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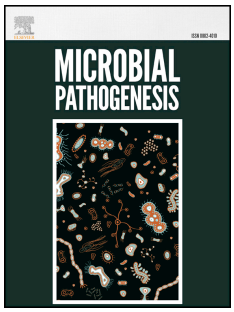

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Multiple roles of putrescine and spermidine in stress resistance and virulence of *Salmonella enterica* serovar Typhimurium

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Abstract

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

1. Introduction

Salmonella enterica serovar Typhimurium (*S*. Typhimurium) is a Gram-negative facultative intracellular pathogen able to cause a wide variety of food- and water-borne diseases ranging from self-limiting gastroenteritis to systemic and life-threatening infections. As *S*. Typhimurium, causes a systemic typhoid-like disease in mice, it serves as an important 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 secretion mediated invasion of the epithelial cell-layer [15], *S*. Typhimurium bacteria are able to escape to the underlying layer where they are taken up by macrophages and 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 bacteria will rapidly spread through the lymphoid and blood systems to the spleen and liver 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 three secretion system and its secreted virulence factors [22]. Inside the macrophages, *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 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 ($ONOO^-$).

Polyamines are small poly-cationic amines present in almost all cell-types. In recent years they have emerged as major modulators of bacterial physiology, including biofilm formation and motility and are essential for virulence of several bacterial pathogens [1, 6, 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 biosynthesis is essential for virulence of *Salmonella* [12, 13, 20]. This observation could be linked to a severe reduction of the polyamine biosynthesis mutant´s ability to invade and survive/replicate inside cultured epithelial cells. However, despite a modest reduction in virulence gene expression [13], the mechanisms for polyamine dependent systemic *Salmonella* infections remain elusive. In recent papers it has been demonstrated that polyamines can protect against oxidative and nitrosative stress in other bacterial species [3, 14] providing a possible link to polyamine dependent virulence of *S*. Typhimurium. In 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 modestly affected, at a level similar to the wild type, by oxidative stress. In contrast, the mutant is severely affected by the presence of two different nitrosative stresses (NO and $ONOO^-$). However, during infection of mice lacking the iNOS system, the polyamine biosynthesis mutant is still highly attenuated pointing to that although polyamines may contribute to resistance against nitrosative stress during infection, polyamines have multiple roles in virulence of *S.* Typhimurium.

2. Materials and methods

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 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 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]

Construction of the polyamine biosynthesis mutant (Δ*speB*;Δ*speC*;Δ*speE*;Δ*speF*) has been previously described [13]. The strain is deleted for the *speB* gene (STM474_3225), the *speC* gene (STM474_3262), the *speF* gene (STM474_0722), and the *speE* gene (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]. Together these genes facilitate the biosynthesis of putrescine and spermidine in *S*. Typhimurium (figure A.1). The spermidine biosynthesis mutant (Δ*speE*), and the uptake mutant (Δ*potCD*) have been deleted for the *speE* gene (STM474_0175) and the *potCD*-genes (STM474_1220, STM474_1219), respectively. Primers used to construct and verify these strains can be found in table A.1. The spermidine biosynthesis and uptake mutant (Δ*speE*;Δ*potCD*) was generated by P22 phage mediated transduction of the *ΔspeE* locus

into the Δ*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 PCR-based sequencing strategy. Primers used for construction and verification of strains are listed in supplemental table 1. Genetic complementation of the biosynthesis mutant was achieved by introducing pACYC-*speB*. We have previously achieved complementation of mice infections phenotypes of the biosynthesis mutant strain with this plasmid [13]. Genetic complementation of the spermidine biosynthesis and transporter mutant was achieved by introducing pACYC-*potCD*. Both plasmids are derivatives of pACYC177 containing the *speB* gene including upstream promoter regions or the *potCD* genes expressed from the promoter of the pACYC177 kanamycin resistance gene. The construction of these plasmids has been described in detail elsewhere [13].

Bacterial strains were maintained in LB-Lennox broth (LB) with 15% glycerol at -80 ℃. LB 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} Na_2HPO_4-$12H_20$, 3.0 g l^{-1} KH_2PO_4, 0.5 g l^{-1} NaCl, 1.0 g l^{-1} NH_4Cl, 0.1 mM $CaCl_2$, 2 mM $MgSO_4$ and 0.4 % w/v glucose. Prior to all experiments the bacteria were grown for 16 hrs, 200 rpm, 37℃ in M9 minimal media to deplete for carry-over polyamines. Where indicated, M9 was supplemented with 100µg ml^{-1} of either putrescine (11.3 mM) or spermidine (6.8 mM), physiologically relevant concentrations [11]. When appropriate, media were supplemented with antibiotics in the following concentrations: 50 µg ampicillin ml^{-1}, 50 µg kanamycin ml^{-1} and 10 µg chloramphenicol ml^{-1}.

2.2. Resistance towards oxidative stress.

Resistance towards reactive oxygen species was investigated for both logarithmic and stationary phase bacterial cultures. For stationary-phase bacteria a disk inhibition assay 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 filter disks were placed in the center of agar plates, 10 µl of 10% H_2O_2 (Sigma Aldrich) was added to the disks, and the plates were incubated at 37℃ overnight. The diameter of the 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 significance. For logarithmic bacteria, after overnight growth in M9, as described above, bacteria were harvested, washed in saline and sub-cultured in M9 media with or without 70 µM H_2O_2. The bacterial cultures were incubated at 37℃ wit h shaking in a Bioscreen C reader (Thermo Labsystems) for 24 hrs. Growth was monitored every 15 minutes for the duration of the experiment. Three replicate assays were performed for each strain/condition.

2.3. Resistance towards nitric oxide stress.

Resistance towards nitric oxide stress was investigated in growth experiments in the presence of either S-Nitrosoglutathione (Sigma-Aldrich) or peroxynitrite (Caymen Chemicals) using logarithmic cultures. To determine the exact concentration of peroxynitrite, absorbance at 302 nm (A) was measured and the concentration C (C = A/ (ε·L)) was calculated based on the extinction coefficient ε = 1670 M^{-1} cm^{-1}. Growth in the presence of S-Nitrosoglutathione was performed similar to growth in the presence of H_2O_2 as described above. For investigations of growth in the presence of peroxynitrite, after overnight growth in M9, as described above, bacteria were harvested, washed in saline

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

2.4. Infection of macrophage-like cells.

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

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 $5x10^2$ CFU for iNOS-/- mice and $5x10^3$ 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].

2.6. Ethics Statement

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

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 ANOVA with correction for multiple comparison with Tukey´s post-test or Students T-test.

3. Results and Discussion

3.1. Polyamines are required for replication in cultured macrophages

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].

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

Figure 1: Intracellular survival and replication of the polyamine biosynthesis mutant inside J774A.1 macrophages. Intracellular replication in J774A.1 macrophage-like cells of the wild type strain, the isogenic polyamine mutant (*ΔspeBCEF*) and the *ΔssaV*-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 time points. The SPI2-mutant is attenuated for intracellular replication as expected. The experiment was 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.

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

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

3.2. Polyamines have minor role in protection against oxidative stress

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).

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

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

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

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

After about 5 hrs of infection of cultured macrophages, the inducible nitric oxide synthase, iNOS, (or NOS2), facilitates production of nitric oxide that is bacteriostatic towards intracellular *Salmonella* [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 *S*. 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.

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

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).

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 ($5x10^3$ CFU for C57BL/6 and $5x10^2$ 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 two-sample t-test analysis.

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.

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

In the polyamine biosynthesis mutant, both putrescine and spermidine biosynthesis genes 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 other molecules. Hence, the two polyamines may have distinct physiological roles in the 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 the polyamines contributes differently to virulence of *S*. Typhimurium, we constructed a spermidine biosynthesis mutant (*ΔspeE*), a spermidine transport mutant (*ΔpotCD*), and a spermidine biosynthesis and spermidine transport double mutant (*ΔspeE*;*ΔpotCD*). This double mutant is unable to synthesize and import spermidine, but retains the ability to

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

During competitive infection of both C57BL/6 mice and iNOS-/- mice, the *ΔspeE*;*ΔpotCD* mutant was severely and significantly attenuated with competitive indices versus the wild type of 0.1 (P=0.0002) and 0.14 (P=0.0002), respectively (Fig 4). Interestingly, neither the spermidine biosynthesis mutant (*ΔspeE*) nor the spermidine transport mutant (*ΔpotCD*) is attenuated for infection of mice, indicating functional redundancy for providing sufficient spermidine during infection between the biosynthesis- and uptake-pathways. This is further supported by the ability of the *potCD* genes to complement the virulence of the *ΔspeE*;*ΔpotCD* mutant when provided *in trans*. These data could suggest that the presence of either the PotABCD transporter or the SpeE spermidine biosynthesis enzyme is sufficient for virulence. However, our previous results of attenuation of the polyamine biosynthesis mutant (*ΔspeB;ΔspeC;ΔspeE*;*ΔspeF*) as well as attenuation of a complete polyamine transporter mutant, a *ΔpotCD;ΔpotE;ΔpotI* mutant strain, [13] contradict this notion and point to a highly sensitive requirement for polyamines for systemic infection where even minor imbalances from a few genetic lesions of polyamine transport or biosynthesis genes cause virulence attenuation.

Further phenotypic characterization of the spermidine biosynthesis and transporter mutant (*ΔspeE*;*ΔpotCD*) revealed that, in contrast to the putrescine and spermidine biosynthesis mutant (*ΔspeB*;*ΔspeC*;*ΔspeE*;*ΔspeF*), the spermidine mutant (*ΔspeE*;*ΔpotCD*) 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).

Figure 5: Phenotypic characterization of the spermidine mutant. A) Growth of the wild type strain, and the isogenic spermidine mutant (*ΔspeE;ΔpotCD*) in M9 minimal media. After overnight growth in M9 minimal media bacteria were subcultured into fresh M9 and 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 μM H_2O_2.The differences in final biomass reached between (A) and (B) are most likely due 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 times. The experiments were performed three times with similar results. D) Intracellular replication in J774A.1 macrophage-like cells of the wild type strain, the spermidine mutant and the *ΔssaV*-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 time points. The experiment was 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.

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.

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

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

Competing interests

The authors declare that they have no competing interests.

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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504

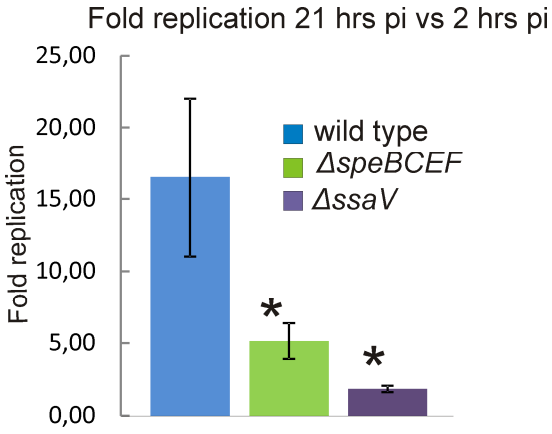
505 **Supplemental figure A1:** Graphic presentation of the polyamine biosynthesis pathways
506 present in *S*. Typhimurium, reviewed in [21]. SAM: S-adenosylmethionine.

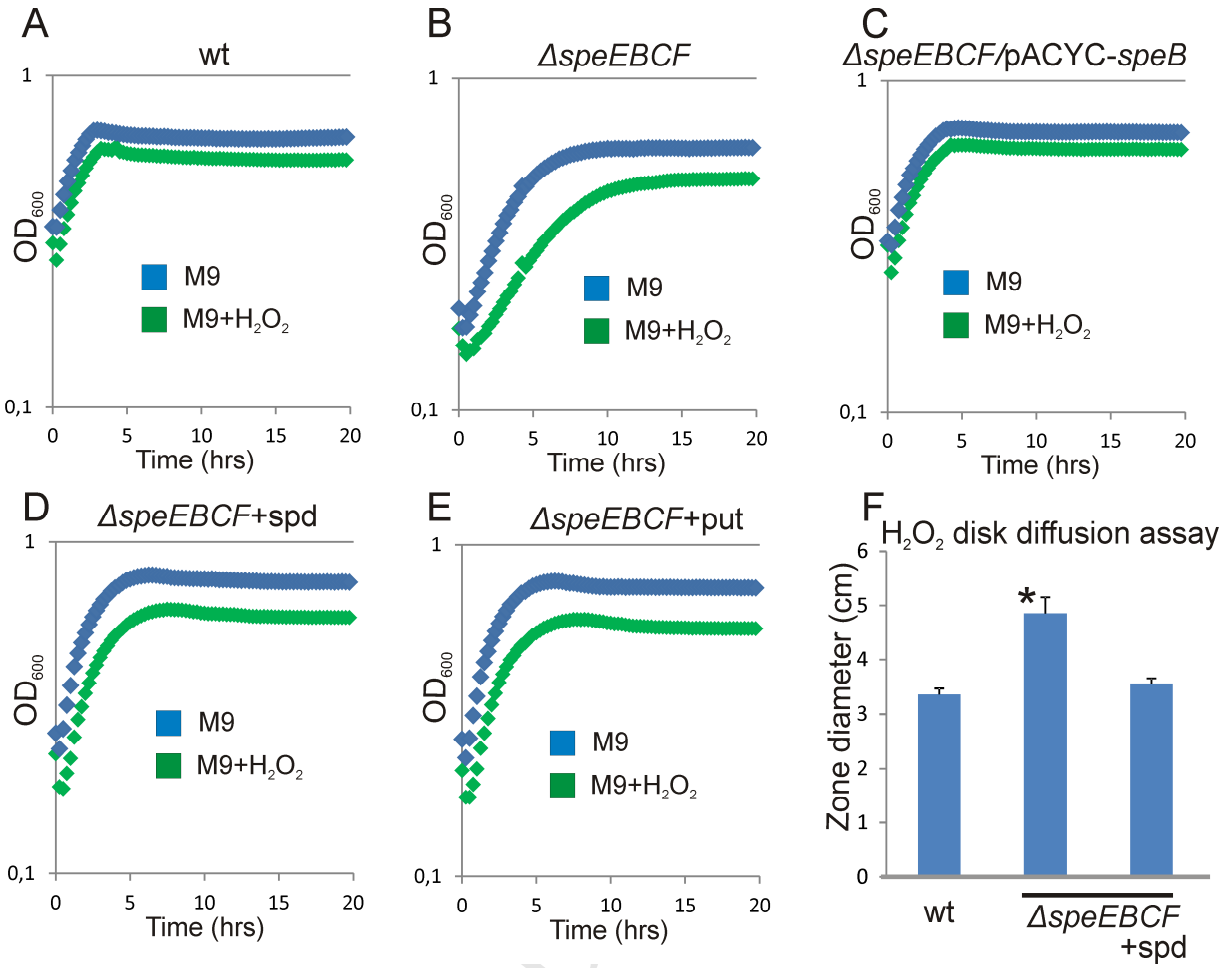
507

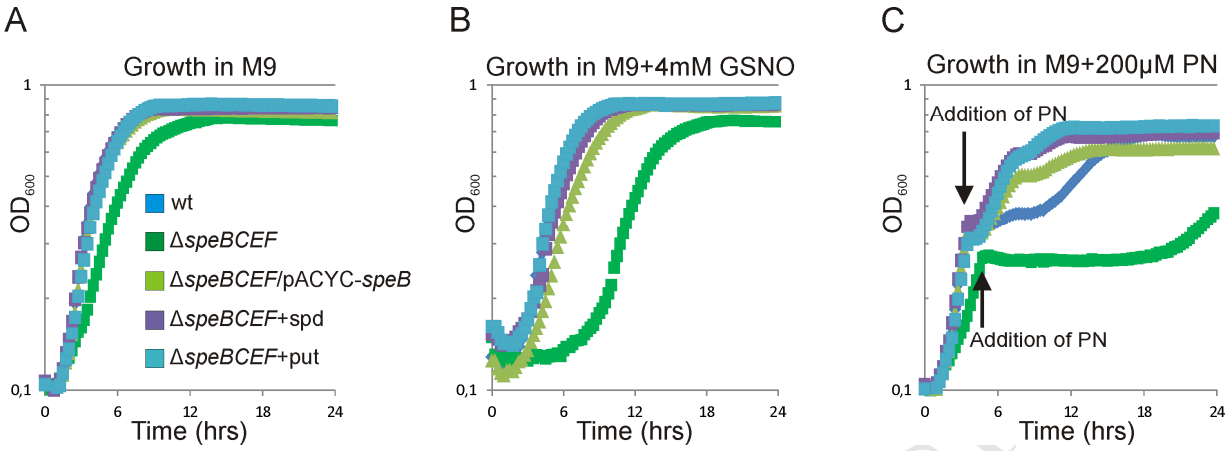
508 **Supplemental figure A2:** CFU counts for indicated strains at indicated time points post
509 infection of macrophages.

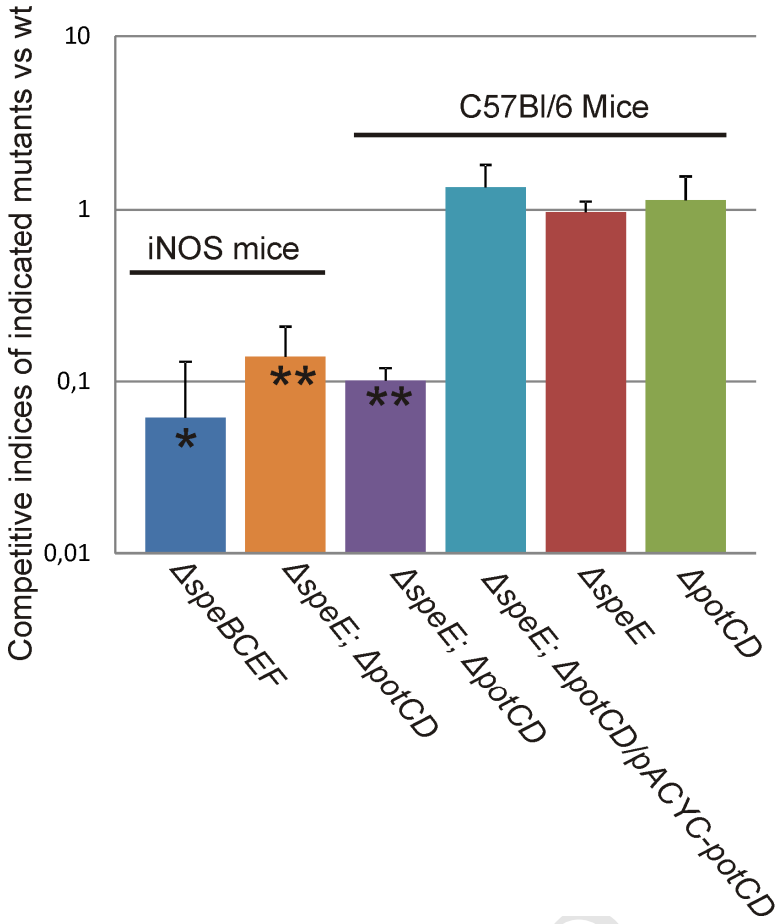
Table 1. Strains used in the study.

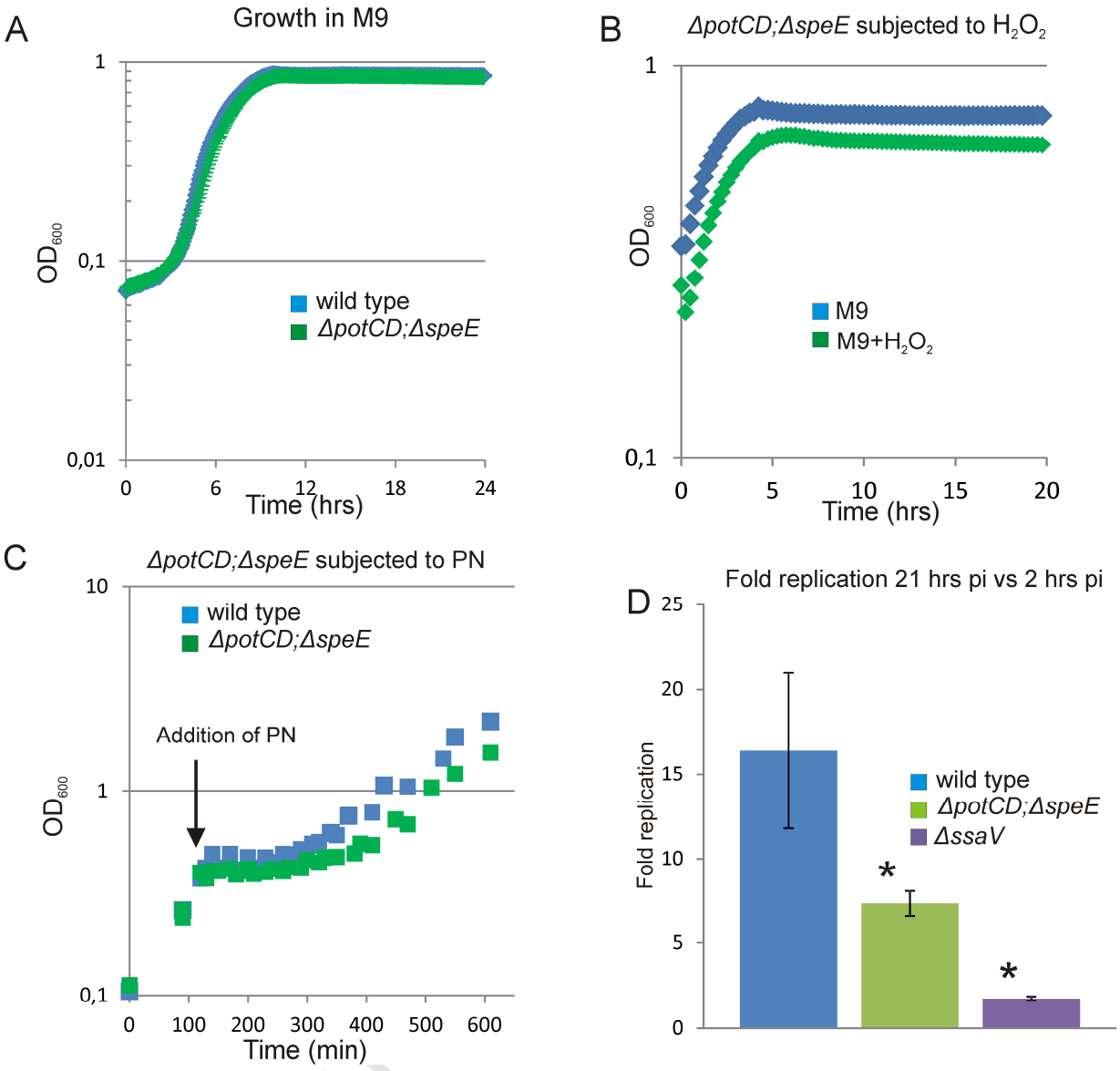
Strain	**Relevant genotype**	**Reference**
S. Typhimurium ST4/74	virulent reference strain	[24]
S. Typhimurium KP1274	Restriction deficient strain, used for introduction of plasmids.	[7]
LJ268	ST4/74::Δ*potCD*;Δ*speE*. Kan^R.	This work.
LJ318	ST4/74::Δ*speB*;Δ*speC*; Δ*speE*;Δ*speF*. Cam^R, Kan^R.	[13]
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]




A
wt
B
ΔspeEBCF
C
ΔspeEBCF/pACYC-speB
D
ΔspeEBCF+spd
E
ΔspeEBCF+put
F
H2O2 disk diffusion assay
OD600
Time (hrs)
M9
M9+H2O2
Zone diameter (cm)
wt
ΔspeEBCF
+spd






A
Growth in M9
1
0,1
0,01
OD600
0
6
12
18
24
Time (hrs)
wild type
ΔpotCD;ΔspeE
B
ΔpotCD;ΔspeE subjected to H2O2
1
0,1
OD600
0
5
10
15
20
Time (hrs)
M9
M9+H2O2
C
ΔpotCD;ΔspeE subjected to PN
10
1
0,1
OD600
0
100
200
300
400
500
600
Time (min)
wild type
ΔpotCD;ΔspeE
Addition of PN
D
Fold replication 21 hrs pi vs 2 hrs pi
25
20
15
10
5
0
Fold replication
wild type
ΔpotCD;ΔspeE
ΔssaV
*
*

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.