metabolism including auxotrophy in intracellular bacteria and their host organisms (34, 35). However, the differences in the isotopologue profiles of Asp and Glu suggested that a fraction of these amino acids was made \textit{de novo} from other host carbon sources, which are not \textsuperscript{13}C-enriched or are less \textsuperscript{13}C-enriched, in particular from metabolites feeding the TCA and contributing one \textsuperscript{13}C atom (Fig. 7). In a potential scenario, Asp and Glu are required for downstream biosynthetic pathways and must therefore be refilled by own bacterial biosynthesis. This is reasonable because it was postulated that the glutamate-aspartate transaminase reaction is associated with the TCA, anabolic, and gluconeogenetic pathways (39, 68). Thus, the respective enzyme is highly active in medium grown \textit{L. pneumophila} strains (68) and Glu plays a key metabolic role \textit{in vitro}, is rapidly taken up, metabolized, and distributed in various cellular fractions within \textit{Legionella}. Furthermore, Glu is the only amino acid that permits growth when added to chemically defined medium containing just eight essential amino acids (12, 24, 68). These observations illustrate that Glu is a key metabolic amino acid for the lifestyle of \textit{Legionella}. We did preliminary \textit{in vitro} experiments using \textsuperscript{15}N-Glu revealing transfer of label into Ala and Asp, respectively (data not shown). Indeed, this suggests activity of the Glu/Pyr and/or Glu/Asp transaminases (Fig. 7). Nevertheless, Wieland \textit{et al.} (26) found that the addition of Glu to \textit{L. pneumophila} infected human macrophages has only a minor supporting effect on intracellular replication of \textit{Legionella}. Cys, Gln, Ser, Met, and Arg were better inducers of bacterial replication. Interestingly, the enzymes of the Asp biosynthesis pathway were induced during growth within the LCV of human macrophages, supporting our hypothesis that Asp is synthesized by \textit{Legionella} during \textit{in vivo} growth (33). Our model is also supported by the fact that, under \textit{in vitro} conditions, \textit{L. pneumophila} is auxotrophic for Phe and Tyr (23), and in \textit{A. castellanii}-grown \textit{L. pneumophila} strains, both amino acids exhibited the same \textsuperscript{13}C labeling pattern as the respective \textit{A. castellanii}-derived amino acids. Thus, we could demonstrate for the first time a direct usage of many \textit{A. castellanii}-derived amino acids for protein biosynthesis in \textit{L. pneumophila}, corroborating other findings suggesting that host-derived amino acids are taken up by \textit{L. pneumophila} and are important for the growth of the bacteria within the LCV of the host cell (16, 26, 28, 33). Indeed, the induction of amino acid transporter genes was observed for several pathogens. Therefore, the \textit{in vivo} recruitment of host amino acids seems to be a widely used strategy during intracellular replication (69–73). As prominent examples, the usage of amino acids from the host has been shown for \textit{L. monocytogenes} and \textit{Francisella tularensis}, both replicating within the cytosol of the host cell (73–75).

We also analyzed four different mutant strains of \textit{L. pneumophila} Paris. The inactivated genes encode regulators (\textit{fleQ} and \textit{rpoN}) or enzymes (\textit{zwf} and \textit{keto}) of different metabolic pathways. \textit{RpoN} is an alternative \textit{σ} factor (\textit{σ25}) that needs an activator protein, like FleQ, FleR, or PilR for transcription initiation (56, 76). Our recently published transcriptome data of \textit{in vitro} grown \textit{L. pneumophila} Paris strains revealed that \textit{RpoN} influences the expression of genes relevant for arginine biosynthesis, glycolysis, gluconeogenesis, and the PHB synthesis pathway (43, 77). Additionally, both regulatory proteins are necessary for full \textit{in vivo} fitness in \textit{A. castellanii} (55, 56). The \textit{Δketo} strain lacks \textit{lpp1788}, which is one of two homologous \(β\)-ketothiolase genes (\textit{lpp1555} and \textit{lpp1788}), putatively catalyzing the acetylation of acetyl-CoA. Our \textit{in vitro} experiments showed for the \textit{Δketo} strain a slightly delayed growth in AYE medium but not in intracellular growth within \textit{A. castellanii} (Fig. 4). However, despite these differences in growth behavior, the amino acid labeling pattern of all examined \textit{Legionella} strains grown in the LCV of \textit{13}C-prefixed \textit{A. castellanii} showed comparable results. Interestingly, also the \textsuperscript{13}C excess and labeling patterns of amino acids analyzed from the \textit{Δzwf} strain grown within \textit{A. castellanii} did not change from that of the wild type strain, whereas we recently showed significant differences under \textit{in vitro} conditions using \textit{[U-\textsuperscript{13}C_6]glucose} as a tracer (23). In \textit{in vitro}, \textit{L. pneumophila} is able to metabolize glucose via the Entner-Doudoroff pathway. However, during the replicative phase within the LCV, \textit{L. pneumophila} does not seem to use external glucose, because the reduced labeling of amino acids in the \textit{Δzwf} mutant strain observed \textit{in vitro} was not visible \textit{in vivo} when \textit{[U-\textsuperscript{13}C_6]glucose} was added to the Ac buffer during infection (preliminary results; data not shown). This is in good agreement with earlier results (37) and our own data (preliminary results; data not shown), indicating that \textit{L. pneumophila} metabolize glucose predominantly in the nonreplicative transmissive phase of growth. Thus, it can be speculated whether glucose or related carbohydrates are used during the transmissive phase in the cytosolic compartment of the host cells.

\textit{PHB} is the universal energy and carbon storage compound of \textit{Legionella} and could be detected in the dichloromethane extracts of the \textit{Legionella} fraction (F2) via \textsuperscript{13}C NMR spectroscopy or MS spectrometry of 3-hydroxybutyrate in the hydrolysate (7, 23, 78). In \textit{in vitro} labeling experiments of \textit{Legionella} with \textit{[U-\textsuperscript{13}C_3]serine} and \textit{[U-\textsuperscript{13}C_6]glucose} confirmed the known PHB formation from acetyl-CoA (23). Furthermore, PHB can be detected within \textit{Legionella} in varying amounts from replicative to transmissive phase depending on the carbon source (2, 16, 78–80). When using glucose or Ser as \(13\text{C}\)-labeled substrate, the \textsuperscript{13}C excess value of PHB of the \textit{Δketo} mutant strain was only 25–28% of that of the wild type strain. On the other hand, using glucose as the substrate, the \textsuperscript{12}C excess value of the \textit{ΔfleQ} and \textit{ΔrpoN} mutant strains was 140–160% of that of the wild type strain. Although the \textsuperscript{13}C excess values of \textit{PHB} in \textit{vivo} were low, the \textsuperscript{13}C excess values of the \textit{Δketo} mutant strains were comparable with that of the \textit{in vitro} experiments. The increased \textit{PHB} labeling in the \textit{ΔrpoN} mutant \textit{in vitro} may be explained by transcriptome data showing that the regulatory proteins FleR and \textit{RpoN} repress the expression of genes putatively encoding acetocetyl-CoA reductases (\textit{lpp0621}, \textit{lpp2322}, and \textit{lpp0620}) and therefore \textit{PHB} synthesis (43). The determined low \textit{in vivo} \textsuperscript{13}C incorporation rate into \textit{PHB} of wild type \textit{Legionella} is in accordance to the growth phase, because in our experiments the bacteria were harvested before they reached the cytosol,
this time interval corresponds to the replicative growth phase where PHB synthesis is still low.

As demonstrated for the analyzed amino acids and in contrast to in medium grown bacteria (23), there was no difference in the $^{13}$C excess values of PHB from in A. castellanii grown $Δzwf$ mutant strain and wild type (23). This result again corroborates the major usage of host-derived amino acids but not of glucose by Legionella during the replicative phase in the LCV.

Conclusion—Numerous studies about the usage of amino acids as carbon and energy source have been carried out in vitro and to a lesser extend in vivo, but the detection was always of indirect nature or based on transcriptome data. In our study, we have used $^{13}$C isotopologue profiling, which directly reflects metabolic pathways and fluxes. On the basis of these data, we show that Legionella recruits a large set of amino acids including Ala, Asp, Glu, Ser, Phe, Tyr, Pro, and Gly from the Acanthamoeba cytosol surrounding the LCV and incorporates these amino acids directly into bacterial protein. In addition, the data indicate that Ala, Asp, and Glu are also synthesized or co-metabolized by the bacteria from other host-derived carbon bodies. These results illustrate special features of intracellular replication. Because Legionella naturally is not able to replicate outside of the LCV, nutrition supply inside of the specialized vacuole is one of the fundamental aspects of the host-pathogen interaction. Pathogenesis research progressively focuses on the formerly neglected topic of metabolism, because colonization of host cell tissues requires the adaptation of bacterial metabolism. Knowledge about the bacterial metabolic routes used in vivo could identify essential enzymes for the bacterial metabolism and, therefore, promising targets for antibiotic development.

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