Microbial Pathogenesis 95 (2016) 117–123

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

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

Irene Cartas Espinel [a], Priscila Regina Guerra [a], Lotte Jelsbak [a, b, *]

[a] Department of Veterinary Disease Biology, Faculty of Life Sciences, University of Copenhagen, Frederiksberg C, Denmark
[b] Department of Science, Systems and Models, Roskilde University, Roskilde, Denmark

ARTICLE INFO

*Article history:*
Received 4 February 2016
Received in revised form
9 March 2016
Accepted 22 March 2016
Available online 1 April 2016

*Keywords:*
Polyamines
Nitric oxide stress
Virulence
Intracellular pathogens

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 Ref. [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

* Corresponding author. Department of Science, Roskilde University, Universitetsvej 1, DK-4000 Roskilde, Denmark.
*E-mail address:* ljelsbak@ruc.dk (L. Jelsbak).

http://dx.doi.org/10.1016/j.micpath.2016.03.008
0882-4010/

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].

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 (Fig. 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 °C. 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 h, 200 rpm, 37 °C in M9 minimal media to deplete for carry-over polyamines. Where indicated, M9 was supplemented with 100 μg $ml^{-1}$ 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 h 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 °C 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 °C with shaking in a Bioscreen C reader (Thermo Labsystems) for 24 h. Growth was monitored every 15 min 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 ($C = A/(\varepsilon \cdot L)$) was calculated based on the extinction coefficient $\varepsilon = 1670\ M^{-1}\ cm^{-1}$. 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

**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] |