Atypical *Salmonella enterica* serovars in murine and human infection models: Is it time to reassess our approach to the study of salmonellosis?

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16 **Abstract**

17

18 Nontyphoidal *Salmonella* species are globally disseminated pathogens and the predominant
19 cause of gastroenteritis. The pathogenesis of salmonellosis has been extensively studied
20 using *in vivo* murine models and cell lines typically challenged with *Salmonella* Typhimurium.
21 Although serovars Enteritidis and Typhimurium are responsible for the most of human
22 infections reported to the CDC, several other serovars also contribute to clinical cases of
23 salmonellosis. Despite their epidemiological importance, little is known about their infection
24 phenotypes. Here, we report the virulence characteristics and genomes of 10 atypical *S.*
25 *enterica* serovars linked to multistate foodborne outbreaks in the United States. We show that
26 the murine RAW 264.7 macrophage model of infection is unsuitable for inferring human
27 relevant differences in nontyphoidal *Salmonella* infections whereas differentiated human THP-
28 1 macrophages allowed these isolates to be further characterised in a more relevant, human
29 context.

30 *Salmonella* is a zoonotic pathogen responsible for illnesses on a global scale and poses a
31 significant burden to public health[1]. Invasive salmonellae, such as the host-restricted
32 *Salmonella enterica* (*S. enterica*) serovars Typhi and Paratyphi cause fever in humans killing
33 nearly 217,000 people worldwide[2]. Infection with nontyphoidal *Salmonella* (NTS) such as *S.*
34 *enterica* serovars Enteritidis and Typhimurium results in bacteraemia and gastroenteritis and is
35 estimated to cause 155,000 deaths annually[3].
36
37 *Salmonella* are non-fastidious bacteria that can survive outside the host in a range of food
38 matrices, low-moisture conditions as well as food processing environments. In humans,
39 infection commonly occurs following the ingestion of contaminated food or water. Sources for
40 contamination vary and can range from the presence of the bacterium on raw produce to the
41 shedding of *Salmonella* in the faecal and urinary excretions of reservoir animals[4,5].
42
43 Upon entering the host, salmonellae are challenged by a series of adverse conditions including
44 the low pH environment of the stomach, the membrane disrupting properties of bile in the small
45 intestine and a battery of phagocytic host immune cells such as macrophages[6,7]. Deployment
46 of a Type III Secretion System (T3SS) apparatus is fundamental to the pathogenesis of
47 *Salmonella* and enables the bacterium to translocate effector proteins into the host cell
48 cytoplasm[8]. The acquisition of Salmonella Pathogenicity Island (SPI) encoded virulence factors
49 *via* horizontal gene transfer followed by evolution has enabled this microorganism to exploit a
50 privileged replicative niche, avoiding the host innate immune system within intracellular
51 vesicles called *Salmonella*-Containing Vacuole (SCV)[9,10]. The protection afforded by the SCV
52 allows *Salmonella* to thrive and sets in motion a cycle of infection whereby the bacterium can

53 proliferate and basolaterally reinfect epithelial cells as well as become engulfed by additional,
54 localised phagocytic cells.
55
56 The importance of *Salmonella* in both clinical and public health settings has fuelled research
57 into the virulence mechanisms and associated pathogenicity of this bacterium[11,12]. Many of
58 these studies use *S.* Typhimurium infection in mice as a model for typhoid fever due to the
59 similar pathology observed including intestinal and extraintestinal lesions that frequently occur
60 in human hosts infected with typhoidal serovars[13]. The *in vitro* transcriptome of *S*. Typhimurium
61 has been characterised extensively by sequencing-based methods[14,15]. Similarly, the *ex vivo*
62 response of *S*. Typhi and *S*. Typhimurium during infection of macrophages has also been
63 elucidated[16–18].
64
65 Although *S.* Typhimurium infection of mice results in symptoms mimicking human typhoid
66 fever, this serovar, along with *S.* Enteritidis is predominantly associated with gastroenteritis in
67 humans. Despite the fact that over 2,610 serovars of *Salmonella* have been reported to date,
68 few studies are available to describe the infection arising from other *S. enterica* serovars[19–22].
69
70 In this study, 10 atypical *S. enterica* serovars cultured from multistate foodborne outbreaks in
71 the U.S. were characterised both pheno- and genotypically. Differences in the nature of the
72 infection process and the underlying virulence determinants were noted. We identified factors
73 that further explain the ability of these atypical *S. enterica* serovars to cause foodborne
74 outbreaks.

75 **Results**

76

77 ***In vitro* characterisation of the isolates**

78

79 To simulate the host-specified challenges ingested salmonellae encounter along the
80 alimentary canal during infection in an *in vitro* laboratory environment, isolates (Table 1) were
81 characterised for their acid tolerance and swim/swarm motility. In addition, differences in
82 susceptibility to salts of the main acid constituents of bile were determined by Minimum
83 Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) assays.

84

85 The acid tolerance response of *Salmonella* is a complex defence mechanism employed by the
86 pathogen to defend against the acid shock experienced in the stomach[6]. Increased numbers
87 for the majority of isolates were recorded following 1 and 2 hours of growth at pH 2.5 before
88 significant reductions in viable numbers occurred at 4 hours (Supplementary Fig. S1). Motility
89 has been suggested as a virulence determinant with respect to invasion with notable non-
90 motile and/or host adapted exceptions[23–26]. After 24 hours of incubation at 21 °C, *S*. Bareilly
91 CFSAN001111 and *S*. Javiana CFSAN000905 exhibited a reduced swim phenotype when
92 compared to *S*. Typhimurium ST4/74 ($P < 0.001$) whereas *S*. Newport CFSAN003345 and *S*.
93 Saintpaul CFSAN004090 exhibited an increased swim phenotype ($P < 0.001$). At 37 °C, *S*.
94 Anatum CFSAN003959 and *S*. Tennessee CFSAN001387 exhibited a reduced swim
95 phenotype after 8 and 24 hours when compared to *S*. Typhimurium ST4/74 ($P < 0.001$)
96 (Supplementary Fig. S2). In the absence of glucose as a carbon source, no swarm motility was
97 observed for any of the isolates regardless of temperature or incubation time (Supplementary

98 Fig. S2). During digestion, contraction of the gall bladder releases bile into the small intestine.
99 *Salmonella* Weltevreden CFSAN001415 showed a two-fold difference in susceptibility to
100 sodium deoxycholate (DOC) in comparison to *S*. Typhimurium ST4/74 although the MBC was
101 comparable to the other isolates (Supplementary Table S1).
102
103 **Intracellular survival of atypical *S. enterica* serovars in murine and human macrophages**
104
105 *Salmonella* Typhimurium pathogenesis has been extensively studied *in vivo* using murine
106 models and *ex vivo* using murine cell lines (such as J774.2 and RAW 264.7). However, the
107 differences in the ability of *Salmonella* to survive and replicate within human macrophages is
108 currently not well described[27]. Differentiated human monocyte cell lines (including THP-1 and
109 U937) have been used to explore the replication of *S. enterica* serovars Enteritidis and
110 Typhimurium. Few studies describing the bacterial replication in human macrophages of
111 atypical NTS serovars have been reported despite their epidemiological importance and their
112 contributions to clinical cases of salmonellosis[27,28].
113
114 To study the ability of the isolates to survive phagocytosis, infections were performed using
115 murine RAW 264.7 and differentiated human THP-1 macrophages by the gentamicin
116 protection assay adapted from protocols previously described[29–32]. *Salmonella* Typhimurium
117 14028S and ST4/74 were included as reference strains in all infection assays. Infections were
118 carried out at a Multiplicity Of Infection (MOI) of 10:1. Viable internalised bacteria were
119 enumerated at 2, 4, 8 and 24 Hours Post Infection (HPI) in RAW 264.7 and 2, 4, 8, 24 and 168
120 HPI in THP-1 macrophages.

121 Of the 10 atypical serovars tested in this study, all isolates were found to persist within RAW
122 264.7 macrophages for 24 HPI with many of these increasing in number over the course of the
123 infection. In the case of *S*. Weltevreden CFSAN001415, a 1-Log_{10} decrease in intracellular
124 bacteria between 2 and 24 HPI was recorded though this isolate was still recoverable. In
125 contrast, *S*. Tennessee CFSAN001387 exhibited a 1-Log_{10} increase in intracellular bacteria
126 between 2 and 24 HPI. Similar numbers of viable bacteria were recorded for *S*. Typhimurium
127 14028S and ST4/74 (Fig. 1a).
128
129 The highest and lowest mean Colony Forming Units (CFU)/mL for all 10 atypical serovars are
130 shown (Supplementary Table S2). When the Log_2 fold change was calculated, *S*. Weltevreden
131 CFSAN001415 recorded the largest decrease at 8 and 24 HPI (Fig. 1b). Of note, *S*. Bareilly
132 CFSAN001111 showed an unusual infection profile in that it had the highest mean CFU/mL at
133 2 HPI, possibly indicative of increased invasiveness, despite normalisation of the infections by
134 centrifuging the cell culture plates. The latter observation was maintained for all subsequent
135 time points. However, the mean CFU/mL at 4, 8 and 24 HPI for *S*. Bareilly CFSAN001111
136 were lower than other isolates.
137
138 When compared to *S*. Typhimurium ST4/74, significant differences were observed in the
139 infection profile of specific isolates at individual time points (Supplementary Table S3). Time
140 courses longer than 24 hours were not possible in RAW 264.7 as this resulted in cell death or
141 proliferation of the macrophages themselves which would skew the MOI. Although differences
142 between these isolates were observed in RAW 264.7 macrophages, the inability to consistently
143 extend the time course of the assay beyond 24 HPI limited the utility of this *ex vivo* murine

144 model to discern any further differences in the ability of the isolates to survive and persist
145 following phagocytosis. To mitigate this limitation and differentiate the isolates in a human
146 relevant context, additional assays were carried out in differentiated THP-1 macrophages.
147
148 Of the 10 atypical serovars tested in this study, all persisted within THP-1 macrophages for 24
149 HPI with the majority exhibiting no significant changes in viable numbers up to this time point.
150 As with RAW 264.7 macrophages, the only exception noted when infecting THP-1
151 macrophages was recorded for *S*. Weltevreden CFSAN001415 which exhibited a 1-Log_{10}
152 decrease in mean CFU/mL between 2 and 24 HPI but remained recoverable. Upon extending
153 this assay beyond 24 hours to 168 HPI, equivalent to 7 days, all serovars with the exception of
154 *S*. Anatum CFSAN003959 and both *S*. Typhimurium 14028S and ST4/74 reference strains
155 were recoverable with the majority exhibiting a 2-Log_{10} decrease in intracellular bacteria.
156 Exceptions to this were noted for *S*. Cubana CFSAN002050, *S.* Heidelberg CFSAN002063
157 and *S*. Tennessee CFSAN001387 which showed a 1-Log_{10} decrease in intracellular bacteria
158 between 2 and 168 HPI with *S*. Tennessee CFSAN001387 displaying the smallest decrease of
159 all isolates. Conversely, *S*. Weltevreden CFSAN001415 demonstrated the largest decrease in
160 bacterial cell numbers at 168 HPI but unlike *S*. Anatum CFSAN003959 and both *S*.
161 Typhimurium 14028S and ST4/74 reference strains, it could still be recovered at the end of the
162 assay (Fig. 1a).
163
164 The highest and lowest mean CFU/mL among the 10 study isolates are shown
165 (Supplementary Table S2). In THP-1 macrophages, *S*. Weltevreden CFSAN001415 had the
166 lowest mean CFU/mL at 24 and 168 HPI of the recoverable isolates as observed in the Log_2

167 fold change (Fig. 1b). As observed in RAW 264.7 macrophages, *S*. Tennessee CFSAN001387
168 had the highest mean CFU/mL at 24 HPI as well as 168 HPI in THP-1 macrophages. When
169 compared to *S*. Typhimurium ST4/74, significant differences were observed in the infection
170 profiles of specific isolates at individual time points (Supplementary Table S2).
171
172 Fewer significant differences were observed between isolates when compared to S.
173 Typhimurium ST4/74 infection in human THP-1 macrophages versus murine RAW 264.7
174 macrophages highlighting the potential unsuitability of the murine model for inferring human
175 relevant differences between isolates in NTS infection. Overall, the viable intracellular bacteria
176 recorded at later time points, including 24 HPI, was significantly higher in RAW 264.7 than
177 THP-1. Bacterial cell numbers reached as high as 1 x 10^7 mean CFU/mL in RAW 264.7 for
178 some atypical serovars compared with values that did not exceed 1 x 10^5 mean CFU/mL in
179 THP-1 for all isolates. This 2-Log_{10} difference supports our observations showing the inability
180 of RAW 264.7 to clear infecting bacteria compared with THP-1 macrophages.
181
182 The viability of both murine and human macrophages following infection with each of the
183 selected bacterial isolates was measured using colorimetric assays to measure extracellular
184 Glucose 6-Phosphate (G6P) and Lactate DeHydrogenase (LDH) activities compared with
185 uninfected control macrophages. No significant differences were observed in host cell viability
186 following a MOI of 10:1 (Supplementary Fig. S4 and Supplementary Fig. S5). This is in
187 agreement with recent studies that used flow cytometry based techniques to quantify apoptosis
188 in macrophages infected with different *Salmonella* strains[27,33].
189

190 As *S*. Tennessee CFSAN001387 was noted to be the most proliferative isolate in THP-1
191 macrophages, exhibiting the lowest reduction in viable intracellular bacteria between 2 and 168
192 HPI, further assays were performed to directly compare to *S.* Typhimurium ST4/74 with CFU
193 being enumerated at 2, 4, 8, 24, 48, 72, 96, 120, 144 and 168 HPI. This was done to
194 determine when *S.* Typhimurium ST4/74 was no longer recoverable compared with *S*.
195 Tennessee CFSAN001387. *Salmonella* Typhimurium ST4/74 exhibited an overall 1-Log_{10}
196 reduction between 2 and 48 HPI with an additional 2-Log_{10} reduction in viable bacteria by 72
197 hours. After 96 and 120 HPI, *S.* Typhimurium ST4/74 was barely detected and was
198 unrecoverable at 144 and 168 HPI. In comparison, *S*. Tennessee CFSAN001387 exhibited an
199 overall 1-Log_{10} reduction between 2 and 168 HPI, similar to the previous infections (Fig. 1c).
200 Differences between these two isolates were significant at multiple individual time points
201 (Supplementary Table S4).
202
203 **Host response to atypical *S. enterica* infection by murine and human macrophages**
204
205 As there were notable differences exhibited by these *Salmonella* serovars in their ability to
206 replicate and survive within murine and human macrophages, the host response to infection
207 was investigated by quantifying proinflammatory cytokine and infection relevant chemokine
208 release using standard immunoassay protocols.
209
210 In RAW 264.7, increased CCL2 and CCL3 chemokine release was observed at 4, 8 and 24
211 HPI with *S*. Tennessee CFSAN001387 and *S*. Weltevreden CFSAN001415 when compared to
212 uninfected control macrophages. When comparing infection with these 10 atypical serovars

213 (Table 1) to *S.* Typhimurium ST4/74, increased proinflammatory cytokine (including IL6, IL10
214 and TNF) and chemokine (including CCL2, CCL3 and CXCL10) release was observed (Fig. 2)
215 (Supplementary Table S5).
216
217 In THP-1, increased proinflammatory cytokine (including CXCL8, IL1B, IL6 and TNF), cytokine
218 (including CSF2, IL1A, IL12B and VEGFA) and chemokine (including CCL2, CCL3, CCL4 and
219 CXCL10) release was recorded at 8, 24 and 168 HPI across a selection of these isolates in
220 comparison with uninfected control macrophages. As with RAW 264.7, when comparing
221 infection with the 10 atypical serovars in this study to *S*. Typhimurium ST4/74 in THP-1,
222 significant increased proinflammatory cytokine and chemokine release was observed, further
223 differentiating the isolates by the innate host response (Fig. 3) (Supplementary Table S5).
224
225 *Salmonella* Heidelberg CFSAN002063 stimulated the release of CCL3, CSF2, CXCL8,
226 CXCL10, IL1A, IL1B, IL6, IL12 and TNF to levels in excess of those observed for *S*.
227 Typhimurium ST4/74, particularly at 8 HPI and from 4 to 168 HPI with respect to TNF
228 (Supplementary Fig. S7). In the gentamicin protection assays it was noted that although both
229 *S*. Tennessee CFSAN001387 and *S*. Weltevreden CFSAN001415 could be recovered at 168
230 HPI from THP-1 macrophages, these two isolates exhibited different infection profiles. The
231 former displayed the smallest reduction in viable intracellular bacteria over the time course of
232 the infection whereas the latter displayed the largest reduction in viable numbers. This can be
233 accounted for in the overlapping yet contrasting cytokine profile of THP-1 macrophages
234 following infection with these isolates. *Salmonella* Tennessee CFSAN001387 and *S*.
235 Weltevreden CFSAN001415 stimulated the release of CCL3, CCL4, IL1A, IL6, IL12B and TNF

236 to levels in excess of those observed for *S*. Typhimurium ST4/74 (Fig. 4a, Fig. 4b and Fig. 4c).
237 In addition, *S*. Weltevreden CFSAN001415 stimulated the release of CCL2, CXCL10, CSF2
238 and IL1B triggering a broader response in comparison to that observed for *S*. Tennessee
239 CFSAN001387 (Fig. 4a, Fig. 4b and Fig. 4c).
240
241 Overall, the human macrophages mounted a much greater proinflammatory response to
242 infection in comparison to the murine model (Fig. 4c). A homolog of the human *CXCL8* gene is
243 absent in mice. However, the murine *Cxcl1* gene codes for a functionally homologous
244 protein[34]. The latter was not released from infected RAW 264.7 to the levels observed for
245 CXCL8 in THP-1 in this study. As all infections were carried out in pure macrophage cultures,
246 the true effect of the observed chemokine release cannot be fully appreciated in this
247 experimental model as the activation and/or recruitment of other phagocytic cells in a coculture
248 population or an *in vivo* environment would have an impact on bacterial survival.
249
250 **Distribution and similarity of SPI proteins from atypical *S. enterica* serovars in**
251 **comparison to *S*. Typhimurium ST4/74**
252
253 Whole genome sequencing was used to facilitate a comparative analysis of all 10 atypical
254 serovars to elucidate the genetic variation among them with particular focus on virulence
255 determinants and SPI loci gene content[35,36]. Sequencing was performed using the Illumina
256 MiSeq and Pacific Biosciences RS II sequencing platforms. *De novo* assemblies were
257 performed on these data and the N50 length for Illumina sequenced assemblies ranged from
258 403 to 763 kbp with an average N50 length of 586 kbp (Supplementary Table S6). These

259 genome sequences were used to determine the relationships between the isolates to identify
260 key differences that may explain the phenotypes observed in the previous experiments.
261 Genetic diversity was characterised by amino acid sequence similarity that identified highly
262 conserved SPI regions between these isolates despite the broad range of serovars in addition
263 to distinct variable regions and/or absence of key effector proteins in specific isolates (Fig. 5
264 and Supplementary Fig. S8).
265
266 SPI-1 and its associated T3SS have been extensively implicated in *Salmonella* virulence and
267 the ability of this pathogen to invade host eukaryotic cells, trigger inflammation and transport
268 effector proteins[37,38]. Sequence variation at the amino acid level in comparison to *S*.
269 Typhimurium ST4/74 was greatest in AvrA, OrgB, SptP, SipD, InvB and SL2883 proteins.
270 Several SPI-1 encoded genes were absent in many of the atypical serovars but present in *S*.
271 Typhimurium ST4/74 though not yet fully characterised (Fig. 5).
272
273 The AvrA protein has been previously shown to be crucial, playing a role in the inhibition of the
274 antiapoptotic NF-κB pathway[39]. In *S*. Typhimurium 14028S, *S*. Anatum CFSAN003959, *S*.
275 Javiana CFSAN000905 and *S*. Newport CFSAN003345, AvrA showed differences at the
276 amino acid sequence level which may affect its ability to function as a protease[40]. SptP is a
277 tyrosine phosphatase involved in the inhibition Raf activation and the subsequent MAP kinase
278 pathway[41]. Its identification here is consistent with the phenotype observed for *S*. Heidelberg
279 CFSAN002063 that stimulated high levels of TNF release at 4, 8, 24 and 168 HPI in THP-1
280 macrophages. A number of proteins, including STM2901, STM2902, STM2903, SL2883,
281 STM2904 and STM2905 that are SPI-1 associated in *S*. Typhimurium ST4/74 showed mixed

282 conservation, either being highly similar in the majority of isolates or were unidentifiable in *S*.
283 Anatum CFSAN003959, *S*. Javiana CFSAN000905 and *S*. Newport CFSAN003345.
284
285 SPI-2 and its associated T3SS contributes to the ability of *Salmonella* to translocate effectors
286 across the membrane of the SCV when the bacterium is internalised in epithelial cells and
287 macrophages[42,43]. The integral function of SPI-2 for intracellular survival can be observed by
288 the degree of amino acid sequence conservation across all atypical serovars (Fig. 5). As these
289 isolates were implicated in multistate foodborne outbreaks and, as demonstrated above,
290 capable of surviving within both RAW 264.7 and THP-1 macrophages, this observation is
291 consistent with the expressed phenotype and raises the question as to whether or not potential
292 differences in the expression of some/all of these genes may further explain the differences
293 shown in intracellular survival. Sequence variation at the amino acid level in comparison to *S*.
294 Typhimurium ST4/74 was greatest in SsaB, SseB, SseC and SseD. Loss of the effector protein
295 SsaB (SpiC) has been shown to promote defective virulence phenotypes due to an inability to
296 translocate all SPI-2 effectors[44]. This may explain the observed infection phenotype in *S*.
297 Anatum CFSAN003959, the only isolate among the 10 atypical serovars studied to be
298 unrecoverable at 168 HPI in THP-1 macrophages, as it had the lowest amino acid sequence
299 similarity for SsaB.
300
301 SseBCD proteins function as a translocon that facilitate the secretion of effector proteins by
302 intracellular *Salmonella*[45]. Both *S*. Cubana CFSAN002050 and *S*. Tennessee CFSAN001387
303 display differences in all three SseBCD proteins with *S*. Tennessee CFSAN001387 being least
304 similar at the amino acid sequence level when compared to *S*. Typhimurium ST4/74.

305 *Salmonella* Tennessee CFSAN001387 was shown in this study to survive within THP-1
306 macrophages significantly better in comparison to *S*. Typhimurium ST4/74.
307
308 High levels of similarity to *S*. Typhimurium ST4/74 were observed for SPI-3 across the isolates
309 with the exception of the complete loss or major differences in STM3752, SugR and RhuM as
310 reported previously[35]. SPI-4 and its associated Type I Secretion System (T1SS) have been
311 implicated in adhesion, contributing to intestinal inflammation in animal models[46,47]. *Salmonella*
312 Newport CFSAN003345 and *S*. Weltevreden CFSAN001415 exhibited low levels of amino acid
313 sequence similarity to *S*. Typhimurium ST4/74 with respect to SiiE which has been shown to
314 be important for persistent infection in macrophages[48]. SPI-5 encodes many SPI-1 and SPI-2
315 T3SS targeted effector proteins. Sequence variation was observed for CopR, PipA, STM1089,
316 STM1093 in multiple isolates with mutations in PipA having previously been implicated in
317 enteric salmonellosis[49].

318 **Discussion**

319

320 Studies aimed at elucidating the host response to NTS serovars including Enteritidis and
321 Typhimurium have been facilitated by the availability of murine infection models. This study
322 focussed on atypical serovars of this genus availing of isolates cultured from foodborne
323 outbreaks, the majority of which are frequently listed in the top 20 serovars responsible for
324 laboratory-confirmed human cases of salmonellosis as reported annually by the CDC[50]. These
325 data identify key differences between isolates related to their ability to survive within
326 macrophages and highlight the potential unsuitability of the widely used murine macrophage
327 model to infer human relevant distinctions between isolates. We have shown that for NTS
328 serovars, distinct differences in the inflammatory response of human macrophages can further
329 differentiate these microorganisms in a manner that was not possible with murine
330 macrophages (Fig. 3). Furthermore, the reference strains *S.* Typhimurium 14028S and ST4/74
331 that are often included in *in vitro* research emerged as the biological outliers in many respects
332 with regards to their infection phenotype (Fig. 1). In the case of specific proinflammatory
333 cytokines such as TNF, RAW 264.7 macrophages responded to infection with *S*. Typhimurium
334 14028S and ST4/74, *S*. Tennessee CFSAN001387 and *S*. Weltevreden CFSAN001415
335 whereas THP-1 macrophages displayed a broader proinflammatory response to the range of
336 serovars studied (Fig. 4).

337

338 These data suggest more differences than previously acknowledged for *S. enterica* serovars
339 with implications for public health. In agreement with this notion, key differences were identified
340 in established virulence determinants of *Salmonella* such as SPI gene content bridging the gap

341 between the observed phenotypes and the underlying genotypes. Further work will be required
342 to understand the full scope of other potential targets within the genomes of these isolates
343 such as the accessory gene content unique to individual strains, many of which are currently
344 poorly characterised.

345 **Methods**

346

347 **Bacterial isolates and culture methods**

348

349 Environmental and food isolates were collected from different countries between 2005 and
350 2012 by the U.S. Food and Drug Administration inspections as part of compliance actions[51].
351 The clinical isolate was obtained from the Washington State Department of Health (Table 1).
352 All *Salmonella* isolates were stored at -80 °C in Lysogeny Broth (LB) broth (Sigma-Aldrich)
353 supplemented with 15 % [v/v] glycerol. Working cultures were prepared by streaking isolates
354 and restreaking individual colonies onto Mueller-Hinton (MH) agar (Sigma-Aldrich).

355

356 Individual colonies from restreaked isolates were used to inoculate 5 mL MH broth (Sigma-
357 Aldrich) and grown overnight at 37 °C with orbital shaking at 200 RPM. Overnight cultures
358 were then used in the subsequent experiments as detailed below.

359

360 **Acid resistance**

361

362 The ability to survive in a low pH culture medium was assessed for all isolates as described
363 previously[52]. Briefly, bacterial cultures were individually grown overnight (18 hours) without
364 shaking in 5 mL buffered MH broth (MES hydrate, 2 % [w/v], pH 5) containing 0.4 % [w/v]
365 glucose at 37 °C. A volume of 333 µL of overnight culture was centrifuged at 10,000 RPM for
366 10 minutes. The supernatant was removed and the cell pellet recovered and resuspended in 2
367 mL prewarmed, buffered MH broth (MES hydrate, 2 % [w/v], pH 2.5) containing 0.4 % [w/v]

368 glucose and incubated at 37 °C in a 24 well plate. Time points were taken at 0 hours (before
369 acidification), 1, 2 and 4 hours post-acidification. At each time point, 10 µL of the culture was
370 diluted in 1,990 µL of maximum recovery diluent (MRD) medium (Oxoid) in a fresh 24 well
371 plate and incubated for 30 minutes at room temperature to allow the bacteria to recover.
372 Samples were then decimally diluted in phosphate buffered saline (PBS) (Sigma-Aldrich) and
373 100 µL aliquots of these dilutions were plated directly onto LB agar. Agar plates were
374 incubated for 18 hours at 37 °C before enumeration of the CFU.
375
376 **Motility assays**
377
378 Swim and swarm motility was assessed for all isolates as described previously[52]. Briefly, swim
379 motility plates (MH broth containing 0.3 % [w/v] agar) were stab inoculated. Swarm motility
380 plates (MH broth containing 0.6 % [w/v] agar) were inoculated by spotting 1 µL of overnight
381 culture. Inoculated plates were then incubated at 21 °C (ambient room temperature) for 8 and
382 24 hours and 37 °C for 8 hours. The diameter of visible colony spread was measured in mm
383 from three directions and the average value recorded.
384
385 **Tolerance to bile salts**
386
387 The MIC and MBC for sodium deoxycholate (DOC) and sodium cholate was determined for all
388 isolates using the broth dilution method according to Clinical and Laboratory Standard Institute
389 (CLSI) guidelines adapted from the protocol as described previously[53].
390

391 **Bacterial growth curves**

392

393 The growth of all isolates in LB broth was assessed using a Multiskan FC microplate
394 photometer (Thermo Fisher Scientific) (Supplementary Fig. S3). Measurements were taken
395 every 15 minutes over 24 hours at OD_{620} nm. The instrument was kept at 37 °C with shaking
396 during kinetic intervals.

397

398 **Preparation of bacterial inoculum for infection**

399

400 The MIC and MBC for gentamicin (CN) was determined for all isolates using the broth dilution
401 method according to CLSI guidelines. Susceptibility or resistance was classified according to
402 the lower working concentration of 20 µg/mL used in the gentamicin protection assays as
403 detailed below.

404

405 Inoculum stocks for each isolate were prepared by streaking and restreaking individual
406 bacterial isolates onto LB agar. Individual colonies from restreaked isolates were taken and
407 used to inoculate 5 mL LB broth before growth overnight at 37 °C with orbital shaking at 200
408 RPM. Overnight cultures were centrifuged at 5,500 RCF for 10 minutes. The supernatant was
409 discarded and the bacterial cell pellet resuspended in 5 mL PBS before centrifugation again at
410 5,500 RCF for 10 minutes. Finally, the pellet was resuspended in 5 mL of PBS (15 % [v/v]
411 glycerol) solution before aliquoting (250 µL/microcentrifuge tube) and freezing at -80 °C.
412 Representative inoculum stocks for each isolate were decimally diluted in PBS and 100 µL

413 aliquots of the dilutions were plated onto LB agar. Agar plates were incubated for 18 hours at
414 37 °C before enumeration of the CFU.
415
416 ***Ex vivo* gentamicin protection assay**
417
418 The ability to survive and proliferate following phagocytosis by murine RAW 264.7 and human
419 THP-1 macrophages was assessed for all gentamicin susceptible isolates using *S.*
420 Typhimurium 14028S and ST4/74 as reference strains adapted from protocols as described
421 previously[30,54].
422
423 RAW 264.7 macrophages were grown in antibiotic-free Dulbecco's Modified Eagle's Medium
424 (DMEM) (Sigma-Aldrich) supplemented with 10 % [v/v] heat inactivated foetal bovine serum
425 (FBS) and incubated at 37 °C in a humidified atmosphere with 5 % CO_2. THP-1 monocytes
426 were grown in antibiotic-free RPMI 1640 media (Sigma-Aldrich) supplemented with 10 % [v/v]
427 heat inactivated FBS and incubated at 37 °C in a humidified atmosphere with 5% CO_2. Cell
428 viability was assessed using trypan blue and a Bio-Rad TC20 automated cell counter.
429
430 Cells were subcultured and 1 mL was directly seeded into 24 well plates at a density of 1 x 10^5
431 cells/mL per well. THP-1 monocytes were differentiated to adherent macrophages by
432 supplementing media with 20 ng/mL phorbol 12-myristate 13-acetate (PMA) for 5 days.
433
434 Prior to infection, inoculum stocks for each bacterial isolate to be assessed were diluted in
435 complete media to 1 x 10^6 bacteria/mL for a MOI of 10:1 and incubated at 37 °C for 1 hour.

436 Macrophages were washed 3 times with 1 mL Hank's Balanced Salt Solution (HBSS) before 1
437 mL of the bacterial suspension prepared as outlined above, was added to each well with 1 mL
438 of complete media being added to uninfected control wells. These 24 well plates were
439 centrifuged at 300 RCF for 5 minutes at room temperature (21 °C) before incubation at 37 °C
440 with 5 % CO_2 for 1 hour to allow for phagocytosis.
441
442 Following phagocytosis, the cells were washed 3 times with 1 mL HBSS. A volume of 1 mL
443 complete media supplemented with 100 µg/mL gentamicin was added to each well before
444 incubation at 37 °C with 5% CO_2 for 1 hour to kill external bacteria. After 1 hour, cells were
445 washed 3 times with 1 mL HBSS. Another volume of 1 mL of complete media supplemented
446 with 20 µg/mL gentamicin was then added to each well before incubation at 37 °C with 5 %
447 CO_2 for the desired time points.
448
449 Time points were processed by washing the cells 3 times with 1 mL HBSS before 1 mL 1 %
450 [v/v] Triton X-100 PBS solution was added to the infected cells prior to incubation at room
451 temperature for 10 minutes. Lysed supernatants were decimally diluted in PBS and 100 µL
452 aliquots of the dilutions were plated onto LB agar. Agar plates were incubated for 18 hours at
453 37 °C before enumeration of the CFU.
454
455
456
457

458 **Relative viability assay for RAW 264.7 and THP-1 by glucose-6-phosphate**
459 **dehydrogenase and lactate dehydrogenase**
460
461 Relative viability of infected macrophages was determined by comparison to uninfected control
462 cells using the Vybrant Cytotoxicity Assay kit (Life Technologies) and Pierce LDH Cytotoxicity
463 Assay kit (Life Technologies) to measure extracellular glucose-6-phosphate dehydrogenase
464 (G6PD) [EC 1.1.1.49] and lactate dehydrogenase (LDH) [EC 1.1.1.27] activity in cell culture
465 supernatants according to manufacturer's instructions. All samples and standards were
466 assayed in duplicate.
467
468 **Cytokine quantification**
469
470 A panel of proinflammatory cytokines and infection relevant chemokines were quantified from
471 the supernatants of infected RAW 264.7 and THP-1 macrophages (Supplementary Table 10).
472 For RAW 264.7 supernatants, targets were quantified at 0 hours (before infection) and 1, 2, 4,
473 8 and 24 HPI. For THP-1 supernatants, targets were quantified at 0 hours (before infection)
474 and 1, 2, 4, 8, 24 and 168 HPI.
475
476 RAW 264.7 cytokine/chemokine release was measured using a multiplex magnetic bead
477 based kit (Life Technologies) and the Luminex 200 xMAP platform (Luminex). Similarly, THP-1
478 cytokine/chemokine release was measured using an electrochemiluminescence based V-
479 PLEX kit (Meso Scale Discovery) and the Sector Imager 2400 platform (Meso Scale
480 Discovery). The measured levels of many targets were below the range of detection in RAW

481 264.7 supernatant samples and for the purposes of this study were recorded at the lower level
482 of detection for the assay used when comparing to *S*. Typhimurium ST4/74 infection or
483 infected THP-1 macrophages. Assays were performed according to the manufacturer's
484 protocol for cell culture supernatant samples. All samples and standards were assayed in
485 duplicate.
486
487 **Bacterial whole genome sequencing**
488
489 Whole genome sequencing was carried out as described previously[55]. Briefly, genomic DNA
490 (gDNA) was purified from overnight cultures of bacterial isolates grown in Trypticase Soy Broth
491 (TSB) (Becton Dickinson) incubated at 37 °C using the DNeasy blood and tissue kit (Qiagen).
492 Libraries were prepared using 1 ng gDNA with the Nextera XT kit (Illumina) and sequenced
493 using the MiSeq platform (Illumina) with a V2 kit (2 x 250 bp).
494
495 Three isolates, namely *S*. Cubana CFSAN002050, *S*. Tennessee CFSAN001387 and *S.*
496 Weltevreden CFSAN001415 were further sequenced using the Pacific Biosciences (PacBio)
497 RS II platform. *Salmonella* Cubana CFSAN002050 was sequenced as described
498 previously[56,57]. For *S*. Tennessee CFSAN001387 and *S*. Weltevreden CFSAN001415, libraries
499 using 6 µg gDNA were sheared to a size of 10 kb using g-TUBEs (Covaris Inc., Woburn, MA)
500 according to the manufacturer's instructions. The SMRTbell 10-kb template libraries were
501 constructed using DNA Template Prep Kit 1.0 with the 10-kb insert library protocol (Pacific
502 Biosciences, Menlo Park, CA, USA) and sequenced using the P4-C2 chemistry on 3 single-
503 molecule real-time (SMRT) cells with a 240-minute collection protocol along with Stage Start.

504 **Whole genome assembly and annotation**

505

506 For Illumina MiSeq data, Jellyfish (version 2.2.6) was used to generate a *k*-mer spectrum
507 before inspecting the quality of the reads using FastQC (version 0.11.5)[58,59]. Error correction
508 was performed using BFC (version r181)[60]. A relaxed sliding window trim for an average Phred
509 quality score of 10 was performed using Trimmomatic (version 0.36) before the genomes were
510 *de novo* assembled with SPAdes (version 3.7.1) using the default *k*-mer size selection for 250
511 bp reads and the automatic coverage cutoff threshold[61,62]. The quality of the subsequent
512 assemblies was assessed using Bandage (version 0.8.0) and QUAST (version 4.1)[63,64].
513 Contigs were excluded from the assembly if they were shorter than 200 bp.

514

515 Analysis of the PacBio data was implemented using SMRT Analysis 2.3.0. The best *de novo*
516 assembly was established with PacBio Hierarchical Genome Assembly Process (HGAP 3.0)
517 program using the continuous-long-reads from the three SMRT cells. The assembly outputs
518 from HGAP produced circular contiguous sequences with overlapping regions at the end that
519 can be identified using dot plots in Gepard (version 1.40)[65]. Genomes were checked manually
520 for even sequencing coverage. Afterwards the interim consensus sequence was used to
521 determine the final consensus and accuracy scores using Quiver consensus algorithm[66].

522

523 All sequences and assemblies are publicly available with accession numbers provided (Table
524 1) and are submitted for annotation using the NCBI Prokaryotic Genome Automatic Annotation
525 Pipeline (PGAAP)[67].

526

527 **Sequence analysis**

528

529 *Salmonella* Pathogenicity Island (SPI) gene content was compared to *S*. Typhimurium ST4/74
530 for all isolates (Fig. 5 and Supplementary Fig. S8). Homologous amino acid sequences for
531 each protein were identified and generated using BLAST+ (version 2.3.0) and Biopython
532 (version 1.66)[68–70]. Amino acid sequence similarity was assessed using a Needleman-Wunsch
533 global alignment through the EMBOSS analysis software (version 6.6.0)[71].

534

535 **Pan-genome analysis**

536

537 To limit bias among different annotation tools for downstream analyses, additional annotation
538 of all isolates included in this study was performed using Prokka (version 1.11)[72–79]. Presence
539 or absence of protein sequences from all strains was determined using the pan-genome
540 pipeline Roary (version 3.6.1) (Supplementary Fig. S9b and Supplementary Fig. S9c)[80–83].
541 Visualisation of the pan-genome data was performed using Anvi'o (version 1.2.3)
542 (Supplementary Fig. S9a)[84,85].

543

544 **Statistics**

545

546 The R statistical computing environment was used for all statistical analyses[86,87]. Multiple
547 comparisons for normally distributed data were performed by one-way ANOVA where
548 appropriate and *post hoc* analysis of significance was inspected by Tukey's range test.

549

Code availability

Data and code to reproduce the analyses and manuscript figures are available[88].

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554

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757 **Author information**

758

759 **Contributions**

760

761 D.H. and M.M. designed experiments. D.H. and M.H. performed experiments and analysed

762 data. T.M. performed experiments. D.H. and S.F. wrote the manuscript. All authors discussed

763 the results and contributed to the revision of the manuscript.

764

765 **Competing interests**

766

767 The authors declare no competing financial interests.

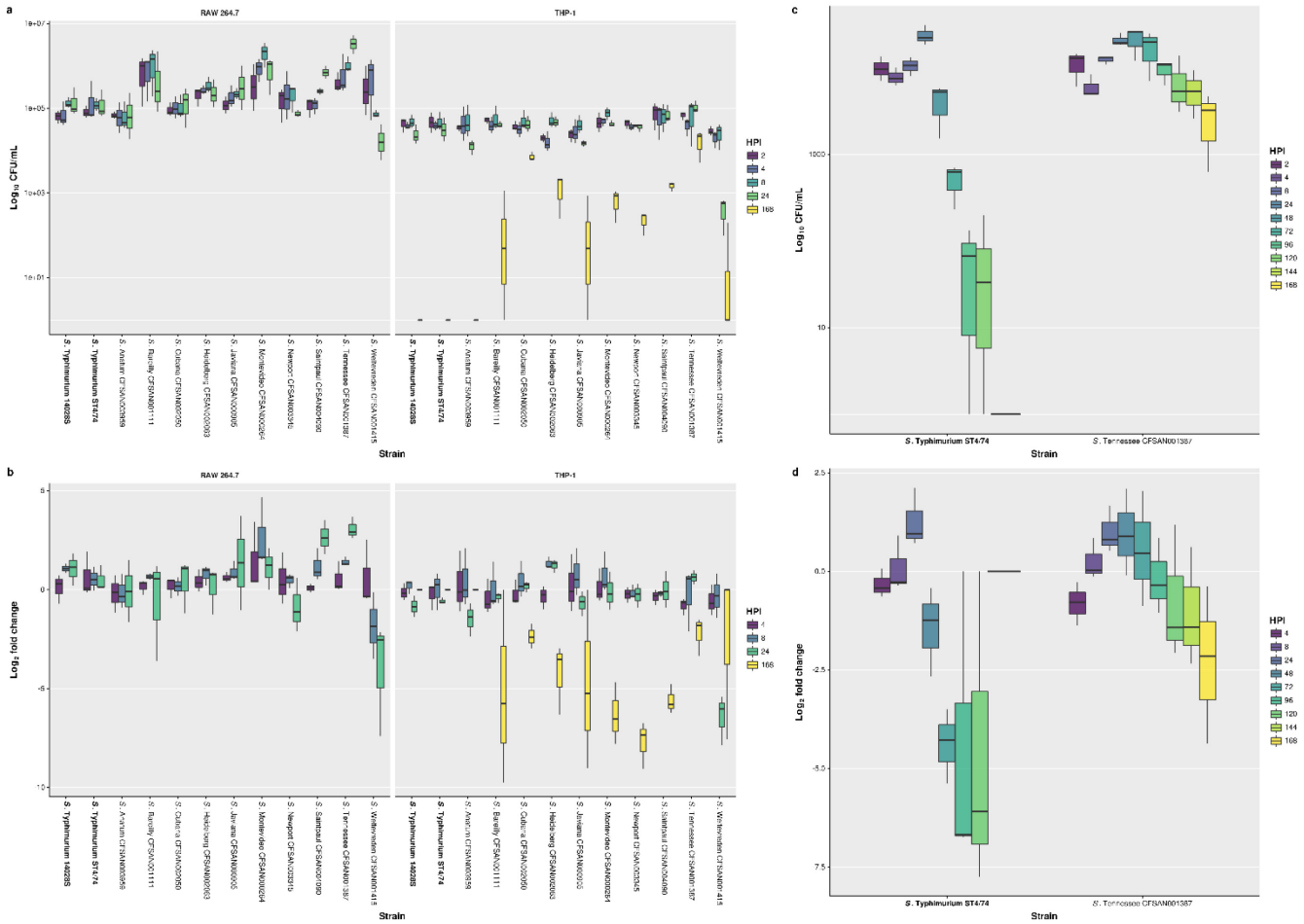
768 **Tables**

S. enterica serovar	Strain	Outbreak	Location	Year	Isolation Source
Anatum	CFSAN003959	Papaya	Mexico	2012	Papaya
Bareilly	CFSAN001111	Tuna	India	2012	Tuna scrape, frozen
Cubana	CFSAN002050	Alfalfa sprouts	USA - Arizona	2012	Alfalfa sprouts
Heidelberg	CFSAN002063	Chicken	USA - Washington	2012	Clinical
Javiana	CFSAN000905	Green onion	Mexico	2009	Canal water
Montevideo	CFSAN000264	Black pepper	USA - Rhode Island	2010	Black pepper
Newport	CFSAN003345	Eastern shore sampling	USA - Virginia	2011	Goose faeces
Saintpaul	CFSAN004090	Jalapeño/serrano pepper	Mexico	2008	Jalapeño pepper
Tennessee	CFSAN001387	Peanut butter	USA - Georgia	2007	Peanut butter
Typhimurium	**14028S**				
Typhimurium	**ST4/74**				
Weltevreden	CFSAN001415	Prison tuna	USA - Virginia	2005	Tuna

769

770 **Table 1** https://dx.doi.org/10.6084/m9.figshare.3364726.v1

771 **Figures**



772

773 **Fig. 1: Survival of isolates following phagocytosis by RAW 264.7 and THP-1**

774 **macrophages assessed by gentamicin protection assay**

775

776 **A)** Survival of isolates following phagocytosis by RAW 264.7 macrophages at 2, 4, 8 and 24

777 HPI and THP-1 macrophages at 2, 4, 8, 24 and 168 HPI reported as Log_{10} CFU/mL. **B)**

778 Survival of isolates following phagocytosis by RAW 264.7 macrophages at 4, 8 and 24 HPI and

779 THP-1 macrophages at 4, 8, 24 and 168 HPI reported as Log_2 fold change comparing to 2

780 HPI. Results for isolates in the study correspond to the mean of three independent assays (*n* =

781 3) with duplicate technical replicates. Results for reference strains *S*. Typhimurium 14028S

782 and ST4/74 correspond to the mean of six independent assays (*n* = 6) with duplicate technical
783 replicates. **C)** Survival of *S*. Tennessee CFSAN001387 in comparison with *S*. Typhimurium
784 ST4/74 following phagocytosis by THP-1 macrophages at 2, 4, 8, 24, 48, 72, 96, 120, 144 and
785 168 HPI reported as Log_{10} CFU/mL. **D)** Survival of *S*. Tennessee CFSAN001387 in
786 comparison with *S*. Typhimurium ST4/74 following phagocytosis by THP-1 macrophages at 4,
787 8, 24, 48, 72, 96, 120, 144 and 168 HPI reported as Log_2 fold change comparing to 2 HPI.
788 Results correspond to the mean of three independent assays (*n* = 3) with triplicate technical
789 replicates. The lower and upper hinges correspond to the 25th and 75th percentiles with the
790 whiskers extending ± 1.5 times the range between first and third quartiles.
791 https://dx.doi.org/10.6084/m9.figshare.3364726.v1

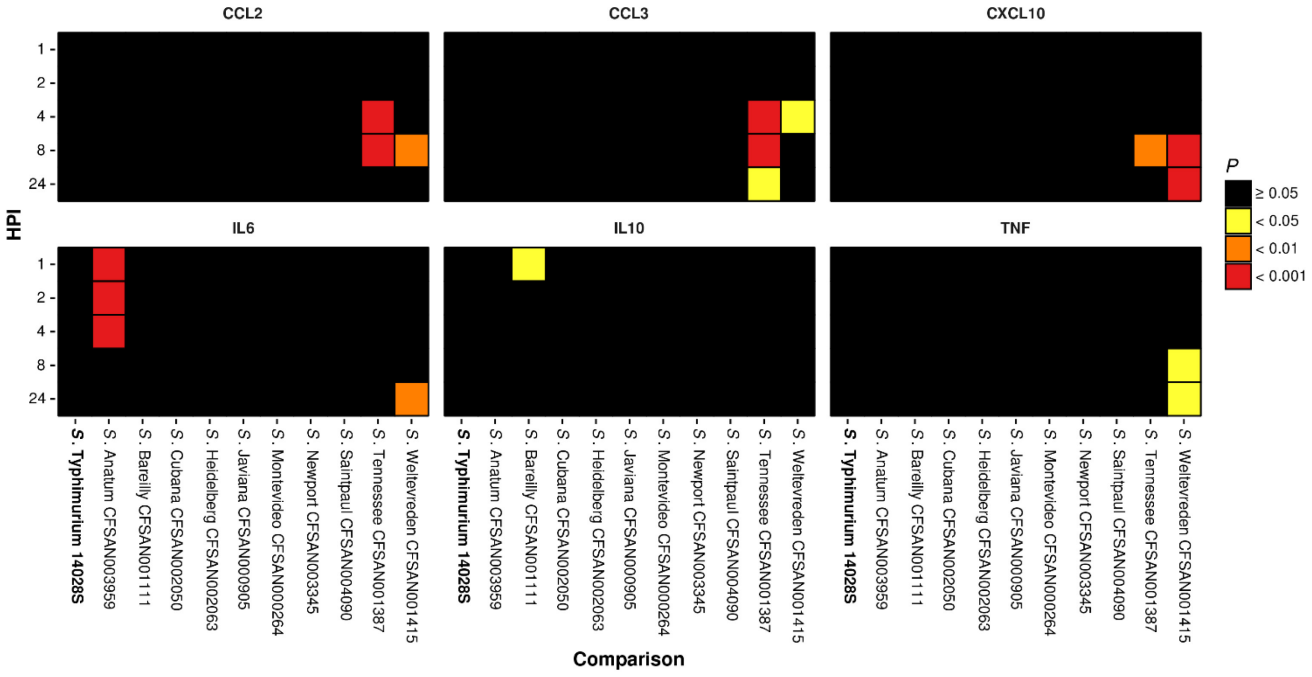


Fig. 2: Significant RAW 264.7 chemokine and proinflammatory cytokine release following infection with isolates when compared to *S*. Typhimurium ST4/74 infection

Increased release of chemokine and proinflammatory cytokine proteins by RAW 264.7 macrophages at 1, 2, 4, 8 and 24 HPI with isolates when compared to *S*. Typhimurium ST4/74 as determined by one-way ANOVA. Values correspond to adjusted probability (*P*) as determined by *post hoc* analysis of significance using Tukey's range test. Differences were deemed significant by arbitrary cut-offs at < 0.05, 0.01 and 0.001.

https://dx.doi.org/10.6084/m9.figshare.3364726.v1

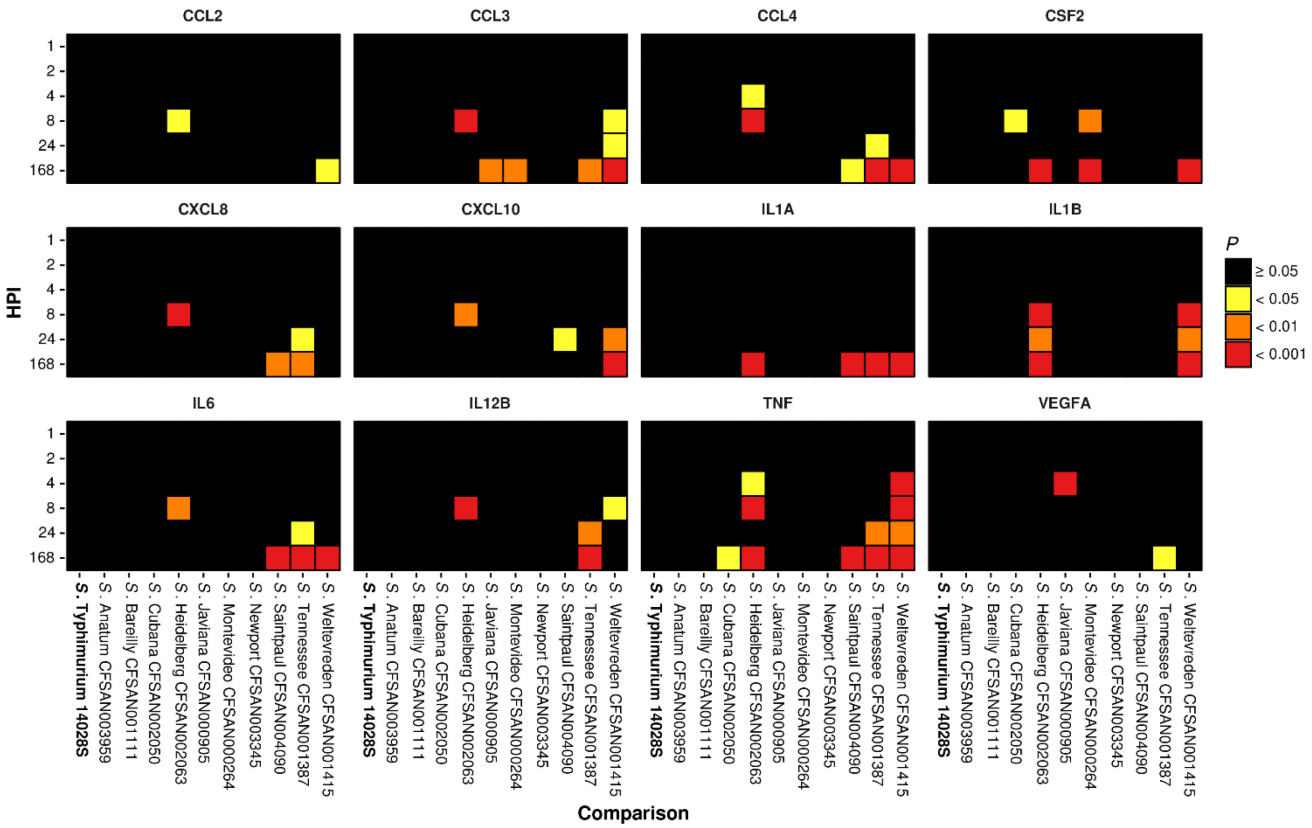
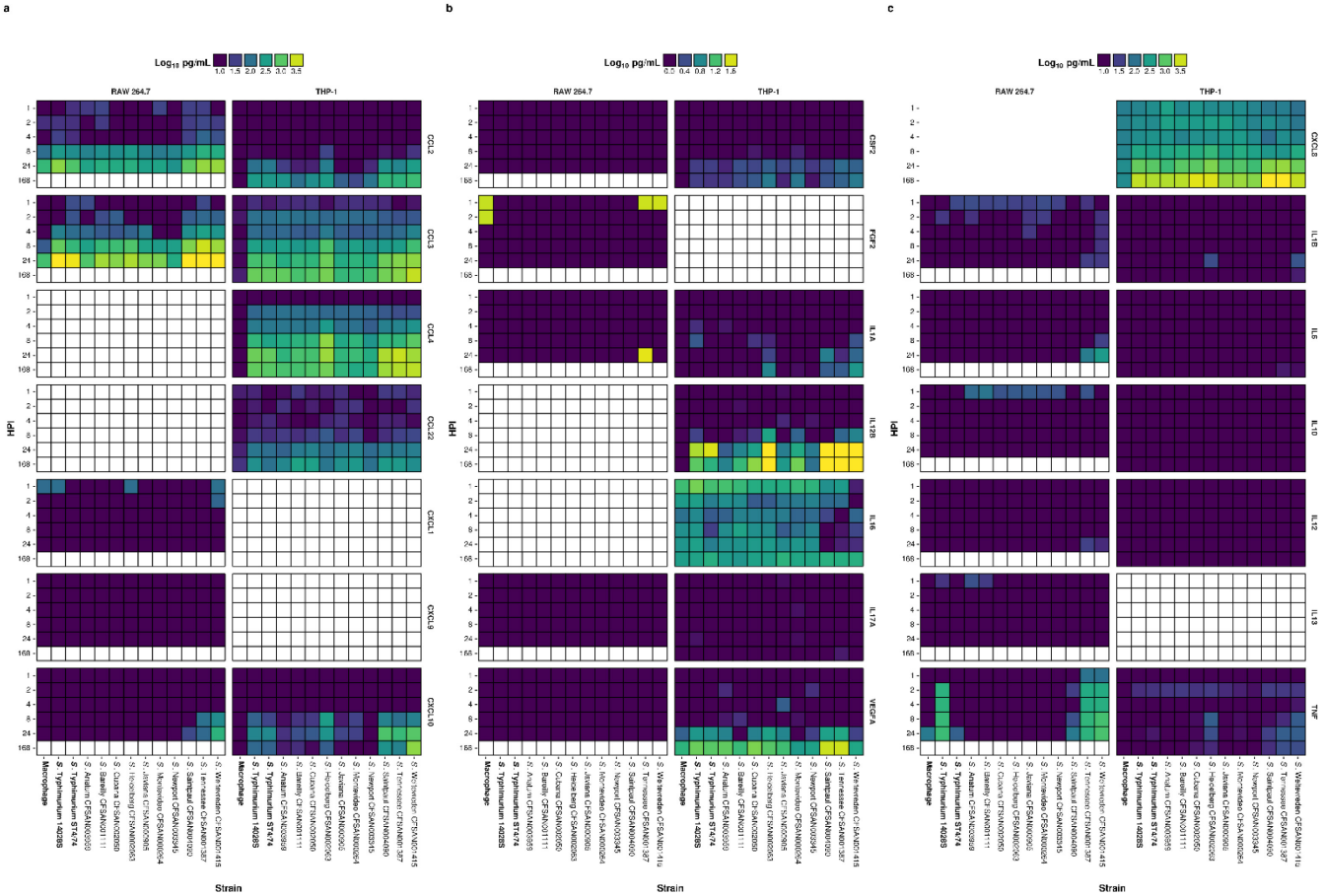


Fig. 3: Significant THP-1 chemokine, cytokine and proinflammatory cytokine release following infection with isolates when compared to *S*. Typhimurium ST4/74 infection

Increased release of chemokine, cytokine and proinflammatory cytokine proteins by THP-1 macrophages at 1, 2, 4, 8, 24 and 168 HPI with isolates when compared to *S*. Typhimurium ST4/74 as determined by one-way ANOVA. Values correspond to adjusted probability (*P*) as determined by *post hoc* analysis of significance using Tukey's range test. Differences were deemed significant by arbitrary cut-offs at < 0.05, 0.01 and 0.001.

https://dx.doi.org/10.6084/m9.figshare.3364726.v1


a
b
c
Log10 pg/mL
1.0 1.5 2.0 2.5 3.0 3.5
0.0 0.4 0.8 1.2 1.6
RAW 264.7
THP-1
CCL2
CCL3
CCL4
CCL22
CXCL1
CXCL9
CXCL10
CSF2
FGF2
IL1A
IL12B
IL16
IL17A
VEGFA
CXCL8
IL1B
IL6
IL10
IL12
IL13
TNF
hpi
1
2
4
8
24
168
Macrophage
S. Typhimurium 14028S
S. Typhimurium ST474
S. Anatum CFSAN030949
S. Saintpaul CFSAN030950
S. Cubana CFSAN032050
S. Heidelberg CFSAN032063
S. Javiana CFSAN030945
S. Newport CFSAN030945
S. Saintpaul CFSAN030950
S. Wichita CFSAN000064
S. Infantis

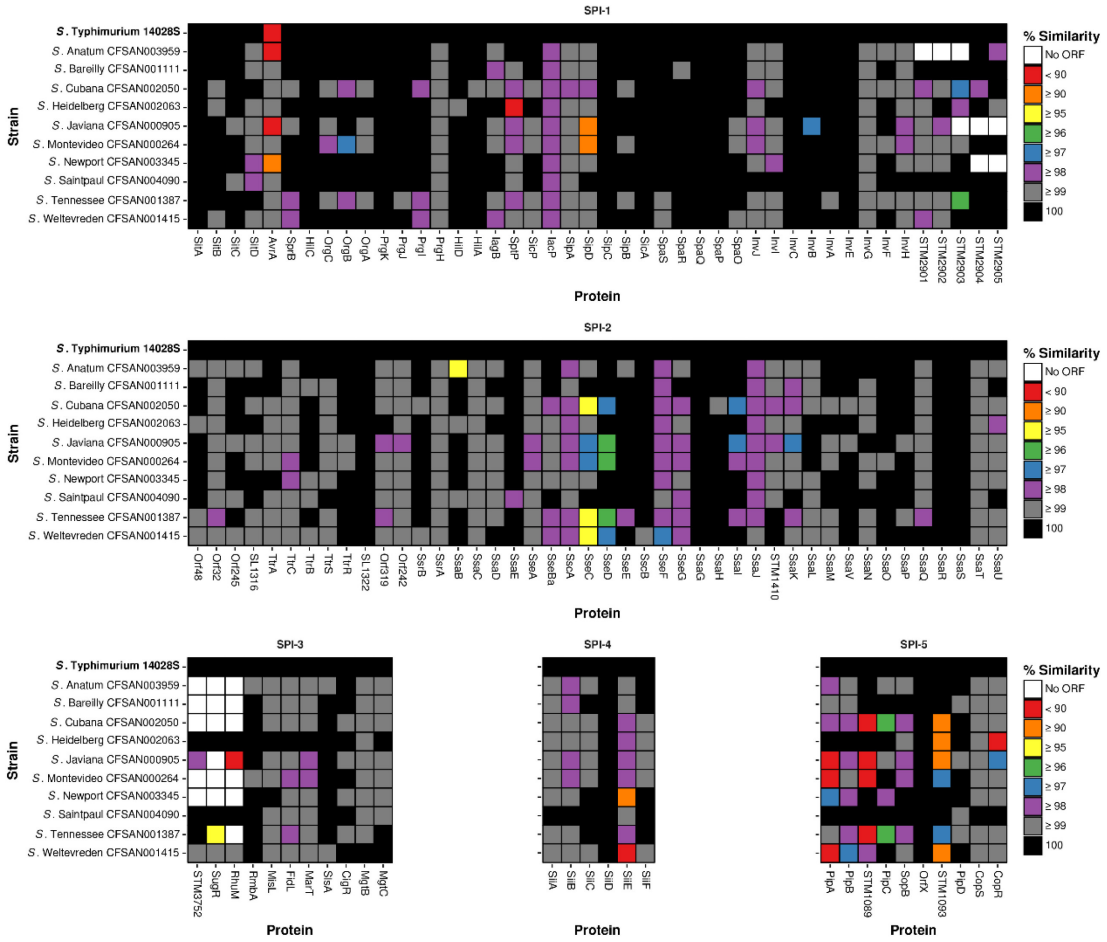


Fig. 5: Sequence variability within SPI-1 to SPI-5

The amino acid sequence similarity for SPI gene content of each isolate included in this study was compared with *S.* Typhimurium ST4/74.

https://dx.doi.org/10.6084/m9.figshare.3364726.v1