Intracellular *Salmonella* Paratyphi A is motile and differs in the expression of flagella-chemotaxis, SPI-1 and carbon utilization pathways in comparison to Intracellular *S*. Typhimurium

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ABSTRACT

Although *Salmonella* Typhimurium (STM) and *Salmonella* Paratyphi A (SPA) belong to the same phylogenetic species, share large portion of their genome and express many common virulence factors, they differ vastly in their host specificity, the immune response they elicit, and the clinical manifestations they cause. In this work, we compared for the first time their intracellular trascriptomic architecture and cellular phenotypes during epithelial cell infection. While transcription induction of many metal transport systems, purines, biotin, PhoPQ and SPI-2 regulons was common in both intracellular SPA and STM, we identified 234 differentially expressed genes that showed distinct expression patterns in intracellular SPA vs. STM. Surprisingly, clear expression differences were found in SPI-1, motility and chemotaxis, and carbon (mainly citrate, galactonate and ethanolamine) utilization pathways, indicating that these pathways are regulated and possibly function differently, during their intracellular phase. Moreover, we show that the induction of flagella genes by intracellular SPA leads to cytosolic motility, a conserved trait specific to SPA. To the best of our knowledge, this is the first report of a flagellum-dependent intracellular motility of any *Salmonella* serovar in living host cells. Importantly, we demonstrate that the elevated expression of SPI-1 and motility genes by intracellular SPA results in increased invasiveness of SPA, following exit from host cells. We propose that such changes prime SPA towards new cycles of host cell infection and contribute to the ability of SPA to disseminate beyond the intestinal lamina propria of the human host, during enteric fever.

44 **IMPORTANCE**

45 *Salmonella enterica* is a ubiquitous, facultative intracellular animal and human pathogen.
46 Although non-typhoidal *Salmonella* (NTS) and typhoidal *Salmonella* serovars belong to the same
47 phylogenetic species and share many virulence factors, the disease they cause in humans is very
48 different. While the underlying mechanisms for these differences are not fully understood, one possible
49 reason expected to contribute to their different pathogenicity is a distinct expression pattern of genes
50 involved in host-pathogen interactions. Here, we compared the global gene expression and the
51 intracellular behavior, during epithelial cell infection of *S*. Paratyphi A (SPA) and *S*. Typhimurium (STM),
52 as prototypical serovars of typhoidal and NTS, respectively. Interestingly, we identified different
53 expression patterns in key virulence and metabolic pathways, together with intracellular motility and
54 increased invasiveness of SPA, following exit from infected cells. We hypothesize that these differences
55 are pivotal to the invasive and systemic disease developed following SPA infection in humans.

56

57 **INTRODUCTION**

58 *Salmonella enterica* (*S. enterica*) is an abundant Gram-negative, facultative intracellular animal
59 and human pathogen. This highly diverse bacterial species contains more than 2600 antigenically distinct
60 serovars (biotypes) that are different in their host specificity and the disease they cause. The developed
61 clinical manifestation is the result of multiple factors, but largely depends on the characteristics of the
62 infecting serovar and the immunological status of the host (1). Many of the *S. enterica* serovars, including
63 the ubiquitous *S. enterica* serovar Typhimurium (*S.* Typhimurium) are generalist pathogens and are
64 capable of infecting a broad range of host species. Infection of immunocompetent humans by non-
65 typhoidal *Salmonellae* (NTS) typically leads to a self-limiting, acute inflammatory gastroenteritis,
66 confined to the terminal ileum and colon. In contrast, a few serovars including Typhi, Paratyphi A and
67 Sendai, collectively referred to as 'typhoidal salmonellae' are restricted to the human host and known as
68 the causative agents of enteric (typhoid) fever. In most cases, enteric fever is a non-inflammatory,
69 systemic life-threatening disease, presented as bacteremia and dissemination of the pathogen to
70 systemic sites such as the spleen, liver and lymph nodes (2-4).

71 *S. enterica* infections are still considered a significant cause of mortality and morbidity with an
72 annual incidence of over 27 million cases of enteric fever (5), and 78.7 million cases of gastroenteritis (6)
73 worldwide. In recent years, the global prevalence of *S.* Paratyphi A (SPA) is increasing and in some
74 countries (especially in eastern and southern Asia), SPA infections are accountable for up to 50% of all
75 enteric fever cases (7, 8). The lack of a commercial SPA vaccine and the increased occurrence of antibiotic
76 resistant strains illuminate SPA as a significant public health concern that is still an understudied
77 pathogen (9).

78 Active invasion into eukaryotic non-phagocytic cells is one of the key virulence-associated
79 phenotypes of all *S. enterica* serovars. This unique capability facilitates *Salmonella* intestinal epithelium

80 crossing of the small intestine (10) and is mediated by a designated type three secretion system (T3SS)
81 encoded in the *Salmonella* Pathogenicity Island (SPI)-1. This sophisticated syringe-like nanosystem is
82 evolutionary related to the flagellar apparatus (11) and used to translocate an array of effector proteins
83 directly into the host cell cytoplasm. Translocated effectors by T3SS-1 trigger cytoskeletal
84 rearrangements and *Salmonella* penetration of intestinal barriers (12). In addition, T3SS-1 and its
85 associated effectors significantly contribute to intestinal inflammation (13) that helps *Salmonella* to
86 compete with the gut microbiota (14).

87 After penetrating the lamina propria into the submucosa and mesenteric lymph nodes,
88 *Salmonella* are taken up by phagocytic cells such as macrophages and dendritic cells. Within host cells,
89 *Salmonella* are compartmentalized into a modified intracellular phagosome, known as the *Salmonella*
90 containing vacuole (SCV). Inside the SCV *Salmonella* manipulates the phagosome-lysosome membrane
91 fusion and other cellular pathways, and starts to replicate intracellularly (13). These activities require a
92 second T3SS encoded by genes on SPI-2 and the translocation of a distinct set of effectors proteins (15).

93 Typhoid fever disease and host-response to *S*. Typhi infection is largely attributed to the function
94 of the Vi polysaccharide capsule and its associated regulator, TviA encoded on SPI-7 (16-19). Nonetheless,
95 since SPA does not harbor the SPI-7, nor expresses the Vi capsule, different mechanisms are expected to
96 contribute to the clinically indistinguishable enteric fever disease caused by SPA and *S*. Typhi. Previously,
97 we showed that in *Salmonella* culture grown in LB to the late logarithmic phase, SPI-1 genes and T3SS-1
98 effectors are expressed and secreted at significantly lower levels by SPA compared to *S*. Typhimurium
99 (STM) (20), and demonstrated differences in the regulatory setup of the flagella-chemotaxis pathway
100 between these serovars (21, 22).

101 Here, to further understand the different pathogenicity of SPA vs. STM as prototypic NTS, we set
102 out to compare their transcriptional architecture, during non-phagocytic host cell infection. Surprisingly,

103 in sharp contrast to the expression pattern in LB grown culture, we show that intracellular SPA is motile
104 and induces the expression of SPI-1 and flagellar genes, compared to intracellular STM. In addition, we
105 demonstrate that SPA and STM diverge in the intracellular expression of genes for citrate and
106 ethanolamine metabolism and in their ability to utilize these carbon sources in vitro. We hypothesize
107 that these differences are pivotal for the distinct diseases resulting from SPA vs. STM infection, and their
108 different interactions with the human host.

109

110 **RESULTS**

111

112 **The intracellular transcriptomic landscape of *S*. Typhimurium**

113 Although STM and SPA belong to the same species, share about 89 % of their genes and express
114 many common virulence factors (23), the disease they cause in immunocompetent humans is very
115 different. One possible reason, expected to contribute to their different pathogenicity is a distinct
116 expression of genes involved in host-pathogen interactions. To test this hypothesis, we compared the
117 transcriptomic architecture and gene activity of SPA or STM in a disease-relevant context of epithelial
118 cell infection. We chose to sample *Salmonella* infection at 8 h p.i. when the pathogen is considered to be
119 well engaged in replication and optimally adapted to the intracellular environment (24). Thus, HeLa cells
120 were infected with fluorescent STM and SPA for 8 h and sorted by flow cytometry to isolate *Salmonella*-
121 infected cells. Isolated RNA was deep-sequenced (RNA-Seq), aligned to the corresponding *Salmonella*
122 genome and compared to the transcriptome of *Salmonella* cultures growing extracellularly in rich LB
123 medium.

124 RNA extracted from three independent infection experiments of HeLa cells infected with STM have
125 generated 496-575 million RNA-Seq reads (human and bacterial reads) per experiment. Of which, 4.9-

126 6.6 million sequence reads, assigned to non-rRNA bacterial genes were used for intracellular bacterial
127 transcriptome analysis. Three independent extracellular STM cultures grown in LB, were used as a
128 reference control, from which we obtained 4.3-13.8 million RNA-seq informative reads that were
129 assigned to non-rRNA bacterial genes. To analyze the STM gene expression, we calculated for each gene
130 the number of transcripts per kilobase million (TPM) from its feature read count counts. The expression
131 level threshold was set to a TPM value of 10 or above (25). Altogether, we identified 1,018 distinct genes
132 that were exclusively expressed by STM during intracellular infection of HeLa cells and 191 distinct genes
133 that were expressed only when STM was grown in LB. 3,127 transcripts were commonly expressed at
134 both conditions (Fig. 1A and Table S1).

135 RNA-seq successfully identified 365 upregulated and 465 downregulated genes that were changed
136 by at least twofold inside epithelial cells at 8 h p.i. (adjusted p-value ≤ 0.05) by STM, relative to their
137 expression in LB (Fig. 2A and Table S2). In agreement with previous studies (14, 26, 27), during STM
138 infection, at least 27 genes belonging to the flagella-motility regulon and several genes (*fimAWYZ*) from
139 the type I fimbriae were significantly repressed. Interestingly, the expression of genes encoding the Saf
140 fimbriae (*safABCD*) were upregulated during HeLa cell infections by STM.

141 In contrast to the flagella and the *fim* genes, at least 31 genes from the PhoPQ regulon were highly
142 induced intracellularly (Fig. 2B). The two-component system PhoPQ, orchestrates *Salmonella* adaptation
143 to the intracellular milieu and regulates a wide array of genes central for *Salmonella* virulence including
144 those encoded on SPI-2 (28, 29). Correspondingly, we identified upregulation of the SPI-2 regulon
145 including *ssaBCDEGHIJKLMVNOPRSTU*, *sseABCDEFGIJL*, *sseK3*, *sspH2*, *pipB, pipB2*, and *steC* genes (Fig.
146 2B). These results are consistent with the known notion that SPI-2 expression is induced during
147 intracellular replication of *Salmonella* (15, 30) and with previous reports that studied STM expression
148 during macrophages infection (31, 32). Other PhoP-regulated genes including *pgtE* (involved in bacterial

149 resistance to antimicrobial peptides), *pagN* (encoding an adhesion/invasion protein), *phoN* (acid
150 phosphatase) and *pagCDJKO* were also induced (Fig. 2B).

151 Iron is an essential micronutrient required by nearly all bacterial species, including *Salmonella.*
152 Limiting the availability of the nutrient metals to intracellular pathogens is one of the main mechanisms
153 of nutritional immunity. In return, *Salmonella* uses a variety of high-affinity iron uptake systems to
154 compete with the host for essential transition metals required for its intracellular growth (33). Indeed,
155 we identified elevated expression of multiple iron (*entABEFH* and *iroNBCDE*), iron/ manganese (*sitABCD*),
156 zinc (*zntR*, *zitT* and *znuA*), and magnesium (*mgtAB*) acquisition system genes, highlighting the essentiality
157 of these metal ions for STM intracellular replication (Fig. 2B).

158 More than 100 suitable carbon substrates as well as various nitrogen, phosphorus and sulfur sources
159 are available to invading pathogens, at different niches of the vertebrate hosts (34, 35). Interestingly,
160 multiple genes belonging to the ethanolamine operon (*eutQTMGAC*) and genes involved in glycerol
161 (*glpABDK*), and maltose (*malEKM*) metabolism were particularly induced during STM infection (Fig. 2B).
162 Nevertheless, the most highly induced metabolic gene was *uhpT,* encoding a hexose phosphate
163 transporter that was upregulated by more than 40-fold at 8 h p.i. These results suggest that sugar
164 phosphates are major substrates required for cytosolic intracellular proliferation of STM within HeLa
165 cells, but also that ethanolamine, glycerol and maltose could be utilized as alternative carbon sources in
166 epithelial cells.

167 Similarly, biotin (*bioABCDF*), purine (*nrdEFHI* and *purDEHKLT*), and sulfate/thiosulfate (*cysACHITW*
168 and *sufABCDES*) biosynthetic metabolic pathways were highly transcribed intracellularly by STM.
169 Moreover, genes involved in biosynthesis of the amino acids histidine (*hisACDFH* and *hutG*), tryptophan
170 (*trpABDE*), and glutamine (*glnAGKL*), and genes that catalyze the cleavage of N-acetylneuraminic acid

171 (sialic acid; Neu5Ac) to form pyruvate and N-acetylmannosamine (ManNAc) (*nanAEKT* and *nagB*) were
172 also upregulated by STM during HeLa cell infections (Fig. 2B).

173 A large number of genes involved in phosphate metabolism were identified to be induced within
174 HeLa cells. For example, the transcriptional levels of *pstABCS* (encoding an ATP-dependent phosphate
175 uptake system), which is responsible for inorganic phosphate uptake during phosphate starvation,
176 *ugpABCEQ, phoR* and *apeE* were markedly increased. The *ugp* operon is involved in hydrolysis of diesters
177 during their transport at the cytoplasmic side of the inner membrane (36). Thereby, sn-glycerol-3-
178 phosphate (G3P) is generated and used as a phosphate source. The *upg* operon is regulated by a two-
179 component system, PhoBR, which also regulates the outer membrane esterase, ApeE (37). Additional
180 genes belonging to the PhoB regulon, such as *phnSVWU* encoding the phosphonatase pathway, which is
181 responsible for breaking down phosphonate and yielding cellular phosphate (38) were also significantly
182 induced.

183 Collectively, these results suggest that STM likely experiences iron, biotin, purine and phosphate
184 deprivation in host epithelial cells and induces the expression of genes involved in their transport and
185 utilization. In addition, these results imply that ethanolamine, glycerol and maltose are available sources
186 of carbon for STM in epithelial cells.

187 **The intracellular transcriptomic landscape of *S*. Paratyphi A**

188 RNA-Seq analysis was similarly applied for intracellular SPA. 313.8 to 805.9 million dual-RNASeq
189 reads were obtained from three independent HeLa cell cultures infected with SPA. Out of these, 14.6 to
190 36.6 million sequence reads were assigned to non-rRNA SPA genes and used to determine the
191 intracellular SPA transcriptome (Table S3). As a reference control, RNA extracted from SPA grown in LB
192 from three independent cultures generated 7.3 to 23 million informative reads (assigned to non rRNA
193 SPA genes). Overall, we identified 322 genes that were exclusively expressed (TPM ≥10) by SPA during

194 infection of HeLa cells compared to 576 genes that were expressed only by extracellular SPA in LB culture.
195 2,995 SPA genes were expressed at both conditions (Fig. 1B and Table S3).

196 At 8 h p.i. of HeLa cells, 322 genes were upregulated and 579 genes were down-regulated (Fig. 3A
197 and Table S4) by twofold or more (adjusted p-value ≤ 0.05). Like in intracellular STM, the genes for type
198 I (Fim) fimbriae (*fimCIWYZ*) and additional fimbria clusters including Bcf (*bcfDE*), the Curli (*csgBCDE*), Stb
199 (*stbBE*), Stc (*stcABC*), Stf (*stfAD*), Sth (*sthABCD*), and Stk (*stkABC*) were all strongly downregulated
200 intracellularly (Fig. 3B). Noteworthy, the downregulation of type I (*fimACIZ*), Curli (*csgBDE*) and Bcf
201 (STM14_0037 and *bcfB*) fimbriae was more pronounced in SPA than in STM (Fig. S1), indicating that the
202 SPA fimbriome is robustly repressed, during intracellular replication, while some basal level of expression
203 of these fimbrial genes is still maintained during STM infection.

204 In contrast, but consistent with our findings for intracellular STM, genes belonging to the PhoPQ
205 (including polymyxins resistance) and SPI-2 regulons were significantly induced by intracellular SPA.
206 Moreover, various transition metals import systems including manganese and iron transporter (*sitABCD*),
207 enterobactin biosynthesis system (*entABCDEF* and *fepAG*) and the iron ABC transporter IroC were
208 considerably upregulated during SPA HeLa cell infection (Fig. 3B).

209 In addition, the expression of several metabolic pathways that were significantly elevated during
210 STM infection were induced by intracellular SPA as well, including biotin (*bioABCDF*) and purine
211 (*purCDEHKLT*) biosynthesis genes. Further overlap in the intracellular transcriptome of these pathogens
212 includes several amino acid biosynthesis pathways such as glutamine (*glnAGKLPQ*), tryptophan
213 (*trpABCDE*) and histidine (*hisAFGHI*) that were upregulated in both intracellular STM and SPA.

214 Nevertheless, comparison of the intracellular transcriptome between STM and SPA, during HeLa
215 cells infection, identified 234 differentially expressed genes (DEGs; Table S5), indicating that some
216 pathways may function differently during the intracellular phase of STM vs. SPA. These differences

217 included the induction of several amino acids biosynthesis genes which expression was more pronounced
218 in intracellular SPA than in STM. These include biosynthesis genes of glycine (*glyASU*), isoleucine
219 (*ilvCDEG*), leucine (*leuABCD*), lysine (*lysAC* and *sucBD*), methionine (*metCEFHKLR*), serine (*serAC*), and
220 threonine (*thrBC*). In contrast, the threonine degradation pathway (*tdcABCDEG*) was found to be
221 repressed in intracellular SPA. These results may suggest that SPA relies more than STM on amino acids
222 as an available carbon and energy source during epithelial cell infection. Additional pathways that were
223 found upregulated in SPA, but not in STM are the oxidative phosphorylation (*nuoEFGHIJKLMN* and
224 *atpCD*), and peptidoglycan cell wall formation (*murACDEG*) pathway genes (Fig. 3B). Remarkably, further
225 differences between the intracellular transcriptome of SPA vs. STM included different expression profiles
226 of the SPI-1, motility and chemotaxis, and carbon utilization regulons as detailed in the following sections.

227

228 **Differences in carbon catabolism during intracellular infection of *S*. Paratyphi A and *S*. Typhimurium**

229 In order to survive, persist and replicate in host cells, intracellular pathogens must adapt their
230 metabolic pathways to the specific physical conditions and available nutrients found in the intracellular
231 environment. Carbon catabolism provides bacteria with energy by means of reducing equivalents, ATP
232 and essential biosynthetic precursors. Different studies have suggested that hexose monosaccharides
233 such as glucose and glucose-6P are the main source of carbon during *Salmonella* infection (39-43).
234 Comparison of the metabolic gene expression between intracellular STM and SPA revealed significant
235 differences in the expression profile of several carbon catabolic pathways. For example, the gene *uhpT*
236 encoding an antiporter for external hexose 6-phosphate and internal inorganic phosphate (44) presented
237 about 90-fold higher expression by intracellular STM than SPA (Fig. 4A). This different expression might
238 be because the sensor-regulator system encoded by *uhpABC* is defective by pseudogene formation in
239 SPA (45). Similarly, the gene coding for galactonate permeases (*dgoT*) was upregulated in intracellular

240 STM, but not in SPA, suggesting that STM utilizes more hexose 6-phosphate and galactonate than SPA as
241 a carbon source during intracellular growth.

242 Similarly, RNA-Seq identified that at least seven genes involved in citrate metabolism including
243 *citCDEFTX* and *citC2* exhibited higher transcription levels in intracellular STM than SPA (Fig. 4A).
244 Independent qRT-PCR further confirmed that the intracellular expression of *citA* and *citX* encoding
245 citrate-proton symporter and apo-citrate lyase phosphoribosyl-dephospho-CoA transferase, respectively
246 is lower by about 3- and 10-fold in SPA infected cells vs. STM (Fig. 4B).

247 STM can utilize ethanolamine as a sole source of carbon, nitrogen, and energy in a cobalamin
248 (vitamin B12)-dependent manner. Ethanolamine may be important to STM growth in the host, since it is
249 derived from the membrane phospholipid phosphatidylethanolamine that is particularly prevalent in the
250 gastrointestinal tract. This process involves breakdown of the precursor molecule
251 phosphatidylethanolamine by phosphodiesterases to glycerol and ethanolamine (46). In STM,
252 ethanolamine catabolism involves 17 genes organized in the *eut* operon, controlled by the transcriptional
253 activator EutR (47). At least six ethanolamine catabolism genes (*eutSPQTGH*) were expressed at higher
254 levels by intracellular STM compared to SPA (Fig. 4A). These differences were independently verified by
255 qRT-PCR that showed significantly reduced expression of *eutG, eutP* and *eutQ* genes in intracellular SPA
256 vs. STM (Fig. 4B).

257 Collectively, these observations suggest that STM is able to better utilize intracellular citrate,
258 ethanolamine and galactonate as intracellular carbon sources than SPA, and that SPA may use an
259 alternative carbon source such as amino acids during intracellular growth.

260 To further test this hypothesis, we compared the growth of SPA and STM in a defined M9 medium
261 supplemented with ethanolamine or citrate as a carbon source. Growth under anaerobic conditions in
262 M9 medium, supplemented with ethanolamine, without amino acids, did not allow bacterial growth of

263 neither STM nor SPA. Adding casamino acids, without additional carbon source, resulted in a minimal
264 and comparable growth of both STM and SPA, likely due to the utilization of amino acids as a restricted
265 carbon source. Nevertheless, adding ethanolamine as a *bona fide* carbon source to the medium resulted
266 with increased and rapid growth of STM, but with a minor improvement of SPA growth (relative to its
267 growth on amino acids only; Fig. 4C), indicating the ability of STM, but not SPA to efficiently ferment
268 ethanolamine. Replacing the carbon source with citrate under aerobic growth conditions allowed
269 delayed, but substantial growth of STM, in comparison to restricted and slower growth of SPA (Fig. 4D).
270 These results are concurring with the expression data and indicate a better ability of STM than SPA to
271 utilize both ethanolamine and citrate as a sole carbon source. These differences may also contribute to
272 a higher intracellular replication pace of STM than SPA found in HeLa cells (Fig. S2).

273

274 **Intracellular *S*. Paratyphi A expresses SPI-1 genes at higher levels than intracellular *S*.**
275 **Typhimurium**

276 Previously we showed that under extracellular aerobic growth conditions in LB, SPI-1 gene
277 expression, as well as secretion of SPI-1-T3SS effector proteins, occur at significantly lower levels in SPA
278 compared to STM (20). Here, in sharp contrast, we found that intracellular SPA transcribed significantly
279 higher levels of SPI-1 and T3SS-1-effector genes than intracellular STM (Fig. 5A). This was clearly evident
280 (log 2 change ≥ 2 and p-value ≤ 0.05) for at least 14 SPI-1 genes including the SPI-1 regulators *hilA* and
281 *hilD*, the effector genes *sopBDF*, the T3SS-1 structural genes *invCHIJ* and *spaOPQ*, *sigE* encoding a
282 chaperon (a.k.a. *pipC*) and *iagB* (Table S5).

283 Independent qRT-PCR was used to verify these observations and demonstrated that intracellular SPA
284 transcribed *hilA*, *hilD*, *invH*, *sopB*, *sopD* and *spaO* at 4- to 27-fold higher levels than STM (Fig. 5B).
285 Moreover, Western blotting against a 2HA-tagged version of SopB and SopE2 further indicated elevated

286 translation of these two SPI-1 effectors in intracellular SPA vs. STM (Fig. 5C). Collectively these results
287 indicate that SPA expresses SPI-1 genes at higher levels during infection of non-phagocytic cells, in
288 comparison to intracellular STM.

289

290 **Intracellular *S*. Paratyphi A is motile and expresses flagella-chemotaxis genes at higher levels**
291 **than *S*. Typhimurium**

292 Functional flagella are necessary for *Salmonella* motility and invasion of host cells (21, 48, 49). In *S.*
293 *enterica,* more than 50 genes are involved in the assembly and motion of flagella, organized in one
294 regulon that composes three ordered regulatory phases corresponding to classes I (early), II (middle),
295 and III (late) (50).

296 Previous studies have shown that the genes coding the flagellar machinery, as well as many genes
297 involved in chemotaxis, are strongly repressed during STM intracellular lifestyle (27, 31, 32). Here, RNA-
298 Seq analysis has demonstrated that at least 38 out of 50 chemotaxis and flagella genes, belonging to class
299 II and III of the flagella regulon are expressed at significantly higher levels in intracellular SPA compared
300 to STM (Fig. 6A and Table S5). These include the flagellin gene *fliC* that was expressed at 11-fold higher
301 levels by SPA vs. STM, and many other flagellar genes including *flgC* that demonstrated 64-fold difference
302 in its expression between intracellular SPA vs. STM. In addition to the flagellar genes, SPA residing in the
303 host cell presented much higher expression levels of chemotaxis genes (e.g. *cheB, cheR, cheM, cheW,*
304 *cheZ*, and *cheY*) than intracellular STM.

305 Independent qRT-PCR analysis confirmed these results and showed that in sorted infected HeLa
306 cells, the expression of *fliC, fliZ, flgM, flgF, cheA* or *cheB* was 8- to 130-fold higher in intracellular SPA
307 than in intracellular STM (Fig. 6B). In agreement with the RNA-seq and qRT-PCR analyses, Western

308 blotting using anti-FliC antibodies demonstrated significantly higher translation of FliC in intracellular SPA
309 vs. STM (Fig. 6C). Taken together, these findings uncover significantly higher expression levels of motility
310 and chemotaxis genes by SPA vs. STM within infected HeLa cells.

311 To further analyze if the increased expression of motility genes by intracellular SPA has
312 physiological consequences, we investigated the possible presence of flagella in intracellular SPA.
313 Immunostaining of intracellular SPA at 8 h p.i. indeed indicated the presence of flagella filaments on
314 intracellular SPA (Fig. S3). Flagella staining was absent for a Δ*fliC* strain of SPA, and restored in the
315 plasmid-complemented SPA Δ*fliC*. No immunolabeling of flagella was observed for intracellular STM WT
316 or STM Δ*fliC* Δ*fljB* (Fig. S3).

317 **Motile intracellular SPA are more inclined to replicate in the cytoplasm rather than the SCV**

318 Analysis of host cells infected by STM or SPA WT or T3SS-2-deficient strains was performed. To
319 distinguish *Salmonella* in the SCV from *Salmonella* in other compartments or in the cytosol, and to enable
320 live-cell imaging of infected host cells, we used LAMP1-GFP expressing HeLa host cells (Fig. 7, Movies 1,
321 4). Lifeact-GFP expressing HeLa cells were used to investigate potential interactions with the host cell
322 actin cytoskeleton (Movie 1-3).

323 More than 80% of STM WT were present in SCV with associated *Salmonella*-induced filaments (SIFs;
324 Fig. 7A, Fig.8). STM WT was observed residing in SCV and the majority of infected host cells showed
325 formation of extensive SIF networks. The STM *ssaV* strain was typically present in SCV, but tubular
326 endosomal filaments were almost absent. A smaller proportion of host cells (ca. 10%) harbored cytosolic
327 STM, and for these cells, massive cytosolic proliferation or hyper-replication was evident (Fig. 7A). For
328 intracellular SPA WT, more heterogeneous phenotypes were observed (Fig. 7BCDE). SPA was located
329 residing in the SCV, or present in the cytosol without association with LAMP1-positive membranes.

330 Intracellular SPA also induced SIF in a T3SS-2-dependent manner, but the frequency of SIF-positive cells
331 was much lower with ca. 9% for SPA WT compared to more than 80% for STM WT (Fig. 7BC, Fig. 8). SPA-
332 infected host cells often contained thin SIF, that are likely composed of single-membrane tubules, in
333 contrast to SIF with more intense LAMP1-GFP signal, indicating double-membrane tubules (Fig. 7BC). For
334 SPA WT, SIF formation was significantly less frequent, yet dependent on T3SS-2. The frequency of SPA
335 associated with SCV marker LAMP1 was highly reduced compared to STM. As intracellular SPA was
336 observed to reside in tightly enclosing SCV (51), we considered flagella expression by vacuolar SPA less
337 likely than by cytosolic bacteria. Because transcriptional analyses showed expression of flagellar genes
338 by intracellular SPA, flagella filaments were detected on intracellular SPA, and a major proportion of
339 infected HeLa cells (~ 40%) harbored cytosolic SPA, we investigated the functional consequences of
340 flagella expression by live-cell imaging of *Salmonella*-infected host cells (Movies 1-4). Indeed, we
341 observed actively motile intracellular SPA in about 40% of the infection host cells.

342 As motility in intracellular microbes typically is mediated by polymerization of host cell actin by
343 microbial surface proteins (52), we investigated F-actin dynamics in SPA-infected HeLa Lifeact-GFP cells
344 (Movie 1). No association of F-actin with static or motile intracellular SPA was evident, and F-actin trails
345 as observed for intracellular motile *Listeria monocytogenes* (53) were absent in SPA WT infected cells.
346 No cytosolic motility was observed for intracellular STM. To control if cytosolic motility is linked to the
347 observed flagella expression, we used a SPA *fliC* mutant strain. This non-motile mutant completely lacked
348 cytosolic motility, but showed increased presence in SCV (Fig. 8, Movie 1). Cytosolic motility of SPA was
349 not affected in the T3SS-deficient Δ*ssaR* strain (Fig. 8, Movie 1). Live-cell imaging at high frame rates
350 (Movies 2 and 3) demonstrated the dynamics of intracellular motility of SPA in real time, with
351 characteristic alternations between straight swimming and stops with tumbling. Analyses of the vectors

352 of individual SPA indicated collisions with the host cell plasma membrane, but there were no indications
353 for formations of protrusions as observed for intracellular motile *L. monocytogenes* (53).

354 We next set out to analyze the broader relevance of intracellular motility of SPA and investigated
355 a set of clinical SPA isolates (22), and SPA reference strains of SARB collection (54). We observed that all
356 SPA strains tested showed intracellular motility in HeLa cells (Movie 4). Expression of flagella genes and
357 presence of flagella filaments was previously demonstrated for STM in dead host cells extruded from
358 polarized epithelial monolayers (55). As these experiments were performed with STM strain SL1344, we
359 also analyzed this strain in our experimental setting. As for STM ATCC 14028, also STM SL1344 showed
360 cytosolic hyper-replication in infected HeLa cells, but cytosolic motility was absent (Movie 4).

361 These data confirm that the expression of flagella by intracellular SPA leads to flagella-mediated
362 cytosolic motility, and that this motility is a conserved trait specific to SPA.

363 **Intracellular SPA are primed for reinfection of host cells**

364 We set out to investigate potential functional consequences of the increased expression of SPI-1
365 and motility genes by intracellular SPA. We hypothesized that increased expression of SPI-1, higher
366 amounts of T3SS-1 effector proteins, and motility could affect the interaction of SPA released from
367 infected cells with naïve host cells. As host cell decay and release of the intracellular *Salmonella* is
368 asynchronous, and quantification of new infection events is difficult in gentamicin protection assays, we
369 set up a re-invasion assay that provides experimental control. HeLa cells were infected by invasive
370 *Salmonella*, at a defined time point of 8 h p.i., host cells were osmotically lysed, and released bacteria
371 were used to reinfect new epithelial cells. The number of viable bacteria in inoculum, released from
372 initially infected host cells, and the number of bacteria in the second round of invasion were quantified
373 (Fig. 9A).

374 We determined similar levels of invasion by SPA after microaerobic culture and STM after aerobic
375 subculture (3.25% and 3.41% of inoculum, respectively), while STM after microaerobic subculture
376 invaded less efficiently (0.47%). Interestingly, 6.88% of the SPA released from HeLa cells at 8 h p.i. were
377 capable of reinvasion of naïve HeLa cells. In contrast, only 0.91% of released STM were able to reinvade,
378 and reinvasion of STM after microaerobic culture was lower with 0.75%. These data indicate that higher
379 expression of SPI-1 genes and bacterial motility by intracellular SPA results in increased invasiveness of
380 SPA following exit from host cells (Fig. 9B).

381

382 **DISCUSSION**

383 Despite high genetic similarity, typhoid and non-typhoidal *Salmonella* serovars differ hugely in their
384 host specificity, the immune response they elicit in humans, and the clinical manifestations they cause
385 (56). Previously, we showed differences in host cell invasion and SPI-1 expression between STM and SPA,
386 following extracellular growth in rich medium LB under aerobic conditions (20) and demonstrated that
387 elevated physiological temperature affects differently their motility and host cell entry (22, 51).

388 In this work, we focused on their intracellular phase of infection and have taken an unbiased deep
389 RNA-Seq approach to get a panoramic view of the intracellular STM and SPA transcriptomic architecture,
390 and to identify differences in the activity of their genes in the intracellular milieu. Following transition
391 from extracellular to the intravacuolar environment, *Salmonella* undergoes extensive adaptation and
392 global modulation of gene expression to respond to the intracellular environment, however the
393 nutritional contents of the SCV and the metabolic pathways required during intracellular growth are
394 largely undefined. Amino acids and purines seem to be deficient since previous studies have shown that
395 many auxotrophic *Salmonella* mutants are unable to grow intracellularly and are severely attenuated for

396 virulence in mice (57-60). These observations suggest that intracellular *Salmonella* depend on *de novo*
397 synthesis of metabolic precursors.

398 Recently, Liu and colleagues have applied a proteomic approach to determine the expression of
399 STM proteins within HeLa cells at 6 (61) and 18 h (27) p.i. Also, the STM transcriptome inside
400 macrophages (31, 32) and epithelial cells (26) has been reported previously. Nevertheless, a global
401 transcriptomic comparison between SPA and STM during intracellular infection has not been published
402 thus far.

403 Previous efforts to identify changes in gene expression using quantitative proteomic approach (LC-
404 MS/MS analyses) have revealed that among about 3,000 detected proteins, the expression of 100
405 *Salmonella* proteins significantly changed (61 were upregulated and 39 proteins were downregulated)
406 during the transition of STM from the extracellular to the intracellular niche of HeLa cells, at 6 h p.i. (61).
407 A microarray hybridization-based technique has identified 605 upregulated and 616 downregulated STM
408 genes at 6 h p.i. of HeLa cells (26). Our current transcriptomic analysis identified 830 differentially
409 expressed STM genes (365 upregulated and 465 downregulated) that were altered at 8 h p.i., relative to
410 their extracellular expression. Similar numbers of DEGs were also detected for SPA, which presented 322
411 upregulated and 579 downregulated genes, during epithelial cell infection.

412 In agreement with these previous analyses, we observed substantial downregulation in the
413 expression of genes associated with SPI-1, Type I fimbriae, flagella and chemotaxis systems in STM, but
414 significant upregulation of STM genes involved in metal ions uptake, purines and biotin synthesis. In fact,
415 nearly all known *Salmonella* iron-, zinc-, or manganese-responsive pathways were found to be
416 intracellularly induced. Since the SCV is characterized by low levels of magnesium, manganese, iron and
417 zinc metals (62) and given the pivotal role of these metal ions in *Salmonella* infection (63, 64),
418 upregulation of iron, zinc and manganese acquisition system genes is not surprising. Nonetheless, these

419 results highlight the importance of biotin and purines to the intracellular phase of *Salmonella* during
420 infection. This notion is consistent with (i) SCVs of both macrophage and epithelial cells are limited for
421 purines and pyrimidines (65); (ii) the avirulent phenotype of purine auxotrophic mutants of *S*.
422 Typhimurium and *S*. Dublin in mice (59); and (iii) the reported contribution of biotin sulfoxide reductase
423 to oxidative stress tolerance and virulence of *Salmonella* in mice (66).

424 Interestingly, in contrast to the proteomics (61) and the microarray-based transcriptome analyses
425 (26), we were also able to identify significant upregulation of SPI-2 and PhoPQ regulon genes both in STM
426 and SPA. These differences may reflect the time point differences (8 h p.i. in our analysis vs. 6 h p.i. in
427 previous analyses), or the superior sensitivity of the current RNA-Seq techniques.

428 While the change in the expression of metal transport systems, purines, biotin, PhoPQ and SPI-2
429 regulons was common to both SPA and STM, intriguing differences in the expression profile of SPI-1,
430 motility and chemotaxis, and carbon utilization pathways (mainly citrate, galactonate and ethanolamine)
431 were identified between intracellular SPA and STM. Ethanolamine is a ubiquitous molecule within
432 vertebrate hosts and serves as a carbon and nitrogen source for bacteria in the intestine, as well as within
433 epithelial cells (67). *Salmonella* can utilize ethanolamine, as an exclusive source of carbon and nitrogen,
434 generating acetaldehyde and ammonia. The ammonia can then provide a cellular supply of reduced
435 nitrogen, while the acetaldehyde can be converted into the metabolically useful compound acetyl-CoA
436 (68). Our data indicated much higher expression of multiple ethanolamine utilization genes by STM vs.
437 SPA during host cell infection and more efficient utilization *in-vitro*. Recently, it was shown that
438 ethanolamine metabolism in the intestine enables STM to establish infection and that EutR, the
439 transcription factor of the ethanolamine cluster in *Salmonella* directly activates the expression of SPI-2
440 in the intracellular environment (69, 70).

441 The sugar acid galactonate is another example of an available carbon and energy source that is
442 produced in the mammalian gut. The gut microbiota releases sugar acids including galactonate from
443 ingested polysaccharides present in nutrients by the host, or from simple sugars as catabolic
444 intermediates of metabolism (71). These results may suggest that STM utilizes galactonate, citrate and
445 ethanolamine more effectively than SPA as available carbon sources, and that STM may better
446 coordinate ethanolamine metabolism and virulence via EutR during infection. This notion is consistent
447 with our *in-vitro* data showing that while STM is able to utilize ethanolamine and citrate as a sole carbon
448 source, SPA cannot grow or grows much slower on these sources. Previous studies have shown that
449 deficiency for citrate lyase activity (*citF*) leads to significant reduction in STM survival inside macrophages
450 (72) and that an STM mutant that cannot utilize citrate is highly attenuated in the mouse (73). Similarly,
451 ethanolamine is released by host tissues during inflammation and a study by Thiennimitr and colleagues
452 in mice has shown that ethanolamine metabolism provides a growth advantage to STM during intestinal
453 colonization (70). Since SPA provokes a noninflammatory disease, it is possible that SPA has lost the
454 ability to utilize ethanolamine as it provides no advantage during noninflammatory infection.

455 It is well established that the expression of the flagella and chemotaxis are highly repressed by
456 intracellular STM (27, 31, 61, 74), supporting a nonmotile state of intracellular STM. In agreement with
457 these reports, we have also observed extensive repression of the entire motility-chemotaxis regulon in
458 intracellular STM, however we found significantly higher expression of this regulon in intracellular SPA.
459 Similar observations were also obtained for the evolutionary related T3SS-1 genes. Most salmonellae are
460 motile and express peritrichous flagella around its membrane. Like T3SS-1, *Salmonella* motility is playing
461 an important role in host colonization and virulence (75, 76). Previous reports have shown a regulatory
462 association between T3SS-1 and the flagella in STM (77). Subsequent to STM entry into host cells, the
463 intracellular expression of T3SS-1 (27, 31, 78) as well as flagella production and chemotaxis (27, 31, 61,
464 79) genes were shown to be highly repressed, possibly to prevent inflammatory response by the

465 NAIP/NLRC4 inflammasome (80). Therefore, it was unexpected to find that the flagella regulon is readily
466 expressed during SPA intracellular infection and that it maintains an intracellular motility during epithelial
467 cell infection.

468 Intracellular motility is long known in bacterial pathogens such as *Listeria monocytogenes, Shigella*
469 *flexneri*, *Rickettsia* spp., and *Burkholderia* spp., that all exhibit intracellular actin-based motility to spread
470 from cell to cell (81). Nonetheless, to the best of our knowledge, this is the first report of a flagellum-
471 dependent intracellular motility of any *Salmonella* serovar in living host cells. While we did not find
472 evidence for intercellular spread, it is possible that this mechanism facilitates escape of SPA from the SCV
473 to the cytoplasm. Previous studies have shown that a small subpopulation of hyper-replicating STM
474 resides within the cytosol of epithelial cells and serves as a reservoir for dissemination. These motile
475 bacteria retain both SPI-1 and flagella expression, but present a low expression of SPI-2 genes, and
476 therefore are primed for invasion (82). This expression profile is largely similar to the intracellular profile
477 of SPA. Indeed, we showed that intracellular SPA possesses higher ability to reinfect non-phagocytic cells
478 than intracellular STM. We propose that elevated expression of motility-chemotaxis and SPI-1 genes in
479 addition to flagella-mediated motility prime SPA towards a new cycle of host cell infection, supporting
480 systemic dissemination of this pathogen in the human body. These phenotypes may contribute to the
481 invasive nature of paratyphoid fever and the ability of SPA to disseminate beyond the intestinal lamina
482 propria of the human host. Future work has to reveal which cells or tissues of infected host organisms
483 are affected by intracellular motility of SPA. However, such analyses are hampered by the lack of animal
484 models for infection. Infection models with human organoids (83), for examples based on gall bladder
485 epithelial organoids (84) as a tissue type important for SPA persistence and dissemination, may offer new
486 perspectives for analyses of cellular interactions.

487

488 **MATERIAL AND METHODS**

489

490 **Bacterial strains, growth conditions and data sets.** Bacterial strains utilized in this study are listed
491 in **Table S6** . *S*. Typhimurium 14028 S (85) and *S*. Paratyphi A strain 45157, an epidemic strain, responsible
492 for a paratyphoid outbreak in Nepal (9) were used as the wild-type strains. Bacterial cultures were
493 routinely maintained in Luria-Bertani (LB; BD Difco) liquid medium at 37°C. An M9 medium [33.7 mM
494 Na_2HPO_4, 22 mM KH_2PO_4, 8.5 mM NaCl, 18.7 mM NH_4Cl, 12.1% Trizma base, pH=7.4] supplemented with
495 2 mM $MgSO_4$, 0.3 mM $CaCl_2$, 3.7 µM thiamine, 4.1 µM biotin, 0.134 mM EDTA, 31 µM $FeCl_3$, 6.2 µM $ZnCl_2$,
496 7.6 nM $CuCl_2$, 4.2 nM $CoCl_2$, 1.62 µM H_3BO_3, 8.1 nM $MnCl_2$, with or without 0.1% Casamino acids, and
497 with one of the following carbon sources glucose (11 mM), ethanolamine hydrochloride (51 mM) or
498 sodium citrate (34 mM) was used as a minimal growth medium.

499 For HeLa cell infections, *Salmonella* cultures were grown to the stationary phase under
500 microaerobic conditions (see below). The final dataset for RNA-seq included 12 samples resulting from
501 three independent growth in LB or infection experiments that were used for RNA extraction: (i) *S*.
502 Typhimurium 14028 S bacterium grown in LB medium (hereafter named STM_m) (ii) *S*. Paratyphi A strain
503 45157 grown in LB medium (hereafter named SPA_m) (iii) Typhimurium 14028 S bacterium infected Hela-
504 cells (hereafter named STM_i) and (iv) *S*. Paratyphi A strain 45157 bacterium in Hela-cells (hereafter
505 named SPA_i).

506 **Infection of HeLa cells.** Human epithelial HeLa (ATCC CCL-2) cells were purchased from the
507 American Type Culture Collection and were cultured in a high-glucose (4.5 g/l) DMEM supplemented with
508 10% FBS, 1 mM pyruvate and 2 mM L-glutamine at 37 °C in a humidified atmosphere with 5% CO_2. Cells
509 were seeded at 6×10^6 cells/ml in a onewell plate (Greiner) tissue culture dish 18 h prior to bacterial
510 infection and infected at multiplicity of infection (MOI) of ~200 (bacteria per cell). For RNA extraction,
511 five plates for *S.* Typhimurium and six plates for *S.* Paratyphi A were used. *Salmonella* strains carrying the

512 pBR-GFP2 plasmid were grown in 2 ml LB containing 20 µg/ml tetracycline at 37 ˚C with shaking (250
513 rpm) for 6-8 h, and then diluted 1:75 into 10 ml LB supplemented with 20 µg/ml tetracycline and grown
514 without shaking at 37 ˚C for 16 h. Bacterial cultures were centrifuged for 5 min at 4,629 × g, at room
515 temperature (RT), and resuspended in 3 ml prewarmed complete DMEM. 3 ml of DMEM suspended
516 Salmonellae (~2.5×10^9 CFU) were used to replace the medium of seeded cells and plates were
517 centrifuged for 5 minutes at 142 × g at RT. After centrifugation, 7 ml of prewarmed DMEM were added
518 and plates were incubated at 37 ˚C under 5% CO_2 atmosphere. At 2 h post infection (p.i.) the medium
519 was aspirated, cells were washed three times with 10 ml PBS containing $CaCl_2$ and $MgCl_2$ (PBS +/+), and
520 incubated with 10 ml prewarmed DMEM containing 100 µg/ml gentamicin. After 60 min, the media was
521 replaced with DMEM containing 10 µg/ml gentamicin and cells were further incubated as before. At 8 h
522 p.i., the cells were washed three times with 10 ml PBS +/+ and trypsinized with 2 ml prewarmed trypsin
523 at 37 ˚C. When the cells were detached, prewarmed DMEM was added to quench the trypsin. The cells
524 were transferred to a 50 ml conical tube, precipitated by 5 min centrifugation at 247 x g and washed in
525 15 ml PBS+/+. Finally, the cells were resuspended in 1 ml / plate fixation reagent (RNAlater: RNAprotect
526 350:1), incubated at RT for 20 min, and then stored at 4 °C until sorting.

527 HeLa cell lines stably transfected for expression of LAMP1-GFP or Lifeact-GFP have been
528 described before (86) and were maintained and infected as described above.

529 **FACS sorting and RNA extraction.** RNA was extracted from three independent infection
530 experiments of HeLa cells with STM strain ATCC 14028S or SPA 45157. As controls, RNA was also
531 extracted from three independent cultures of uninfected cells and from bacterial cultures grown in LB.
532 HeLa cells (infected or uninfected) were fixed, centrifuged at 247 × g for 5 min and resuspended in 3 ml
533 PBS+/+. The cells were filtered through 30 µm pre-separation filter into 5 ml FACS tubes (SARSTEDT) and
534 sorted on a BD-FACSAria IIu (100 µm nozzle; ND filter 2) at a rate of approximately 300 cells/sec. 2-

535 2.5×10^6 GFP-positive (infected) HeLa cells were collected and centrifuged at 247 × g for 5 min. RNA
536 extraction was performed using Hybrid-R RNA Purification System (GeneAll). RNA samples were treated
537 with RNase-free DNase (Qiagen) according to manufacturer's protocol, followed by ethanol precipitation
538 and Ribosomal RNA depletion that was performed by Epidemiology Ribo-Zero Gold rRNA Removal Kit
539 (Illumina).

540 **RNA sequencing.** RNAseq was performed at the GeT-PlaGe core facility, INRAE Toulouse. RNA-
541 seq libraries were prepared using the Illumina TruSeq Stranded mRNA Library Prep, without a poly-A
542 mRNA selection, according to the manufacturer's protocol. Briefly, RNAs were fragmented to generate
543 double-stranded cDNA and adaptors were ligated. 11 cycles of PCR were applied to amplify the libraries
544 and their quality was assessed using a Fragment Analyser. Libraries were quantified by qPCR using the
545 Kapa Library Quantification Kit (Roche). To reach 5 million bacterial reads for each sample, as
546 recommended in (87), RNA-seq reads were generated from three lanes of an Illumina HiSeq3000 and
547 two lanes of an Illumina NOVAseq6000 using a paired-end read length of 2×150 bp. Ribosomal RNA
548 (rRNA) reads were filtered out using sortmerna-2.1b (88) against Silva and Rfam databases. The raw reads
549 data for this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI
550 under accession number PRJEB46495 and as specified in Table S7.

551 **Genome annotation and quality control.** A flowchart summarizing the RNA-seq pipeline and
552 bioinformatic analyses is shown in Fig. S4. Annotation of SPA 45157 and STM 14028S genomes was
553 performed using EugenePP 1.2 (89) with general and targeted evidence to predict coding genes and
554 noncoding features (Figure S1). Functional prediction was produced with InterProscan v5.15-54.0 with
555 GO terms, COG version COG2014 (90), REBASE v708 (91), IslandViewer4 (92) and PHAST (93).

556 Structural and regulatory non-coding RNA were annotated using RNAspace software v1.2.1 (94)
557 together with the following tools: RNAmmer 1.2 for rRNA (95), tRNAScan-SE 1.23 for tRNA (96) and blastn

558 2.2.25 against RFAM 10.0 for other ncRNA (97). The gff3 files produced by Eugene and RNA annotation
559 were merged by a semi-manually procedure using BEDTools-2.26.0 (98).

560 FastQC v0.11.7 was used for quality control of the raw sequence data. Cutadapt 1.8.3 was used
561 to remove Illumina Truseq adaptors and to filter out all reads with length shorter than 36 nucleotides.

562 **Reads mapping and counting.** The remaining, non rRNA reads were mapped with STAR-2.6.0c
563 (99), with default parameters, against a "hybrid" genome composed by the Ensembl GRCh38 human
564 primary assembly (release 92) and the SPA (accession number CP076727) or STM (accession number
565 CP001362 - CP001363) genomes. In addition, in order to analyze bacterial samples, we indexed each
566 bacterial genome separately by decreasing the parameter "genomeSAindexNbases" to 10 for STM and
567 to 9 for SPA, while producing the alignment (.bam) files sorted by coordinates. Samtools flagstat v1.8 was
568 used to obtain mapping statistics. Then we added read groups to trace the origin of each read with
569 AddOrReplaceReadGroups of Picard tools v2.18.2. Next, we merged the bam files that originated from
570 resequencing of the same sample using the MergeSamFiles command of Picard tools. The featureCounts
571 command from the Subread-1.6.0 tool (100) was used to compute fractional counting for all annotated
572 genes.

573 **Clustering of orthologous genes.** Orthologous genes were computed using Roary v.3.11.2 (101)
574 with default parameters (minimum of 95% identity between coding sequences of the same cluster)
575 except for the "-s" option that avoids paralog splitting in different clusters. Based on the orthologous
576 groups, we produced raw count matrices that compare the expression level between orthologous genes
577 of SPA and STM. We also added groups including paralog genes on either SPA or STM genome into the
578 raw count matrix to sum up the raw reads count of each putative paralog gene.

579 **Differential Gene expression analysis.** Normalization and differential analysis were carried out
580 according to the DESeq2 model and package (102) using adjusted p-value with FDR correction and the

581 Wald test to infer the probability value. Genes that had too few reads (the sum of reads for all the
582 samples was less than 10) were discarded. To compare DEGs between SPA and STM, we created a count
583 matrix composed of the shared protein-coding and ncRNA genes between these two serovars. The
584 heatmaps were created by applying DESeq2 variance stabilizing transformations (the vst command) on
585 the counts followed by subtracting the mean of the gene across all samples. The heatmaps were drawn
586 using the ComplexHeatmap R package (103).

587 **Reverse transcription and quantitative real-time PCR (qRT-PCR).** 500 ng of RNA was reverse-
588 transcribed using qScript cDNA synthesis kit (Quanta-bio). Real-time PCR was performed as previously
589 described (20) on a StepOnePlus Real-Time PCR System (Applied Biosystems). Relative quantity of
590 transcripts was calculated as $2^{-\Delta\Delta Ct}$, using the *rpoD* gene as an endogenous normalization control.

591 **Western Blotting.** HeLa cells were infected with *Salmonella* strains carrying pBR-GFP2 plasmid
592 and pWSK29 carrying HA-tagged alleles of *sopE2* and *sopB*. The infection procedure was performed as
593 above until the fixation step. Cell fixation was performed by incubation with 5 ml of 4% formaldehyde
594 for 15 min under gentle shaking. Five ml of PBS +/+ were added and the cells were centrifuged for 5 min
595 at 2,700 × g. Subsequently, the cells were washed three times with PBS +/+ and finally resuspended in
596 PBS +/+ containing 1% BSA. The cells were stored at 4 °C until sorting. 1×10^6 GFP-positive cells were
597 centrifuged for 5 min at 247 × g, and resuspended in 100 µl sample buffer. Western blot of the infected
598 cells was performed as previously described (104) using the anti-2HA (Abcam ab18181; 1:1,000), anti-
599 FliC (Abcam ab93713; 1:200,000) and anti-DnaK (Abcam ab69617; 1:10,000). Proteins were quantified
600 relative to DnaK using ImageJ.

601 **Host cell infection and live-cell imaging.** HeLa cells stably transfected with LAMP1-GFP were
602 seeded in surface-treated 8-well slides (ibidi) 24 or 48 h prior to infection to reach ~80% confluency
603 (~80,000 cells) and were then used for infection. STM strains for infection experiments were subcultured

604 from an overnight culture (1:31) in fresh LB medium and grown for 3.5 h at 37°C. For infection, SPA strains
605 were grown for 8 h under aerobic conditions, subcultured (1:100) in fresh LB medium and stationary-
606 phase subcultures were grown for 16 h under microaerophilic conditions as described in (21). Bacteria
607 were adjusted to an optical density of 0.2 at 600 nm in PBS and used for infection with the respective
608 MOI (between 25 and 100, dependent on strain). Bacteria were centrifuged onto the cells for 5 min at
609 500 × g to synchronize infection and the infection was allowed to proceed for 25 min. After washing
610 thrice with PBS, cells were incubated in medium containing 100 µg × ml^{-1} of gentamicin to kill extracellular
611 bacteria. Afterwards, the cells were maintained in medium supplemented with 10 µg × ml^{-1} gentamicin
612 for the ongoing experiment. Live-cell imaging was performed with Cell Observer Spinning Disc
613 microscope (Zeiss) equipped with a Yokogawa Spinning Disc Unit CSU-X1a5000, an incubation chamber,
614 63× objective (α-Plan-Apochromat, NA 1.4), two ORCA Flash 4.0 V3 cameras (Hamamatsu) and
615 appropriate filters for the respective fluorescence proteins.

616 **Immunostaining of infected cells.** HeLa cells stably transfected with LAMP1-GFP were seeded in
617 surface-treated 24-well plates (TPP) on coverslips 24 h or 48 h prior infection to reach ~80% confluency
618 (~180,000 cells) and were used for infection. Cultivation of bacteria and infection of HeLa cells was
619 carried out as described above. After the desired incubation time the cells were washed thrice with PBS
620 and fixed with 3% PFA in PBS for 15 min at RT, followed by immunostaining. Fixed cells were washed
621 thrice with PBS and incubated in blocking solution (2% goat serum, 2% bovine serum albumin, 0.1%
622 saponin, 0.2 % Triton X-100) for 30 min. Cells were stained 1 h at RT with primary antibodies against
623 *Salmonella* (H:i, BD Difco 228241; H:a, Sifin TR1401; O:5, Sifin TR5303). After washing thrice with PBS,
624 cells were incubated with the appropriate secondary antibodies for 1 h at RT. Coverslips were mounted
625 with Fluoroshield (Sigma) and sealed with Entellan (Merck). Microscopy of fixed samples was either
626 performed using a Cell Observer Spinning Disc microscope (Zeiss) as described above, or using a Leica

627 SP5 confocal laser-scanning microscope using 100× objective (HCX PL APO CS, NA 1.4-0.7) and polychroic
628 mirror TD 488/543/633.

629 **Reinvasion assays.** HeLa cells were seeded in surface-treated 6-well plates (TPP) 24 h or 48 h prior
630 to infection to reach ~100% confluency (1×10^6 cells) on the day of infection. Infection cultures of STM
631 and SPA strains were grown as either 3.5 h aerobic or 16 h microaerobic subcultures and used for
632 infection as described above. At 1 h p.i., cells were washed thrice with PBS and lysed using 0.1% Triton
633 X-100. CFU were determined by plating serial dilutions of lysates and inoculum on Mueller-Hinton II (MH)
634 agar and incubated overnight at 37 °C. The percentage of internalized bacteria of the inoculum (from
635 subcultures) was calculated. 8 h p.i., cells were washed twice with PBS, once with ice-cold ddH_2O, and
636 lysed using ddH_2O. Bacteria liberated from host cells by hypotonic lysis were used to infect naïve HeLa
637 cells, and 1 h p.i. cells were washed and lysed and CFU were determined as described above. The
638 percentage of internalized bacteria of the inoculum (from liberated bacteria) was calculated.

639

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651 genomes.

652

653 **FIGURE LEGENDS**

654 **Figure 1. The number of STM and SPA genes expressed in LB medium and during intracellular**
655 **growth.** *Salmonella* RNA-seq reads were aligned to *S*. Typhimurium 14028S and *S*. Paratyphi A 45157
656 genomes and normalized by transcripts per million (TPM) transformation. Genes with TPM ≥ 10 were
657 considered as expressed. The number of expressed genes in STM (**A**) and SPA (**B**) growing in LB medium
658 to the late logarithmic phase and within HeLa cells at 8 h p.i. is shown.

659 **Figure 2. DEGs of intracellular *S*. Typhimurium compared to growth in LB medium. (A)** Volcano
660 plots showing the fold change (log2 ratio) in the expression of STM genes grown intracellularly in HeLa
661 cells (8 h p.i.) vs. the growth in LB medium (X-axis) plotted against the -$\log_{10}$ adjusted p-value (Y-axis).
662 Each dot on the plot represents the mean value (from three independent cultures) of one gene. Genes
663 that were changed by more than twofold are colored in red. (**B**) Heatmap based on RNA-Seq results
664 showing the relative transcription of STM genes of interest during intracellular infection (C1 to C3) vs.
665 their extracellular expression in LB medium (M1 to M3).

666 **Figure 3. DEGs of intracellular *S*. Paratyphi A compared to growth in LB medium. (A)** Volcano
667 plots showing the fold change (log2 ratio) in the expression of SPA genes grown intracellularly in HeLa
668 cells (8 h p.i.) vs. the growth in LB medium (X-axis) plotted against the -$\log_{10}$ adjusted p-value (Y-axis).
669 Each dot on the plot represents the mean value (from three independent cultures) of one gene. Genes
670 that were changed by more than twofold are colored in red. (**B**) Heatmap based on RNA-Seq results
671 showing the relative transcription of SPA genes of interest during intracellular infection (C1 to C3) vs.
672 their extracellular expression in LB medium (M1 to M3).

673 **Figure 4. Differences in carbon utilization gene expression between STM and SPA.** (**A**) Heatmap
674 of carbon catabolism gene expression by intracellular STM vs. intracellular SPA. Pseudogenes in the SPA
675 genome are marked with an asterisk. (**B**) The fold change in the transcription of the glucose-6P (*uhpT*),

676 citrate (*citA* and *citX*), and ethanolamine (*eutP, eutQ* and *eutG*) utilization pathways in intracellular STM
677 relative to their expression in intracellular SPA was determined using qRT-PCR. RNA was extracted from
678 FACS-sorted intracellular salmonellae 8 h p.i. The mean and the standard error of the mean of 3-8
679 independent qRT-PCR reactions are shown. Student t-test was used to determine statistical significance.
680 **, $p < 0.005$; ***, $p < 0.0001$; ND, not detected. (**C**) Growth of STM and SPA in M9 in the presence (+) or
681 absence (-) of casamino acids (AA) and ethanolamine (ETA), under anaerobic conditions. (**D**) Growth of
682 STM and SPA in M9 supplemented with citrate as a sole carbon source.

683 **Figure 5. Intracellular SPA expresses higher levels of SPI-1 genes than intracellular STM.** (**A**) Heat
684 map of RNA-Seq results showing the relative transcription of SPI-1 genes in three independent HeLa cell
685 infections with STM and SPA. (**B**) The fold change in the transcription of six SPI-1 genes (*hilA, hilD, invH,*
686 *sopB, sopD* and *spaO*) in intracellular SPA relative to their expression in intracellular STM was determined
687 by qRT-PCR. The results show the mean of 3-6 qRT-PCR reactions and the error bars indicate the SEM.
688 (**C**) SPA and STM strains expressing GFP and a 2HA-tagged version of the SPI-1 effectors SopE2 and SopB
689 were used to infect HeLa cells. At 8 h p.i., cells were fixed and FACS-sorted for infected (GFP-positive
690 cells). Infected HeLa cells (1×10^6 for each sample) were collected and separated on 12% SDS-PAGE.
691 Western blotting using anti-hemagglutinin (HA) tag antibodies was used to detect the intracellular
692 expression of SopE2 and SopB. Anti-DnaK antibodies were used as a loading control.

693 **Figure 6. Intracellular SPA expresses higher levels of motility-chemotaxis genes than intracellular**
694 **STM.** (**A**) Heat map of RNA-Seq results showing the relative transcription of the motility-chemotaxis
695 regulon in three independent HeLa cells infections with STM and SPA. (**B**) The fold change in the
696 transcription of six flagellum genes (*fliC, fliZ, glgM, flgF, cheA* and *cheB*) in intracellular SPA relative to
697 their expression in intracellular STM was analyzed by qRT-PCR. The results show the mean of 3-5
698 independent reactions and the error bars indicate the SEM. (**C**) SPA and STM strains expressing GFP were

699 used to infect HeLa cells. At 8 h p.i., cells were fixed and FACS-sorted. Equal numbers of 1×10^6 infected
700 HeLa cells were collected, lysed, and proteins were separated on 12% SDS-PAGE. Western blotting using
701 anti-FliC antibodies were used to detect synthesis of FliC by intracellular *Salmonella*. Anti-DnaK
702 antibodies were used as a loading control.

703 **Figure 7. Distinct phenotypes of intracellular SPA.** HeLa cells stably transfected for expression of
704 LAMP1-GFP (green) were infected with STM WT (**A**, MOI 25) or SPA WT (**B-E**, MOI 75), each harboring
705 pFPV-mCherry for constitutive expression of mCherry (red). Live-cell imaging was performed at 8 h p.i.
706 Images are shown as maximum intensity projection. Arrowheads indicate subpopulations with distinct
707 phenotypes in infected cells: SIF (green), thin SIF (green with asterisk), *Salmonella* residing in SCV
708 (yellow), cytosolic *Salmonella* (red), hyper-replicating cytosolic *Salmonella* (red with asterisk). Scale bars:
709 10 µm (overview), 5 µm (inset).

710 **Figure 8. Quantification of intracellular phenotypes.** HeLa cells stably expressing LAMP1-GFP
711 (green) were infected with *Salmonella* strains expressing mCherry (red). Quantification of intracellular
712 phenotypes was performed with a Zeiss Cell Observer with 63x objective. From 6-8 h p.i. at least 100
713 infected cells per strain (for SPA *fliC* at least 50) were examined for intracellular phenotype. Data
714 represent the means with standard deviations for three biological replicates for each strain and
715 condition. Statistical analysis was performed with One-way ANOVA and is indicated as n.s., not
716 significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

717 **Figure 9. SPA shows higher invasion after release out of infected cells. (A)** HeLa cells were infected
718 with STM WT or SPA WT at MOI 5, and invasion by each strain was determined by gentamicin protection
719 assays. At 8 h p.i., one portion of infected cells was lysed by hypotonic conditions, and the released
720 bacteria were used to reinfect naive HeLa cells. (**B**) Intracellular CFU counts were determined 1 h p.i.
721 after initial infection (invasion, black bars), or 1 h p.i. after infection with *Salmonella* released from host
722 cells at 8 h p.i. (reinvasion, grey bars). Depicted are means and SD of three biological replicates, each

723 performed in triplicate. For statistical analysis, Student's t-test was performed. Significance is indicated
724 as n.s., not significant; *, $p < 0.05$; **, $p < 0,01$; ***, $p < 0.001$.

725

726 **SUPPLEMENTARY MATERIALS**

727 **Fig. S1. Expression of fimbriae genes in intracellular STM vs. SPA.** (**A**) Heat map of RNA-Seq
728 results showing the relative transcription of genes encoding curli (Csg), Fim, or Bcf fimbriae in three
729 independent HeLa cells infections with STM and SPA. (**B**) The x-fold change in the expression of *csgA* and
730 *csgD* in intracellular SPA relative to their expression in intracellular STM was analyzed by qRT-PCR. The
731 results show the means of 4-8 independent reactions and the error bars indicate the SEM.

732 **Fig. S2. Intracellular replication of SPA and STM in HeLa cells.** Intracellular growth of STM vs. SPA
733 in HeLa cells at 2, 4, 6, 8 and 24 h p.i. Bacterial replication was determined by the gentamicin protection
734 assay. Replication was calculated as the ratio between the intracellular bacteria (CFU) recovered at each
735 time point and the number of CFU at 2 p.i. The means and the SEM of four independent infections are
736 shown.

737 **Fig. S3. Formation of flagella by SPA within the host cell cytoplasm.** HeLa cells stably expressing
738 LAMP1-GFP (green) were infected with SPA WT, SPA *fliC*, SPA *fliC* [*fliC*], STM WT, or STM *fliC fljB* as
739 indicated, each constitutively expressing mCherry (red). Infected cells were fixed 8 h p.i. and
740 subsequently immunolabeled with serovar-specific antibody against FliC (blue). Scale bars: 10 µm.

741 **Fig. S4. A flowchart of RNA-seq and bioinformatic analyses.** The different steps and main tools
742 used for the bioinformatic analyses illustrated. RNA extracted from STM and SPA grown in culture and
743 from infected cells was subjected to NGS on Illumina sequencing. RNA-seq reads were cleaned and
744 quality controlled using FastQC. Cutadapt was used to remove Illumina Truseq adaptors and to filter out
745 all reads shorter than 36 nucleotides. SortMeRNA was used to filter out ribosomal RNA reads from raw

746 data. Human and bacterial genomes were merged into “Hybrid genomes” and indexed with STAR.
747 Bacterial genomes were reannotated using EugenePP. STAR was used to align reads to reference
748 genomes and sort resulting bam files by coordinates. To compute fractional counting for all annotated
749 coding and non-coding genes the Subread tool with parameters “-s 2 (reversely stranded), -p (count
750 fragments instead of reads) -M, -O, --fraction” was used. Clustering of *Salmonella* homologous genes was
751 computed using Roary. Normalization and differential expressed genes analysis were carried out
752 according to the DESeq2 model and package using adjusted P-value and the Wald test to infer the
753 probability value. To obtain DEGs between STM and SPA a count matrix composed of the shared and
754 coding genes and ncRNAs between these two serovars was created.

755

756 **TABLES**

757 **Table S1**. The transcriptome of STM grown in LB and intracellularly. The RNA-seq reads obtained from
758 STM grown on LB or extracted from HeLa cells infected with STM were mapped to the STM 12048S
759 genome. This table shows the normalized (TPM) featurecount counts of reads assigned to the STM genes.
760 This table also lists the genes that were found expressed in LB only, intracellularly only, and at both
761 conditions.

762 **Table S2**. DEGs of intracellular STM vs. LB.

763 **Table S3**. The transcriptome of SPA grown in LB or intracellular. The RNA-seq reads obtained from SPA
764 grown on LB or extracted from HeLa cells infected with SPA were mapped to the SPA 45157 genome.
765 This table shows the normalized (TPM) featurecount counts of reads assigned to the SPA genes. This
766 table also lists the genes that were found expressed in LB only, intracellular only, and under both
767 conditions.

768 **Table S4**. DEGs of intracellular SPA vs. LB.

769 **Table S5**. DEGs between intracellular STM and intracellular SPA.

770 **Table S6**. Bacterial strains and primers utilized in this study.

771 **Table S7**. RNA-Seq metadata and accession numbers of the 12 samples analyzed in this study.

772

773 **MOVIES**

774 **Movie 1. Live-cell imaging of STM and SPA strains reveals intracellular motility of SPA.** To
775 investigate intracellular motility, HeLa cells stably expressing LAMP1-GFP (green) or LifeAct-GFP (green)
776 as indicated were infected with SPA WT, SPA *ssaR*, SPA *fliC*, or STM ATCC 14028s WT, each harboring
777 pFPV-mCherry for constitutive expression of mCherry (red). Individual clips of about 30 seconds are
778 shown successively. Time stamps in upper left corner indicate sec.ms. Scale bars: 10 µm.

779 **Movie 2. Real time imaging of intracellular motility of SPA WT.** HeLa cells stably expressing
780 LifeAct-GFP (green) were infected with SPA WT expression mCherry (red) and imaged at a frame rate of
781 ~40 frames per second. Time stamps in upper left corner indicate sec.ms. Scale bar: 10 µm.

782 **Movie 3. Real time imaging of intracellular motility of SPA WT.** HeLa cells stably expressing
783 LifeAct-GFP (green) were infected with SPA WT expression mCherry (red) and imaged at a frame rate of
784 ~40 frames per second. Time stamps in upper left corner indicate sec.ms. Scale bar: 10 µm.

785 **Movie 4. Live-cell imaging of further intracellular STM and SPA strains.** HeLa cells stably
786 expressing LAMP1-GFP (green) were infected with STM SL1344 WT, or various isolates of SPA as indicated
787 in the lower left corner of movie sections. All strains harbored pFPV-mCherry for constitutive expression
788 of mCherry (red) Time stamps in upper left corner indicate sec.ms. Scale bars: 10 µm.

789

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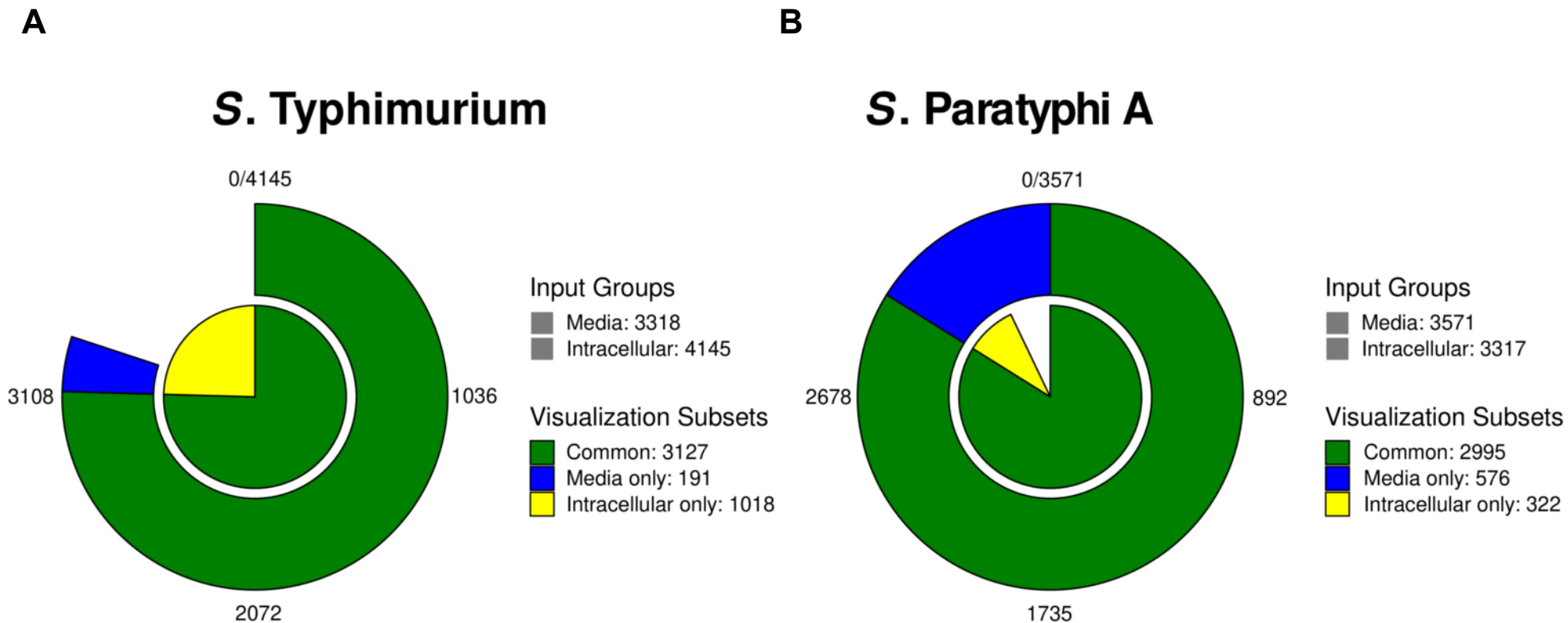

A
S. Typhimurium
0/4145
3108
1036
2072
Input Groups
Media: 3318
Intracellular: 4145
Visualization Subsets
Common: 3127
Media only: 191
Intracellular only: 1018
B
S. Paratyphi A
0/3571
2678
892
1735
Input Groups
Media: 3571
Intracellular: 3317
Visualization Subsets
Common: 2995
Media only: 576
Intracellular only: 322

Fig. 1

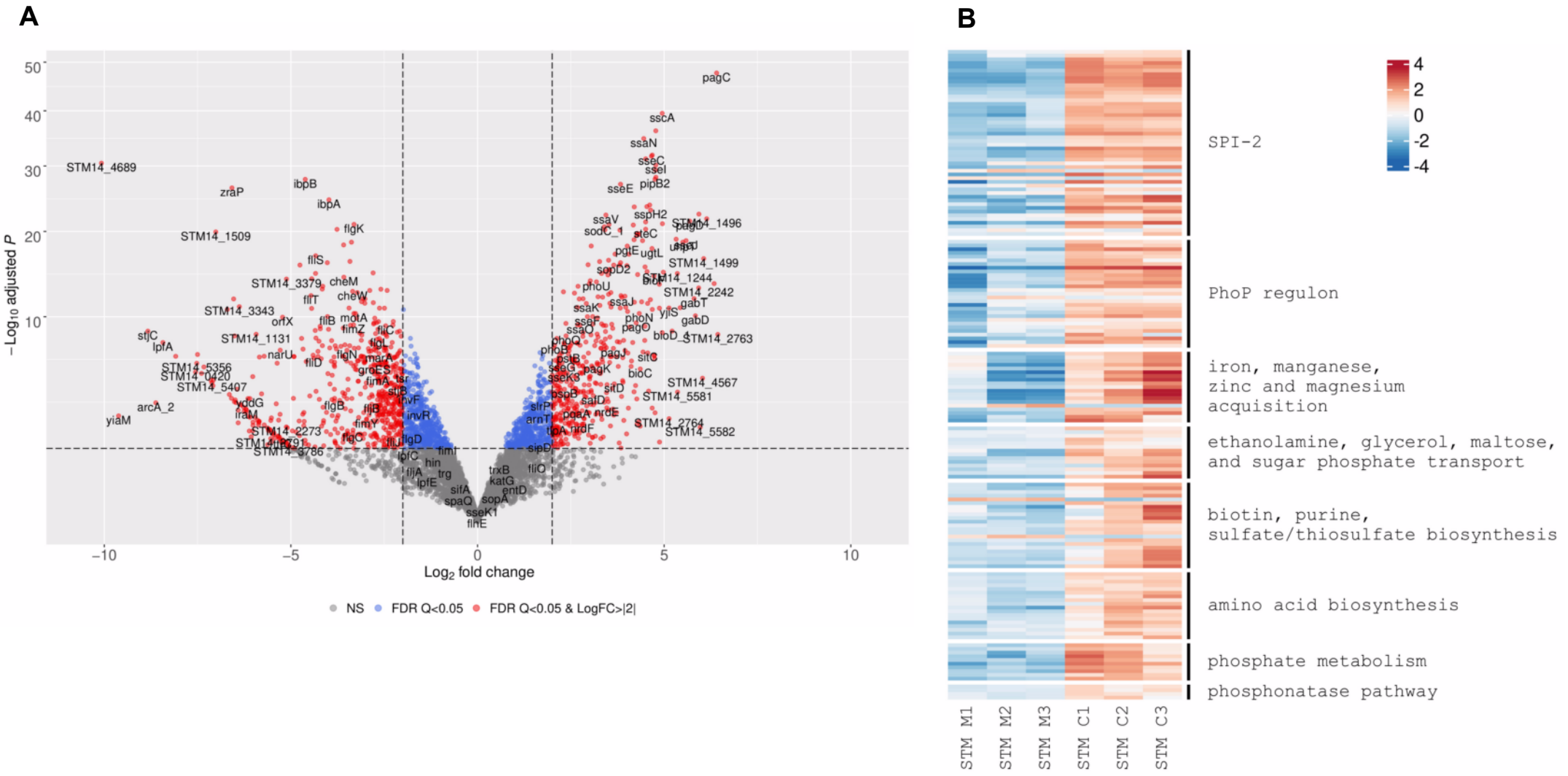

A
B
−Log10 adjusted P
Log2 fold change
NS
FDR Q<0.05
FDR Q<0.05 & LogFC>|2|
SPI-2
PhoP regulon
iron, manganese, zinc and magnesium acquisition
ethanolamine, glycerol, maltose, and sugar phosphate transport
biotin, purine, sulfate/thiosulfate biosynthesis
amino acid biosynthesis
phosphate metabolism
phosphonatase pathway
STM M1
STM M2
STM M3
STM C1
STM C2
STM C3

Fig. 2

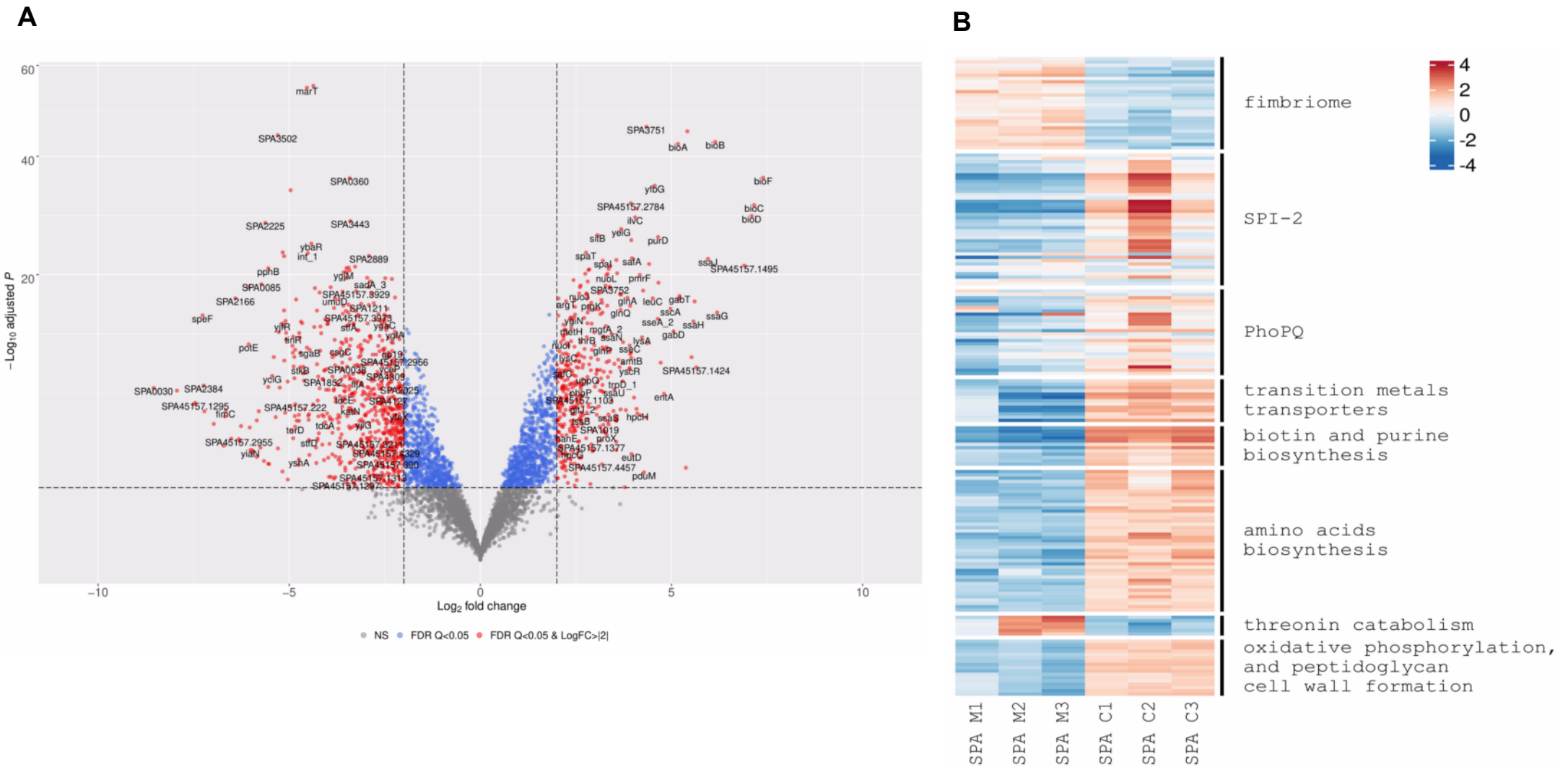

A
B
−Log10 adjusted P
Log2 fold change
NS
FDR Q<0.05
FDR Q<0.05 & LogFC>|2|
fimbriome
SPI-2
PhoPQ
transition metals transporters
biotin and purine biosynthesis
amino acids biosynthesis
threonin catabolism
oxidative phosphorylation, and peptidoglycan cell wall formation
SPA M1
SPA M2
SPA M3
SPA C1
SPA C2
SPA C3

Fig. 3

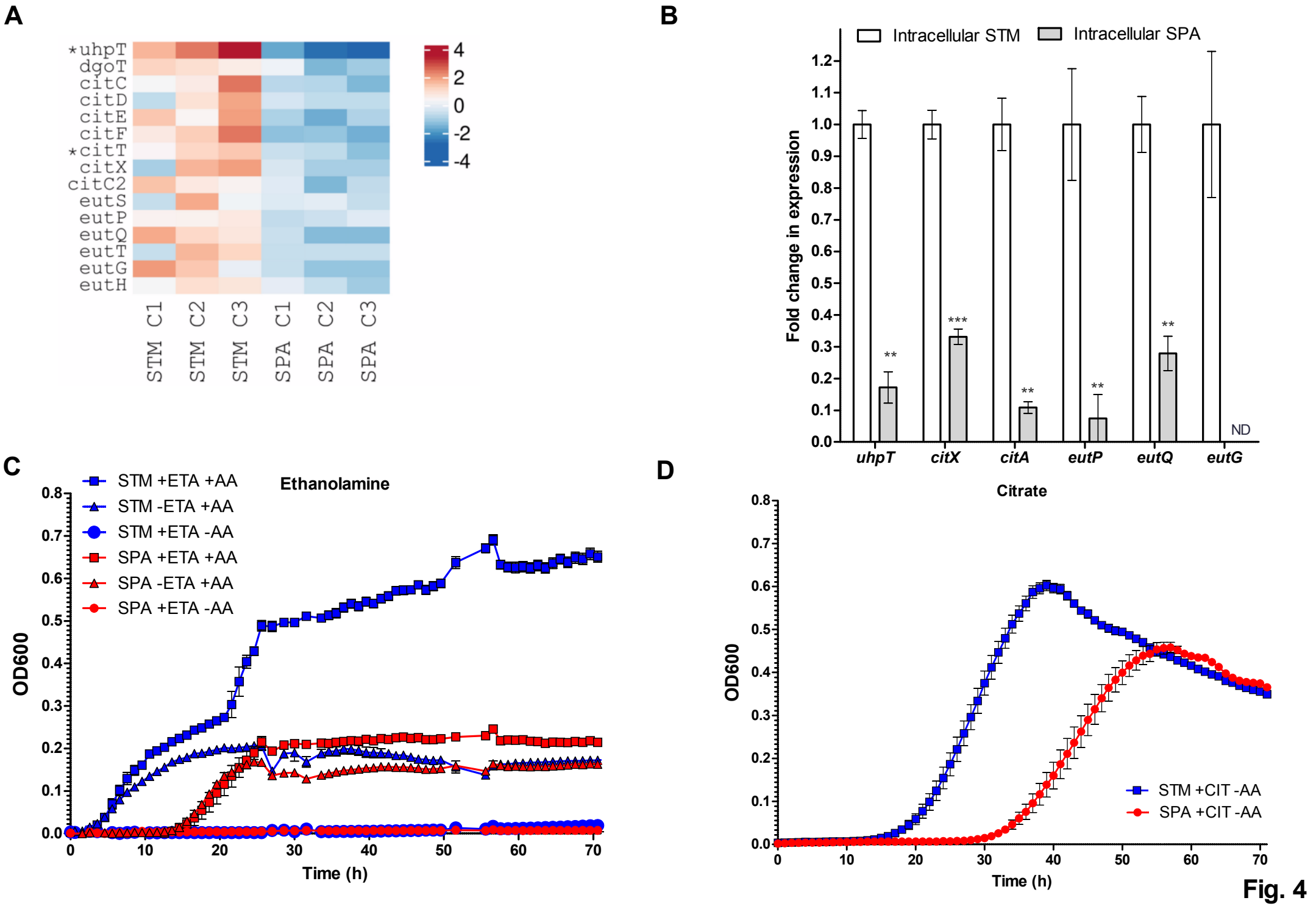
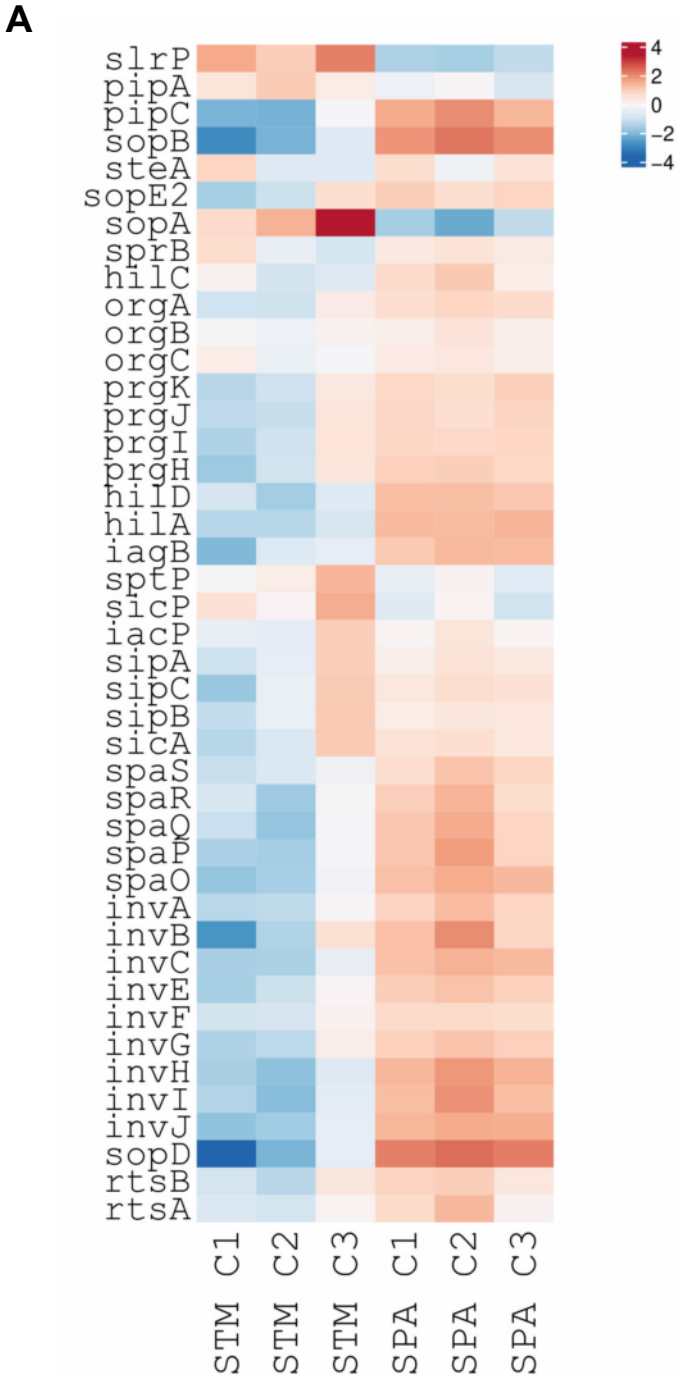

A
*uhpT
dgoT
citC
citD
citE
citF
*citT
citX
citC2
eutS
eutP
eutQ
eutT
eutG
eutH
STM C1
STM C2
STM C3
SPA C1
SPA C2
SPA C3
4
2
0
-2
-4
B
Intracellular STM
Intracellular SPA
Fold change in expression
uhpT
citX
citA
eutP
eutQ
eutG
ND
C
Ethanolamine
STM +ETA +AA
STM -ETA +AA
STM +ETA -AA
SPA +ETA +AA
SPA -ETA +AA
SPA +ETA -AA
OD600
Time (h)
D
Citrate
STM +CIT -AA
SPA +CIT -AA
OD600
Time (h)

Fig. 4


A
slrP
pipA
pipC
sopB
steA
sopE2
sopA
sprB
hilC
orgA
orgB
orgC
prgK
prgJ
prgI
prgH
hilD
hilA
iagB
sptP
sicP
iacP
sipA
sipC
sipB
sicA
spaS
spaR
spaQ
spaP
spaO
invA
invB
invC
invE
invF
invG
invH
invI
invJ
sopD
rtsB
rtsA
STM C1
STM C2
STM C3
SPA C1
SPA C2
SPA C3
4
2
0
−2
−4

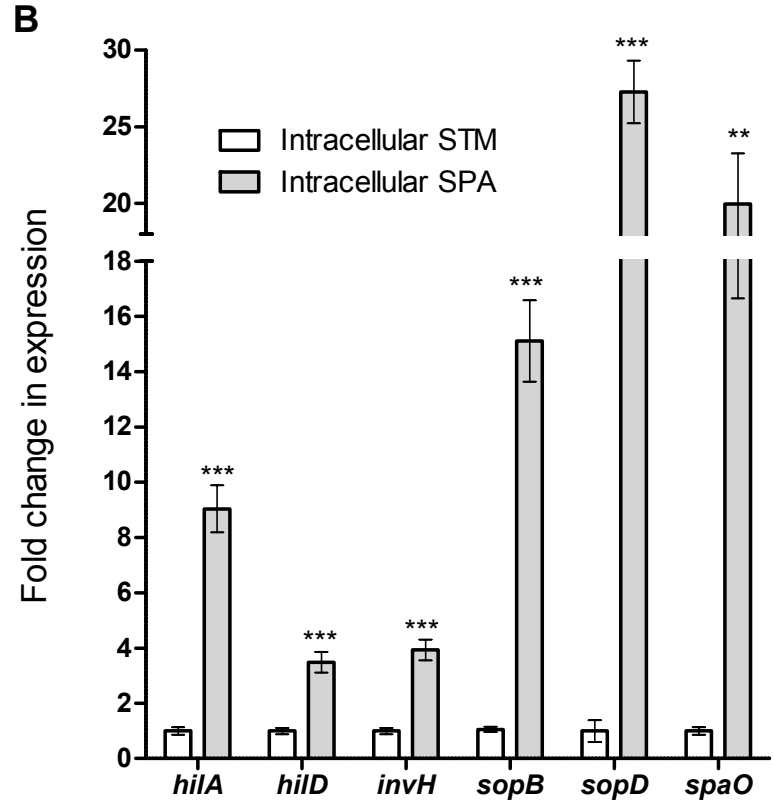
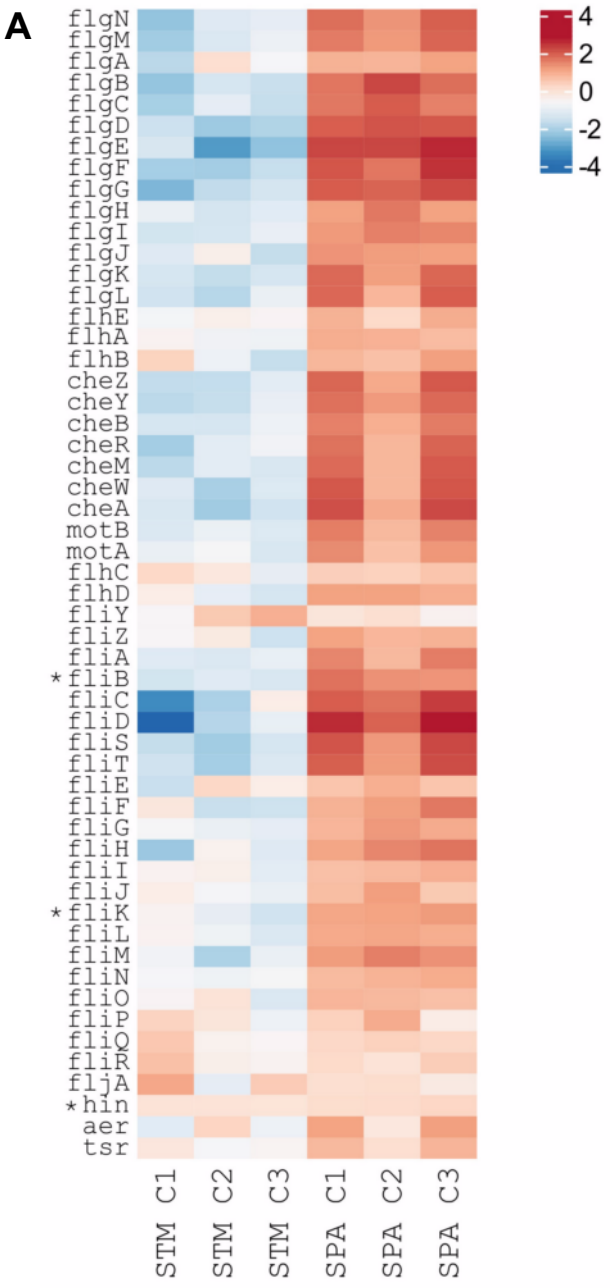
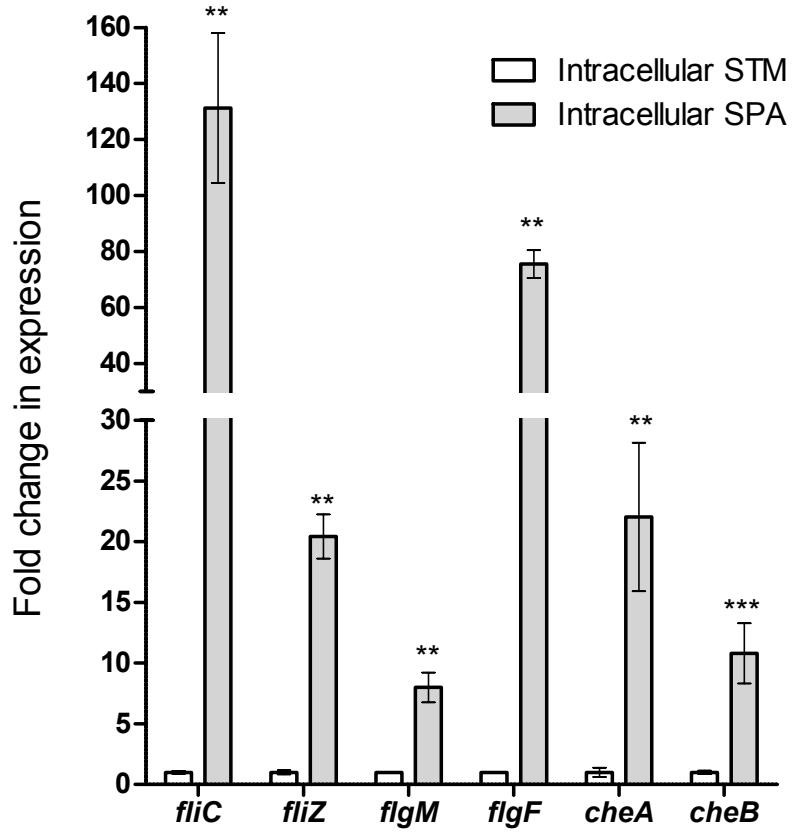

B
Fold change in expression
Intracellular STM
Intracellular SPA
hilA
hilD
invH
sopB
sopD
spaO
***
**
C
HeLa/ STM
HeLa/ SPA
SopE2-HA
DnaK
SopB-HA

Fig. 5


A
flgN
flgM
flgA
flgB
flgC
flgD
flgE
flgF
flgG
flgH
flgI
flgJ
flgK
flgL
flhE
flhA
flhB
cheZ
cheY
cheB
cheR
cheM
cheW
cheA
motB
motA
flhC
flhD
fliY
fliZ
fliA
*fliB
fliC
fliD
fliS
fliT
fliE
fliF
fliG
fliH
fliI
fliJ
*fliK
fliL
fliM
fliN
fliO
fliP
fliQ
fliR
fljA
*hin
aer
tsr
STM C1
STM C2
STM C3
SPA C1
SPA C2
SPA C3
4
2
0
-2
-4


B
Intracellular STM
Intracellular SPA
Fold change in expression
160
140
120
100
80
60
40
30
25
20
15
10
5
0
**
**
**
**
**
***
fliC
fliZ
flgM
flgF
cheA
cheB

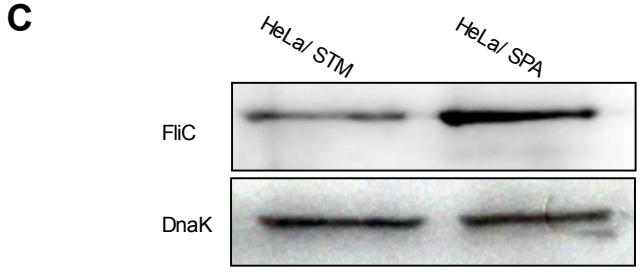
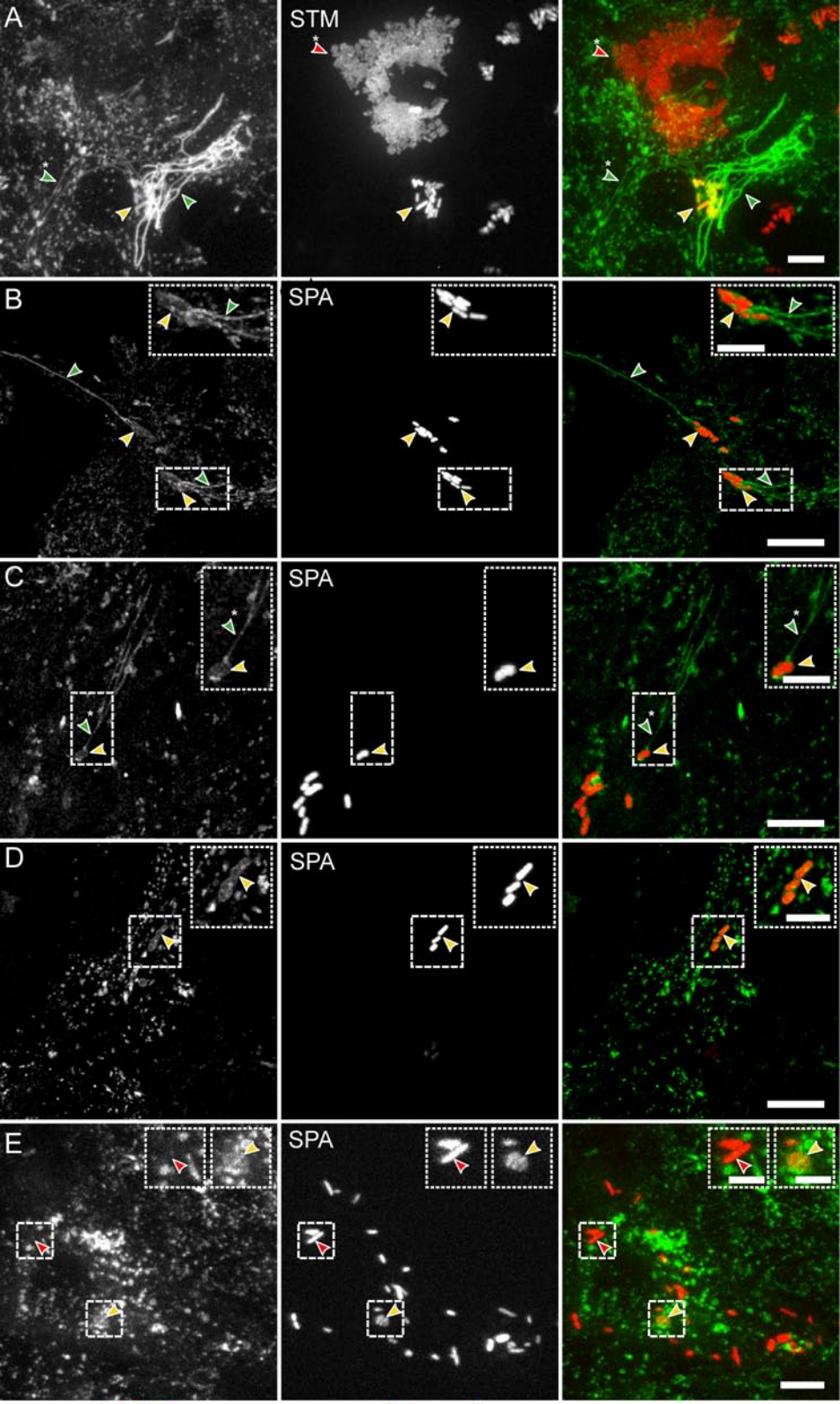
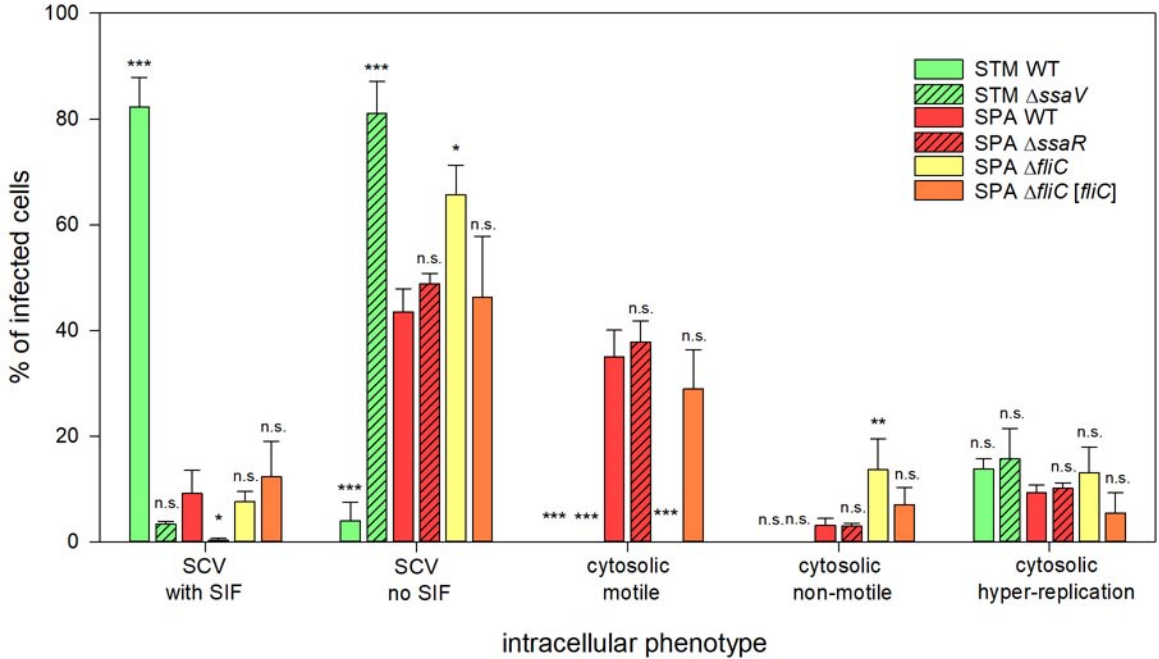
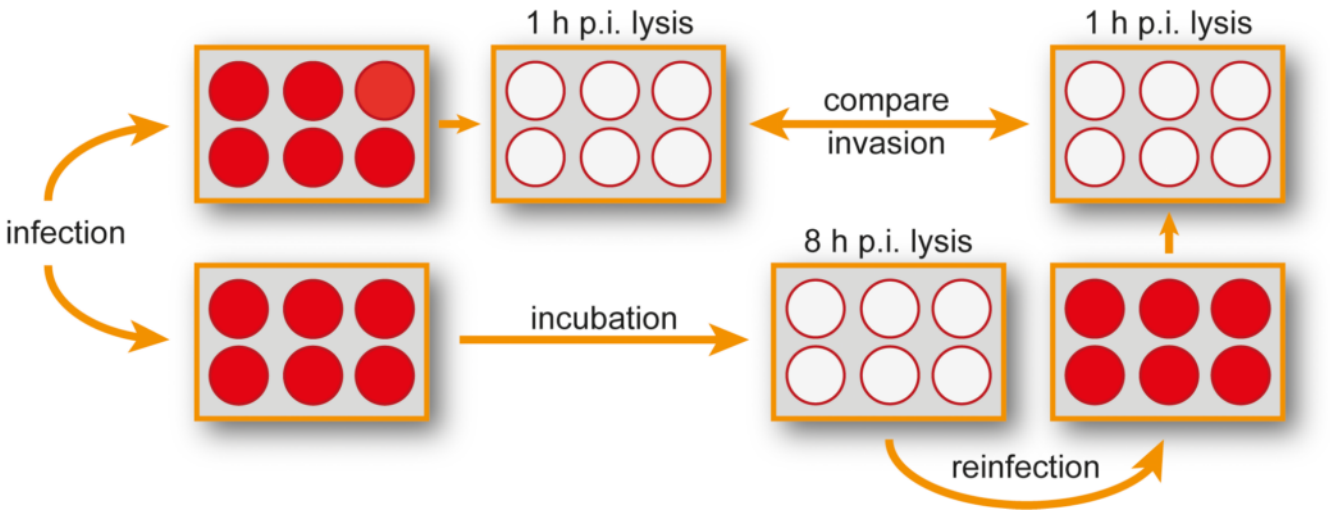

C
HeLa/ STM
HeLa/ SPA
FliC
DnaK

Fig. 6

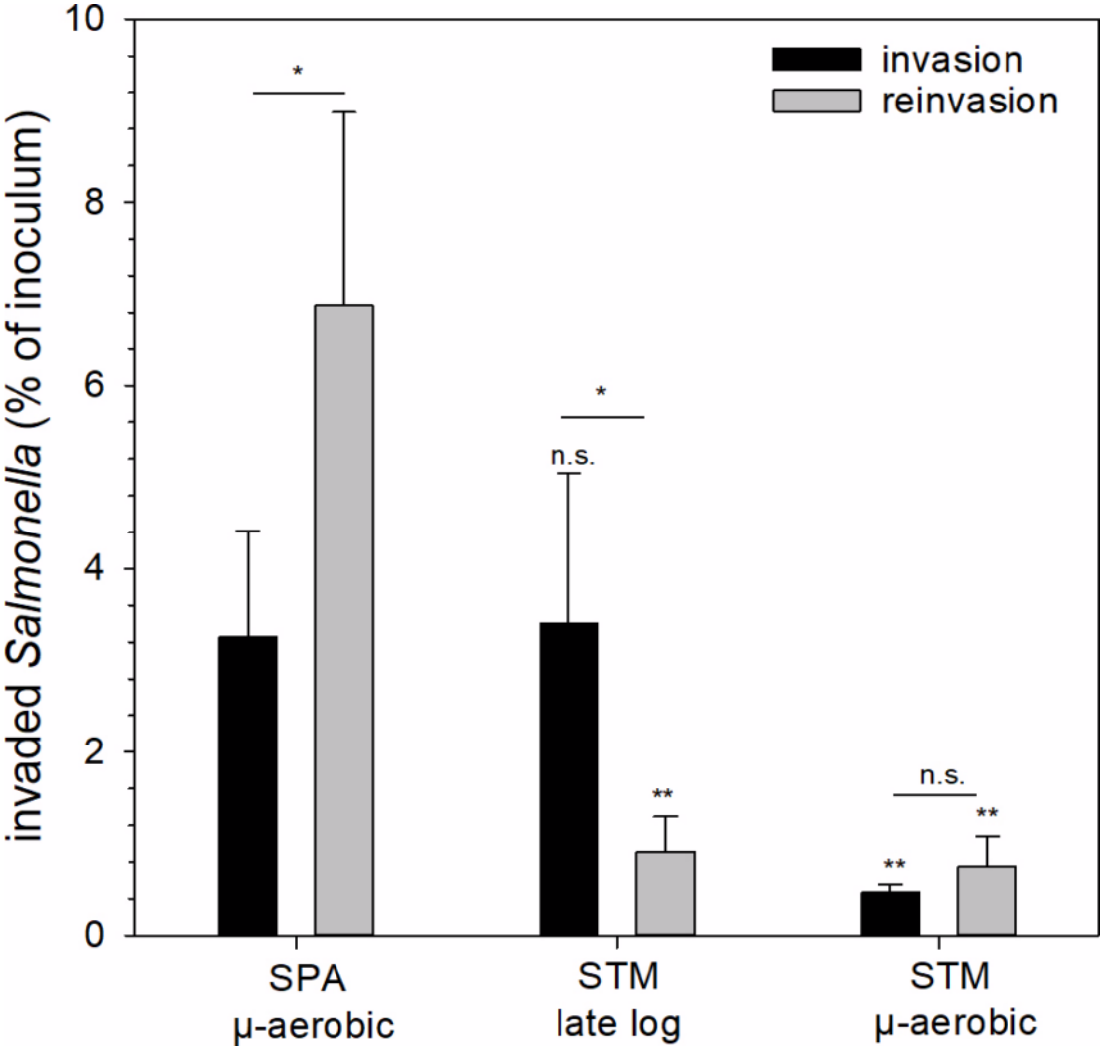

A
STM
B
SPA
C
SPA
D
SPA
E
SPA
LAMP1
Salmonella
merge


100
80
60
40
20
0
% of infected cells
STM WT
STM ΔssaV
SPA WT
SPA ΔssaR
SPA ΔfliC
SPA ΔfliC [fliC]
***
n.s.
*
n.s.
n.s.
***
n.s.
*
n.s.
***
***
n.s.
***
n.s.
n.s.
n.s.
n.s.
**
n.s.
n.s.
n.s.
n.s.
n.s.
n.s.
SCV
with SIF
SCV
no SIF
cytosolic
motile
cytosolic
non-motile
cytosolic
hyper-replication
intracellular phenotype

A


1 h p.i. lysis
1 h p.i. lysis
compare
invasion
infection
8 h p.i. lysis
incubation
reinfection

B


invasion
reinvasion
invaded Salmonella (% of inoculum)
10
8
6
4
2
0
*
*
n.s.
**
n.s.
**
**
SPA
μ-aerobic
STM
late log
STM
μ-aerobic

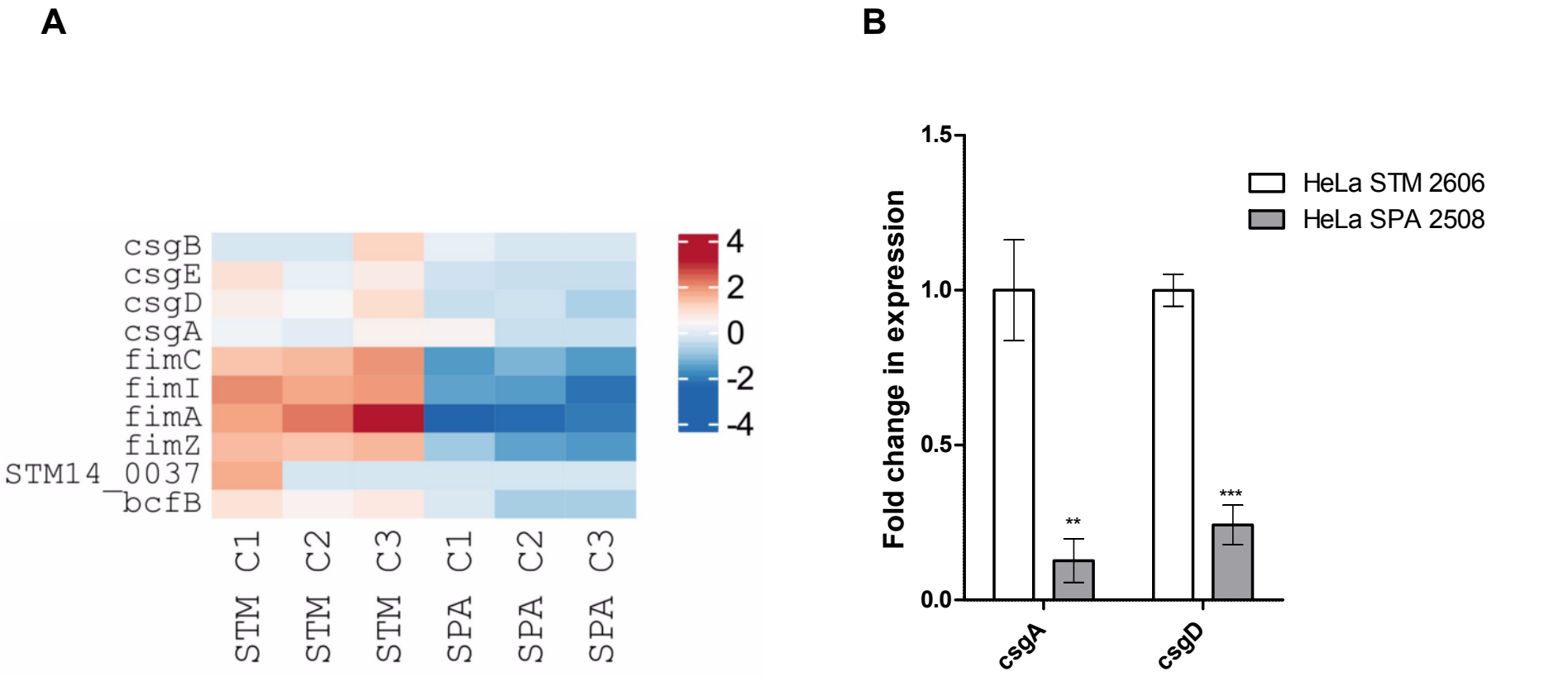

A
csgB
csgE
csgD
csgA
fimC
fimI
fimA
fimZ
STM14_0037
bcfB
STM C1
STM C2
STM C3
SPA C1
SPA C2
SPA C3
4
2
0
-2
-4
B
Fold change in expression
1.5
1.0
0.5
0.0
HeLa STM 2606
HeLa SPA 2508
**
***
csgA
csgD

Fig. S1

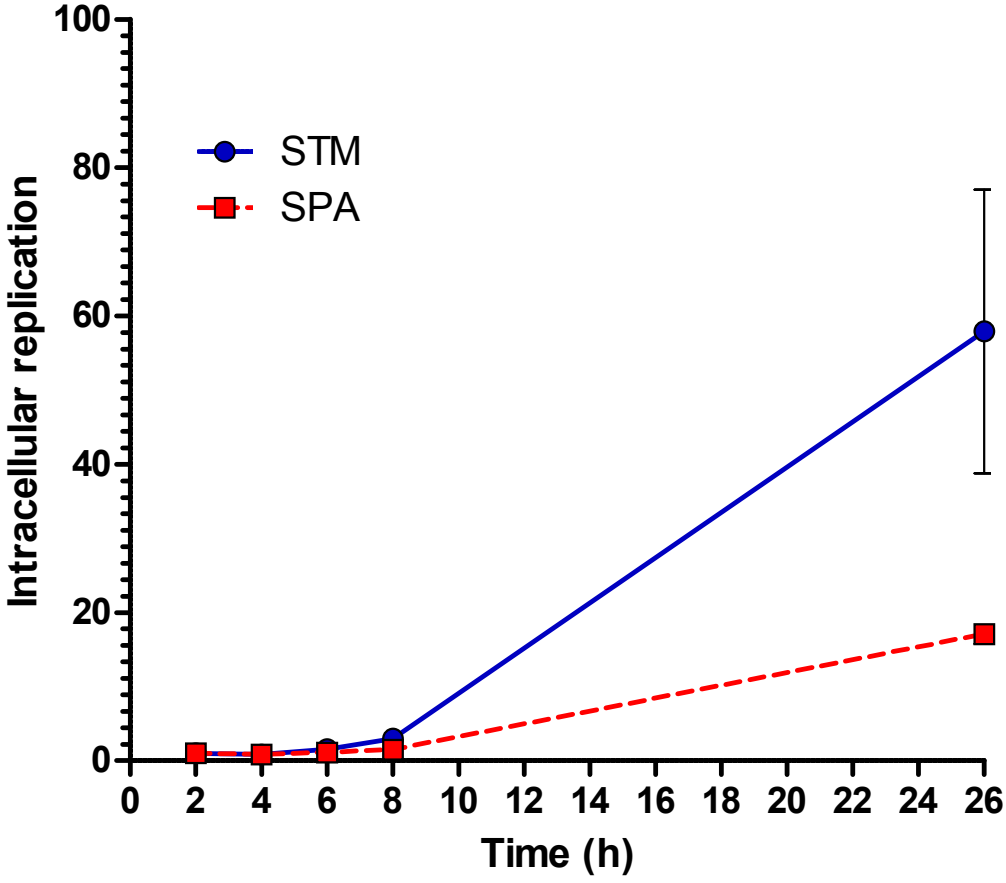
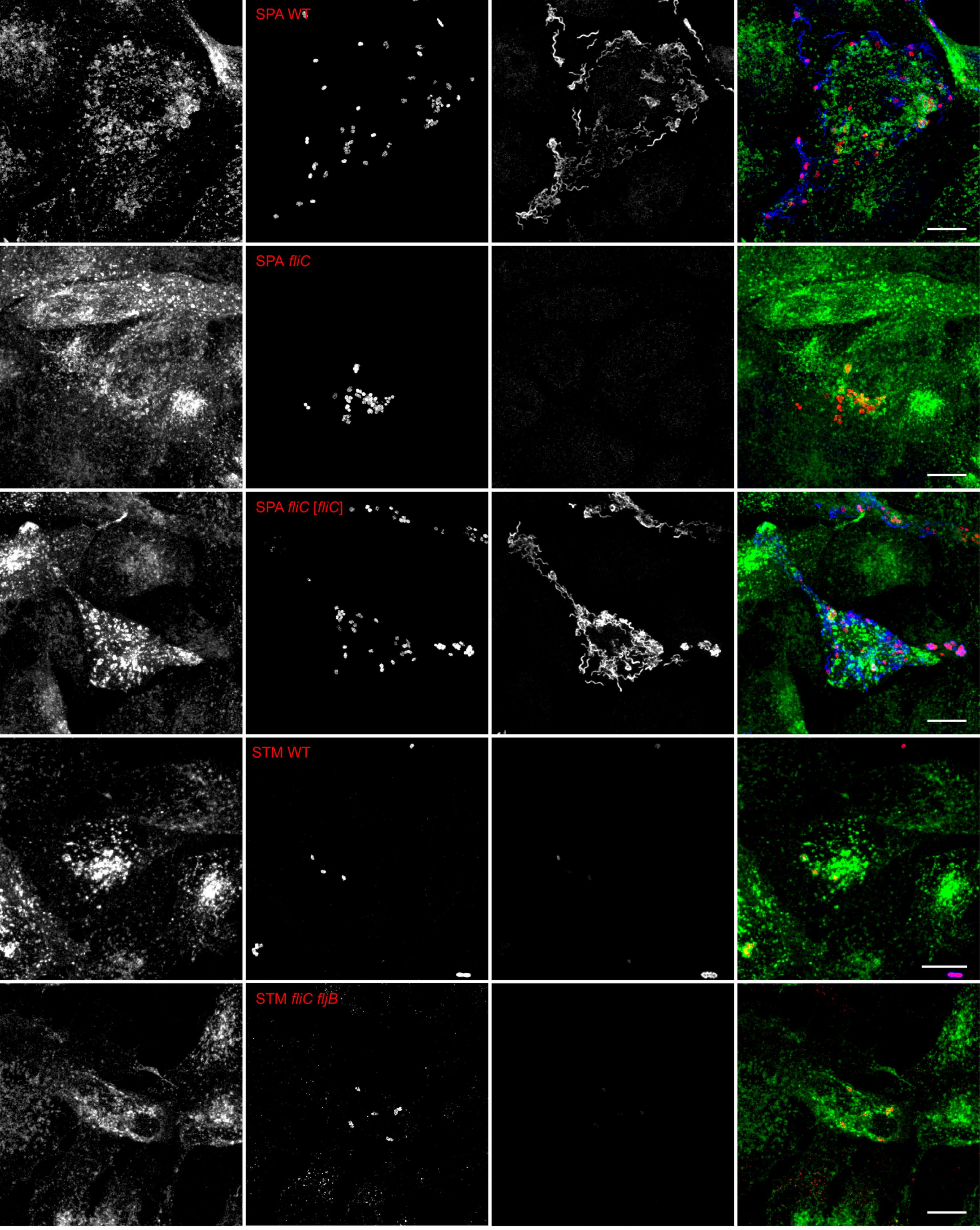

100
80
60
40
20
0
Intracellular replication
STM
SPA
0
2
4
6
8
10
12
14
16
18
20
22
24
26
Time (h)

Fig. S2


SPA WT
SPA fliC
SPA fliC [fliC]
STM WT
STM fliC fljB
LAMP1
Salmonella
flagella
merge

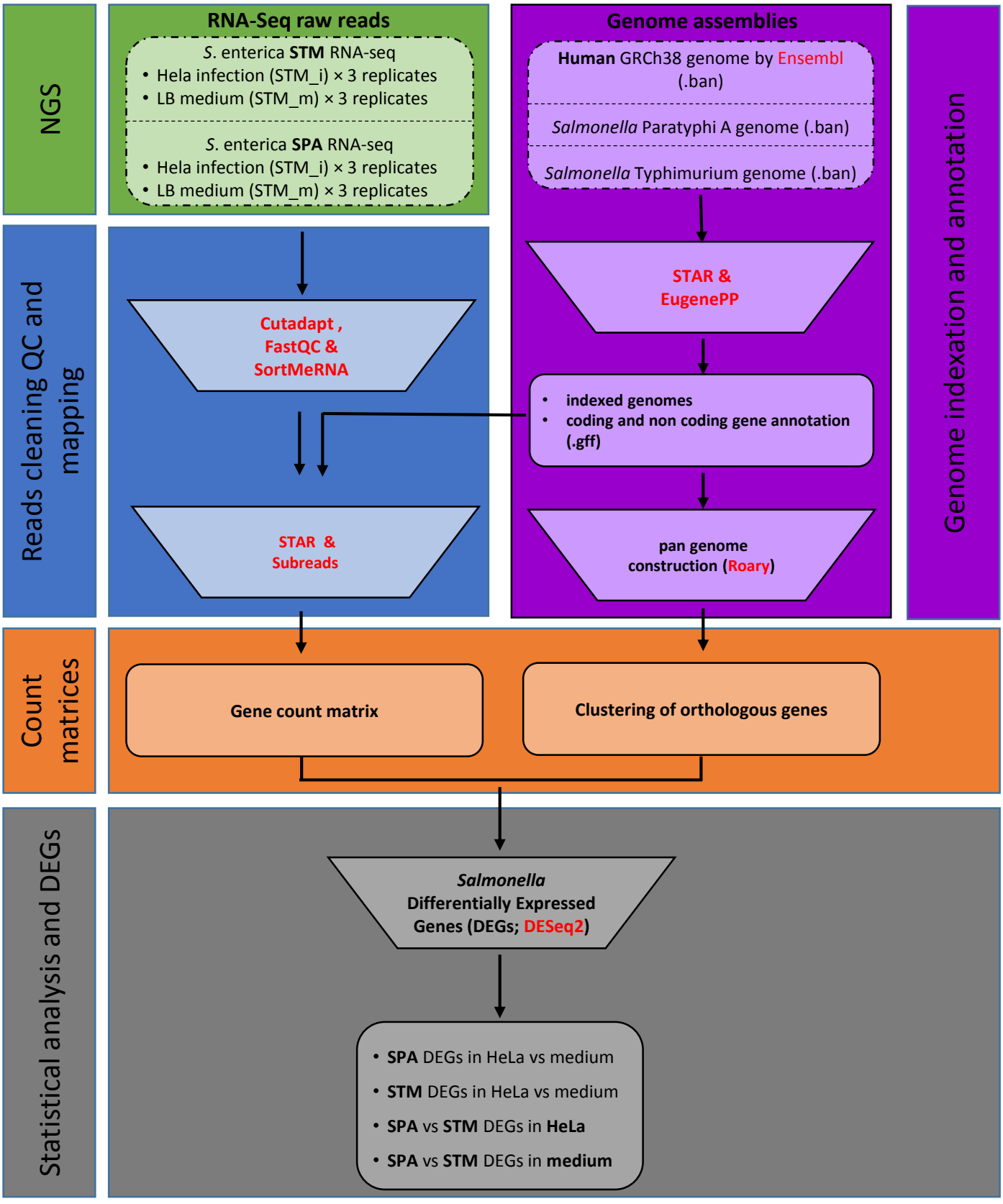

NGS
RNA-Seq raw reads
S. enterica STM RNA-seq
• Hela infection (STM_i) × 3 replicates
• LB medium (STM_m) × 3 replicates
S. enterica SPA RNA-seq
• Hela infection (STM_i) × 3 replicates
• LB medium (STM_m) × 3 replicates
Genome assemblies
Human GRCh38 genome by Ensembl (.ban)
Salmonella Paratyphi A genome (.ban)
Salmonella Typhimurium genome (.ban)
Genome indexation and annotation
Reads cleaning QC and mapping
Cutadapt , FastQC & SortMeRNA
STAR & EugenePP
• indexed genomes
• coding and non coding gene annotation (.gff)
STAR & Subreads
pan genome construction (Roary)
Count matrices
Gene count matrix
Clustering of orthologous genes
Statistical analysis and DEGs
Salmonella Differentially Expressed Genes (DEGs; DESeq2)
• SPA DEGs in HeLa vs medium
• STM DEGs in HeLa vs medium
• SPA vs STM DEGs in HeLa
• SPA vs STM DEGs in medium

Fig. S4