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1 Multiple roles of putrescine and spermidine in stress resistance and virulence
2 of *Salmonella enterica* serovar Typhimurium

3
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11
12 Keywords: Polyamines; nitric oxide stress; virulence; intracellular pathogens.

13
14
15 **Abstract**

16 Polyamines (putrescine and spermidine) are small-cationic amines ubiquitous in nature
17 and present in most living cells. In recent years they have been linked to virulence of
18 several human pathogens including *Shigella* spp and *Salmonella enterica* serovar
19 Typhimurium (*S. Typhimurium*). Central to *S. Typhimurium* virulence is the ability to
20 survive and replicate inside macrophages and resisting the antimicrobial attacks in the
21 form of oxidative and nitrosative stress elicited from these cells. In the present study, we
22 have investigated the role of polyamines in intracellular survival and systemic infections of
23 mice. Using a *S. Typhimurium* mutant defective for putrescine and spermidine
24 biosynthesis, we show that polyamines are essential for coping with reactive nitrogen
25 species, possibly linking polyamines to increased intracellular stress resistance. However,
26 using a mouse model defective for nitric oxide production, we find that polyamines are
27 required for systemic infections independently of host-produced reactive nitrogen species.
28 To distinguish between the physiological roles of putrescine and spermidine, we

29 constructed a strain deficient for spermidine biosynthesis and uptake, but with retained
30 ability to produce and import putrescine. Interestingly, in this mutant we observe a strong
31 attenuation of virulence during infection of mice proficient and deficient for nitric oxide
32 production suggesting that spermidine, specifically, is essential for virulence of *S.*
33 Typhimurium.

34

35 **1. Introduction**

36 *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) is a Gram-negative facultative
37 intracellular pathogen able to cause a wide variety of food- and water-borne diseases
38 ranging from self-limiting gastroenteritis to systemic and life-threatening infections. As *S.*
39 Typhimurium, causes a systemic typhoid-like disease in mice, it serves as an important
40 model for studying the often fatal human illness typhoid fever. Virulence of *S.* Typhimurium
41 is dependent on the ability to survive and replicate inside host cells. Following type three
42 secretion mediated invasion of the epithelial cell-layer [15], *S.* Typhimurium bacteria are
43 able to escape to the underlying layer where they are taken up by macrophages and
44 dendritic cells of the host immune system, reviewed in [16]. Inside these cells, *S.*
45 Typhimurium is able to survive and replicate within a membrane bound compartment. The
46 bacteria will rapidly spread through the lymphoid and blood systems to the spleen and liver
47 resulting in a life-threatening systemic infection. Formation of the intracellular niche and
48 replication is facilitated by the *Salmonella* Pathogenicity Island 2 (SPI2) encoded type
49 three secretion system and its secreted virulence factors [22]. Inside the macrophages,
50 *Salmonella* is sequentially exposed to the antimicrobial activity of first the NADPH oxidase
51 generating a burst of the bactericidal superoxide (O_2^-) radicals followed by a more
52 sustained activation of the inducible nitric oxide synthase (iNOS) generating the

53 bacteriostatic nitric oxide (NO) radicals. Combination of superoxide and NO can produce
54 the highly reactive and bactericidal product peroxynitrite (ONOO⁻).

55

56 Polyamines are small poly-cationic amines present in almost all cell-types. In recent years
57 they have emerged as major modulators of bacterial physiology, including biofilm
58 formation and motility and are essential for virulence of several bacterial pathogens [1, 6,
59 9, 13, 18-20]. Acquisition of polyamines can be mediated by either biosynthesis (Fig. A.1)
60 or uptake by dedicated transporters. We have previously shown that polyamine
61 biosynthesis is essential for virulence of *Salmonella* [12, 13, 20]. This observation could be
62 linked to a severe reduction of the polyamine biosynthesis mutant's ability to invade and
63 survive/replicate inside cultured epithelial cells. However, despite a modest reduction in
64 virulence gene expression [13], the mechanisms for polyamine dependent systemic
65 *Salmonella* infections remain elusive. In recent papers it has been demonstrated that
66 polyamines can protect against oxidative and nitrosative stress in other bacterial species
67 [3, 14] providing a possible link to polyamine dependent virulence of *S. Typhimurium*. In
68 the present study, we have investigated the role of polyamines in protection against
69 oxidative and nitrosative stress. We find that the polyamine biosynthesis mutant is only
70 modestly affected, at a level similar to the wild type, by oxidative stress. In contrast, the
71 mutant is severely affected by the presence of two different nitrosative stresses (NO and
72 ONOO⁻). However, during infection of mice lacking the iNOS system, the polyamine
73 biosynthesis mutant is still highly attenuated pointing to that although polyamines may
74 contribute to resistance against nitrosative stress during infection, polyamines have
75 multiple roles in virulence of *S. Typhimurium*.

76

77 **2. Materials and methods**78 *2.1. Bacterial strains and growth conditions.*

79 A list of strains used in the current study can be found in table 1. *S. Typhimurium* ST4/74
80 was used as wild-type strain in all experiments. This strain has been described previously
81 and its virulence is well defined [24].

Strain	Relevant genotype	Reference
<i>S. Typhimurium</i> ST4/74	virulent reference strain	[24]
<i>S. Typhimurium</i> KP1274	Restriction deficient strain, used for introduction of plasmids.	[7]
LJ268	ST4/74:: $\Delta potCD$; $\Delta speE$. Kan ^R .	This work.
LJ318	ST4/74:: $\Delta speB$; $\Delta speC$; $\Delta speE$; $\Delta speF$. Cam ^R , Kan ^R .	[13]
LJ328	ST4/74:: $\Delta speB$; $\Delta speC$; $\Delta speE$; $\Delta speF$ /pACYC- <i>speB</i> . Amp ^R , Cam ^R , Kan ^R .	[13]
LJ251	ST4/74:: $\Delta potCD$.	This work.
LJ336	ST4/74:: $\Delta potCD$; $\Delta speE$ /pACYC- <i>potCD</i> . Amp ^R , Kan ^R .	This work.
LJ238	ST4/74:: $\Delta speE$. Cam ^R .	This work.
LJ607	ST4/74:: $\Delta ssaV$. Kan ^R .	[25]

82

83 Construction of the polyamine biosynthesis mutant ($\Delta speB$; $\Delta speC$; $\Delta speE$; $\Delta speF$) has
84 been previously described [13]. The strain is deleted for the *speB* gene (STM474_3225),
85 the *speC* gene (STM474_3262), the *speF* gene (STM474_0722), and the *speE* gene
86 (STM474_0175). These genes were deleted by Lambda-Red mediated recombination
87 using either pKD3 or pKD4 as templates for the PCR reaction, as previously described [5].
88 Together these genes facilitate the biosynthesis of putrescine and spermidine in *S.*
89 *Typhimurium* (figure A.1). The spermidine biosynthesis mutant ($\Delta speE$), and the uptake
90 mutant ($\Delta potCD$) have been deleted for the *speE* gene (STM474_0175) and the *potCD*-
91 genes (STM474_1220, STM474_1219), respectively. Primers used to construct and verify
92 these strains can be found in table A.1. The spermidine biosynthesis and uptake mutant
93 ($\Delta speE$; $\Delta potCD$) was generated by P22 phage mediated transduction of the $\Delta speE$ locus

94 into the $\Delta potCD$ mutant as previously described [13]. In some cases the resistance gene
95 was removed by use of the pCP20 encoded flippase [5]. All strains were verified using a
96 PCR-based sequencing strategy. Primers used for construction and verification of strains
97 are listed in supplemental table 1. Genetic complementation of the biosynthesis mutant
98 was achieved by introducing pACYC-*speB*. We have previously achieved
99 complementation of mice infections phenotypes of the biosynthesis mutant strain with this
100 plasmid [13]. Genetic complementation of the spermidine biosynthesis and transporter
101 mutant was achieved by introducing pACYC-*potCD*. Both plasmids are derivatives of
102 pACYC177 containing the *speB* gene including upstream promoter regions or the *potCD*
103 genes expressed from the promoter of the pACYC177 kanamycin resistance gene. The
104 construction of these plasmids has been described in detail elsewhere [13].

105

106 Bacterial strains were maintained in LB-Lennox broth (LB) with 15% glycerol at -80 °C. LB
107 agar plates (LB + 1.5 % agar) were used for growth on solid media. If not stated otherwise,
108 growth in liquid medium was performed in M9 medium containing 12.8 g l⁻¹ Na₂HPO₄-
109 12H₂O, 3.0 g l⁻¹ KH₂PO₄, 0.5 g l⁻¹ NaCl, 1.0 g l⁻¹ NH₄Cl, 0.1 mM CaCl₂, 2 mM MgSO₄ and
110 0.4 % w/v glucose. Prior to all experiments the bacteria were grown for 16 hrs, 200 rpm,
111 37°C in M9 minimal media to deplete for carry-over polyamines. Where indicated, M9 was
112 supplemented with 100µg ml⁻¹ of either putrescine (11.3 mM) or spermidine (6.8 mM),
113 physiologically relevant concentrations [11]. When appropriate, media were supplemented
114 with antibiotics in the following concentrations: 50 µg ampicillin ml⁻¹, 50 µg kanamycin ml⁻¹
115 and 10 µg chloramphenicol ml⁻¹.

116

117 *2.2. Resistance towards oxidative stress.*

118 Resistance towards reactive oxygen species was investigated for both logarithmic and
119 stationary phase bacterial cultures. For stationary-phase bacteria a disk inhibition assay
120 was performed. Bacteria were grown 16 hrs in M9 media at 37°C with shaking (200 rpm).
121 The next day 100 µl of overnight culture was spread on M9 agar plates. Sterile 13-mm
122 filter disks were placed in the center of agar plates, 10 µl of 10% H₂O₂ (Sigma Aldrich) was
123 added to the disks, and the plates were incubated at 37°C overnight. The diameter of the
124 zone of growth inhibition was measured. Three replicate assays were performed for each
125 strain, and the data were subjected to Student's t-test to evaluate their statistical
126 significance. For logarithmic bacteria, after overnight growth in M9, as described above,
127 bacteria were harvested, washed in saline and sub-cultured in M9 media with or without 70
128 µM H₂O₂. The bacterial cultures were incubated at 37°C with shaking in a Bioscreen C
129 reader (Thermo Labsystems) for 24 hrs. Growth was monitored every 15 minutes for the
130 duration of the experiment. Three replicate assays were performed for each
131 strain/condition.

132

133 2.3. Resistance towards nitric oxide stress.

134 Resistance towards nitric oxide stress was investigated in growth experiments in the
135 presence of either S-Nitrosoglutathione (Sigma-Aldrich) or peroxyntirite (Caymen
136 Chemicals) using logarithmic cultures. To determine the exact concentration of
137 peroxyntirite, absorbance at 302 nm (A) was measured and the concentration C ($C = A/$
138 $(\epsilon \cdot L)$) was calculated based on the extinction coefficient $\epsilon = 1670 \text{ M}^{-1} \text{ cm}^{-1}$. Growth in the
139 presence of S-Nitrosoglutathione was performed similar to growth in the presence of H₂O₂
140 as described above. For investigations of growth in the presence of peroxyntirite, after
141 overnight growth in M9, as described above, bacteria were harvested, washed in saline

142 and sub-cultured in M9 media. The bacterial cultures were incubated at 37°C with shaking.
143 When the cultures had entered logarithmic phase ($OD_{600} \sim 0.4$) 200 μM peroxyntirite was
144 added to the cultures and they were re-incubated at 37°C with shaking for 24 hrs. Growth
145 was monitored every 15 minutes for the duration of the experiment using a Bioscreen C
146 reader. Three replicate assays were performed for each strain/condition.

147

148 *2.4. Infection of macrophage-like cells.*

149 Intracellular survival/replication was investigated using J774A.1 macrophage-like cells as
150 previously described [26]. Essentially, J774A.1 cells were infected with bacteria in a
151 multiplicity of infection of 5:1. After 25 min of infection cells were washed and either cells
152 were lysed in the presence of 0.1 % (v/v) Triton-X to release intracellular bacteria (time
153 point 0h post infection) or treated with 100 $\mu\text{g ml}^{-1}$ gentamicin for two hours to kill
154 extracellular bacteria (time point 2h post infection). At this point cells were lysed, as
155 described, or further incubated in the presence of 25 $\mu\text{g ml}^{-1}$ gentamicin for 19h (time point
156 21 h post infection) and then lysed. The number of intracellular bacteria was determined at
157 each time point by CFU ml^{-1} calculations (supplementary figure). A SPI2 mutant (ΔssaV)
158 [25] unable to propagate intracellularly [4, 23] was used as control.

159

160 *2.5. Infection of mice.*

161 Infection of 6 week old $i\text{NOS}^{-/-}$ (B6.129P2-*Nos2tm1Lau/J*) mice (Jackson Laboratory) or
162 C57BL/6 mice was done as described previously [13]. Briefly, four mice were infected
163 intraperitoneally with a 1:1 mixture of 5×10^2 CFU for $i\text{NOS}^{-/-}$ mice and 5×10^3 CFU for
164 C57BL/6 mice of each the wild type and the mutant strain. After 6 days mice were
165 euthanized by cervical dislocation and spleens were removed aseptically and mechanically

166 homogenized in saline. Serial dilutions were made and plated on LB agar plates. 100
167 randomly picked colonies of both the inoculum and the spleen samples from each mouse
168 were streaked on LB agar plates containing the appropriate antibiotic to determine the
169 proportion of the mutant to the wild type strain. The competitive index was calculated as
170 reported [13].

171

172 *2.6. Ethics Statement*

173 All mouse experiments were reviewed and approved by the Copenhagen University animal
174 experimentation unit and conducted with permission from the Animal Experiments
175 Inspectorate (<http://www.dyreforsogstilsynet.dk>) under license number 2013-15-2934-
176 00761 in accordance with Danish law LBK 474 af 15/05/2014 (Animal experimentation and
177 welfare act).

178

179 *2.7. Statistical analyses.*

180 Outliers with a significance of 0.05 were identified by Grubb's outlier test and removed
181 before further analysis. Statistical significance of differences between datasets was
182 determined using the GraphPad Prism® software, version 5.0 (GraphPad) using either
183 ANOVA with correction for multiple comparison with Tukey's post-test or Students T-test.

184

185 **3. Results and Discussion**

186 *3.1. Polyamines are required for replication in cultured macrophages*

187 We have previously reported that a polyamine-biosynthesis mutant is unable to cause
188 systemic disease in a mouse model of typhoid fever and furthermore that the mutant was
189 unable to replicate inside cultured epithelial cells during an 8 hr period post infection [13].

190 However, as the mutant also was severely reduced for invasion of epithelial cells and as
191 macrophages is the primary cell type that *S. Typhimurium* is associated with during
192 systemic infections, the ability of the polyamine mutant to replicate inside cultured
193 macrophage-like cells, J774A.1 was investigated, using a gentamicin protection assay as
194 previously described [25]. Intracellular bacteria were enumerated at t=2 hrs and t=21 hrs
195 post infection (Supplemental figure 2). During this 19 hr period, the wild type replicated
196 16.5 fold with some variation between experiments (Fig. 1). This is consistent with
197 previous observations [25]. The negative control, the Δ ssaV-mutant (SPI2-deficient),
198 exhibited a 2 fold replication as expected [4]. The polyamine mutant was able to replicate
199 5.1 fold, a significant reduction compared to the wild type (P=0.0018), but not completely
200 deficient for intracellular replication (Fig. 1).

201

202 **Figure 1: Intracellular survival and replication of the polyamine biosynthesis mutant**
203 **inside J774A.1 macrophages.** Intracellular replication in J774A.1 macrophage-like cells
204 of the wild type strain, the isogenic polyamine mutant (Δ speBCEF) and the Δ ssaV-mutant
205 (SPI2-mutant) was assayed between 2 hrs post infection and 21 hrs post infection (pi).
206 The bars shows fold change in intracellular CFU between these two time points. The SPI2-
207 mutant is attenuated for intracellular replication as expected. The experiment was
208 performed four times with similar results. Shown is an average of the results. Error-bars
209 are standard deviation * Indicate significant differences to the wild type, P< 0.05.

210

211 Compared to our previous results on the polyamine mutant's inability to replicate inside
212 epithelial cells, the present results could indicate that either the mutant has a delayed
213 onset of replication not sampled in the shorter time-frame of the epithelial assay (8 hrs) or
214 that the reduced ability to infect the epithelial cells affect the subsequent intracellular
215 replication. However, for both macrophages and epithelial cells we conclude that the

216 polyamine biosynthesis mutant is significantly reduced for intracellular replication, a
217 hallmark of *S. Typhimurium* systemic spread.

218

219 3.2. Polyamines have minor role in protection against oxidative stress

220 One of the first antimicrobial responses of phagocytic cells is the production of the
221 bactericidal superoxide by the NADPH oxidase. In *E. coli* it has been shown that
222 polyamines protect against oxidative stress [3], and it is therefore possible that the
223 reduced intracellular survival of the polyamine mutant is linked to reduced oxidative stress-
224 resistance. To investigate this hypothesis, bacterial cultures of the wild type and the
225 polyamine biosynthesis mutant were subjected to oxidative stress (Fig. 2).

226

227 **Figure 2: Oxidative stress survival of the polyamine biosynthesis mutant.** Indicated
228 strains were grown in M9 minimal media with (green symbols) or without (blue symbols) 70
229 μM H_2O_2 (graphs A to E). For D and E 6.8 μM spermidine (spd) or 11.3 μM putrescine
230 (put), respectively, were added to the cultures to test for exogenous complementation. F)
231 Disk inhibition assay. The indicated strains were spread on M9-agar-plates and a disk
232 soaked in H_2O_2 was placed on top of the agar plate. Where indicated 6.8 μM spermidine
233 (spd) was added to the agar plate. After overnight incubation, the growth inhibition zone
234 diameter was measured. All experiments were performed three times with similar results.
235 For A to E a representative experiment is shown, for F, an average of three experiments is
236 shown, error bars are standard deviation. * Indicate significant differences to the wt, $P <$
237 0.00001.

238

239 During exponential growth, the peroxide H_2O_2 was added to actively growing cultures of
240 the wild type and polyamine mutant (Fig. 2A, B, C, D, and E). In all conditions tested, the
241 cultures are affected by oxidative stress. All strains reach a lower optical density when
242 grown in the presence of H_2O_2 in stationary phase compared to growth without H_2O_2 . For

243 the polyamine biosynthesis mutant, the growth-rate is also reduced in response to the
244 stress, however, not at a statistically significant level (Fig. 2B). The slight reduction in
245 growth rate is complemented by a plasmid expressing the putrescine biosynthesis gene
246 (*speB*) (Fig. 2C), and by addition of either spermidine (Fig. 2D) or putrescine (Fig. 2E) to
247 the cultures, respectively. In contrast, when stationary cultures are exposed to peroxide
248 using a disk inhibition assay there is a statistically significant ($P < 0.00001$) reduced
249 tolerance towards H_2O_2 in the polyamine biosynthesis mutant (Fig. 2F). In conclusion,
250 polyamines have a minor role in protection against peroxide stress in *S. Typhimurium*.

251

252 3.3. Polyamines biosynthesis is required for growth in the presence of reactive nitrogen 253 species

254 After about 5 hrs of infection of cultured macrophages, the inducible nitric oxide synthase,
255 iNOS, (or NOS2), facilitates production of nitric oxide that is bacteriostatic towards
256 intracellular *Salmonella* [17], whereas the reaction of NO and superoxide generates the
257 bactericidal compound peroxynitrite $ONOO^-$. To investigate if polyamines are involved in
258 coping with reactive nitrogen species in *S. Typhimurium*, bacterial cultures of the wild type
259 and the polyamine biosynthesis mutant were subjected to two types of reactive nitrogen
260 species, S-Nitrosoglutathione (GSNO), a bacteriostatic compound (Fig. 3B) and
261 peroxynitrite (PN), a bactericidal compound (Fig. 3C).

262

263 **Figure 3: Nitrosative stress survival.** Indicated strains were grown in M9 (A), M9 with 4
264 mM S-Nitrosoglutathione (GSNO) (B) or in M9 with 200 μ M peroxynitrite (PN) (C). PN was
265 added to logarithmic cultures at the indicated time. The experiments were performed three
266 times with similar results.

267

268 Addition of S-Nitrosoglutathione to growing cultures severely inhibits growth of the
269 polyamine mutant compared to the wild type (Fig. 3B). This inhibition is relieved by
270 addition of putrescine or spermidine to the cultures and by genetic complementation of
271 putrescine biosynthesis (the *speB*-gene). Similarly, addition of peroxyntirite to growing
272 cultures completely attenuates growth of the polyamine mutant (Fig. 3C). Together, these
273 data show that in *S. Typhimurium*, polyamines are essential for protection against the toxic
274 effects of reactive nitrogen species. The biochemical and genetic complementation of
275 these phenotypes strongly indicate that the increased stress sensitivity of the polyamine
276 biosynthesis mutant is, in fact, caused by the lack of polyamines in this strain when grown
277 in media without polyamines. Further, recovery from peroxyntirite stress (Fig 3C) is
278 enhanced, compared to the wild type, by the exogenous addition of polyamines as well as
279 by the presence of the putrescine biosynthesis gene, *speB*, encoded *in trans*. This could
280 indicate that polyamines confer partial protection against peroxyntirite stress in *S.*
281 *Typhimurium*. Similarly, in uropathogenic *E. coli* it has been reported that polyamines
282 rescue growth in the presence of reactive nitrogen species [2].

283

284 *3.4. Polyamine biosynthesis is required for systemic infection of mice independently of* 285 *host-produced nitric-oxide*

286 We have previously shown that polyamines are required for virulence of *S. Typhimurium* in
287 the mouse model of systemic infection [13]. To investigate if the reduced virulence of the
288 polyamine mutant is linked to its increased sensitivity to reactive nitrogen species, mice
289 deficient for iNOS were infected with equal amounts of the wild type and the polyamine
290 biosynthesis mutant (Figure 4).

291

292 **Figure 4: Mice infections.** C57BL/6 or iNOS^{-/-} mice (as indicated above the bars) were
293 infected via the intraperitoneal route with equal numbers of indicated mutant and wild type
294 bacteria (5×10^3 CFU for C57BL/6 and 5×10^2 CFU for iNOS^{-/-}). After 6 days, mice were
295 sacrificed and spleens were removed. Competitive indices (C.I.) were calculated as
296 previously described [13]. The results are shown as mean values, error-bars are standard
297 deviation. Significant differences from 1.0 (*P<0.005, **P<0.001) were determined by two-
298 sample t-test analysis.

299

300 After 6 days of infection mice were euthanized and bacteria recovered from the spleens
301 were used to determine the competitive index as described previously [13]. The
302 competitive index of the polyamine mutant versus the wild type was calculated to be
303 0.06 ± 0.06 , significantly different from 1.0, P=0.002, indicating that polyamine
304 biosynthesis is required for virulence of *S. Typhimurium* even in the absence of host
305 produced reactive nitrogen species.

306

307 3.5. Spermidine biosynthesis and transport is required for systemic infections of mice

308 In the polyamine biosynthesis mutant, both putrescine and spermidine biosynthesis genes
309 are deleted. However, as the two polyamines differ in their net charge, with spermidine
310 having three amine groups and putrescine only two [10], it could affect interactions with
311 other molecules. Hence, the two polyamines may have distinct physiological roles in the
312 cell. In support of this, a distinct role for spermidine in virulence of the related intracellular
313 pathogen *Shigella spp*, has previously been reported [1]. Hence, to investigate if either of
314 the polyamines contributes differently to virulence of *S. Typhimurium*, we constructed a
315 spermidine biosynthesis mutant ($\Delta speE$), a spermidine transport mutant ($\Delta potCD$), and a
316 spermidine biosynthesis and spermidine transport double mutant ($\Delta speE; \Delta potCD$). This
317 double mutant is unable to synthesize and import spermidine, but retains the ability to

318 synthesize and import putrescine. Accordingly, this strain enables investigations into the
319 specific role of spermidine during infection. Construction of a putrescine defective strain
320 that retains the ability to synthesize spermidine is not possible as putrescine is the
321 substrate for spermidine biosynthesis (figure A.1).

322 During competitive infection of both C57BL/6 mice and iNOS^{-/-} mice, the $\Delta speE;\Delta potCD$
323 mutant was severely and significantly attenuated with competitive indices versus the wild
324 type of 0.1 (P=0.0002) and 0.14 (P=0.0002), respectively (Fig 4). Interestingly, neither the
325 spermidine biosynthesis mutant ($\Delta speE$) nor the spermidine transport mutant ($\Delta potCD$) is
326 attenuated for infection of mice, indicating functional redundancy for providing sufficient
327 spermidine during infection between the biosynthesis- and uptake-pathways. This is
328 further supported by the ability of the *potCD* genes to complement the virulence of the
329 $\Delta speE;\Delta potCD$ mutant when provided *in trans*. These data could suggest that the
330 presence of either the PotABCD transporter or the SpeE spermidine biosynthesis enzyme
331 is sufficient for virulence. However, our previous results of attenuation of the polyamine
332 biosynthesis mutant ($\Delta speB;\Delta speC;\Delta speE;\Delta speF$) as well as attenuation of a complete
333 polyamine transporter mutant, a $\Delta potCD;\Delta potE;\Delta potI$ mutant strain, [13] contradict this
334 notion and point to a highly sensitive requirement for polyamines for systemic infection
335 where even minor imbalances from a few genetic lesions of polyamine transport or
336 biosynthesis genes cause virulence attenuation.

337

338 Further phenotypic characterization of the spermidine biosynthesis and transporter mutant
339 ($\Delta speE;\Delta potCD$) revealed that, in contrast to the putrescine and spermidine biosynthesis
340 mutant ($\Delta speB;\Delta speC;\Delta speE;\Delta speF$), the spermidine mutant ($\Delta speE;\Delta potCD$) does not
341 have a growth defect in M9 (Fig 5A) and it is not more sensitive to oxidative (5B) or

342 nitrosative stress (5C) than the wild type, whereas replication in macrophages was slightly
343 and significantly reduced for the spermidine mutant (7.4 fold replication) compared to the
344 wild type (16.5 fold replication), $P=0.0442$ (Fig 5D).

345

346 **Figure 5: Phenotypic characterization of the spermidine mutant.** A) Growth of the wild
347 type strain, and the isogenic spermidine mutant ($\Delta speE; \Delta potCD$) in M9 minimal media.
348 After overnight growth in M9 minimal media bacteria were subcultured into fresh M9 and
349 re-incubated. Growth was monitored using a Bioscreen C plate reader. B) The spermidine
350 mutant was grown in M9 minimal media with (green symbols) or without (blue symbols) 70
351 μM H_2O_2 . The differences in final biomass reached between (A) and (B) are most likely due
352 to the fact that different stocks of M9-media were used for these experiments. C) Growth in
353 M9 with 200 μM peroxyntirite (PN). PN was added to logarithmic cultures at the indicated
354 times. The experiments were performed three times with similar results. D) Intracellular
355 replication in J774A.1 macrophage-like cells of the wild type strain, the spermidine mutant
356 and the $\Delta ssaV$ -mutant (SPI2-mutant) was assayed between 2 hrs post infection and 21 hrs
357 post infection (pi). The bars shows fold change in intracellular CFU between these two
358 time points. The experiment was performed four times with similar results. Shown is an
359 average of the results. Error-bars are standard deviation * Indicate significant differences
360 to the wild type, $P < 0.05$.

361

362

363 Together, our data signify that spermidine, independently of putrescine, is promoting
364 virulence in *S. Typhimurium*. Furthermore, our *in vitro* growth experiments point to that
365 attenuation of virulence of the spermidine deficient strain is not related to a growth defect
366 in the absence of spermidine. Interestingly, the results also signify that putrescine alone is
367 not sufficient for virulence of *S. Typhimurium*.

368

369

370 **4. Conclusions**

371 It has previously been reported that in *S. Typhimurium* polyamine biosynthesis genes are
372 induced during infection of mammalian cells indicating a role for polyamines in intracellular
373 replication [8]. In a previous study, we showed that polyamine biosynthesis is indeed
374 essential for systemic infection of mice [13]. In the present study we have investigated if
375 polyamines confer protection against oxidative and nitrosative stress and whether this
376 ability contributes to the reduced virulence of a polyamine biosynthesis mutant [13]. While
377 we find that polyamines are essential for coping with nitrosative stress during growth in M9
378 we do not see an improved infection potential of the polyamine biosynthesis mutant in
379 mice lacking the ability to produce reactive nitrogen species (*iNOS*^{-/-} mice) compared to
380 our previous observations with mice proficient for this. This indicates that while polyamine
381 mediated stress-resistance possibly contribute to virulence of *S. Typhimurium*, polyamines
382 have additional roles in *S. Typhimurium* during infection. Interestingly, our results also
383 reveal a unique role for spermidine during infection. This is similar to the reported
384 spermidine requirement of the related enteric pathogen *Shigella* spp. [1].

385

386 The essentiality for polyamines during infection of several bacterial pathogens is
387 underscored by the diverse mechanisms employed to secure acquisition of polyamines
388 during infection. For example, in *Legionella pneumophila*, a water borne intracellular
389 pathogen, intracellular replication depends on polyamines [18]. This bacterium does not
390 have the functional genes for polyamine biosynthesis, but instead uses a unique
391 chaperone to recruit a polyamine biosynthesis protein, S-adenosylmethionine
392 decarboxylase, from the host cell. In another example, the intracellular enteric pathogen
393 *Shigella* spp. accumulates spermidine due to evolutionary inactivation of the gene
394 encoding the spermidine acetyltransferase, an enzyme catalyzing the conversion of

395 spermidine into the physiologically inert acetylspermidine [1]. And in *S. Typhimurium*, the
396 requirement for polyamines during infection is controlled by regulation of gene expression
397 of polyamine biosynthesis genes [8]. Furthermore, in the present and previous studies [12,
398 13, 20], we have shown that minor imbalances by single gene deletions only have
399 negligible effects, if any, on virulence, whereas, deletions of multiple polyamine genes
400 results in virulence attenuation. These results point to functional redundancies between
401 the polyamine genes, and an absolute requirement for sufficient supplies of polyamines
402 during infection, accommodated either through uptake, biosynthesis or both. In conclusion,
403 polyamines are required for virulence of several intracellular pathogens. Furthermore,
404 these pathogens have evolved distinct mechanisms (regulation of biosynthesis genes,
405 sequestration of host cell biosynthesis enzymes, and reduced degradation of polyamines)
406 to meet their shared requisite for polyamines during infection.

407

408 **Competing interests**

409 The authors declare that they have no competing interests.

410

411 **Authors contributions**

412 ICE carried out experiments, analysed the results and drafted the manuscript. PRG carried
413 out experiments and analysed the data. LJ conceived the study, carried out experiments,
414 analysed the data, provided materials, and wrote the manuscript. All authors read and
415 approved the final manuscript.

416

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505 **Supplemental figure A1:** Graphic presentation of the polyamine biosynthesis pathways
506 present in *S. Typhimurium*, reviewed in [21]. SAM: S-adenosylmethionine.

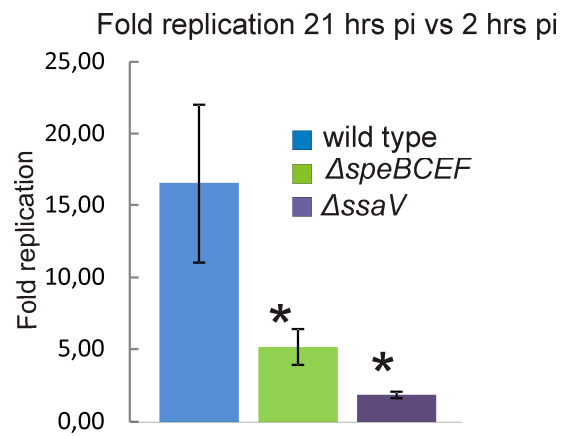
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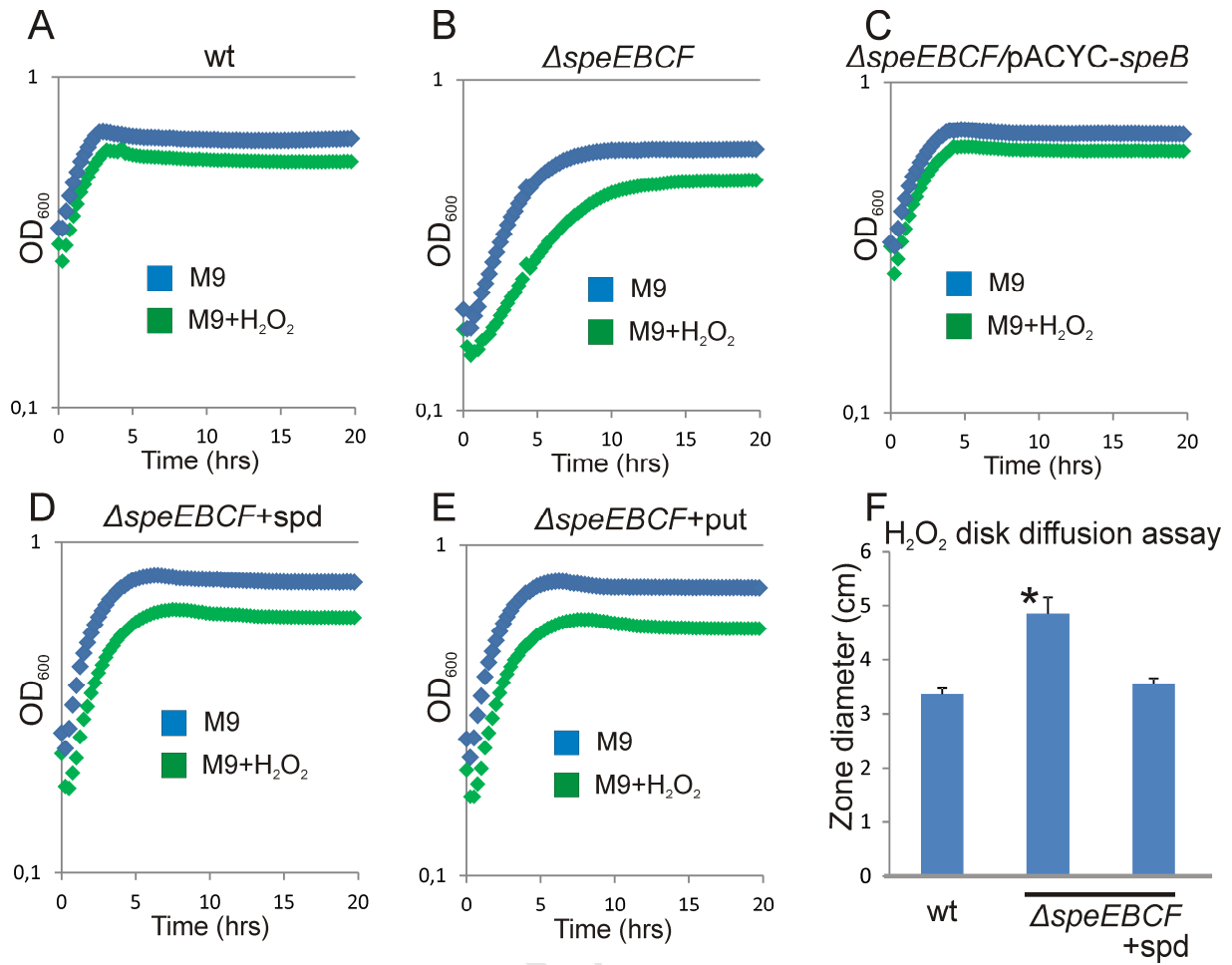
508 **Supplemental figure A2:** CFU counts for indicated strains at indicated time points post
509 infection of macrophages.

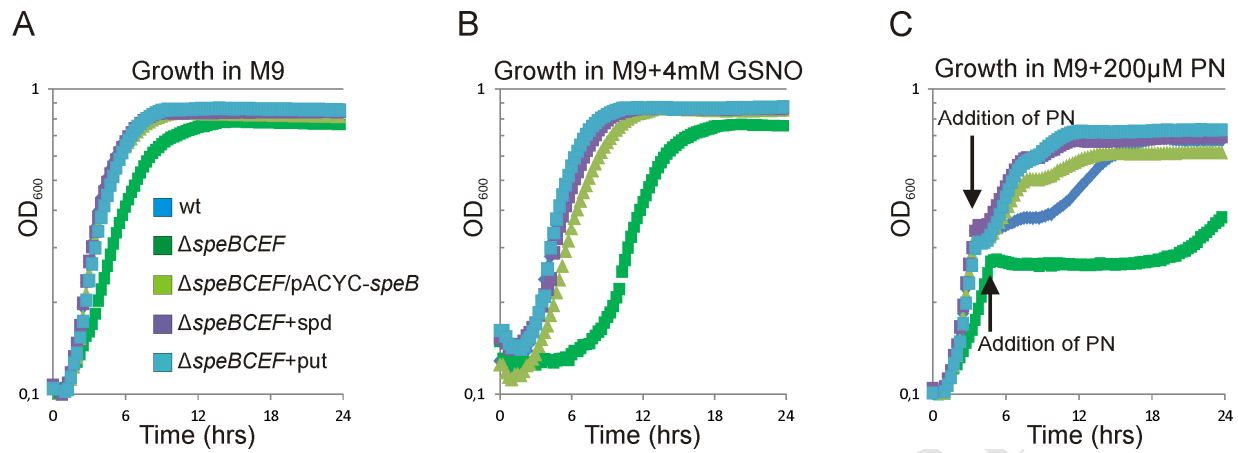
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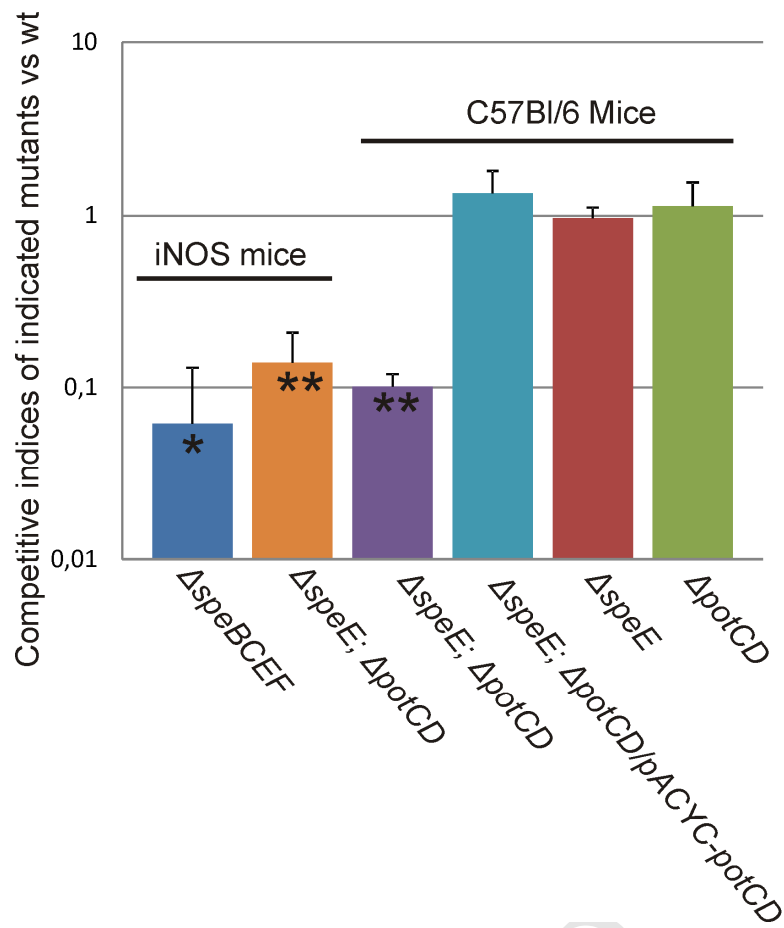
Table 1. Strains used in the study.

Strain	Relevant genotype	Reference
S. Typhimurium ST4/74	virulent reference strain	[24]
S. Typhimurium KP1274	Restriction deficient strain, used for introduction of plasmids.	[7]
LJ268	ST4/74:: Δ potCD; Δ speE. Kan ^R .	This work.
LJ318	ST4/74:: Δ speB; Δ speC; Δ speE; Δ speF. Cam ^R , Kan ^R .	[13]
LJ328	ST4/74:: Δ speB; Δ speC; Δ speE; Δ speF/pACYC-speB. Amp ^R , Cam ^R , Kan ^R .	[13]
LJ251	ST4/74:: Δ potCD.	This work.
LJ336	ST4/74:: Δ potCD; Δ speE/pACYC- potCD. Amp ^R , Kan ^R .	This work.
LJ238	ST4/74:: Δ speE. Cam ^R .	This work.
LJ607	ST4/74:: Δ ssaV. Kan ^R .	[25]



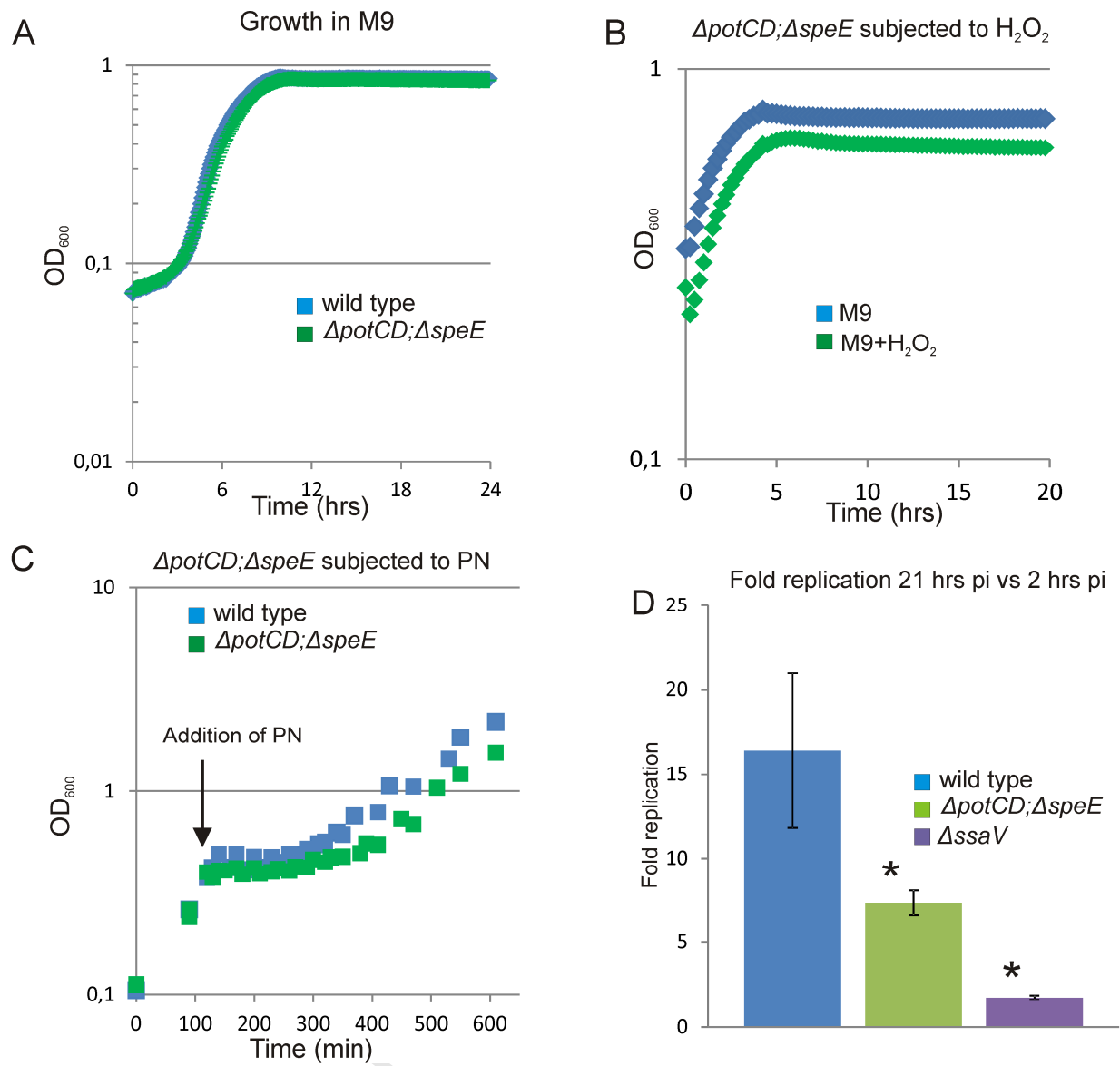






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Highlights for the manuscript entitled “Multiple roles of putrescine and spermidine in stress resistance and virulence of *Salmonella enterica* serovar Typhimurium” by authors Irene Cartas Espinel, Priscila Regina Guerra, and Lotte Jelsbak.

- Polyamines are required for resistance against nitrosative stress, but not oxidative stress.
- Polyamines are required for intracellular replication in macrophage-like cells.
- Polyamines are required for virulence in mice deficient for nitric oxide synthase.
- Spermidine is essential for virulence in mice, even in the presence of functional putrescine biosynthesis genes.