Intracellular *Salmonella* metabolism

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Abstract

Growth of *Salmonella* inside infected host cells is a key aspect of their ability to cause local enteritis or systemic disease. This growth depends on exploitation of host nutrients through a large *Salmonella* metabolism network with hundreds of metabolites and enzymes. Studies in cell culture infection models are unraveling more and more of the underlying molecular and cellular mechanisms, but also show striking *Salmonella* metabolic plasticity depending on host cell line and experimental conditions. In vivo studies have revealed a qualitatively diverse, but quantitatively poor, host-*Salmonella* nutritional interface, which on one side makes *Salmonella* fitness largely resilient against metabolic perturbations, but on the other side severely limits *Salmonella* biomass generation and growth rates. This review discusses goals and techniques for studying *Salmonella* intracellular metabolism, summarizes main results and implications, and proposes key issues that could be addressed in future studies.

Introduction

Salmonella enterica is a Gram-negative bacterium and a close relative of *Escherichia coli*. There are more than two 2300 different *Salmonella* serovars, many of which can cause local intestinal disease (enteritis, diarrhea) in a broad range of hosts. By contrast, some serovars can also disseminate from the gut and cause systemic disease in a host-specific manner (enteric fever / (para)typhoid fever, non-typhoidal salmonellosis NTS). *Salmonella* cause major mortality and morbidity worldwide (Havelaar *et al.*, 2015, Keestra-Gounder *et al.*, 2015, LaRock *et al.*, 2015, Wain *et al.*, 2015). Vaccines for prevention of human disease are available for the single serovar Typhi, and have only moderate protective efficacy. Antimicrobial chemotherapy becomes less and less effective due to rapidly increasing multidrug resistance, and both the US Center for Disease Control and the WHO include *Salmonella* among the most serious infectious disease threats for human health. In addition to better vaccines and novel antibiotics, interference with transmission by supplying clean drinking water can dramatically reduce incidence of enteric fever, and better control of *Salmonella* carriage in animal livestock might largely prevent diarrheal disease and NTS.

There are two main types of *Salmonella* infection, causing either enteritis or systemic disease. Both infections start with ingestion of *Salmonella*-contaminated food or water. A large part of the ingested *Salmonella* are killed by stomach acid, bile, and intestinal defensins (Wotzka *et al.*, 2017). Neutrophils migrating through the gut mucosa also efficiently kill *Salmonella* in the gut lumen, and neutrophils and macrophages in the gut mucosa kill efficiently invading *Salmonella*. Nevertheless, if the infectious dose is large enough (i.e, higher than 1’000 to 100’000 colony-forming units) and competition by resident microbiota is overcome, surviving *Salmonella* can overwhelm these defenses and cause disease in the intestine. During enteritis, *Salmonella* proliferates mostly in the gut lumen, although some *Salmonella* invade the intestinal mucosa and proliferate in gut epithelial cells (Wotzka *et al.*,

2017), and this is strongly enhanced in neonate mice (Zhang *et al.*, 2014). Intra-epithelial *Salmonella* proliferation leads to inflammasome activation and extrusion of infected cells (Knodler *et al.*, 2014, Sellin *et al.*, 2014, Zhang *et al.*, 2014), which might contribute to the overall inflammation response which ultimately leads to water loss to the lumen and diarrhea (Darwin *et al.*, 1999). However, the importance of this epithelial proliferation for enteritis pathology and *Salmonella* overall fitness and transmission is still unclear.

Salmonella serovars that can cause systemic disease on the other hand, enter intestinal Peyer's patches and solitary intestinal lymphoid tissue. In mouse models, *Salmonella* are rarely found in epithelial cells of adult hosts (Halle *et al.*, 2007, Zhang *et al.*, 2014), whereas in neonate mice, which largely lack differentiated M-cells, *Salmonella* mostly invade epithelial cells and probably disseminate from there to internal organs (Zhang *et al.*, 2014). Just a few clones can successfully establish themselves in these tissues and disseminate systemically (Meynell, 1957, Lim *et al.*, 2014). Within host tissues, *Salmonella* resides and proliferates predominantly in tissue macrophages but also other cell types (Burton *et al.*, 2014). Spleen and liver are major target organs but other tissues are also infected. From the liver, *Salmonella* can reach the gallbladder where they proliferate in gallbladder epithelial cells (and on gallstones, if present). Infected cells activate the inflammasome and are extruded (Knodler *et al.*, 2010). Released *Salmonella* then reach together with bile the intestine and ultimately feces, thus closing the transmission cycle.

Taken together, intracellular *Salmonella* proliferation occurs during both diarrheal and systemic disease. The metabolic conditions and mechanisms that drive this intracellular growth are the focus of extensive research efforts since a better overview of the *Salmonella*-host metabolic landscape might open new windows for antimicrobial development. In this review, we discuss some aspects of this exciting field. Additional aspects are covered in other informative recent reviews (Eisenreich *et al.*, 2010, Dandekar *et al.*, 2012, Eisenreich *et*

71 *al.*, 2013, Dandekar *et al.*, 2014, Eisenreich *et al.*, 2015). For *Salmonella* metabolism during
72 extracellular stages, an excellent review has recently been published (Rivera-Chavez *et al.*,
73 2015).

74

75 **Metabolism as a basis for *Salmonella* fitness, virulence, and persistence**

76 *Salmonella* in vivo proliferation requires de novo synthesis of biomass components such as
77 proteins, carbohydrates, lipids and nucleic acids from small molecule precursors. *Salmonella*
78 can obtain these precursors from internal storage such as glycogen or lipids (for a limited
79 number of divisions) or directly from the host microenvironment (e.g., amino acids), and/or
80 synthesize them from a few basic host carbon (such as acetate, glycerol, glucose), nitrogen
81 (e.g., ammonium), sulfur (e.g., sulfate), and phosphor (e.g., phosphate) sources. How
82 *Salmonella* salvages these nutrients from the host is still a matter of debate and will be
83 discussed later on. Nutrient uptake and conversion into biomass requires substantial energy,
84 and finding and exploiting a suitable energy source is the single most important metabolic
85 activity of growing cells (Abu Kwaik *et al.*, 2015). Counterintuitively, *Salmonella* seems to
86 diminish ATP production under some host conditions by direct inhibition of the ATP
87 synthase with MgtC (Lee *et al.*, 2013), a protein that is required for wild-type level
88 *Salmonella* fitness (Alix *et al.*, 2008). This could be a special adaptation to limiting
89 magnesium availability by releasing ATP-bound magnesium for ribosome stabilization
90 (Pontes *et al.*, 2016).

91 Nutrient availability and *Salmonella* metabolic capabilities will modulate overall
92 biomass generation and proliferation (Monahan *et al.*, 2016) and thus *Salmonella* fitness in
93 host tissues during infection. Identifying and quantifying relevant nutrients as well as the
94 corresponding catabolic and anabolic *Salmonella* pathways is therefore of fundamental

95 importance for understanding the disease process and to identify opportunities for specific
96 enzyme inhibitors as urgently needed novel antimicrobials to control infection. Moreover,
97 *Salmonella* metabolic mutants might be useful as live attenuated vaccines (Tennant *et al.*,
98 2015), or as cancer therapeutics (Wang *et al.*, 2016).

99 It is important to note that fitness (the capability to produce viable offspring) does not
100 always correlate with virulence (the capability to cause host pathology). Indeed, *Salmonella*
101 mutants with severe fitness defects nevertheless still cause wild-type level gut pathology in
102 single-strain infections in the mouse enteritis model (Winter *et al.*, 2010, Thiennimitr *et al.*,
103 2011, Faber *et al.*, 2017). Inhibiting the corresponding enzymes would thus have limited
104 impact on disease progression. By contrast, several metabolic mutations (*galE, purA, aroA,*
105 *aroCD, manA*, etc.) abrogate both *Salmonella* in vivo fitness as well as systemic virulence in
106 the mouse typhoid fever model and in human volunteers (Bumann *et al.*, 2000), highlighting
107 metabolism as a key targetable aspect of systemic salmonellosis.

108 Metabolism is not only required for proliferation but also for maintaining essential
109 basal activities even in non-dividing *Salmonella* cells. Identification of such "dormancy"-
110 associated metabolic activities could provide a basis for specific targeting of *Salmonella*
111 persisters that can survive even prolonged antimicrobial chemotherapy (Harms *et al.*, 2016).
112 However, such pathways seem to be quite rare with the possible exception of fatty acid
113 biosynthesis (Barat *et al.*, 2012).

114 Finally, *Salmonella* metabolic enzymes might be important for disease progression
115 not because of their catalytic activity, but rather due to unrelated so-called moonlighting
116 functions (Henderson, 2014). So far, there is only one example of such a moonlighting
117 enzyme with a role in *Salmonella* virulence (the sugar transport protein and metabolic
118 regulator $EIIA^{Glc}$ that activates the second type 3 secretion system encoded on *Salmonella*

pathogenicity island 2, (Maze *et al.*, 2014). It is possible that additional enzymes have relevant moonlighting functions as is the case for other pathogens (Henderson, 2014), and this is an important caveat for "simple" metabolic interpretations of enzyme defect phenotypes.

Goals for studying intracellular *Salmonella* metabolism

One of the major challenges in studying *Salmonella* in vivo metabolism and identifying potential antimicrobial targets is the strong interplay between *Salmonella* and the mammalian host cells, which provide a very complex metabolic microenvironment for intracellular *Salmonella*. These host cells contain thousands of different metabolites and use versatile regulatory mechanisms to modulate metabolite concentrations and metabolic fluxes. Furthermore, they can dramatically alter their metabolism in various cell differentiation and activation states which strongly influences nutrient availability and hence *Salmonella* fitness. The interplay works in both directions, with the host cell providing nutrients for *Salmonella*, and *Salmonella* simultaneously perturbing the host metabolic network by consuming certain metabolites and releasing waste products (Olive *et al.*, 2016).

In addition to these direct interactions, *Salmonella* releases waste products, produces stimulatory components such as lipopolysaccharide, and secretes numerous virulence factors directly into the host cell cytosol. These *Salmonella* activities modulate host cell physiology in a wide variety of aspects including host cell synthesis of toxic molecules that affect *Salmonella* metabolism. This includes nitric oxide, which can block respiration (Husain *et al.*, 2008); itaconic acid, which inhibits the glyoxylate shunt (Michelucci *et al.*, 2013); and superoxide/peroxide that damages several bacterial components whose repair requires supportive metabolism (Slauch, 2011, Burton *et al.*, 2014). Importantly, many of these host /

143 *Salmonella* interactions are highly heterogeneous with strong cell-to-cell variation on both
144 the host cell and the *Salmonella* side (Helaine *et al.*, 2014b, Bumann, 2015, Knodler, 2015,
145 Kreibich *et al.*, 2015, Mills *et al.*, 2017, Saliba *et al.*, 2017).

146 To understand how this complex and changing metabolic landscape supports and/or
147 limits *Salmonella* fitness, we need to address the following questions: (i) which nutrients are
148 available for intracellular *Salmonella*, and which host cell supply routes ensure sufficient
149 replacement of consumed nutrients? (ii) which pathways do *Salmonella* employ to utilize
150 these nutrients for energy production and biomass generation, and to what extent can
151 *Salmonella* compensate for perturbations of these processes by employing alternative,
152 partially redundant pathways? (iii) what are the consequences for host cell physiology and
153 metabolism? (iv) what is the level of heterogeneity in host and *Salmonella* metabolism, and
154 are there particularly important subsets with unusual properties?

155

156 **Methods for investigating intracellular *Salmonella* metabolism**

157 The most direct experimental technique to monitor host and *Salmonella* metabolism is
158 metabolomics, which can reveal both metabolite concentrations and metabolic fluxes
159 (Zampieri *et al.*, 2017). Infected cells contain both host and *Salmonella* metabolites, and
160 available separation techniques are too slow compared to the turnover rates for most
161 metabolites, making assignment difficult (except for a few kingdom-specific metabolites such
162 as peptidoglycan and lipid precursors, and secondary metabolites). Mass imaging techniques
163 might offer a solution, but spatial resolution is still insufficient for distinguishing intracellular
164 *Salmonella* from surrounding host cell contents (Petras *et al.*, 2017).

165 Even if we could determine metabolite concentrations in *Salmonella* and their
166 microenvironment, such data would not necessarily reflect their relative importance.

Nutrients might be present at low concentration but still have high turnover rates with vigorous *Salmonella* salvage and rapid host replenishment, resulting in an important contribution to *Salmonella* fitness. Instead of metabolite concentrations, metabolic fluxes are therefore more relevant. To obtain such data, one can follow the fate of isotope-labelled glucose and the incorporation of each atom into various amino acids (that are stably retained as part of proteins) (Sauer, 2006, Eisenreich *et al.*, 2015). Amino acid labeling patterns reflect their origin at various central carbon metabolism intermediates, enabling the reconstruction of major fluxes in these central pathways. Interpretation of results for intracellular *Salmonella* can be complicated due to direct transfer of labeled host amino acids into *Salmonella* (Gotz *et al.*, 2010). Such approaches mostly focus on incorporation of ^{13}C and/or ^{15}N isotope labels into *Salmonella* biomass. In addition, it can be useful to detect waste products generated by fundamentally important energy conversion pathways in the cell culture medium (Kentner *et al.*, 2014, Garcia-Gutierrez *et al.*, 2016). All these techniques might be also applicable for in vivo analysis as *Salmonella* can be purified from infected host tissues using flow cytometry (Becker *et al.*, 2006, Steeb *et al.*, 2013). Data interpretation will need to take into account the increased complexity of whole organism metabolism of labeled nutrients. An exciting recent study tracked incorporation of deuterium-labelled drinking water of *Leishmania*-infected mice into macromolecules of the parasites (Kloehn *et al.*, 2015).

Complementary information can be obtained from enzyme proteomics. A general finding from various studies has been the large number and high expression levels of many metabolic enzymes indicating substantial *Salmonella* resource allocation into metabolism. Absolute quantification yields enzyme copy numbers per *Salmonella* cell (Steeb *et al.*, 2013). Combination with tabulated turnover numbers k_{cat} yields maximal reaction rates v_{max} for hundreds of metabolic conversions providing large-scale information on feasible metabolic pathways activities (Steeb *et al.*, 2013, Schubert *et al.*, 2015). Interpretation of such data is,

however, complicated by the fact that many enzymes have broader substrate spectrum besides their normally considered specificities ("substrate promiscuity", e.g., pentoses in addition to hexoses) (Khersonsky *et al.*, 2010). In some cases, such secondary reactions might be actually more relevant. Furthermore, a substantial fraction of metabolic reactions is non-enzymatic, and protein data thus can offer only incomplete coverage of metabolic networks (Keller *et al.*, 2015).

An additional layer of complexity is provided by the observation that the majority of metabolic reactions are dispensable for *Salmonella* fitness, since alternative pathways and supplementation by host metabolites often provide partial redundancy, or pathway products are not required (Becker *et al.*, 2006, Bumann, 2009, Steeb *et al.*, 2013). Several genome-scale genetic screens and numerous more focused studies have revealed a few metabolic mutations with severe phenotypes. This may indicate true pathway relevance, or artefacts such as toxicity of truncated gene products, polar effects on downstream genes, or in particular accumulation of toxic upstream intermediates. Such artefacts can be minimized by clean deletion of genes encoding enzymes at metabolism branching points that lead into the pathway of interest ("first committed step"), with other branches buffering potential buildup of toxic precursors. Access to essential biomass precursors can be inferred from auxotrophic strains that depend on external supplementation. Interpretation should consider wasteful *Salmonella* degradation of such precursors (e.g. dispensable *Salmonella* lysine decarboxylase, which lowers lysine availability below biomass needs (Steeb *et al.*, 2013)). Another complication could be moonlighting functions (see above). Single mutants can only identify non-redundant pathways. To determine the relevance of much more frequent partially redundant pathways, multiple mutations must be combined, and epistasis can provide further insights into pathway architecture (Ideker *et al.*, 2012).

Understanding the metabolism network underlying *Salmonella* fitness during infection requires genome-scale integration of all complementary data sets. The consensus in silico reconstruction of *Salmonella* metabolism that was obtained in a jamboree involving more than fifty researchers provides a suitable basis for this (Thiele *et al.*, 2011). It accounts for 1270 metabolic genes, 2201 metabolic reactions that were curated for thermodynamically feasible reversibility, and 1110 metabolites. This reconstruction reveals all known metabolic pathways and their interconnectivity as a basis for studying the entire *Salmonella* metabolism network in different external conditions and internal states.

The substantial progress in understanding intracellular *Salmonella* metabolism is encouraging but one crucial, previously neglected aspect is just emerging. All experimental and in silico methods are based on population averages, but recent work has shown extensive heterogeneity between infected host cells and individual *Salmonella* cells in terms of microenvironments, stress, gene expression, overall metabolic activities, growth rates, and cell fates (Helaine *et al.*, 2014b, Bumann, 2015, Knodler, 2015, Kreibich *et al.*, 2015, Mills *et al.*, 2017, Saliba *et al.*, 2017). Differential nutrient access seems to cause heterogeneous *Salmonella* growth rates during systemic infection in vivo, with important consequences for disease progression and tolerance against antimicrobial chemotherapy (Claudi *et al.*, 2014). New approaches for *Salmonella* single-cell and subpopulation analysis will be needed to unravel this fascinating new aspect of host-*Salmonella* interactions.

Metabolic patterns of intracellular *Salmonella* in in vitro cell culture models

Salmonella metabolism in cell culture infections has been covered in excellent recent reviews (Dandekar *et al.*, 2014, Eisenreich *et al.*, 2015), and we just summarize some findings here. Cell culture infections recapitulate a central hallmark of salmonellosis, intracellular

240 *Salmonella* replication in host cells. Compared to in vivo studies, cell culture infections
241 enable detailed experimental analysis with a broad spectrum of techniques. Cell culture
242 conditions such as medium composition and oxygen tension can be freely adjusted according
243 to the specific research questions, and defined pulses of isotopically labeled nutrients enable
244 tracking of the kinetics of metabolite conversion in both host and *Salmonella* cells. Another
245 advantage is the focus on a single host cell type simplifying analysis and interpretation of
246 results compared to complex in vivo situations.

247 The results obtained so far indicate a major role of glucose as carbon/energy source,
248 under some, but not all experimental conditions (Bowden *et al.*, 2009, Holzer *et al.*, 2012,
249 Steeb *et al.*, 2013, Bowden *et al.*, 2014, Dandekar *et al.*, 2014, Eisenreich *et al.*, 2015, Popp
250 *et al.*, 2015, Garcia-Gutierrez *et al.*, 2016, Liu *et al.*, 2017, Singh *et al.*, 2017). Peptides/
251 amino acids (Popp *et al.*, 2015, Singh *et al.*, 2017) and other nutrients such as C3 (pyruvate
252 and/or glycerol) or C2 (acetate and/or fatty acids) metabolites (Gotz *et al.*, 2010) are also
253 available to intracellular *Salmonella*. *Salmonella* degrades these nutrients with its central
254 carbon metabolism pathways such as glycolysis (Bowden *et al.*, 2014, Garcia-Gutierrez *et al.*,
255 2016) and the TCA cycle (Bowden *et al.*, 2010), and uses various intermediates of these
256 pathways to synthesize several amino acids in addition to amino acids obtained from the host
257 cell (Gotz *et al.*, 2010). However, results have been quite diverse depending on specific host
258 cells and conditions (Fig. 1A). Clarification of the most relevant setting should be a priority
259 of future work (see below).

260 *Salmonella* can also directly modulate host amino acid abundance. *Salmonella*
261 catabolism of host asparagine causes T cell suppression in vitro, but has no detectable impact
262 in vivo (Kullas *et al.*, 2012), suggesting other reasons for fitness contributions of *Salmonella*
263 asparagine catabolism (Jelsbak *et al.*, 2014). *Salmonella* induces host arginase II expression
264 (Lahiri *et al.*, 2008), in addition to "stealing" of host arginine by ArgT-mediated uptake (Das

et al., 2010). Both processes could deprive host cells of a precursor for generating antimicrobial nitric oxide, but *Salmonella* subsets experience substantial nitric oxide levels during infection (Burton *et al.*, 2014) suggesting sufficient arginine availability. Moreover, *Salmonella* arginine deiminase contributes to in vivo fitness without affecting host nitric oxide production (Choi *et al.*, 2012), and arginine seems to serve as *Salmonella* carbon/nitrogen source (Steeb *et al.*, 2013). *Salmonella* also induces host tryptophan catabolites with immunomodulatory properties in the bloodstream of humans and mice (Blohmke *et al.*, 2016).

One major question is how the host supplies nutrients for intracellular *Salmonella* (Fig. 1B). One fascinating mechanism could be the formation of a large membrane network called *Salmonella*-induce filaments (Sif) (Liss *et al.*, 2017). *Salmonella* can induce Sif with effector proteins that it injects into the host cell cytosol using the type 3 secretion system encoded on *Salmonella* pathogenicity island 2 (SPI-2). Sif continuously merge with endosomes providing a gateway between extracellular metabolites, which the host cell takes up by pinocytosis, and the *Salmonella*-containing vacuole (Drecktrah *et al.*, 2007, Liss *et al.*, 2017). Indeed, this can be the major nutrient delivery pipeline for driving intracellular *Salmonella* growth (Holzer *et al.*, 2012, Liss *et al.*, 2017). An additional host cell nutrient supply pathway is chaperone-mediated autophagy providing peptides directly to the *Salmonella*-containing vacuole (Singh *et al.*, 2017), which works independently of any extracellular nutrient supply.

Future goals for in vitro cell culture studies

An important goal is to define meaningful criteria for in vitro cell culture conditions that reproduce key aspects of host-*Salmonella* interactions during salmonellosis. Recent studies

have revealed dramatic differences in *Salmonella* metabolism and virulence mechanisms depending on particular host cell types and experimental conditions, and it remains often unclear which patterns are most pertinent for understanding actual disease processes.

Most studies used cancer cell lines that are easy to cultivate and grow, and are permissive for *Salmonella* proliferation. However, compared to primary cells these cancer cells have distorted metabolism (respiration/fermentation, pentose pathway, lipid biosynthesis/β-oxidation, etc.) (Pavlova *et al.*, 2016), which might affect the metabolism of intracellular *Salmonella*. The major model for epithelial cell infections are HeLa cells that are cervix carcinoma cells carrying the Human Papilloma virus 18 genome. *Salmonella* infection biology in HeLa cells differs in important aspects from (possibly more relevant) polarized epithelial cells, even when compared under identical conditions (Fig. 1A). This includes differential overall growth rates; relevance of glucose and other nutrients; divergent roles of glycolysis, overflow metabolism leading to acetate secretion, purine biosynthesis, chorismate biosynthesis (a key pathway for classifying biosafety of *Salmonella* mutants); and essentiality/dispensability of Sif formation (Holzer *et al.*, 2012, Bowden *et al.*, 2014, Lorkowski *et al.*, 2014, Popp *et al.*, 2015, Garcia-Gutierrez *et al.*, 2016, Liu *et al.*, 2017, Singh *et al.*, 2017).

Similarly, *Salmonella* metabolism and virulence mechanisms show remarkable differences depending on the macrophage activation status and source of host cells (cancer cell lines, primary bone marrow-derived macrophages) (Fig. 1A). Examples include the differential relevance of glucose for *Salmonella* nutrition; divergent roles of *Salmonella* TCA cycle and overflow metabolism; and differential requirements for Sif (Bowden *et al.*, 2009, Lathrop *et al.*, 2015, Popp *et al.*, 2015, Garcia-Gutierrez *et al.*, 2016) and extracellular small metabolites that could be supplied via Sif (Singh *et al.*, 2017). Interestingly, one study suggested that SPI-2 and its role in Sif formation are actually dispensable for intracellular

314 growth in phagocytes in vivo. Instead, SPI-2 seems to play a major role in *Salmonella* cell-to-
315 cell spreading within host tissues (Grant *et al.*, 2012), which is not the focus of most in vitro
316 cell culture studies although it is an essential part of *Salmonella* in vivo fitness. Another
317 particularly concerning discrepancy is the apparent dispensability of *Salmonella* respiration
318 for growth in macrophages in vitro (Garcia-Gutierrez *et al.*, 2016), whereas aerobic
319 respiration is one of the key metabolic activities supporting *Salmonella* fitness in vivo
320 (Turner *et al.*, 2003, Steeb *et al.*, 2013).

321 Another caveat concerning in vitro *Salmonella* studies is the common use of cell
322 culture media that contain non-physiological metabolite concentrations, which might affect
323 both host cell and intracellular Salmonella metabolism. Intestinal epithelial cells (enterocytes)
324 that are one important infected host cell type in vivo, do for example not normally depend on
325 glucose but rather on microbiota-derived short-chain fatty acids as energy source although
326 this can change during gut dysbiosis (Rivera-Chavez *et al.*, 2016).

327 To deal with this complexity and to ensure that the results are meaningful for
328 understanding disease mechanisms in salmonellosis, we propose to derive a couple of
329 decisive benchmarks from in vivo studies, and to use these benchmarks for establishment of
330 appropriate in vitro cell culture models. As an example, *Salmonella* fitness in macrophage
331 cell cultures should depend on respiratory pathways as it does in vivo. Glucose should
332 contribute as a carbon and energy source, but only as one limited contribution among several
333 nutrients (Bowden *et al.*, 2009, Steeb *et al.*, 2013) (whereas it could be more relevant during
334 chronic infections (Eisele *et al.*, 2013)). Benchmarks for metabolic pathways relevant for
335 *Salmonella* fitness in epithelial cells in vivo are still largely lacking, but their establishment
336 could be straightforward with *Salmonella* mutants in the mouse enteritis and/or neonate
337 infection models. In particular, the role of central metabolic pathways and host cell processes
338 such as Sif formation or chaperone-activated autophagy should be clarified in vivo. Once

such criteria are established, host cell types and culture conditions could be optimized in an iterative manner until key aspects of *Salmonella* intracellular metabolism in vivo are faithfully reproduced in vitro. A final step could be to reproduce the intracellular environment, and *Salmonella* exploitation of accessible nutrients as well as defense against toxic molecules, in axenic cultures (without any host cell). This would be particularly useful for screening of novel antimicrobials under meaningful conditions.

A second focus of future studies of *Salmonella* in vitro cell culture infection models should be on heterogeneity. Most previous investigations have determined average properties, but recent studies clearly revealed striking differences between individual *Salmonella* cells but also substantial cell-to-cell variation among host cells. *Salmonella* subsets with divergent growth rates and metabolic activities exist in infected macrophages (Helaine *et al.*, 2010, Claudi *et al.*, 2014, Helaine *et al.*, 2014a, Diacovich *et al.*, 2016), and properties and fates of individual *Salmonella*-infected macrophages also highly variable (Avraham *et al.*, 2015, Saliba *et al.*, 2016, Thurston *et al.*, 2016, McQuate *et al.*, 2017). Striking heterogeneity has also been observed in epithelial HeLa cells. Most *Salmonella* remain initially in phagosomes where they slowly proliferate, but some *Salmonella* escape to the host cell cytosol where they can vigorously proliferate and overgrow the phagosomal *Salmonella* subpopulation, (Knodler *et al.*, 2010, Knodler, 2015). While their differential growth rates must correspond to striking differences in biomass generation and the entire metabolism network (Knodler *et al.*, 2010, Knodler, 2015, Wrande *et al.*, 2016), and recent evidence reveals distinct gene expression patterns and genetic determinants for the two subpopulations (Knodler *et al.*, 2010, Knodler, 2015, Wrande *et al.*, 2016), most HeLa infection studies merely determined average properties. Such data initially reflect mostly phagosomal *Salmonella*, but later predominantly the overgrowing cytosolic *Salmonella* subset. Consequently, data for early time points largely ignore the properties of a nevertheless particularly important *Salmonella* subset (the cytosolic

escapers), and differences along the time course could be misinterpreted as changing metabolic patterns of *Salmonella* cells, instead of changing contributions of two distinct subsets. It is possible that cytosolic *Salmonella* resemble cytosolic *Shigella*, which rely on conversion of host-derived pyruvate to acetate as the major energy conversion pathway (Kentner *et al.*, 2014). In particular, *Salmonella* can grow even without glycolysis in these cells, excretes large amounts of acetate, and partially depends on acetyl-CoA to acetate conversion (Bowden *et al.*, 2014, Garcia-Gutierrez *et al.*, 2016, Liu *et al.*, 2017).

Traditional bulk average read-outs neglect this heterogeneity and could thus result in misleading interpretations. Single-cell approaches increasingly unravel the molecular differences between *Salmonella* and host cell subsets, but the corresponding metabolic patterns are still largely unknown.

Salmonella **metabolism during systemic infections**

In vivo models reflect relevant conditions during salmonellosis compared to more ambiguous cell culture conditions. For practical reasons most studies used a typhoid fever model of systemic salmonellosis in genetically susceptible mice. This model differs in some important aspects from human systemic salmonellosis (Santos *et al.*, 2001), but the limited available information suggests at least similarities in *Salmonella* metabolism as compared to genetically resistant mice (Steeb *et al.*, 2013) and human volunteers immunized with attenuated *Salmonella enterica* serovar Typhi (Bumann *et al.*, 2000). A major drawback of in vivo studies is the severely restricted number of suitable experimental methods for investigating metabolism, and the much higher complexity of the host microenvironment with multiple cell types and strong inflammation dynamics. Under these circumstances, there are at present essentially only two applicable methods to study *Salmonella* metabolism;

388 proteomics to determine enzyme copy numbers, and competitive infections to determine
389 fitness defects of metabolic mutants. On the other hand, contributions of many research
390 groups over more than three decades have accumulated an astonishing amount of information
391 that enabled us to derive a genome-scale metabolic reconstruction of *Salmonella* metabolism
392 in infected mouse spleen (Steeb *et al.*, 2013) (Fig. 2). Subsequent publications corrected
393 some aspects (hydrogen oxidation not required (Maier *et al.*, 2013, Maier *et al.*, 2014);
394 reconsideration of proline biosynthesis mutants for inferring limited proline availability (Lee
395 *et al.*, 2014); contribution of asparagine deamination and polyamine synthesis for *Salmonella*
396 fitness (Jelsbak *et al.*, 2014)). However, this did not have much impact on the overall
397 properties of the *Salmonella* metabolism network in vivo.

398 One striking finding is the large complexity of the in vivo host-*Salmonella* nutritional
399 interface (Fig. 2). *Salmonella* has access to more than fifty diverse host nutrients comprising
400 multiple carbon/energy and nitrogen sources, all amino acids (with limiting amounts for
401 proline), many provitamins, and inorganic nutrients. Although an individual nutrient,
402 glycerol, plays a key role, the broad availability of many different nutrients makes
403 *Salmonella* metabolism largely resilient against perturbations. This in part explains the
404 seemingly paradoxical finding that *Salmonella* invests major resources into metabolic
405 enzymes suggesting a crucial importance of metabolism for *Salmonella* fitness in vivo, yet
406 only few metabolic mutations show remarkable infection phenotypes (Becker *et al.*, 2006,
407 Bumann, 2009, Steeb *et al.*, 2013). Many other microbial pathogens likely have access to
408 similar complex host nutrients based on widespread auxotrophies for amino acids,
409 nucleosides, and (pro)vitamins (Steeb *et al.*, 2013).

410 *Salmonella* degrades many of these nutrients primarily through the Embden-Meyerhof
411 pathway (and to some extent also pentose-phosphate and Entner-Doudoroff), followed by the
412 TCA cycle coupled with aerobic respiration involving ubiquinone as the main energy

providing pathway. Anaerobic respiration is dispensable (Craig *et al.*, 2013) although *Salmonella* expresses several enzymes that could mediate such energy conversion pathways in vivo (Steeb *et al.*, 2013). Vulnerable "Achilles heels" are rare in *Salmonella* metabolism and are almost entirely restricted to biosynthesis of essential biomass components that are not provided in sufficient amounts, or not at all, by the host such as peptidoglycan, riboflavin, unsaturated fatty acids, ubiquinone, etc. (Becker *et al.*, 2006, Bumann, 2009, Steeb *et al.*, 2013).

As discussed above, *Salmonella* has access to a wide variety of different host-nutrients. However, this does not necessary imply favorable conditions as available amounts could be still scarce. Quantitative simulation of the entire *Salmonella* metabolic network based on all available experimental data indeed suggested a severe overall nutrient limitation of *Salmonella* growth, suggesting that although the host microenvironment is qualitatively rich (providing many different nutrients), it is quantitatively poor (nutrients are available in only scarce amounts) resulting in slow growth with an average generation time around 6 h (Becker *et al.*, 2006, Claudi *et al.*, 2014).

Salmonella metabolism does not only depend on nutrient access. Host cells can also attack *Salmonella* with toxic molecules such as nitric oxide and reactive oxygen species (ROS), that could interfere with *Salmonella* metabolism and consume reducing equivalents for detoxification and repair. Nitric oxide can block *Salmonella* respiration (Husain *et al.*, 2008), but exposed *Salmonella* upregulate the detoxifying enzyme NO dioxygenase HmpA which lowers nitric oxide levels sufficiently to prevent fitness defects (Burton *et al.*, 2014). Similarly, *Salmonella* that are exposed to moderate levels of reactive oxygen species in resident macrophages, upregulate catalases and peroxidases that together with generally expressed superoxide dismutase ensure uncompromised fitness (Burton *et al.*, 2014). By contrast, neutrophils attack *Salmonella* with much higher levels of ROS that overwhelm

438 *Salmonella* defenses (Burton *et al.*, 2014, Schurmann *et al.*, 2017). Both *Salmonella* defenses
439 against nitric oxide and ROS require reducing equivalents but this puts only small additional
440 demands on *Salmonella* metabolism (in the range of 10% of total electron flow). Activated
441 macrophages also express cis-aconitate decarboxylase (Irg1) (Michelucci *et al.*, 2013), which
442 produces itaconate. Itaconate could inhibit the *Salmonella* glyoxylate shunt, but
443 concentrations are probably too low for fitness impairment, especially since *Salmonella* does
444 not depend on the glyoxylate shunt during acute infections (Fang *et al.*, 2005, Kim *et al.*,
445 2006). On the other hand, itaconate also inhibits host succinate dehydrogenase resulting in
446 succinate accumulation, which increases inflammatory responses (Cordes *et al.*, 2016) and
447 modulates host fatty acid metabolism and ROS production (Hall *et al.*, 2013).

448 As might be expected from the striking variation among both *Salmonella* and infected
449 host cells in vitro (see above), *Salmonella* growth and metabolism is also highly
450 heterogeneous in vivo (Claudi *et al.*, 2014, Helaine *et al.*, 2014a, Bumann, 2015). This is in
451 part a consequence of differential stress conditions and *Salmonella* toxin expression, but
452 varying access to host nutrients and inhomogeneous activities across the entire *Salmonella*
453 metabolism network are also involved. The individual relevance of the many metabolic
454 difference is still largely unclear, but growth patterns of a purine auxotrophic mutant indicate
455 divergent *Salmonella* access to purines in vivo. Interestingly, nutrient access seems to be
456 rather homogeneous within one *Salmonella* microcolony in one host cell, but can be very
457 different in neighboring host cells suggesting that host cell properties might modulate nutrient
458 supply (Claudi *et al.*, 2014).

459

460 **Future goals for in vivo studies**

461 A better understanding of heterogeneous *Salmonella* metabolism is clearly required to
462 unravel host and *Salmonella* molecular mechanisms that enable rapid growth of some
463 *Salmonella* subsets driving disease progression, but cause only slow to moderate growth of
464 other subsets which enables these *Salmonella* cells to tolerate antibiotics exposure (Claudi *et*
465 *al.*, 2014). New single-cell techniques will be required to characterize qualitative and
466 quantitative differences in nutrient access and metabolic pathway activities in the various
467 *Salmonella* subsets. Apart from growth rate, other aspects of metabolism could also influence
468 antimicrobial susceptibility and tolerance, and this could be highly relevant for explaining the
469 surprisingly slow *Salmonella* eradication from infected mice and humans (Waddington *et al.*,
470 2014, Dobinson *et al.*, 2017) with antibiotics that are highly effective against *Salmonella* in
471 vitro.

472 Finally, a major enigma is still the mechanism of host nutrient delivery to intracellular
473 *Salmonella*. Elegant in vitro studies suggest a major role in SPI-2 inducing Sif that connect
474 the extracellular fluid to the *Salmonella*-containing vacuole (Liss *et al.*, 2017). However,
475 nutrients that actually reach *Salmonella* in vivo do not show a typical signature of blood
476 metabolites but rather suggest predominant nutrient release by degradation of host
477 macromolecules (Steeb *et al.*, 2013), as would occur in lysosomes or autophagosomes.
478 Moreover, one study challenges the role of SPI-2 (and therefore Sif) for intracellular
479 *Salmonella* growth in vivo (Grant *et al.*, 2012). Further work might (i) determine the in vivo
480 occurrence of Sif around *Salmonella*-containing vacuoles in macrophages, (ii) determine
481 nutrient access in *Salmonella* wild-type and SPI-2 mutants, and (iii) clarify the importance of
482 vesicular trafficking and alternative mechanisms for nutrient delivery to the *Salmonella*-
483 containing vacuoles. Another very recent study proposes that host cell chaperone-mediated
484 autophagy provides peptides (and possibly other host macromolecule degradation products)
485 directly to the *Salmonella*-containing vacuole (Singh *et al.*, 2017). Although this supply route

appears to have limited relevance for fitness of wild-type *Salmonella* in vitro cell culture infections (in contrast to a peptide-dependent mutant), this mechanism might still play a role in vivo. Methods that have been established to purify and analyze the phagosomal membrane from in vitro infected cells (Herweg *et al.*, 2015, Vorwerk *et al.*, 2015), might be applicable to in vivo conditions to obtain informative comprehensive data on suitable marker proteins (in addition to immunohistochemistry data) to address these issues.

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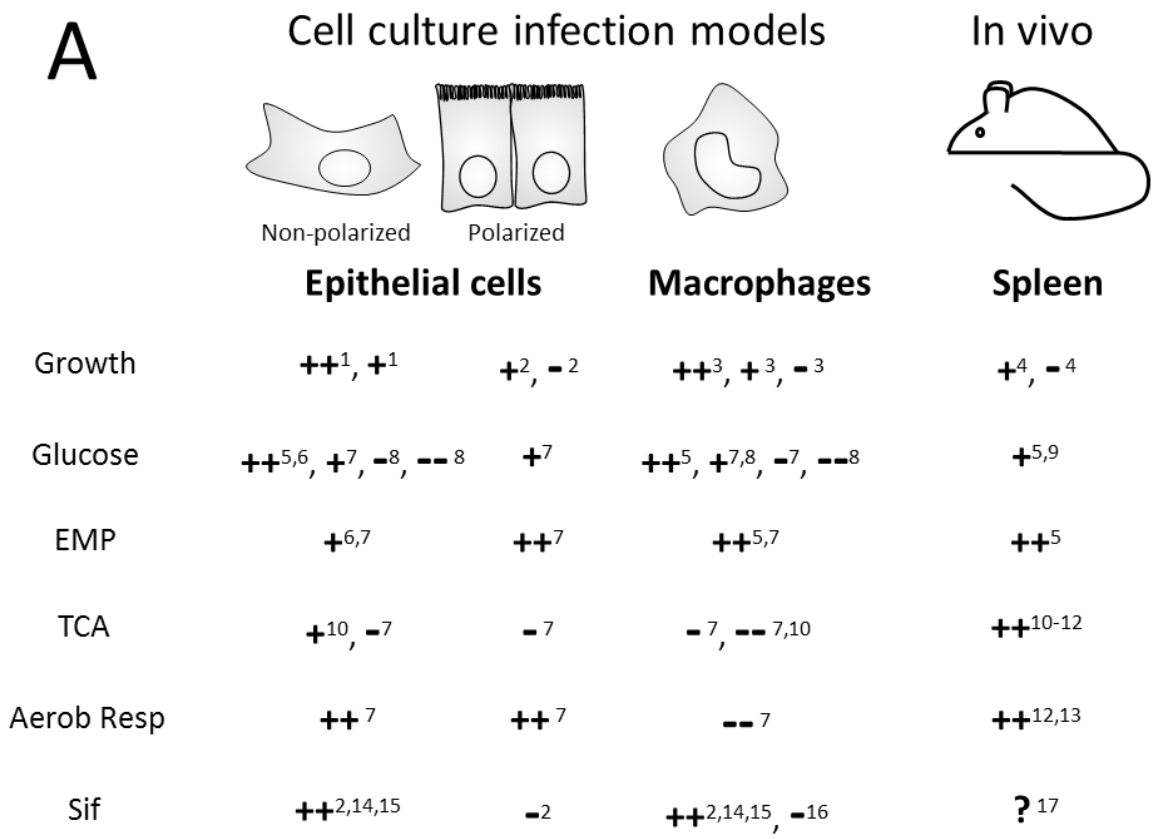
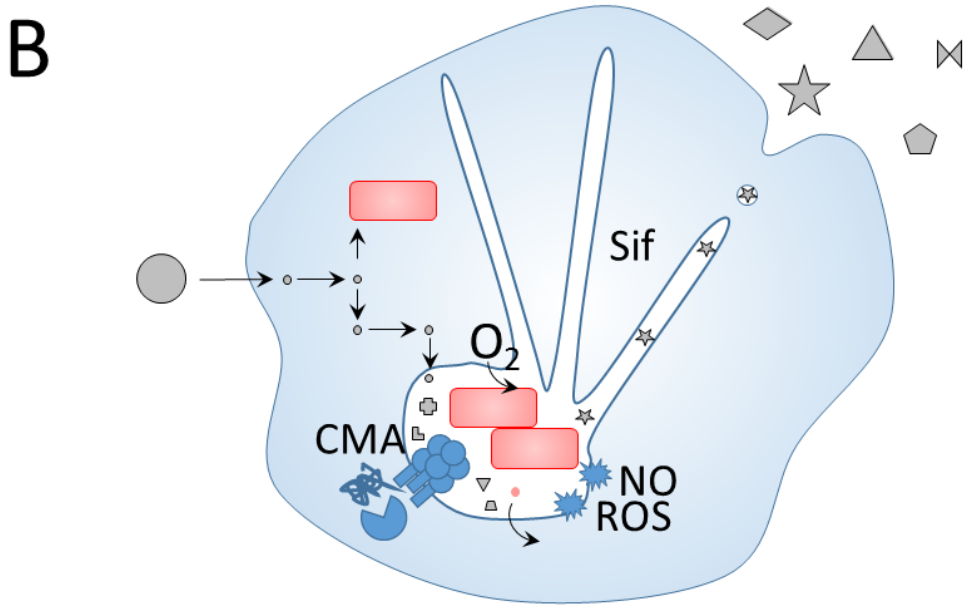
790 **Figures**





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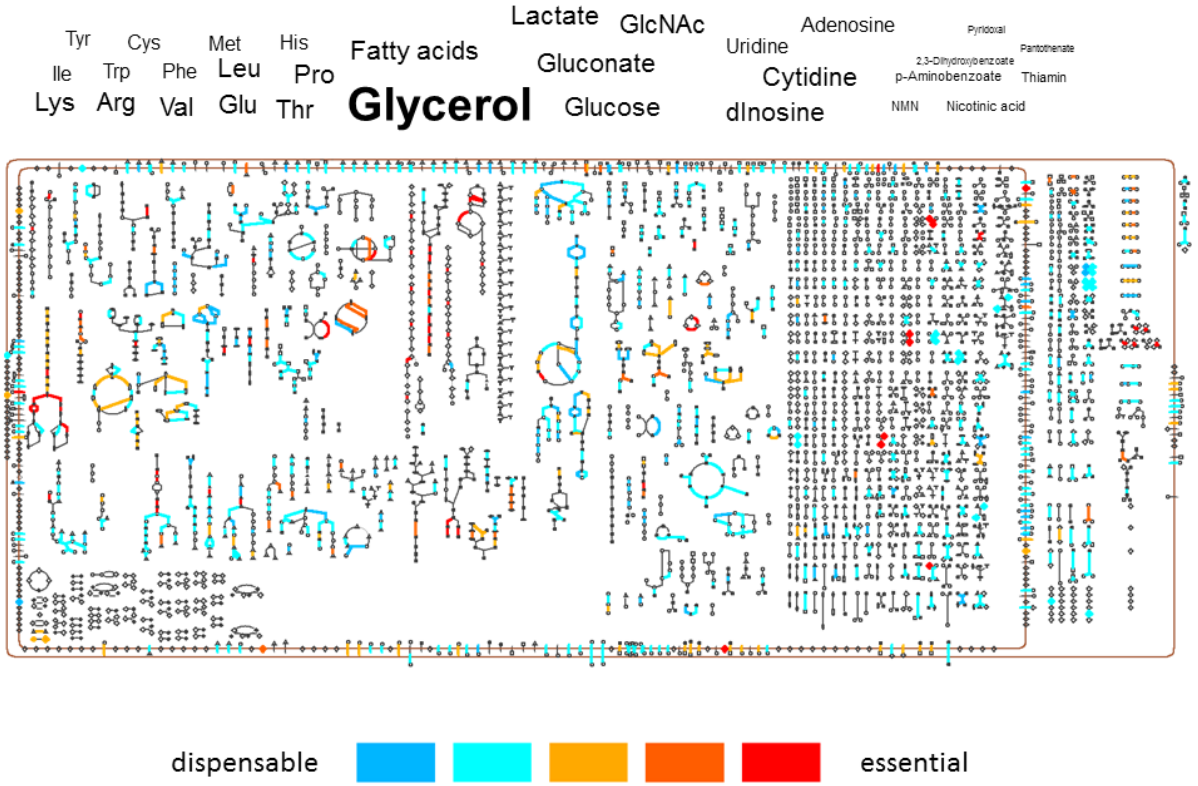
792 **Figure 1**: Evidence for core *Salmonella* metabolic activities and nutrient supply routes.

A) Experimental evidence for *Salmonella* intracellular metabolism in various cell culture models and during systemic salmonellosis in the mouse typhoid fever model. Data for intracellular growth, glucose as a major nutrient, key metabolic pathways (EMP, Embden-Meyerhof-Parnas pathway; TCA, tricarboxylic acid cycle; Aerob Resp, aerobic respiration), and the role of *Salmonella*-induced filamens (Sif) for nutrient supply are shown (++, strong effect; 1, significant but moderate effect, -, no detectable impact; --, growth-diminsihing effect). Data were collected from various studies ([1](Knodler *et al.*, 2010), [2](Holzer *et al.*, 2012), 3(Helaine *et al.*, 2010), [4](Claudi *et al.*, 2014), [5](Bowden *et al.*, 2009), [6](Bowden *et al.*, 2014), [7](Garcia-Gutierrez *et al.*, 2016), [8](Singh *et al.*, 2017), [9](Steeb *et al.*, 2013), [10](Bowden *et al.*, 2010), [11](Tchawa Yimga *et al.*, 2006), [12](Becker *et al.*, 2006), [13](Craig *et al.*, 2013), [14](Popp *et al.*, 2015), [15](Liss *et al.*, 2017), [16](Lathrop *et al.*, 2015), [17](Grant *et al.*, 2012)).

B) Possible supply routes for host nutrients and toxic molecules in *Salmonella*-infected cells. Orange symbols represent various compounds.



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809 **Figure 2**: Experimental evidence for nutrient supply and enzyme essentiality for *Salmonella*
810 during systemic salmonellosis in the mouse typhoid fever model. On top, nutrients are shown
811 with font size corresponding to differential supply rates. The *Salmonella* metabolism network
812 is shown below in a schematic overview with lines (representing enzymes) connecting
813 symbols (metabolites). Enzymes are shown in different colors that represent enzyme
814 relevance for *Salmonella* in vivo fitness. A fully annotated version of this scheme is available
815 at http://www.biozentrum.unibas.ch/personal/bumann/steeb_et_al/index.html.