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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Abstract

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

30 *Salmonella* is a zoonotic pathogen responsible for illnesses on a global scale and poses a
 31 significant burden to public health¹. 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 worldwide². 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 annually³.
 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 cytoplasm⁸. 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

proliferate and basolaterally reinfect epithelial cells as well as become engulfed by additional, localised phagocytic cells.

The importance of *Salmonella* in both clinical and public health settings has fuelled research into the virulence mechanisms and associated pathogenicity of this bacterium^{11,12}. Many of these studies use *S. Typhimurium* infection in mice as a model for typhoid fever due to the similar pathology observed including intestinal and extraintestinal lesions that frequently occur in human hosts infected with typhoidal serovars¹³. The *in vitro* transcriptome of *S. Typhimurium* has been characterised extensively by sequencing-based methods^{14,15}. Similarly, the *ex vivo* response of *S. Typhi* and *S. Typhimurium* during infection of macrophages has also been elucidated^{16–18}.

Although *S. Typhimurium* infection of mice results in symptoms mimicking human typhoid fever, this serovar, along with *S. Enteritidis* is predominantly associated with gastroenteritis in humans. Despite the fact that over 2,610 serovars of *Salmonella* have been reported to date, few studies are available to describe the infection arising from other *S. enterica* serovars^{19–22}.

In this study, 10 atypical *S. enterica* serovars cultured from multistate foodborne outbreaks in the U.S. were characterised both pheno- and genotypically. Differences in the nature of the infection process and the underlying virulence determinants were noted. We identified factors that further explain the ability of these atypical *S. enterica* serovars to cause foodborne outbreaks.

Results

In vitro characterisation of the isolates

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

The acid tolerance response of *Salmonella* is a complex defence mechanism employed by the pathogen to defend against the acid shock experienced in the stomach⁶. Increased numbers for the majority of isolates were recorded following 1 and 2 hours of growth at pH 2.5 before significant reductions in viable numbers occurred at 4 hours (Supplementary Fig. S1). Motility has been suggested as a virulence determinant with respect to invasion with notable non-motile and/or host adapted exceptions^{23–26}. After 24 hours of incubation at 21 °C, *S. Bareilly* CFSAN001111 and *S. Javiana* CFSAN000905 exhibited a reduced swim phenotype when compared to *S. Typhimurium* ST4/74 ($P < 0.001$) whereas *S. Newport* CFSAN003345 and *S. Saintpaul* CFSAN004090 exhibited an increased swim phenotype ($P < 0.001$). At 37 °C, *S. Anatum* CFSAN003959 and *S. Tennessee* CFSAN001387 exhibited a reduced swim phenotype after 8 and 24 hours when compared to *S. Typhimurium* ST4/74 ($P < 0.001$) (Supplementary Fig. S2). In the absence of glucose as a carbon source, no swarm motility was observed for any of the isolates regardless of temperature or incubation time (Supplementary

Fig. S2). During digestion, contraction of the gall bladder releases bile into the small intestine. *Salmonella* Weltevreden CFSAN001415 showed a two-fold difference in susceptibility to sodium deoxycholate (DOC) in comparison to *S. Typhimurium* ST4/74 although the MBC was comparable to the other isolates (Supplementary Table S1).

Intracellular survival of atypical *S. enterica* serovars in murine and human macrophages

Salmonella Typhimurium pathogenesis has been extensively studied *in vivo* using murine models and *ex vivo* using murine cell lines (such as J774.2 and RAW 264.7). However, the differences in the ability of *Salmonella* to survive and replicate within human macrophages is currently not well described²⁷. Differentiated human monocyte cell lines (including THP-1 and U937) have been used to explore the replication of *S. enterica* serovars Enteritidis and Typhimurium. Few studies describing the bacterial replication in human macrophages of atypical NTS serovars have been reported despite their epidemiological importance and their contributions to clinical cases of salmonellosis^{27,28}.

To study the ability of the isolates to survive phagocytosis, infections were performed using murine RAW 264.7 and differentiated human THP-1 macrophages by the gentamicin protection assay adapted from protocols previously described^{29–32}. *Salmonella* Typhimurium 14028S and ST4/74 were included as reference strains in all infection assays. Infections were carried out at a Multiplicity Of Infection (MOI) of 10:1. Viable internalised bacteria were enumerated at 2, 4, 8 and 24 Hours Post Infection (HPI) in RAW 264.7 and 2, 4, 8, 24 and 168 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-Log₁₀ 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-Log₁₀ 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 Log₂ 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-Log₁₀
 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-Log₁₀ 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-Log₁₀ 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 Log₂

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 1×10^7 mean CFU/mL in RAW 264.7 for
178 some atypical serovars compared with values that did not exceed 1×10^5 mean CFU/mL in
179 THP-1 for all isolates. This 2-Log₁₀ 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-Log₁₀
 196 reduction between 2 and 48 HPI with an additional 2-Log₁₀ 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-Log₁₀ 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

(Table 1) to *S. Typhimurium* ST4/74, increased proinflammatory cytokine (including IL6, IL10 and TNF) and chemokine (including CCL2, CCL3 and CXCL10) release was observed (Fig. 2) (Supplementary Table S5).

In THP-1, increased proinflammatory cytokine (including CXCL8, IL1B, IL6 and TNF), cytokine (including CSF2, IL1A, IL12B and VEGFA) and chemokine (including CCL2, CCL3, CCL4 and CXCL10) release was recorded at 8, 24 and 168 HPI across a selection of these isolates in comparison with uninfected control macrophages. As with RAW 264.7, when comparing infection with the 10 atypical serovars in this study to *S. Typhimurium* ST4/74 in THP-1, significant increased proinflammatory cytokine and chemokine release was observed, further differentiating the isolates by the innate host response (Fig. 3) (Supplementary Table S5).

Salmonella Heidelberg CFSAN002063 stimulated the release of CCL3, CSF2, CXCL8, CXCL10, IL1A, IL1B, IL6, IL12 and TNF to levels in excess of those observed for *S. Typhimurium* ST4/74, particularly at 8 HPI and from 4 to 168 HPI with respect to TNF (Supplementary Fig. S7). In the gentamicin protection assays it was noted that although both *S. Tennessee* CFSAN001387 and *S. Weltevreden* CFSAN001415 could be recovered at 168 HPI from THP-1 macrophages, these two isolates exhibited different infection profiles. The former displayed the smallest reduction in viable intracellular bacteria over the time course of the infection whereas the latter displayed the largest reduction in viable numbers. This can be accounted for in the overlapping yet contrasting cytokine profile of THP-1 macrophages following infection with these isolates. *Salmonella* Tennessee CFSAN001387 and *S. 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 *Cxc1* gene codes for a functionally homologous
 244 protein³⁴. 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- κ B pathway³⁹. 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 protease⁴⁰. SptP is a
 277 tyrosine phosphatase involved in the inhibition Raf activation and the subsequent MAP kinase
 278 pathway⁴¹. 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

conservation, either being highly similar in the majority of isolates or were unidentifiable in *S.*
Anatum CFSAN003959, *S. Javiana* CFSAN000905 and *S. Newport* CFSAN003345.

SPI-2 and its associated T3SS contributes to the ability of *Salmonella* to translocate effectors
across the membrane of the SCV when the bacterium is internalised in epithelial cells and
macrophages^{42,43}. The integral function of SPI-2 for intracellular survival can be observed by
the degree of amino acid sequence conservation across all atypical serovars (Fig. 5). As these
isolates were implicated in multistate foodborne outbreaks and, as demonstrated above,
capable of surviving within both RAW 264.7 and THP-1 macrophages, this observation is
consistent with the expressed phenotype and raises the question as to whether or not potential
differences in the expression of some/all of these genes may further explain the differences
shown in intracellular survival. Sequence variation at the amino acid level in comparison to *S.*
Typhimurium ST4/74 was greatest in *SsaB*, *SseB*, *SseC* and *SseD*. Loss of the effector protein
SsaB (*SpiC*) has been shown to promote defective virulence phenotypes due to an inability to
translocate all SPI-2 effectors⁴⁴. This may explain the observed infection phenotype in *S.*
Anatum CFSAN003959, the only isolate among the 10 atypical serovars studied to be
unrecoverable at 168 HPI in THP-1 macrophages, as it had the lowest amino acid sequence
similarity for *SsaB*.

SseBCD proteins function as a translocon that facilitate the secretion of effector proteins by
intracellular *Salmonella*⁴⁵. Both *S. Cubana* CFSAN002050 and *S. Tennessee* CFSAN001387
display differences in all three *SseBCD* proteins with *S. Tennessee* CFSAN001387 being least
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 previously³⁵. 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 macrophages⁴⁸. 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 salmonellosis⁴⁹.

Discussion

Studies aimed at elucidating the host response to NTS serovars including Enteritidis and Typhimurium have been facilitated by the availability of murine infection models. This study focussed on atypical serovars of this genus availing of isolates cultured from foodborne outbreaks, the majority of which are frequently listed in the top 20 serovars responsible for laboratory-confirmed human cases of salmonellosis as reported annually by the CDC⁵⁰. These data identify key differences between isolates related to their ability to survive within macrophages and highlight the potential unsuitability of the widely used murine macrophage model to infer human relevant distinctions between isolates. We have shown that for NTS serovars, distinct differences in the inflammatory response of human macrophages can further differentiate these microorganisms in a manner that was not possible with murine macrophages (Fig. 3). Furthermore, the reference strains *S. Typhimurium* 14028S and ST4/74 that are often included in *in vitro* research emerged as the biological outliers in many respects with regards to their infection phenotype (Fig. 1). In the case of specific proinflammatory cytokines such as TNF, RAW 264.7 macrophages responded to infection with *S. Typhimurium* 14028S and ST4/74, *S. Tennessee* CFSAN001387 and *S. Weltevreden* CFSAN001415 whereas THP-1 macrophages displayed a broader proinflammatory response to the range of serovars studied (Fig. 4).

These data suggest more differences than previously acknowledged for *S. enterica* serovars with implications for public health. In agreement with this notion, key differences were identified 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.

Methods

Bacterial isolates and culture methods

Environmental and food isolates were collected from different countries between 2005 and 2012 by the U.S. Food and Drug Administration inspections as part of compliance actions⁵¹. The clinical isolate was obtained from the Washington State Department of Health (Table 1). All *Salmonella* isolates were stored at -80 °C in Lysogeny Broth (LB) broth (Sigma-Aldrich) supplemented with 15 % [v/v] glycerol. Working cultures were prepared by streaking isolates and restreaking individual colonies onto Mueller-Hinton (MH) agar (Sigma-Aldrich). Individual colonies from restreaked isolates were used to inoculate 5 mL MH broth (Sigma-Aldrich) and grown overnight at 37 °C with orbital shaking at 200 RPM. Overnight cultures were then used in the subsequent experiments as detailed below.

Acid resistance

The ability to survive in a low pH culture medium was assessed for all isolates as described previously⁵². Briefly, bacterial cultures were individually grown overnight (18 hours) without shaking in 5 mL buffered MH broth (MES hydrate, 2 % [w/v], pH 5) containing 0.4 % [w/v] glucose at 37 °C. A volume of 333 µL of overnight culture was centrifuged at 10,000 RPM for 10 minutes. The supernatant was removed and the cell pellet recovered and resuspended in 2 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 previously⁵². 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 previously⁵³.

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 OD₆₂₀ 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 % CO₂. 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% CO₂. 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 10⁵
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 10⁶ 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 % CO₂ 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% CO₂ 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 CO₂ 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

Relative viability assay for RAW 264.7 and THP-1 by glucose-6-phosphate dehydrogenase and lactate dehydrogenase

Relative viability of infected macrophages was determined by comparison to uninfected control cells using the Vybrant Cytotoxicity Assay kit (Life Technologies) and Pierce LDH Cytotoxicity Assay kit (Life Technologies) to measure extracellular glucose-6-phosphate dehydrogenase (G6PD) [EC 1.1.1.49] and lactate dehydrogenase (LDH) [EC 1.1.1.27] activity in cell culture supernatants according to manufacturer's instructions. All samples and standards were assayed in duplicate.

Cytokine quantification

A panel of proinflammatory cytokines and infection relevant chemokines were quantified from the supernatants of infected RAW 264.7 and THP-1 macrophages (Supplementary Table 10). For RAW 264.7 supernatants, targets were quantified at 0 hours (before infection) and 1, 2, 4, 8 and 24 HPI. For THP-1 supernatants, targets were quantified at 0 hours (before infection) and 1, 2, 4, 8, 24 and 168 HPI.

RAW 264.7 cytokine/chemokine release was measured using a multiplex magnetic bead based kit (Life Technologies) and the Luminex 200 xMAP platform (Luminex). Similarly, THP-1 cytokine/chemokine release was measured using an electrochemiluminescence based V-PLEX kit (Meso Scale Discovery) and the Sector Imager 2400 platform (Meso Scale Discovery). The measured levels of many targets were below the range of detection in RAW

264.7 supernatant samples and for the purposes of this study were recorded at the lower level of detection for the assay used when comparing to *S. Typhimurium* ST4/74 infection or infected THP-1 macrophages. Assays were performed according to the manufacturer's protocol for cell culture supernatant samples. All samples and standards were assayed in duplicate.

486

487 **Bacterial whole genome sequencing**

488

Whole genome sequencing was carried out as described previously⁵⁵. Briefly, genomic DNA (gDNA) was purified from overnight cultures of bacterial isolates grown in Trypticase Soy Broth (TSB) (Becton Dickinson) incubated at 37 °C using the DNeasy blood and tissue kit (Qiagen). Libraries were prepared using 1 ng gDNA with the Nextera XT kit (Illumina) and sequenced using the MiSeq platform (Illumina) with a V2 kit (2 x 250 bp).

494

Three isolates, namely *S. Cubana* CFSAN002050, *S. Tennessee* CFSAN001387 and *S. Weltevreden* CFSAN001415 were further sequenced using the Pacific Biosciences (PacBio) RS II platform. *Salmonella* *Cubana* CFSAN002050 was sequenced as described previously^{56,57}. For *S. Tennessee* CFSAN001387 and *S. Weltevreden* CFSAN001415, libraries using 6 µg gDNA were sheared to a size of 10 kb using g-TUBEs (Covaris Inc., Woburn, MA) according to the manufacturer's instructions. The SMRTbell 10-kb template libraries were constructed using DNA Template Prep Kit 1.0 with the 10-kb insert library protocol (Pacific Biosciences, Menlo Park, CA, USA) and sequenced using the P4-C2 chemistry on 3 single-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 algorithm⁶⁶.

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

550 **Code availability**

551

552 Data and code to reproduce the analyses and manuscript figures are available⁸⁸.

553 References

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>

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 Log₁₀ 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 Log₂ 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

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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>

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>