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1 2	Multiple roles of putrescine and spermidine in stress resistance and virulence of <i>Salmonella enterica</i> serovar Typhimurium
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11	
12 13	Keywords: Polyamines; nitric oxide stress; virulence; intracellular pathogens.
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

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

34

35 **1. Introduction**

Salmonella enterica serovar Typhimurium (S. Typhimurium) is a Gram-negative facultative 36 intracellular pathogen able to cause a wide variety of food- and water-borne diseases 37 ranging from self-limiting gastroenteritis to systemic and life-threatening infections. As S. 38 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 is dependent on the ability to survive and replicate inside host cells. Following type three 41 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. Typhimurium is able to survive and replicate within a membrane bound compartment. The 45 bacteria will rapidly spread through the lymphoid and blood systems to the spleen and liver 46 47 resulting in a life-threatening systemic infection. Formation of the intracellular niche and replication is facilitated by the Salmonella Pathogenicity Island 2 (SPI2) encoded type 48 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 generating a burst of the bactericidal superoxide (O2) radicals followed by a more 51 52 sustained activation of the inducible nitric oxide synthase (iNOS) generating the

- bacteriostatic nitric oxide (NO) radicals. Combination of superoxide and NO can produce
 the highly reactive and bactericidal product peroxynitrite (ONOO⁻).
- 55

Polyamines are small poly-cationic amines present in almost all cell-types. In recent years 56 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) or uptake by dedicated transporters. We have previously shown that polyamine 60 biosynthesis is essential for virulence of Salmonella [12, 13, 20]. This observation could be 61 linked to a severe reduction of the polyamine biosynthesis mutant's ability to invade and 62 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 oxidative and nitrosative stress. We find that the polyamine biosynthesis mutant is only 69 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 multiple roles in virulence of S. Typhimurium. 75

76

77 **2. Materials and methods**

- 78 2.1. Bacterial strains and growth conditions.
- 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
- and its virulence is well defined [24].

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

82

Construction of the polyamine biosynthesis mutant ($\Delta speB; \Delta speC; \Delta speE; \Delta speF$) has 83 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 using either pKD3 or pKD4 as templates for the PCR reaction, as previously described [5]. 87 Together these genes facilitate the biosynthesis of putrescine and spermidine in S. 88 Typhimurium (figure A.1). The spermidine biosynthesis mutant ($\Delta speE$), and the uptake 89 90 mutant ($\Delta potCD$) have been deleted for the speE gene (STM474_0175) and the potCDgenes (STM474 1220, STM474 1219), respectively. Primers used to construct and verify 91 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 was removed by use of the pCP20 encoded flippase [5]. All strains were verified using a 95 96 PCR-based sequencing strategy. Primers used for construction and verification of strains are listed in supplemental table 1. Genetic complementation of the biosynthesis mutant 97 98 was achieved by introducing pACYC-*speB*. We have previously achieved complementation of mice infections phenotypes of the biosynthesis mutant strain with this 99 100 plasmid [13]. Genetic complementation of the spermidine biosynthesis and transporter 101 mutant was achieved by introducing pACYC-potCD. Both plasmids are derivatives of pACYC177 containing the speB gene including upstream promoter regions or the potCD 102 genes expressed from the promoter of the pACYC177 kanamycin resistance gene. The 103 104 construction of these plasmids has been described in detail elsewhere [13]. 105 Bacterial strains were maintained in LB-Lennox broth (LB) with 15% glycerol at -80 °C. LB 106 107 agar plates (LB + 1.5 % agar) were used for growth on solid media. If not stated otherwise, growth in liquid medium was performed in M9 medium containing 12.8 g l⁻¹ Na₂HPO₄-108 12H₂0, 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 109 0.4 % w/v glucose. Prior to all experiments the bacteria were grown for 16 hrs, 200 rpm, 110 111 37°C in M9 minimal media to deplete for carry-over polyamines. Where indicated, M9 was supplemented with 100 μ g ml⁻¹ of either putrescine (11.3 mM) or spermidine (6.8 mM), 112 physiologically relevant concentrations [11]. When appropriate, media were supplemented 113 with antibiotics in the following concentrations: 50 µg ampicillin ml⁻¹, 50 µg kanamycin ml⁻¹ 114 and 10 μ g chloramphenicol ml⁻¹. 115

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). The next day 100 µl of overnight culture was spread on M9 agar plates. Sterile 13-mm 121 filter disks were placed in the center of agar plates, 10 μ l of 10% H₂O₂ (Sigma Aldrich) was 122 added to the disks, and the plates were incubated at 37°C overnight. The diameter of the 123 124 zone of growth inhibition was measured. Three replicate assays were performed for each strain, and the data were subjected to Student's t-test to evaluate their statistical 125 significance. For logarithmic bacteria, after overnight growth in M9, as described above, 126 127 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℃ 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.

Resistance towards nitric oxide stress was investigated in growth experiments in the 134 presence of either S-Nitrosoglutathione (Sigma-Aldrich) or peroxynitrite (Caymen 135 136 Chemicals) using logarithmic cultures. To determine the exact concentration of peroxynitrite, absorbance at 302 nm (A) was measured and the concentration C (C = A/ 137 $(\epsilon \cdot L)$) was calculated based on the extinction coefficient $\epsilon = 1670 \text{ M}^{-1} \text{ cm}^{-1}$. Growth in the 138 139 presence of S-Nitrosoglutathione was performed similar to growth in the presence of H_2O_2 140 as described above. For investigations of growth in the presence of peroxynitrite, 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° with shaking.
143	When the cultures had entered logarithmic phase (OD $_{600} {\sim} 0.4$) 200 μM peroxynitrite was
144	added to the cultures and they were re-incubated at 37° 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 μ 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 μ 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 ($\Delta ssaV$)
158	[25] unable to propagate intracellularly [4, 23] was used as control.

159

160 2.5. Infection of mice.

Infection of 6 week old *iNOS^{-/-}* (B6.129P2-*Nos2tm1Lau*/J) mice (Jackson Laboratory) or
C57BL/6 mice was done as described previously [13]. Briefly, four mice were infected
intraperitoneally with a 1:1 mixture of 5x10² CFU for iNOS-/- mice and 5x10³ CFU for
C57BL/6 mice of each the wild type and the mutant strain. After 6 days mice were
euthanized by cervical dislocation and spleens were removed aseptically and mechanically

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

Outliers with a significance of 0.05 were identified by Grubb's outlier test and removed before further analysis. Statistical significance of differences between datasets was 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

systemic disease in a mouse model of typhoid fever and furthermore that the mutant was

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 $\Delta ssaV$ -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

Figure 1: Intracellular survival and replication of the polyamine biosynthesis mutant 202 inside J774A.1 macrophages. Intracellular replication in J774A.1 macrophage-like cells 203 204 of the wild type strain, the isogenic polyamine mutant ($\Delta speBCEF$) and the $\Delta ssaV$ -mutant (SPI2-mutant) was assayed between 2 hrs post infection and 21 hrs post infection (pi). 205 206 The bars shows fold change in intracellular CFU between these two time points. The SPI2mutant is attenuated for intracellular replication as expected. The experiment was 207 208 performed four times with similar results. Shown is an average of the results. Error-bars are standard deviation * Indicate significant differences to the wild type, P< 0.05. 209 210

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

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

bactericidal superoxide by the NADPH oxidase. In *E. coli* it has been shown that

polyamines protect against oxidative stress [3], and it is therefore possible that the

reduced intracellular survival of the polyamine mutant is linked to reduced oxidative stress-

resistance. To investigate this hypothesis, bacterial cultures of the wild type and the

polyamine biosynthesis mutant were subjected to oxidative stress (Fig. 2).

226

Figure 2: Oxidative stress survival of the polyamine biosynthesis mutant. Indicated 227 strains were grown in M9 minimal media with (green symbols) or without (blue symbols) 70 228 229 μ M H₂O₂ (graphs A to E). For D and E 6.8 μ M spermidine (spd) or 11.3 μ M 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 μ M spermidine (spd) was added to the agar plate. After overnight incubation, the growth inhibition zone 233 234 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

During exponential growth, the peroxide H_2O_2 was added to actively growing cultures of the wild type and polyamine mutant (Fig. 2A, B, C, D, and E). In all conditions tested, the cultures are affected by oxidative stress. All strains reach a lower optical density when 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	
250	intracellular Salmonella [17], whereas the reaction of NO and superoxide generates the
250	intracellular <i>Salmonella</i> [17], whereas the reaction of NO and superoxide generates the bactericidal compound peroxynitrite ONOO ⁻ . To investigate if polyamines are involved in
257 258	intracellular <i>Salmonella</i> [17], whereas the reaction of NO and superoxide generates the bactericidal compound peroxynitrite ONOO ⁻ . To investigate if polyamines are involved in coping with reactive nitrogen species in <i>S</i> . Typhimurium, bacterial cultures of the wild type
250 257 258 259	intracellular <i>Salmonella</i> [17], whereas the reaction of NO and superoxide generates the bactericidal compound peroxynitrite ONOO ⁻ . To investigate if polyamines are involved in coping with reactive nitrogen species in <i>S</i> . Typhimurium, bacterial cultures of the wild type and the polyamine biosynthesis mutant were subjected to two types of reactive nitrogen
257 258 259 260	intracellular <i>Salmonella</i> [17], whereas the reaction of NO and superoxide generates the bactericidal compound peroxynitrite ONOO ⁻ . To investigate if polyamines are involved in coping with reactive nitrogen species in <i>S</i> . Typhimurium, bacterial cultures of the wild type and the polyamine biosynthesis mutant were subjected to two types of reactive nitrogen species, S-Nitrosoglutathione (GSNO), a bacteriostatic compound (Fig. 3B) and
257 258 259 260 261	intracellular <i>Salmonella</i> [17], whereas the reaction of NO and superoxide generates the bactericidal compound peroxynitrite ONOO ⁻ . To investigate if polyamines are involved in coping with reactive nitrogen species in <i>S</i> . Typhimurium, bacterial cultures of the wild type and the polyamine biosynthesis mutant were subjected to two types of reactive nitrogen species, S-Nitrosoglutathione (GSNO), a bacteriostatic compound (Fig. 3B) and peroxynitrite (PN), a bactericidal compound (Fig. 3C).

Figure 3: Nitrosative stress survival. Indicated strains were grown in M9 (A), M9 with 4 mM S-Nitrosoglutathione (GSNO) (B) or in M9 with 200µM peroxynitrite (PN) (C). PN was added to logarithmic cultures at the indicated time. The experiments were performed three 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 peroxynitrite 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 peroxynitrite 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 peroxynitrite 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

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

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

291

Figure 4: Mice infections. C57BL/6 or iNOS-/- mice (as indicated above the bars) were infected via the intraperitoneal route with equal numbers of indicated mutant and wild type bacteria (5x10³ CFU for C57BL/6 and 5x10² CFU for iNOS-/-). After 6 days, mice were sacrificed and spleens were removed. Competitive indices (C.I.) were calculated as previously described [13]. The results are shown as mean values, error-bars are standard deviation. Significant differences from 1.0 (*P<0.005, **P<0.001) were determined by twosample t-test analysis.

299

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

306

3.5. Spermidine biosynthesis and transport is required for systemic infections of mice 307 308 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 having three amine groups and putrescine only two [10], it could affect interactions with 310 other molecules. Hence, the two polyamines may have distinct physiological roles in the 311 312 cell. In support of this, a distinct role for spermidine in virulence of the related intracellular pathogen Shigella spp, has previously been reported [1]. Hence, to investigate if either of 313 the polyamines contributes differently to virulence of S. Typhimurium, we constructed a 314 spermidine biosynthesis mutant ($\Delta speE$), a spermidine transport mutant ($\Delta potCD$), and a 315 316 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

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

nitrosative stress (5C) than the wild type, whereas replication in macrophages was slightly
 and significantly reduced for the spermidine mutant (7.4 fold replication) compared to the

wild type (16.5 fold replication), P=0.0442 (Fig 5D).

345

Figure 5: Phenotypic characterization of the spermidine mutant. A) Growth of the wild 346 347 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 mutant was grown in M9 minimal media with (green symbols) or without (blue symbols) 70 350 351 μ M H₂O₂. 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 M9 with 200µM peroxynitrite (PN). PN was added to logarithmic cultures at the indicated 353 354 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 *AssaV*-mutant (SPI2-mutant) was assayed between 2 hrs post infection and 21 hrs post infection (pi). The bars shows fold change in intracellular CFU between these two 357 358 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 to the wild type, P < 0.05. 360

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

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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].

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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, polyamines are required for virulence of several intracellular pathogens. Furthermore, 403 404 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.

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411 Authors contributions

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

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

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

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

Table 1. Strains used in the study.













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.