Role of Host Cell-Derived Amino Acids in Nutrition of Intracellular *Salmonella enterica*

Jasmin Popp, Janina Noster, Kim Busch, Alexander Kehl, Gero zur Hellen, Michael Hensel

Abteilung Mikrobiologie, Universität Osnabrück, Osnabrück, Germany

The facultative intracellular pathogen *Salmonella enterica* resides in a specific membrane-bound compartment termed the *Salmonella*-containing vacuole (SCV). Despite being segregated from access to metabolites in the host cell cytosol, *Salmonella* is able to efficiently proliferate within the SCV. We set out to unravel the nutritional supply of *Salmonella* in the SCV with focus on amino acids. We studied the availability of amino acids by the generation of auxotrophic strains for alanine, asparagine, aspartate, glutamine, and proline in a macrophage cell line (RAW264.7) and an epithelial cell line (HeLa) and examined access to extracellular nutrients for nutrition. Auxotrophies for alanine, asparagine, or proline attenuated intracellular replication in HeLa cells, while aspartate, asparagine, or proline auxotrophies attenuated intracellular replication in RAW264.7 macrophages. The different patterns of intracellular attenuation of alanine- or aspartate-auxotrophic strains support distinct nutritional conditions in HeLa cells and RAW264.7 macrophages. Supplementation of medium with individual amino acids restored the intracellular replication of mutant strains auxotrophic for asparagine, proline, or glutamine. Similarly, a mutant strain deficient in succinate dehydrogenase was complemented by the extracellular addition of succinate. Complementation of the intracellular replication of auxotrophic *Salmonella* by external amino acids was possible if bacteria were proficient in the induction of *Salmonella*-induced filaments (SIFs) but failed in a SIF-deficient background. We propose that the ability of intracellular *Salmonella* to redirect host cell vesicular transport provides access of amino acids to auxotrophic strains and, more generally, is essential to continuously supply bacteria within the SCV with nutrients.

The facultative intracellular life-style is a common virulence strategy among bacterial pathogens, and intracellular life-styles are as diverse as the diseases caused by these pathogens. While some bacteria lyse the host cell membrane compartment and initiate replication in the cytosol, others remain in a host cell-derived membrane compartment that is modified to allow intravacuolar survival and replication. To understand the life-style of bacterial pathogens, it is of central importance to analyze which nutritional limitations are experienced by the pathogen in its intracellular habitat and how the pathogen adapts its metabolism in order to survive and proliferate despite these limitations.

Salmonella enterica is an invasive, facultative, intracellular pathogen responsible for foodborne diseases ranging from localized gastroenteritis to systemic typhoid fever. Inside mammalian host cells, *S. enterica* resides in a specialized membrane-bound compartment, the *Salmonella*-containing vacuole (SCV). The SCV is modified by the action of virulence factors of *S. enterica*, and the function of the type III secretion system (T3SS) encoded by *Salmonella* pathogenicity island 2 (SPI2) and its effector proteins is of central importance (reviewed in reference 1). Despite being secluded from direct access to host cell metabolites, *Salmonella* is able to obtain all nutrients required for rapid proliferation within the SCV. The mechanisms of how *Salmonella* cells within the SCV are able to obtain these nutrients are largely unknown.

Salmonella enterica serovar Typhimurium is commonly used as a model organism for systemic *Salmonella* infections, and various cell culture models and murine infection models can be employed. *S. Typhimurium* is a prototrophic species able to synthesize all cellular macromolecules from simple C sources. *S. Typhimurium* can also synthesize all proteinogenic amino acids from metabolites of the central metabolism. Mutant strains deficient in amino acid biosynthesis are attenuated in intracellular proliferation, such as the *aroA* strain that is deficient in the syn-

thesis of aromatic amino acids (2). This indicates that access to host-derived or extracellular metabolites is limited for these amino acids.

In addition to being building blocks for protein biosynthesis, amino acids have been shown to be important C sources for various intracellular pathogens, such as *Legionella*, *Francisella*, or *Chlamydia* (3–5). *Legionella pneumophila*, for example, exploits the host cell degradation machinery by injecting effector proteins into its host cells, mimicking typical eukaryotic domains to increase the cytosolic level of free amino acids. The presence of intracellular pathogens such as *Shigella* spp. in the cytosol can lead to amino acid starvation of the host cell and was shown to stimulate host cell responses leading to autophagy (6).

Intracellular *S. Typhimurium* cells efficiently manipulate their host cells by a complex set of effector proteins injected via the SPI2-encoded T3SS (SPI2-T3SS) (reviewed in reference 7). Coinciding with the onset of intracellular replication is the formation of tubular membrane compartments that are derived from host

Received 13 May 2015 Returned for modification 9 June 2015

Accepted 28 August 2015

Accepted manuscript posted online 8 September 2015

Citation Popp J, Noster J, Busch K, Kehl A, zur Hellen G, Hensel M. 2015. Role of host cell-derived amino acids in nutrition of intracellular *Salmonella enterica*. Infect Immun 83:4466–4475. doi:10.1128/IAI.00624-15.

Editor: V. B. Young

Address correspondence to Michael Hensel, Michael.Hensel@biologie.uni-osnabrueck.de.

J.P., J.N., and K.B. contributed equally.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/IAI.00624-15>.

Copyright © 2015, American Society for Microbiology. All Rights Reserved.

TABLE 1 *Salmonella enterica* serovar Typhimurium strains used in this study

Strain	Genotype	Relevant defect(s) and/or phenotype ^a	Source or reference
NCTC 12023	Wild type		NCTC, Colindale, UK
MvP377	$\Delta sseJ::aph$	Effector SseJ; Km ^r	50
MvP505	$\Delta sifA::FRT$	<i>aph</i> deleted from MvP509	This study
MvP509	$\Delta sifA::aph$	Effector SifA; Km ^r	51
MvP1165	$\Delta sucAB::FRT$	α -Ketoglutarate dehydrogenase	Popp and Hensel, unpublished
MvP1573	$\Delta ssaV::FRT$	SPI2-T3SS	This study
MvP2096	$\Delta avtA::FRT \Delta yfbQ::FRT \Delta yfdZ::aph$	Valine-pyruvate aminotransferase, putative aminotransferase, putative PLP-dependent aminotransferase; Km ^r	This study
MvP1540	$\Delta aspC::aph$	PLP-dependent aspartate aminotransferase, Km ^r	This study
MvP1670	$\Delta aspC::FRT$	PLP-dependent aspartate aminotransferase	This study
MvP1541	$\Delta glnA::aph$	Glutamine synthetase; Km ^r	This study
MvP1671	$\Delta asnA::aph$	Asparagine synthetase A; Km ^r	This study
MvP1672	$\Delta asnA::FRT$	Asparagine synthetase A	This study
MvP1673	$\Delta asnB::aph$	Asparagine synthetase B; Km ^r	This study
MvP1674	$\Delta asnB::FRT$	Asparagine synthetase B	This study
MvP1675	$\Delta proB::aph$	Glutamate 5-kinase; Km ^r	This study
MvP1676	$\Delta proB::FRT$	Glutamate 5-kinase	This study
MvP1677	$\Delta orf32::aph$	Proline iminopeptidase; Km ^r	This study
MvP1678	$\Delta orf32::FRT$	Proline iminopeptidase	This study
MvP1679	$\Delta proC::aph$	Pyrroline-5-carboxylate reductase; Km ^r	This study
MvP1680	$\Delta proC::FRT$	Pyrroline-5-carboxylate reductase	This study
MvP1683	$\Delta asnA::FRT \Delta asnB::aph$	Asparagine synthetases A and B; Km ^r	This study
MvP1684	$\Delta asnA::FRT \Delta asnB::FRT$	Asparagine synthetases A and B	This study
MvP1685	$\Delta proB::FRT \Delta proC::aph$	Glutamate 5-kinase; Km ^r ; pyrroline-5-carboxylate reductase	This study
MvP1900	$\Delta sifA::FRT \Delta sseJ::aph$	P22 transduction of MvP377 to MvP503, effector SifA, SseJ; Km ^r	This study
MvP2164	$\Delta sifA::FRT \Delta sseJ::FRT$	Deletion of <i>aph</i> cassette from MvP1900	This study
MvP2192	$\Delta sifA::FRT \Delta sseJ::FRT \Delta proC::aph$	P22 transduction of MvP1679 in MvP2164	This study
MvP2203	$\Delta sifA::FRT \Delta sseJ::FRT \Delta proC::FRT$	Deletion of <i>aph</i> cassette from MvP2192, effectors SifA, SseJ, pyrroline-5-carboxylate reductase	This study

^a PLP, pyridoxal phosphate.

cell endosomes, and the most prominent type of *Salmonella*-induced tubules is termed *Salmonella*-induced filaments (SIFs) (reviewed in reference 8). SIFs and the SCV membranes contain characteristic organelle markers that indicate the origin of vesicles mainly from the endolysosomal pathway. Furthermore, interference with the secretory pathway and the Golgi apparatus was observed (7). The various interactions of intracellular pathogens with host cell vesicular transport and their role in the efficient intracellular replication of *S. Typhimurium* raise the question of a role for intracellular *Salmonella* in nutrition. These interactions might play an important role in intracellular nutrition by delivering extracellular or host cell-derived material, and by this strategy, *Salmonella* might also acquire host cell transporters for transportation of nutrients such as amino acids from the host cell cytosol to the SCV lumen.

We wanted to shed light on the relevance of amino acids as a nutritional source for intracellular *Salmonella* and addressed the following three questions. (i) Can auxotrophy for nonessential amino acids be compensated for by the uptake of amino acids from host cells? (ii) Is SIF formation required to access amino acids for growth in the SCV? (iii) Are requirements for amino acids distinct for *Salmonella* strains in various mammalian cell line infection models? We investigated the availability of amino acids for the intracellular growth of *S. Typhimurium* within the SCV. For this purpose, a set of defined mutant strains defective in the biosynthesis of amino acids not essential for the mammalian host cell was generated and analyzed for intracellular replication in an

epithelial cell line (HeLa) and a macrophage cell line (RAW264.7). A subset of these auxotrophic strains was attenuated in intracellular replication, and we investigated whether this defect would be restored by the addition of individual amino acids to the culture medium of host cells. Our data demonstrate that intracellular *Salmonella* cells utilize medium-derived amino acids rather than host cell-derived amino acids. The dependency of auxotrophic *Salmonella* on amino acid supplementation was distinct in different host cells. The access to such amino acids is dependent on the function of the SPI2-T3SS, indicating a role in manipulation of host cell transport for intracellular nutrition.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Salmonella enterica* serovar Typhimurium NCTC 12023 was used as the wild-type (WT) strain, and isogenic mutant strains were constructed by λ Red-mediated mutagenesis (9) (Table 1). Primers and plasmids required to introduce the FLP recombination target (FRT)-flanked resistance cassette and to check for the correct insertion and removal of the cassette by FLP recombinase afterwards are listed in Table S1 in the supplemental material. Mutant alleles were transferred into the fresh strain background or for combination with other mutations by P22 transduction according to standard procedures (10). Transductants were streak purified twice on Luria broth (LB) agar containing selective antibiotics and 10 mM EGTA to prevent the formation of lysogenic strains. Control for lysogeny in transduced strains was performed by streaking onto green plates and by streaking over a P22 lysate to prove that strains can still be infected by phage and therefore are

FIG 1 Schematic overview of selected amino acid biosynthesis pathways in *S. Typhimurium* and their origin from central metabolic pathways. Genes that encode the relevant enzymes are indicated and are further referred to in the text. Intermediates in the proline biosynthesis pathway are indicated by numbers, as follows: ①, γ -glutamyl phosphate; ②, glutamate γ -semialdehyde; ③, Δ^1 -pyrroline-5-carboxylate. Solid arrows indicate single reaction steps, while dotted arrows represent several reactions, with the number of steps given in parentheses. The reaction from ② to ③ occurs spontaneously. α KG, α -ketoglutarate; α KV, α -keto-isovalerate; CIT, citrate; GLC, glucose; OAA, oxaloacetate; PEP, phosphoenolpyruvate; PYR, pyruvate.

nonlysogenic. Positive clones were verified by PCR. Multiple gene deletions were introduced by repeated rounds of λ Red mutagenesis.

Bacteria were grown at 37°C in LB (10 g tryptone, 5 g yeast extract, and 10 g NaCl per liter) or on LB agar plates (additionally 15 g agar) containing carbenicillin and/or kanamycin at 50 µg/ml if required for selection of antibiotic resistance or maintenance of plasmids. For growth measurements, we used LB and minimal medium, referred to as PCN medium (11), containing 10 mM glucose, 4 mM tricine, 100 µM FeCl₃, 376 µM K₂SO₄, 50 mM NaCl, 10 nM Na₂MoO₄, 10 nM NaSeO₃, 4 nM H₃BO₃, 0.3 mM CoCl₂, 0.1 mM CuSO₄, 0.8 mM MnCl₂, 0.1 mM ZnSO₄, 15 mM NH₄Cl, 1 mM MgSO₄, and 10 µM CaCl₂. Here, neutral PCN medium (pH 7.4) containing 80 mM morpholinepropanesulfonic acid (MOPS) and 1 mM phosphate was used.

Growth kinetics. Cultures of bacteria were grown overnight in neutral PCN medium with 1 mM phosphate. Minimal medium for the $\Delta asnAB$ strain was supplemented with 0.8 mM asparagine (Sigma), and medium for the $\Delta proB$ and $\Delta proC$ strains contained 0.8 mM proline (Sigma). Cultures grown overnight were inoculated into neutral PCN medium with 1 mM phosphate to start at an optical density at 600 nm (OD_{600}) of 0.01. The cultures were shaken aerobically in a water bath at 37°C. For growth tests at increased osmolarity, LB was adjusted with 0.4 or 0.65 M NaCl. At time intervals of 1 h, the optical density at 600 nm was measured by using a Shimadzu UV-1202 photometer.

Gentamicin protection assay. RAW264.7 cells were routinely cultured in high-glucose (4.5 g/liter) Dulbecco's modified Eagle's medium (DMEM) containing 4 mM glutamine (Gln) (PAA, Cölbe, Germany) and 6% fetal calf serum (FCS) (Sigma) in a cell culture incubator (37°C in 5% CO₂). HeLa cells were cultured in DMEM containing 4.5 g/liter glucose, 4 mM glutamine (PAA, Cölbe, Germany), and 10% FCS (Sigma) in a cell culture incubator (37°C in 5% CO₂). For infection experiments, cells were seeded 24 or 48 h in advance in surface-treated 24-well dishes (Nunc, Thermo Fisher Scientific, Waltham, MA, USA, or TPP, Trasadingen, Switzerland) to confluence ($\sim 4 \times 10^5$ and 2×10^5 cells for RAW264.7 and HeLa cells, respectively) on the day of infection. With the onset of infection, FCS-free medium was used for the remainder of the experi-

ment. Cells were infected at a multiplicity of infection (MOI) of 1, and infection was performed with cultures of *Salmonella* strains grown aerobically in LB overnight (for RAW264.7 cells) or for 3.5 h to late log phase (for HeLa cells). Bacteria were centrifuged onto the cells for 5 min at $370 \times g$, and infection was allowed to proceed for a further 25 min. Afterwards, cells were washed twice with phosphate-buffered saline (PBS), medium containing 100 $\mu\text{g/ml}$ gentamicin (Genta 100) was added, and cells were incubated for 1 h to kill extracellular bacteria. The concentration of gentamicin was reduced to 10 $\mu\text{g/ml}$ for the rest of the experiment (Genta 10). To determine replication, cells were washed twice with PBS and lysed at 2 or 16 h postinfection (p.i.) by the addition of PBS with 0.1% Triton X-100. Serial dilutions of lysates were plated onto Mueller-Hinton II agar plates and incubated overnight at 37°C . CFU at various time points p.i. were determined.

Extracellular supplementation experiments. (i) Glutamine. For glutamine, gentamicin protection assays were performed, with the exception that glutamine was left out during infection and incubation with Genta 100 medium. A total of 4 mM glutamine (PAA, Cölbe, Germany) or alanyl-glutamine (Glx) (PAA, Cölbe, Germany) was added to Genta 10 medium and maintained for the remaining period of culturing.

(ii) Asparagine and proline. For asparagine and proline, experiments were performed as described above for the gentamicin protection assays except that 0.8 or 2.0 mM asparagine or 0.8 mM proline (Sigma) was added to Genta 10 medium where indicated.

(iii) Succinate. For succinate, experiments were performed as described above for the gentamicin protection assays except that hybridoma express medium (HEM) (PAA, Cölbe, Germany) was used and 24.6 mM succinate was added to the cells with Genta 10 medium where indicated. For one experiment, RAW264.7 cells were precultured in DMEM with additional sodium pyruvate (PAA, Cölbe, Germany) instead of medium without sodium pyruvate.

RESULTS

Generation of auxotrophic *S. Typhimurium* mutant strains.

Based on the knowledge of amino acid biosynthesis pathways in *S.*

enterica and related bacteria (Fig. 1), *S. Typhimurium* strains auxotrophic for the nonessential amino acids alanine, asparagine, aspartate, glutamine, and proline were constructed. For *Escherichia coli*, it has been shown that the major enzymes contributing to alanine biosynthesis are encoded by *avtA*, *alaA* (*yfbQ*), and *alaC* (*yfdZ*). Deletion of these genes led to severe growth defects in minimal medium containing the C source glycerol, glucose, acetate, or succinate without the addition of alanine (12). An *avtA yfbQ yfdZ* triple-deletion strain of *S. Typhimurium* was generated. Asparagine biosynthesis starts from aspartate and is performed by the gene products of *asnA* and *asnB*. In *E. coli*, the loss of either enzyme is compensated for by the other, as long as ammonia is not limiting (13). Thus, we constructed an *asnA asnB* double mutant strain. Glutamine derives from glutamate in a reaction catalyzed by glutamine synthetase (GlnA). For the synthesis of glutamine, neither an alternative pathway nor an alternative enzyme with glutamine synthase activity is known. Glutamine is a proteinogenic amino acid but serves as a donor for amino groups in biosynthetic processes and plays an important role in nitrogen metabolism. It has been shown that nitrogen-limiting conditions are sensed by changes in the glutamine pool (14). The loss of GlnA therefore resulted in glutamine auxotrophy. Like glutamine, proline derives from glutamate in a three-step reaction process catalyzed by ProB, ProA, and ProC. As these three enzymes work sequentially in one pathway and do not represent alternative pathways, knockout of any of these enzymes should result in proline auxotrophy. We generated $\Delta proB$ and $\Delta proC$ strains to block the first and last steps of proline biosynthesis. We also tested a strain deficient in the gene for a putative proline iminopeptidase (*orf32*), an enzyme that removes the N-terminal proline from a peptide (15). Proline iminopeptidase contributes to the virulence of the plant pathogen *Xanthomonas campestris* and is highly active in *Treponema denticola*, a pathogen causing human periodontal disease (16, 17). For the generation of an aspartate-auxotrophic strain, *aspC* was deleted. The *aspC* gene product acts as an aminotransferase, preferentially as an aspartate aminotransferase producing aspartate from oxaloacetate, but also takes part in the biosynthesis of the aromatic amino acids Phe and Tyr by performing transamination reactions. Furthermore, aspartate is a precursor for Thr, Ile, Leu, Met, and Asn. The aromatic amino acid transferase TyrB is mainly involved in the synthesis of aromatic amino acids but was also reported to act as an aspartate aminotransferase (18) (Fig. 1). However, only low levels of aspartate aminotransferase activity were observed in cell extracts from $\Delta aspC$ strains of *E. coli* and *Salmonella*, indicating a key role of *aspC* in aspartate biosynthesis (18, 19).

All mutant strains generated grew well in LB medium. With the exception of strains with a single deletion of *asnA*, *asnB*, or *orf32*, no growth overnight was detected in cultures in minimal medium with glucose (data not shown). Growth of the mutant strains in minimal medium was restored by the addition of Casamino Acids or specific single amino acids (data not shown).

Intracellular replication of auxotrophic *S. Typhimurium* strains in RAW264.7 and HeLa cells. The intracellular proliferation of mutant strains with defects in amino acid biosynthesis pathways in comparison to the *S. Typhimurium* WT was analyzed by using the macrophage-like cell line RAW264.7 as the host cell line. The *ssaV* mutant strain deficient in the SPI2-encoded T3SS served as a negative control, and this strain showed highly attenuated intracellular proliferation (Fig. 2). The *avtA yfbQ yfdZ*,

FIG 2 Role of amino acid biosynthesis pathways in intracellular proliferation of *S. Typhimurium*. Intracellular replication of *S. Typhimurium* WT, $\Delta ssaV$, and various mutant strains impaired in amino acid biosynthesis in RAW264.7 (A) and HeLa (B) cells was determined by gentamicin protection assays. The $\Delta ssaV$ strain defective in the SPI2-encoded T3SS served as a negative control. The $\Delta avtA \Delta yfbQ \Delta yfdZ$ strain is abbreviated *ayy*. Infected cells were lysed at 2 h and 16 h p.i., and serial dilutions were plated onto agar plates for determination of CFU. x-fold intracellular proliferation is calculated as the ratio of CFU at 16 h p.i./CFU at 2 h p.i. The x-fold proliferation of the WT strain was set as 100%, and intracellular proliferation of mutant strains is expressed as a percentage of the value for the WT. Experiments were performed in triplicates, and mean values and standard deviations are given for at least three biological replicates (two biological replicates for the $\Delta asnAB$ strain in HeLa cells). Statistical significances were calculated by Student's *t* test and are indicated as follows: n.s., not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

asnA, *asnB*, and *orf32* mutant strains showed intracellular proliferation comparable to that of the WT, whereas the *glnA*, *aspC*, *proB*, and *proC* mutant strains showed reduced intracellular proliferation. The $\Delta proC$ strain was more severely attenuated than the $\Delta proB$ strain blocked in the introducing step of proline synthesis (Fig. 2A). Proline uptake is one mechanism of osmoprotection in bacteria (reviewed in reference 20). The $\Delta proB$ and $\Delta proC$ strains grew similarly to or even better than WT *Salmonella* in medium with increased osmolarity (see Fig. S1 in the supplemental mate-

TABLE 2 Generation times for *S. Typhimurium* WT and mutant strains auxotrophic for asparagine or proline

Strain	Generation time (min) ^a	
	Expt 1	Expt 2
WT	41.9	45.9
Δ <i>asnAB</i> ^b	42.6	45.0
Δ <i>proB</i> ^c	45.0	44.9
Δ <i>proC</i> ^c	45.7	46.9

^a Strains grown aerobically in PCN minimal medium with 10 mM glucose.

^b Addition of 0.8 mM asparagine.

^c Addition of 0.8 mM proline.

rial). This indicates that increased sensitivity to high osmolarity is unlikely to cause attenuated intracellular proliferation of the Δ *proB* or Δ *proC* strain. The most severe attenuation of intracellular proliferation was observed for the *asnAB* mutant strain, with an intracellular replication rate 4-fold lower than that of the *ssaV* strain (Fig. 2A).

It was reported previously that strains impaired in the biosynthesis of aromatic amino acids and purine biosynthesis showed distinct intracellular replication defects, dependent on the cell line used for infection experiments (21). We also tested the replication of the *avtA yfbQ yfdZ*, *asnAB*, *aspC*, and *proC* mutant strains in the human epithelial cell line HeLa. The *asnAB* and *proC* mutant strains showed similarly reduced intracellular proliferation in HeLa cells as well as in RAW264.7. In contrast, the *avtA yfbQ yfdZ* mutant strain was highly attenuated in replication in HeLa cells, while the *aspC* mutant strain showed replication similar to that of the WT strain, with both phenotypes being reverted in RAW264.7 cells (Fig. 2B). Since identical formulations of DMEM were used for RAW264.7 and HeLa cell cultures, the differences observed are features of the host cells.

Medium amino acids can supplement intracellular proliferation of auxotrophic mutant strains. We next investigated the effect of medium supplementation with specific amino acids on the intracellular proliferation of auxotrophic strains in RAW264.7 cells. Tests for culture growth indicated that the *asnAB*, *proB*, and *proC* mutant strains showed growth rates comparable to that of the WT strain in minimal medium supplemented with asparagine or proline (Table 2).

The cell culture medium DMEM contains all amino acids apart from alanine, asparagine, aspartate, glutamate, and proline. To investigate supplementation in infection experiments, asparagine or proline was added to DMEM 1 h after infection. For the *asnAB* mutant strain, the addition of 0.8 mM asparagine to DMEM cell culture medium resulted in a 35- to 40-fold increase in intracellular proliferation (Fig. 3A). Asparagine supplementation also led to increased intracellular proliferation of the WT and the *ssaV* mutant strain, but these effects were not statistically significant. The addition of larger amounts of asparagine (2 mM) further increased intracellular replication of the *asnAB* mutant strain without significant effects on WT and *ssaV* strains (see Fig. S2 in the supplemental material). For *proB* and *proC* mutant strains, supplementation of medium with 0.8 mM proline restored intracellular proliferation to levels of the WT strain (Fig. 3B). Proline supplementation did not affect the intracellular proliferation of WT and *ssaV* deletion strains, while proliferation of *proB* and *proC* mutant strains was increased 9-fold and 26-fold, respectively.

Glutamine but not alanyl-glutamine restores intracellular replication of glutamine-auxotrophic *Salmonella*. While Gln is a nonessential amino acid for mammalian cells, tumor cell lines are unable to meet the demand of their own biosynthesis and depend on large amounts of extracellular glutamine in the medium (22–24). Besides being a nitrogen and carbon source, the most recently reported results postulate an important role for glutamine in

FIG 3 Complementation of asparagine- and proline-auxotrophic strains by extracellular amino acids. Intracellular replication of the *S. Typhimurium* WT strain, the Δ *ssaV* strain, and mutant strains auxotrophic for asparagine (two independently generated strains) (A) or proline (B) in RAW264.7 cells was determined as described in the legend of Fig. 2. Cell culture medium was not supplemented (–) or was supplemented (+) with 0.8 mM asparagine (A) or 0.8 mM proline (B). Amino acids were added at 1 h p.i., with a change to medium containing 10 μ g/ml gentamicin. Experiments were performed in triplicates, and mean values and standard deviations are given for one biological experiment. Statistical significances were calculated by Student’s *t* test and are indicated as follows: n.s., not significant; *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001.

FIG 4 Complementation of the glutamine-auxotrophic strain by extracellular amino acids. The effect of the addition of glutamine (Gln) or alanyl-glutamine (Glx) on the intracellular replication of *S. Typhimurium* in RAW264.7 cells (A) or HeLa cells (B) was determined as described in the legend of Fig. 2. *S. Typhimurium* WT, $\Delta ssaV$, and glutamine-auxotrophic $\Delta glnA$ strains were used for infection. Cell culture medium was supplemented with 4 mM glutamine (Gln) or alanyl-glutamine (Glx). Amino acids were added at 1 h p.i., i.e., with a change to medium containing 10 μ g/ml gentamicin. Experiments were performed in triplicates, and mean values and standard deviations are given for two (WT strain with Glx, $\Delta ssaV$ mutant with Gln, and $\Delta ssaV$ mutant with Glx) or three ($\Delta glnA$ strain with Gln and $\Delta glnA$ strain with Glx) independent biological experiments. Statistical significances were calculated by Student's *t* test and are indicated as follows: n.s., not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

counteracting acid stress due to high glycolysis rates and the level of lactate production of tumor cell lines (25). For tumor cell lines like RAW264.7 and HeLa cells, glutamine is an essential amino acid, and therefore, cell culture media usually contain 4 mM Gln. Glx is often preferred over Gln in media due to its greater stability, avoiding the formation of harmful ammonia (26). Using cell culture medium containing 4 mM Gln, we observed replication of the $\Delta glnA$ strain varying from 30% to 100% of WT replication (Fig. 2A). Interestingly, we found that virtually no intracellular replication was observed for the $\Delta glnA$ strain when cell culture medium was supplemented with 4 mM Glx instead of Gln (Fig. 4). Intracellular proliferation in RAW264.7 (Fig. 4A) and HeLa (Fig. 4B) cells was 18.6-fold and 18.4-fold higher, respectively, with Gln than with Glx. During extracellular growth, *Salmonella* used both Glx and Gln as amino acid sources, since the *glnA* mutant strain grew with similar rates *in vitro* in DMEM supplemented with 4 mM Gln and in DMEM supplemented with Glx (data not shown). The presence of the additional alanine therefore has no detrimental effect on growth *in vitro*. Replication of the WT strain in host cells was not negatively affected by the use of Glx instead of Gln (Fig. 4).

Medium succinate supplements intracellular proliferation of a tricarboxylic acid (TCA) cycle-deficient strain. To further address the direct access of intracellular *Salmonella* bacteria to nutrients in medium, we considered central metabolites as possible reporters. Succinate was a candidate since a *S. Typhimurium* $\Delta sucAB$ strain is dependent on supplementation with succinate for growth in minimal medium with glucose (J. Popp and M. Hensel, unpublished observations). A *sucAB* mutant strain showed WT levels of intracellular replication in RAW264.7 cells when cultured in DMEM but highly reduced intracellular replication when host cells were cultured in fully synthetic hybridoma

express medium (HEM) (Popp and Hensel, unpublished). Analysis of HEM showed that histidine, cysteine, and lysine are absent (data not shown), but they are present in the DMEM formulation used here. At least lysine biosynthesis in *E. coli* depends on succinyl coenzyme A (succinyl-CoA) (27). We determined the intracellular replication of *Salmonella* WT, $\Delta ssaV$, and $\Delta sucAB$ strains in RAW264.7 cells cultured in HEM with and without the addition of succinate (Fig. 5). The addition of succinate to the extracellular medium partially restored the intracellular replication of the $\Delta sucAB$ strain but had no or only a small effect on the intracellular proliferation of the *ssaV* or WT strain, respectively. This observation indicates that access of intracellular *Salmonella* to molecules present in the external medium is a more general phenomenon.

Role of SIF induction in access to extracellular amino acids. We demonstrated that extracellular amino acid supplementation can complement the intracellular proliferation of auxotrophic *Salmonella* strains. A possible mechanism is the remodeling of the endosomal system in host cells as a function of the SPI2-encoded T3SS. To test this hypothesis, we investigated the effects of *ssaV* or *sifA* defects on the extracellular supplementation of the *proC* strain. *SsaV* is a subunit essential for the function of the SPI2-T3SS, while the effector protein *SifA* is required for the maintenance of the SCV and the formation of a network of so-called *Salmonella*-induced filaments (SIFs). We investigated whether the ability to induce SIF formation affects the intracellular complementation of proline-auxotrophic strains by supplementation with extracellular proline. A *sifA*-deficient strain is unable to induce SIF formation and to maintain the integrity of the SCV (28). As a consequence, release of *Salmonella* bacteria into the host cell cytosol and cytosolic replication was observed. A double mutant strain defective in the SPI2-T3SS effectors *SifA* and *SseJ* also fails to induce SIFs but is not released into the cytosol (29). Cytosolic

FIG 5 Effect of availability of extracellular succinate on intracellular replication of *S. Typhimurium*. RAW264.7 cells were infected with WT, $\Delta ssaV$, and $\Delta sucAB$ strains, and intracellular proliferation was determined as described in the legend of Fig. 2. Hybridoma express medium was used for the period of the infection experiment without (–) or with (+) the addition of 24.6 mM succinate (Suc), added at 1 h p.i. WT replication was set as 100%, and replication of the mutant strains is expressed as a percentage of WT replication. Experiments were performed in triplicates, and mean values and standard deviations are given for at least three biological experiments, with the exception of experiments with the WT strain with succinate and the $\Delta ssaV$ strain with succinate (two replicates). Statistical significances were calculated by Student's *t* test and are indicated as follows: n.s., not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

replication of the *sifA* mutant strain may obscure potential nutritional limitation of the bacterial population in the SCV. Thus, we used the $\Delta sifA \Delta sseJ$ strain background to investigate the fate of *Salmonella* in the SCV dependent on SIF formation. Intracellular proliferation rates of the WT, $\Delta ssaV$, $\Delta sifA \Delta sseJ$, $\Delta proC$, and $\Delta sifA \Delta sseJ \Delta proC$ strains with and without the addition of 0.8 mM proline to the medium were determined (Fig. 6). Proline supplementation slightly increased the intracellular proliferation of WT *Salmonella* but had no effect on the *ssaV* or *sifA sseJ* mutant strain. Intracellular replication of the *proC* mutant strain was augmented 7.8-fold by proline supplementation, while no significant effect on the *sifA sseJ proC* strain was observed (1.08-fold increased replication). These data suggest that the SPI2-T3SS-mediated redirection of endosomal transport in host cells contributes to the access of intracellular *Salmonella* to external amino acids.

DISCUSSION

Various forms of dependency of intracellular pathogens on their host cells are known. Obligate intracellular bacteria such as *Rickettsia* spp. and *Coxiella* spp. (30, 31) have reduced genome sizes and also restricted metabolic capacities, leaving the bacteria to acquire relevant building blocks like amino acids from the host (32). Some facultative intracellular pathogens such as *Legionella pneumophila* and *Francisella tularensis* are auxotrophic for several amino acids and have evolved strategies to increase the intracellular

FIG 6 Effect of *Salmonella*-induced filament formation on complementation by extracellular proline. The intracellular replication of *S. Typhimurium* WT, $\Delta ssaV$, $\Delta sifA \Delta sseJ$, and proline-auxotrophic $\Delta proC$ or $\Delta proC \Delta sifA \Delta sseJ$ strains in RAW264.7 cells was determined as described in the legend of Fig. 2. WT replication was set as 100%, and replication of the mutant strains was calculated as a percentage of WT replication. Cell culture media were used without (–) or with (+) 0.8 mM proline added to the medium at 1 h p.i. Experiments were performed in triplicates, and mean values and standard deviations are given for three biological replicates. Statistical significances were calculated by Student's *t* test and are indicated as follows: n.s., not significant; ***, $P < 0.001$.

lar abundance of free amino acids (4, 33). In contrast, *S. Typhimurium* appears to be independent from its host cell, since this pathogen possesses all relevant central metabolic pathways (glycolysis, pentose-phosphate pathway, and the TCA cycle) and biosynthetic pathways for all amino acids. However, host defense mechanisms like nitric oxide production might provoke Lys and Met auxotrophies, and this would cause the need for uptake from the host cell (34). Accordingly, a defect of the methionine transporter MetD attenuated virulence in a mouse model (34). Furthermore, attenuated virulence was also shown for a *S. Typhimurium* strain deficient in the arginine transporter *argT* (35). ^{13}C isotopologue profiling studies further supported the access of *S. Typhimurium* to host cell-derived amino acids in epithelial and macrophage cell lines (37; Popp and Hensel, unpublished). Therefore, the uptake of amino acids may play an important role in the intracellular life of *Salmonella*, and we analyzed the intracellular phenotypes of various *Salmonella* mutant strains with auxotrophies for nonessential amino acids. Of these, auxotrophies for alanine, asparagine, or proline attenuated intracellular replication in HeLa cells, while aspartate, asparagine, or proline auxotrophies attenuated intracellular replication in RAW264.7 macrophages. Our work demonstrated that a subset of amino-acid-auxotrophic mutant strains of *S. Typhimurium* can be complemented in intracellular proliferation in mammalian host cells by adding amino acids externally to the medium. These amino acids are not present in most cell culture media such as DMEM, used here.

Our data show a strong dependency of *Salmonella* on *de novo* amino acid biosynthesis of asparagine and proline if the respective amino acid is not available in the extracellular medium. In turn, this dependency indicates that intracellular *Salmonella* bacteria are not able to obtain sufficient amounts of the amino acids from

the host cell. This finding is in accordance with previously reported observations by the Groisman group, who suggest that proline-poor conditions in the SCV to which *Salmonella* adapt, mediated by the small amount of Pro-charged tRNAs, are an inductor of expression of the operon for the virulence genes *mgtCBR* (38). Despite the ability to utilize a multiplicity of nutrients for intracellular proliferation (39), the ability to sense the precise composition of the intracellular environment allows rapid and efficient adaptation. Our results do not exclude host cell-derived amino acids as possible nutrients, yet the amounts are likely insufficient to fulfill the requirements for proliferation of *Salmonella*.

The different degrees of attenuation for the auxotrophic strains suggest differences in the intracellular availabilities of amino acids. Interestingly, we found that the alanine-auxotrophic Δ *avtA* Δ *yfbQ* Δ *yfdZ* strain was not affected in replication in RAW264.7 cells but exhibited highly reduced replication in HeLa cells. Therefore, *Salmonella* might have access to alanine in RAW264.7 cells but not in HeLa cells, or the host cell alanine pool is larger in RAW264.7 than in HeLa cells. Alternatively, access to pyruvate could explain the observed difference, since previous work showed that the Δ *avtA* Δ *yfbQ* Δ *yfdZ* mutant had no growth defect if pyruvate is available as a C source (12). There are alternative pathways and enzymes that can compensate for the loss of the major transaminases in this case. For *E. coli*, it was shown that several minor aminotransferases could contribute to alanine biosynthesis that can in the best case compensate for the loss of the major enzymes if pyruvate is available as a carbon source (12). Therefore, this difference might not be due to different abundances of alanine in the two cell lines but rather could be caused by different access to the pyruvate present in the host cell cytosol or internalized from the culture medium.

Mammalian host cells possess transporters for monocarboxylates, like pyruvate and lactate, in the plasma membrane (monocarboxylate transporter [MCT]) and a pyruvate transporter in the mitochondrial membrane (mitochondrial pyruvate carrier [MPC]) (40). By manipulation of host cell trafficking, *Salmonella* might recruit these transporters and integrate them into the SCV to access cytosolic pyruvate. Tumor cell lines have highly upregulated glycolysis, known as the Warburg effect (41), thus producing pyruvate that is subsequently converted to lactate and excreted. Furthermore, we have shown that infection with *Salmonella* triggers increased glycolysis in RAW264.7 cells (Popp and Hensel, unpublished), so pyruvate might be available as a carbon source. It is also possible that *Salmonella* has access to extracellular excreted lactate that is converted into pyruvate by lactate dehydrogenase. Higher glycolytic fluxes and/or lactate excretion in macrophages stimulated by *Salmonella* infection than in HeLa cells may explain the differences. Further work should address this point by analyzing metabolite profiles of uninfected and infected host cells. Furthermore, it would be of interest to determine the growth of the Δ *avtA* Δ *yfbQ* Δ *yfdZ* strain in cell culture medium and cell culture supernatants removed from infected and uninfected RAW264.7 and HeLa cells. Also, the Δ *avtA* Δ *yfbQ* Δ *yfdZ* strain that is additionally defective in lactate dehydrogenase could be tested for further attenuation in RAW264.7 cells.

The opposite situation was observed for the *aspC* mutant strains, showing WT levels of replication in HeLa cells and reduced proliferation in RAW264.7 cells. *Salmonella* might gain efficiently the required amounts of aspartate and amino acids deriving from aspartate from the host cell in HeLa cells yet not in

RAW264.7 cells. Also, a higher level of activity of TyrB could explain this phenotype.

The greater intracellular attenuation of the Δ *asnAB* strain than of proline-auxotrophic *Salmonella* strains indicates more limited access to host-derived asparagine than to proline or a greater requirement for asparagine than for proline. Interestingly, reduced intracellular proliferation of an Δ *asnAB* strain was predicted by a systems biology approach, and a murine systemic infection model confirmed the severe attenuation of the Δ *asnAB* strain (43). The more severe attenuation of the Δ *proC* strain could be caused by the harmful accumulation of Δ^1 -pyrroline-5-carboxylate (P5C) or other side effects, whereas a Δ *proB* strain may partially complete proline biosynthesis from intermediates of the biosynthesis pathway. The addition of larger amounts of extracellular asparagine resulted in a gradual increase in the intracellular proliferation of the *asnAB* mutant strain, indicating a limiting availability of asparagine. The knockout of *asnA* and *asnB* might also have further side effects leading to attenuated replication. However, since asparagine is formed from aspartate in one enzymatic step, it is unlikely that intermediates required for other pathways are missing or that a harmful intermediate(s) accumulates.

Intracellular *S. Typhimurium* bacteria induce the massive remodeling of the host cell endosomal system in a SPI2-T3SS-dependent manner. One remarkable consequence is the formation of extensive tubular membrane compartments, termed SIFs. SIF formation coincides with intracellular replication (44), and mutant strains deficient in SIF induction are also highly attenuated in systemic virulence in a murine model and in intracellular proliferation. However, the role of SIF induction in the physiology of intracellular *S. Typhimurium* is not clear. Continuous interactions of the SCV with the endolysosomal network have been observed (45), and live-cell imaging revealed that the SCV is connected with an extensive network of SIFs (46). These activities may indicate that continuous exchange of the SCV with host cell endosomes is the strategy for the acquisition of nutrients that are limiting intracellular replication. Complementation of amino acid auxotrophies by extracellular amino acids was observed for a SPI2-T3SS-proficient *S. Typhimurium* strain but not for an *sifA sseJ* mutant strain that is unable to induce tubular endosomal aggregates. From these data, we conclude that access to portions of media endocytosed by the host cell is an important part of the nutrition of intracellular *S. Typhimurium*.

The glutamine-auxotrophic strain could be complemented by glutamine or alanyl-glutamine during extracellular growth; however, only glutamine restored intracellular proliferation of the Δ *glnA* strain. At present, we cannot explain the molecular basis of this remarkable difference. For *E. coli* and *Salmonella*, the presence of various peptide permeases for the uptake of di-, tri-, and oligopeptides was reported previously (47, 48). Furthermore, the dipeptide permease Dpp seemed to be functional at a broad range of pHs since the protein binding part of DppA showed similar substrate binding between pH 3.0 and pH 9.5 (48). Therefore, even under the acidic conditions within the SCV, the uptake of alanyl-glutamine by *Salmonella* should be possible, provided that this dipeptide reaches the lumen of the SCV. If manipulation of endolysosomal trafficking is a major pathway to gain nutrients, the impact of the access to glutamine or alanyl-glutamine in medium should be similar for the intracellular growth of the *S. Typhimurium* Δ *glnA* strain. An alternative explanation might be the putative recruiting of host cell transporters for glutamine to the SCV,

similar to the intracellular strategy of *S. Typhimurium* for the acquisition of arginine described previously (35). Such a recruitment mechanism would call for host cell transporters for the uptake of glutamine from the medium. In contrast to glutamine, alanyl-glutamine is hydrolyzed to alanine and glutamine by extracellular aminopeptidases that are released into the medium by the host cell and taken up after cleavage (GlutaMAX; Life Technologies) (49). This results in a constantly lower concentration of available glutamine in the extracellular medium (GlutaMAX; Life Technologies) (49), possibly provoking less uptake of glutamine and lower cytosolic levels in the host cell. Further work has to reveal how *S. Typhimurium* can utilize glutamine and alanyl-glutamine under intracellular conditions.

To conclude, our work demonstrates the requirement of prototrophic metabolism and the full biosynthetic repertoire for the intracellular life-style of *S. Typhimurium* in mammalian host cells. The availabilities of amino acids are distinct in HeLa cells and murine macrophages, and our data support that the SPI2-T3SS-dependent manipulation of host cell endosomal transport is a means of continuous supply of the SCV with medium compounds internalized by the host cell. As a metabolic all-rounder, intracellular *S. Typhimurium* can efficiently utilize internalized amino acids and various C sources for efficient intracellular proliferation.

ACKNOWLEDGMENT

This work was supported by grant HE1964/14-2 as part of the DFG priority program 1316, Host-Adapted Metabolism of Bacteria Pathogens.

REFERENCES

1. Figueira R, Holden DW. 2012. Functions of the *Salmonella* pathogenicity island 2 (SPI-2) type III secretion system effectors. *Microbiology* 158: 1147–1161. <http://dx.doi.org/10.1099/mic.0.058115-0>.
2. Hoiseh SK, Stocker BA. 1981. Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. *Nature* 291:238–239. <http://dx.doi.org/10.1038/291238a0>.
3. Al-Quadani T, Price CT, Abu Kwaik Y. 2012. Exploitation of evolutionarily conserved amoeba and mammalian processes by *Legionella*. *Trends Microbiol* 20:299–306. <http://dx.doi.org/10.1016/j.tim.2012.03.005>.
4. Steele S, Brunton J, Ziehr B, Taft-Benz S, Moorman N, Kawula T. 2013. *Francisella tularensis* harvests nutrients derived via ATG5-independent autophagy to support intracellular growth. *PLoS Pathog* 9:e1003562. <http://dx.doi.org/10.1371/journal.ppat.1003562>.
5. Braun PR, Al-Younes H, Gussmann J, Klein J, Schneider E, Meyer TF. 2008. Competitive inhibition of amino acid uptake suppresses chlamydial growth: involvement of the chlamydial amino acid transporter BrnQ. *J Bacteriol* 190:1822–1830. <http://dx.doi.org/10.1128/JB.101240-07>.
6. Tattoli I, Sorbara MT, Vuckovic D, Ling A, Soares F, Carneiro LA, Yang C, Emili A, Philpott DJ, Girardin SE. 2012. Amino acid starvation induced by invasive bacterial pathogens triggers an innate host defense program. *Cell Host Microbe* 11:563–575. <http://dx.doi.org/10.1016/j.chom.2012.04.012>.
7. LaRock DL, Chaudhary A, Miller SI. 2015. *Salmonella* interactions with host processes. *Nat Rev Microbiol* 13:191–205. <http://dx.doi.org/10.1038/nrmicro3420>.
8. Liss V, Hensel M. 2015. Take the tube: remodelling of the endosomal system by intracellular *Salmonella enterica*. *Cell Microbiol* 17:639–647. <http://dx.doi.org/10.1111/cmi.12441>.
9. Datsenko KA, Wanner BL. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A* 97:6640–6645. <http://dx.doi.org/10.1073/pnas.120163297>.
10. Maloy SR, Stewart VL, Taylor RK. 1996. Genetic analysis of pathogenic bacteria. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
11. Neidhardt FC, Bloch PL, Smith DF. 1974. Culture medium for enterobacteria. *J Bacteriol* 119:736–747.
12. Kim SH, Schneider BL, Reitzer L. 2010. Genetics and regulation of the major enzymes of alanine synthesis in *Escherichia coli*. *J Bacteriol* 192: 5304–5311. <http://dx.doi.org/10.1128/JB.00738-10>.
13. Felton J, Michaelis S, Wright A. 1980. Mutations in two unlinked genes are required to produce asparagine auxotrophy in *Escherichia coli*. *J Bacteriol* 142:221–228.
14. Ikeda TP, Shauger AE, Kustu S. 1996. *Salmonella typhimurium* apparently perceives external nitrogen limitation as internal glutamine limitation. *J Mol Biol* 259:589–607. <http://dx.doi.org/10.1006/jmbi.1996.0342>.
15. Sarid S, Berger A, Katchalski E. 1959. Proline iminopeptidase. *J Biol Chem* 234:1740–1746.
16. Makinen KK, Chen CY, Makinen PL. 1996. Proline iminopeptidase from the outer cell envelope of the human oral spirochete *Treponema denticola* ATCC 35405. *Infect Immun* 64:702–708.
17. Zhang L, Jia Y, Wang L, Fang R. 2007. A proline iminopeptidase gene upregulated in planta by a LuxR homologue is essential for pathogenicity of *Xanthomonas campestris* pv. *campestris*. *Mol Microbiol* 65:121–136. <http://dx.doi.org/10.1111/j.1365-2958.2007.05775.x>.
18. Gelfand DH, Rudo N. 1977. Mapping of the aspartate and aromatic amino acid aminotransferase genes *tyrB* and *aspC*. *J Bacteriol* 130:441–444.
19. Van Dyk TK, LaRossa RA. 1986. Sensitivity of a *Salmonella typhimurium* *aspC* mutant to sulfometuron methyl, a potent inhibitor of acetolactate synthase II. *J Bacteriol* 165:386–392.
20. Wood JM. 1988. Proline porters effect the utilization of proline as nutrient or osmoprotectant for bacteria. *J Membr Biol* 106:183–202. <http://dx.doi.org/10.1007/BF01872157>.
21. Hölzer SU, Hensel M. 2012. Divergent roles of *Salmonella* pathogenicity island 2 and metabolic traits during interaction of *S. enterica* serovar Typhimurium with host cells. *PLoS One* 7:e33220. <http://dx.doi.org/10.1371/journal.pone.0033220>.
22. Fuchs BC, Bode BP. 2006. Stressing out over survival: glutamine as an apoptotic modulator. *J Surg Res* 131:26–40. <http://dx.doi.org/10.1016/j.jss.2005.07.013>.
23. Eagle H. 1955. Nutrition needs of mammalian cells in tissue culture. *Science* 122:501–514. <http://dx.doi.org/10.1126/science.122.3168.501>.
24. Wise DR, Thompson CB. 2010. Glutamine addiction: a new therapeutic target in cancer. *Trends Biochem Sci* 35:427–433. <http://dx.doi.org/10.1016/j.tibs.2010.05.003>.
25. Huang W, Choi W, Chen Y, Zhang Q, Deng H, He W, Shi Y. 2013. A proposed role for glutamine in cancer cell growth through acid resistance. *Cell Res* 23:724–727. <http://dx.doi.org/10.1038/cr.2013.15>.
26. Tritsch GL, Moore GE. 1962. Spontaneous decomposition of glutamine in cell culture media. *Exp Cell Res* 28:360–364. [http://dx.doi.org/10.1016/0014-4827\(62\)90290-2](http://dx.doi.org/10.1016/0014-4827(62)90290-2).
27. Patte J-C. 1996. Biosynthesis of threonine and lysine, p 528–541. In Neidhardt FC, Curtiss R III, Ingraham JL, Lin ECC, Low KB, Magasanik B, Reznikoff WS, Riley M, Schaechter M, Umberger HE (ed), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, DC.
28. Beuzon CR, Meresse S, Unsworth KE, Ruiz-Albert J, Garvis S, Waterman SR, Ryder TA, Boucrot E, Holden DW. 2000. *Salmonella* maintains the integrity of its intracellular vacuole through the action of SifA. *EMBO J* 19:3235–3249. <http://dx.doi.org/10.1093/emboj/19.13.3235>.
29. Ruiz-Albert J, Yu XJ, Beuzon CR, Blakey AN, Galyov EE, Holden DW. 2002. Complementary activities of SseJ and SifA regulate dynamics of the *Salmonella typhimurium* vacuolar membrane. *Mol Microbiol* 44:645–661. <http://dx.doi.org/10.1046/j.1365-2958.2002.02912.x>.
30. Omsland A, Hackstadt T, Heinzen RA. 2013. Bringing culture to the uncultured: *Coxiella burnetii* and lessons for obligate intracellular bacterial pathogens. *PLoS Pathog* 9:e1003540. <http://dx.doi.org/10.1371/journal.ppat.1003540>.
31. Hackstadt T. 1996. The biology of rickettsiae. *Infect Agents Dis* 5:127–143.
32. Casadevall A. 2008. Evolution of intracellular pathogens. *Annu Rev Microbiol* 62:19–33. <http://dx.doi.org/10.1146/annurev.micro.61.080706.093305>.
33. Isberg RR, O'Connor TJ, Heidtman M. 2009. The *Legionella pneumophila* replication vacuole: making a cosy niche inside host cells. *Nat Rev Microbiol* 7:13–24. <http://dx.doi.org/10.1038/nrmicro1967>.
34. Richardson AR, Payne EC, Younger N, Karlinsey JE, Thomas VC, Becker LA, Navarre WW, Castor ME, Libby SJ, Fang FC. 2011. Multiple targets of nitric oxide in the tricarboxylic acid cycle of *Salmonella enterica* serovar Typhimurium. *Cell Host Microbe* 10:33–43. <http://dx.doi.org/10.1016/j.chom.2011.06.004>.
35. Das P, Lahiri A, Lahiri A, Sen M, Iyer N, Kapoor N, Balaji KN, Chakravorty D. 2010. Cationic amino acid transporters and *Salmonella* Typhimurium ArgT collectively regulate arginine availability towards in-

- tracellular *Salmonella* growth. PLoS One 5:e15466. <http://dx.doi.org/10.1371/journal.pone.0015466>.
36. Reference deleted.
 37. Gotz A, Eylert E, Eisenreich W, Goebel W. 2010. Carbon metabolism of enterobacterial human pathogens growing in epithelial colorectal adenocarcinoma (Caco-2) cells. PLoS One 5:e10586. <http://dx.doi.org/10.1371/journal.pone.0010586>.
 38. Lee EJ, Choi J, Groisman EA. 2014. Control of a *Salmonella* virulence operon by proline-charged tRNA^{Pro}. Proc Natl Acad Sci U S A 111:3140–3145. <http://dx.doi.org/10.1073/pnas.1316209111>.
 39. Steeb B, Claudi B, Burton NA, Tienz P, Schmidt A, Farhan H, Maze A, Bumann D. 2013. Parallel exploitation of diverse host nutrients enhances *Salmonella* virulence. PLoS Pathog 9:e1003301. <http://dx.doi.org/10.1371/journal.ppat.1003301>.
 40. Schell JC, Rutter J. 2013. The long and winding road to the mitochondrial pyruvate carrier. Cancer Metab 1:6. <http://dx.doi.org/10.1186/2049-3002-1-6>.
 41. Lopez-Lazaro M. 2008. The Warburg effect: why and how do cancer cells activate glycolysis in the presence of oxygen? Anticancer Agents Med Chem 8:305–312. <http://dx.doi.org/10.2174/187152008783961932>.
 42. Reference deleted.
 43. Jelsbak L, Hartman H, Schroll C, Rosenkrantz JT, Lemire S, Wallrodt I, Thomsen LE, Poolman M, Kilstrup M, Jensen PR, Olsen JE. 2014. Identification of metabolic pathways essential for fitness of *Salmonella* Typhimurium in vivo. PLoS One 9:e101869. <http://dx.doi.org/10.1371/journal.pone.0101869>.
 44. Garcia-del Portillo F, Zwick MB, Leung KY, Finlay BB. 1993. *Salmonella* induces the formation of filamentous structures containing lysosomal membrane glycoproteins in epithelial cells. Proc Natl Acad Sci U S A 90:10544–10548. <http://dx.doi.org/10.1073/pnas.90.22.10544>.
 45. Drecktrah D, Knodler LA, Howe D, Steele-Mortimer O. 2007. *Salmonella* trafficking is defined by continuous dynamic interactions with the endolysosomal system. Traffic 8:212–225. <http://dx.doi.org/10.1111/j.1600-0854.2006.00529.x>.
 46. Zhang Y, Hensel M. 2013. Evaluation of nanoparticles as endocytic tracers in cellular microbiology. Nanoscale 5:9296–9309. <http://dx.doi.org/10.1039/c3nr01550e>.
 47. Payne JW, Smith MW. 1994. Peptide transport by microorganisms. Adv Microb Physiol 36:1–80. [http://dx.doi.org/10.1016/S0065-2911\(08\)60176-9](http://dx.doi.org/10.1016/S0065-2911(08)60176-9).
 48. Smith MW, Tyreman DR, Payne GM, Marshall NJ, Payne JW. 1999. Substrate specificity of the periplasmic dipeptide-binding protein from *Escherichia coli*: experimental basis for the design of peptide prodrugs. Microbiology 145:2891–2901. <http://dx.doi.org/10.1099/00221287-145-10-2891>.
 49. Christie A, Butler M. 1994. Glutamine-based dipeptides are utilized in mammalian cell culture by extracellular hydrolysis catalyzed by a specific peptidase. J Biotechnol 37:277–290. [http://dx.doi.org/10.1016/0168-1656\(94\)90134-1](http://dx.doi.org/10.1016/0168-1656(94)90134-1).
 50. Chakravorty D, Hansen-Wester I, Hensel M. 2002. *Salmonella* pathogenicity island 2 mediates protection of intracellular *Salmonella* from reactive nitrogen intermediates. J Exp Med 195:1155–1166. <http://dx.doi.org/10.1084/jem.20011547>.
 51. Halici S, Zenk SF, Jantsch J, Hensel M. 2008. Functional analysis of the *Salmonella* pathogenicity island 2-mediated inhibition of antigen presentation in dendritic cells. Infect Immun 76:4924–4933. <http://dx.doi.org/10.1128/IAI.00531-08>.