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ORIGINAL ARTICLE

Induction of the viable but nonculturable state of *Salmonella enterica* serovar Enteritidis deficient in (p)ppGpp synthesis

Ramila Cristiane Rodrigues · Evandro Martins · Maria Cristina Dantas Vanetti · Uelinton Manoel Pinto · Míriam Teresinha dos Santos

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Abstract *Salmonella enterica* enters a viable but nonculturable (VBNC) state in adverse environmental conditions. Under nutritional stress, RelA and SpoT proteins synthesize (p)ppGpp, a molecule that controls the expression of genes important for the survival of a cell under those conditions. This study aimed to verify the involvement of (p)ppGpp in the entry of *S. enterica* serovar Enteritidis PT4 578 cells into the VBNC state by evaluating Δ*relA* and Δ*relA*Δ*spoT* mutants. The wild-type and mutant strains deficient in (p)ppGpp synthesis were subjected to osmotic, nutritional and cold stress, after which the cells entered a VBNC state at different time periods, concurrent with reductions in cell diameter, volume and length, and conversion from a bacillary to a coccoid form. No difference in the culturability or cell viability of the wild-type and single and double mutants was observed. Expression of the *rpoS* gene was increased in the double mutant, while both mutant strains presented a reduction in *mreB* gene expression after 25 days under conditions of nutritional and cold stress. Surprisingly, (p)ppGpp was not necessary for induction of the VBNC state in *Salmonella* PT4 578 cells, but may be associated with regulation of genes that control septum formation during cell division, maintaining the bacillary cell morphology (*mreB*) and stress response (*rpoS*). Our findings contribute to the understanding of the mechanisms of resistance and survival of *Salmonella* under adverse conditions.

Keywords (p)ppGpp · VBNC state · Stringent response · Cell filamentation · Septum formation

R. C. Rodrigues · E. Martins
LUNAM Université, Oniris, University of Nantes, Nantes, France

R. C. Rodrigues · E. Martins
Institut National de la Recherche Agronomique (INRA), Nantes, France

M. C. D. Vanetti · M. T. dos Santos
Department of Microbiology, Federal University of Viçosa (UFV), Viçosa, MG, Brazil

U. M. Pinto
Department of Food, Federal University of Ouro Preto (UFOP), Viçosa, MG, Brazil

M. T. dos Santos (✉)
Avenida Peter Henry Rolfs, s/n - Campus Universitário, Viçosa, MG 36570-000, Brazil
e-mail: miriamsantos@ufv.br

Introduction

Salmonella serovars are among the most prevalent pathogens causing foodborne diseases worldwide (CDC 2012). These bacteria grow and survive in numerous environments, sensing and responding to a wide range of external stimuli. Many of these environments induce stress responses in *Salmonella* and generate specific or general adaptation to various adverse conditions (Spector and Kenyon 2012).

The lack of nutrients such as carbon (C), nitrogen (N) or phosphorus (P) is a common environmental condition that such bacteria face. In response to these different nutritional stresses, *Salmonella* produces significant amounts of guanosine 3',5'-bispyrophosphate (ppGpp) and guanosine 3'-diphosphate,5'-triphosphate (pppGpp) that favor cell resistance and/or cell survival (Cashel and Gallant 1969; Chatterji and Ojha 2001). The molecule (p)ppGpp is a low-molecular-weight nucleotide synthesized by two enzymes, RelA (GTP pyrophosphokinase) and SpoT (guanosine 3',5'-bis(diphosphate) 3'-pyrophosphohydrolase) (Neidhardt et al. 1990; Cashel et al. 1996; Wu and Xie 2009).

In response to stress conditions, the (p)ppGpp molecule interacts directly with the RNA polymerase exerting a pleiotrophic regulatory effect in conjunction with the DksA suppressor protein (Hengee 2009). The DksA-(p)ppGpp complex ultimately increases the affinity of the RNA polymerase to alternative σ factors that accumulate in response to a given stress, such as the σ^S factor (Paul et al. 2004; Perederina et al. 2004).

The σ^S factor, also known as RpoS, accumulates under conditions of nutritional stress or entry into the stationary phase of growth (Shen and Fang 2012). It regulates up to 500 genes required for general stress response, including: *bolA,* an important regulator in general stress response; *aidB*, required for adaptive response; *dps*, responsible for the production of a stress response DNA-binding protein with ferritin-like domain; *osmC*, responsible for the production of an osmotically induced protein; and, *uspB*, responsible for production of a universal stress protein*,* among others (Weber et al. 2005; Hengee 2009; Shen and Fang 2012).

Due to the changes in gene expression determined by the accumulation of (p)ppGpp, *Salmonella* withstands diverse environmental conditions (Spector and Kenyon 2012). Under severe stress, the pathogen may undergo a physiological state of dormancy called the viable but nonculturable (VBNC) state (Oliver 2000) as a survival strategy. During this latency state, cells do not grow nor form colonies in routine bacteriological media but remain alive and metabolically active (Oliver 2010). The presence of pathogens in the VBNC state in foods is a public health concern as cells in this state cannot be detected by conventional microbiological analyses, even though they may resume culturability and maintain virulence when present in the host (Oliver 2010). There is evidence that correlates entry into the VBNC state in *Escherichia coli, Salmonella* (Munro et al. 1995) and *Pseudomonas aeruginosa* (Vogt et al. 2011) with synthesis of (p)ppGpp nucleotides.

Cells in the VBNC state show significant morphological changes such as size reduction and transition to the coccoid form (Munro et al. 1995; Albertini et al. 2006; Chiu et al. 2008; Vogt et al. 2011). However, the relation between the accumulation of (p)ppGpp nucleotides and the morphological changes in VBNC cells is insufficiently discussed in the literature.

Therefore, additional studies are needed, considering the importance of understanding the resistance and survival mechanisms of *Salmonella* under adverse conditions, and also to better understand the morphological and metabolic changes of cells deficient in (p)ppGpp synthesis in the VBNC state.

To evaluate the influence of (p)ppGpp on the entry of *Salmonella enterica* cells into the VBNC state and the expression of *mreB genes* related to the cytoskeleton, and *rpoS*, which regulates genes in response to stress, mutants deficient in (p)ppGpp synthesis were subjected to stress conditions and subsequently evaluated for culturability, viability, gene expression and morphological changes.

Materials and methods

Bacteria and culture conditions used

The *Salmonella enterica* serovar Enteritidis PT4 578 strain and the isogenic mutant strains Δ*rel*A and Δ*rel*AΔ*spo*T obtained by Mata (2012) were used in this study. Before each experiment, the wild-type strain was inoculated into Luria-Bertani (LB) broth (pH 7.4) (Miller 1972) for activation and incubated at 37 °C for 18 h. The Δ*rel*A and Δ*rel*AΔ*spo*T mutants were grown in LB broth supplemented with gentamicin, or gentamicin and chloramphenicol, respectively, and incubated at 37 °C for 18 h.

Induction of the viable but nonculturable state

Wild-type and mutant *Salmonella* Enteritidis PT4 578 strains were inoculated into 5 mL of LB broth supplemented with specific antibiotics as required and incubated at 37 °C for 18 h. Cultures were centrifuged at 2,723 × *g* in an Eppendorf 5804 R centrifuge (Eppendorf AG, Hamburg, Germany) to harvest the cells and subsequently washed with 40 mL of 0.85 % (w/v) saline solution for 15 min. After washing, the pellets were resuspended in 1 mL of 0.85 % (w/v) saline solution and inoculated into 1 L of Butterfield's phosphate solution (BPS), prepared according to Association of Official Analytical Chemists (AOAC; 1998), containing 7.35 mmol L^{-1} KH_2PO_4 with or without 0.6-M NaCl. The inoculated flasks were kept at 4 °C for up to 180 days and the cells were evaluated for culturability and viability over time.

Determination of culturability and cell viability

Culturability was assessed for up to 180 days by plating on trypticase soy agar (TSA, Merck) using the microdroplet technique (Morton 2001). The TSA plates were incubated at 37 °C for 24 h. The minimum detection limit of the technique was reduced 10 times to 0.1 CFU mL^{-1} by centrifuging the samples at 2,723 × *g* for 20 min. The TSA plates were incubated at 37 °C for up to 72 h. In the samples that did not show colony formation after 72 h, a volume of 10 mL was inoculated in 10 mL of 2 × brain heart infusion (BHI) broth and incubated at 37 °C for 48 h. A loss in culturability was indicated by the absence of turbidity of the culture medium.

Cell viability was assessed by directly counting viable cells under an epifluorescence microscope using the Live/Dead® BacLight Kit (Molecular Probes) containing the dyes SYTO 9

and propidium iodide, which indicate the integrity of the cell membrane, according to the instructions of the manufacturer. Upon staining, cells were analyzed via epifluorescence microscopy (Olympus BX50 microscope) using a WG filter.

Bacterial RNA isolation and purification

To study the expression of *mreB* and *rpoS* genes in conditions of nutritional and cold stress, the wild-type and mutant *Salmonella* Enteritidis PT4 578 strains were induced to enter the VBNC state in BPS solution at 4 °C for 25 days. After this period, the wild-type and mutant strains were harvested by centrifugation (16,743×*g*, 30 min, 4 °C). The cell pellets were resuspended in BPS solution and centrifuged at 3,300×*g* for 6 min at 4 °C, then resuspended in 1 mL of Tri®Reagent (Sigma-Aldrich, USA) and 0.2 mL of chloroform. After centrifugation at 12,000×*g* for 15 min at 4 °C, the aqueous phase was removed. Total RNA was precipitated in isopropanol, rinsed in 75 % (v/v) cold ethanol and solubilized in 50 μL of sterile water. DNA was degraded by treatment with RQ1 RNase-free DNase (Promega), and then DNA removal was checked by polymerase chain reaction (PCR) analysis using *sdiA* primers (Table 1). Quality and quantity of RNA were checked using a spectrophotometer (Ultrospec® 3000, Pharmacia Biotech, England). The integrity of RNA samples was checked on a 1 % (w/v) agarose gel.

Quantitative real-time reverse transcription PCR

Two hundred ng of total RNA were subjected to first strand cDNA synthesis by using the ImProm-II™ Reverse Transcription System (Promega, Madison, USA) with random hexamer primers, according to the instructions of the manufacturer. Quantitative real-time PCR assay was performed using a BIORAD CFX96 real-time PCR detection system and *sdiA*, *mreB* and *rpoS* primers (Table 1). Composition of the PCR mix was as follows: 1.0 μL of sample, reverse primer (0.2 μM), forward primer (0.2 μM), and 12.5 μL of SYBR Green I Master Mix (Applied Biosystems, FosterCity, CA, USA). The amplification program included an initial denaturing step at 95 °C (10 min), followed by 40 cycles of 95 °C (15 s) and 60 °C (1 min). A negative control (without cDNA) was included in each run. A melting curve was obtained from the first step starting between 60 and 95 °C in order to control specificities of quantitative PCR reactions for each primer pair. The efficiency of the amplifications was determined by running a standard curve with serial dilutions of cDNA. Results were analyzed by using the comparative critical threshold (ΔΔCT) method in which the amount of target RNA was adjusted to a reference [internal target RNA, *sdiA*]. Relative expression level was obtained by the following formula: $2^{-\Delta\Delta CT}$. Quantitative reverse transcription PCR (RT-qPCR) was performed in triplicate.

Scanning electron microscopy

Wild-type and mutant *Salmonella* Enteritidis PT4 578 cells were harvested by centrifugation (11,627×*g* for 10 min at 4 °C) and then washed twice in 0.85 % (w/v) saline solution. Aliquots of the cell suspensions in the log phase and the VBNC state were filtered on a white polycarbonate membrane (Millipore) with a 0.22 μm pore size. The membrane containing the specimens was fixed with 5 % (v/v) glutaraldehyde solution for one hour at room temperature. Then, the samples were washed in 0.05 mol L^{-1} PBS (pH 6.8 to 7.2), and dehydrated in serial concentrations of ethanol. The membranes were transferred to a critical point dryer (CPD®, Bal-Tec, model 030), and the specimens were subsequently sputter coated (Balzers® coater, model FDU 010) and observed under a scanning electron microscope (Leo 1430 VP).

Atomic force microscopy

Wild-type and mutant *Salmonella* Enteritidis PT4 578 cells in the logarithmic growth phase and in the VBNC state were induced in BPS solution supplemented with or without 0.6 M NaCl at 4 °C. Subsequently, the cells were concentrated by centrifugation at 13,000×*g* for 30 min at 4 °C and washed with sodium phosphate buffer (50 mmol L^{-1}, pH 6.5). The pellets were resuspended in sodium phosphate buffer (50 mmol L^{-1}, pH 6.5)\ and the cells were spread onto glass slides (1 cm x 1 cm) that had previously been cleaned, sterilized and air-dried in a laminar flow hood. A poly-L-lysine solution (Sigma-Aldrich, MO, USA) was used to improve adhesion of the cells to the slide. Topography measurements were performed using the tapping mode.

Table 1 Primers used in this study

Primer	Sequence 5'→3'	Amplicon size (pb)
*sdiA*2(RTPCR)-L	ACACAGCGGCTGGAATTTG	100
*sdiA*2(RTPCR)-R	ACGCCGGAGGATAAGTGGTA	100
*mreB*2(RTPCR)-L	CGACGAAGCCATCATTAATTACG	100
*mreB*2(RTPCR)-R	CGGATAAGCGGAACCGATTT	100
*rpoS*1(RTPCR)-L	ATCCGTGCAGTCGAGAAGTT	100
*rpoS*1(RTPCR)-R	GGTTCATAATCGCCCGTTC	100

Results and discussion

Induction of the VBNC state in *Salmonella* Enteritidis PT4 mutant strains deficient in (p)ppGpp synthesis

The VBNC state was induced in *Salmonella* Enteritidis PT4 578 wild-type and mutant strains deficient in (p)ppGpp synthesis after inoculation of each strain into an oligotrophic medium and maintained at a low temperature. The number of cultured cells in the population decreased gradually from approximately 10^6 CFU mL^{-1} to less than 10^0 CFU mL^{-1} in about 180 days (Fig. 1a, c and e). The addition of 0.6 M NaCl to the PBS buffer decreased culturability and reduced the time period for the decline in cell viability (from 10^6 CFU mL^{-1} to less than 10^0 CFU mL^{-1}) to 140 days (Fig. 1b, d and f). Additionally, when comparing individual time periods, the decline in culturability becomes noticeable for all strains when 0.6 M NaCl is added. These results show that the addition of NaCl to the suspension medium influenced the loss of culturability of the wild-type and mutant strains kept under nutritional, osmotic and cold stress, indicating that the addition of more than one stress factor accelerates induction of the VBNC state.

From 140 to 180 days, bacterial viability was maintained at approximately 10^5 cells mL^{-1} (Fig. 1), as determined by using a Live/Dead® BacLight Kit with epifluorescence microscopy. The percentage of viable cells in the population kept in a state of nutritional and cold stress ranged from 72.7 % to 79.0 %, and the addition of osmotic stress reduced the percentage of viable cells to between 72.7 % and 76.2 %. No difference in viability was observed when comparing the wild-type and mutant strains.

We report here, for the first time, that the (p)ppGpp nucleotide seems to be non-essential for *Salmonella* Enteritidis PT4 578 cells to enter into and remain in the VBNC state, as wild-type and both mutant cells demonstrated similar behaviors regarding losses in culturability and viability. Nonetheless, in *E. coli* and *P. aeruginosa*, (p)ppGpp is important for the

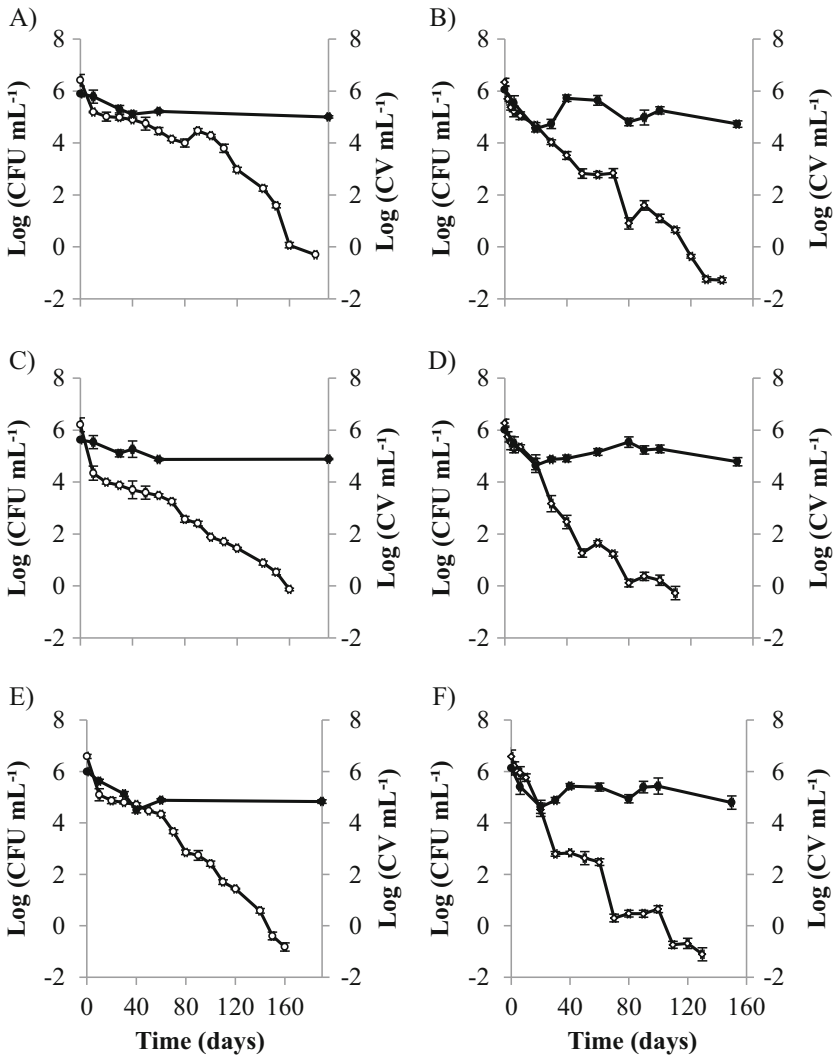

Fig. 1 Culturability (□) and viability (●) of *Salmonella* Enteritidis PT4 578 wild-type and mutant strains deficient in (p)ppGpp synthesis. A, C and E: in BPS solution at 4 °C. B, D and F: in BPS solution supplemented with 0.6 M NaCl at 4 °C. (**A** and **B**) Wild-type; (**C** and **D**) single mutant (Δ*rel*A); (**E** and **F**) double mutant (Δ*rel*AΔ*spo*T). The number of culturable cells was determined by plating the cells on TSA agar using the microdroplet technique followed by incubation at 37 °C for 24 h. The number of viable cells (CV) was determined using the Live/Dead® BacLight Kit with epifluorescence microscopy

maintenance of culturability and cell viability under conditions of nutritional and osmotic stress (Munro et al. 1995; Vogt et al. 2011).

The effect of nutritional and cold stress on the expression of mreB and rpoS genes in *Salmonella* Enteritidis PT4 wild-type and mutant strains deficient in (p)ppGpp synthesis

The analysis of relative gene expression measured by RT-PCR revealed increased expression of *rpoS* and *mreB* genes in the single and double mutant strains of *Salmonella* Enteritidis PT4 578 at time 0 compared to the relative expression of these genes in the wild-type culture (Fig. 2).

After seven days of inoculation in BPS solution, the *relA* single mutant had a drop in the relative expression of *rpoS* of 75.3 % while *mreB* expression was reduced by 93.4 %, compared to the wild-type strain. The double mutant strain still showed increased relative expression of the *mreB* gene when compared to the wild-type (Fig. 2).

However, after 25 days in conditions of nutritional and cold stress, the single and double mutant strains showed a decrease in relative *mreB* gene expression of 88.2 % and 84.5 %, respectively, when compared to the wild-type. This is the first report in the literature that shows *mreB* gene expression in cells in the VBNC state and deficient in the synthesis of (p)ppGpp, indicating that this nucleotide is necessary for *mreB* induction over time, under stressful conditions. On the other hand, as compared to the wild-type, the single and double mutant strains showed a 1.6- and 16.5-fold increase, respectively, in relative expression of the *rpoS* gene .

Salmonella enterica serovar Enteritidis PT4 mutants deficient in (p)ppGpp synthesis showed resistance to the stress conditions to which they were subjected. The resistance displayed by the mutants may be associated with the activity of RpoS sigma factor-dependent promoters and/or the presence of regulators of RpoS sigma factor. This hypothesis is reinforced mainly by the increase in the relative expression of the *rpoS* gene in the single and double mutant strains (Fig. 2).

Our results suggest that, in *S. enterica* serovar Enteritidis PT4, the (p)ppGpp molecule may be necessary for RpoS sigma factor activity. Therefore, cells would need to compensate for the absence of this nucleotide by increasing *rpoS* gene expression in order to maintain viability (Fig. 2c). In fact, in *E. coli,* the (p)ppGpp molecule works by facilitating binding between RpoS sigma factor and the RNA polymerase core (Jishage et al. 2002). It is also possible that other regulators of the RpoS sigma factor, such as the DNA-binding protein Fis and the DksA, a protein responsible for RpoS regulation, may have acted in the mutant strains, favoring resistance to the adverse conditions (Brown et al. 2002; Hirsch and Elliot 2005).

Cell morphology of *Salmonella* Enteritidis PT4 wild-type and mutant strains deficient in (p)ppGpp synthesis

The cellular morphology of the wild-type and the mutant strains during the logarithmic growth phase in LB broth exhibited a bacillary form when observed using scanning electron microscopy (Fig. 3) and a compact and slightly irregular surface using atomic force microscopy (Fig. 4). However, the single and double mutant strains in the logarithmic phase were elongated and formed filaments (Fig. 3b and c), while those in the VBNC state showed slightly rough surfaces, concurrent with a transition from the bacillary to the coccoid form

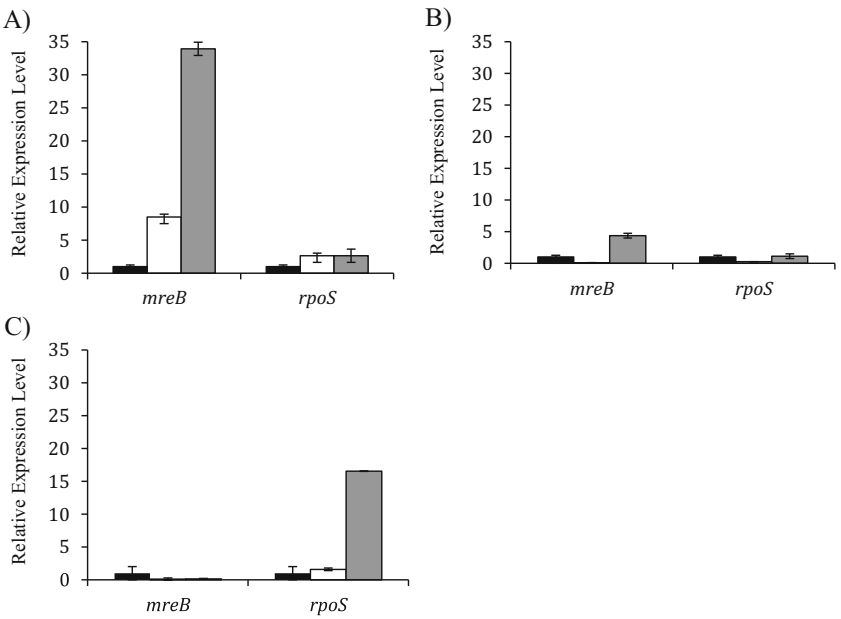

Fig. 2 Relative expression levels of *mreB* and *rpoS* genes of strains of *Salmonella* Enteritidis PT4 578 wild-type and mutants deficient in (p)ppGpp synthesis subjected to nutritional and cold stress at different times. Gene expression has been estimated using RT-qPCR and the comparative critical threshold ($\Delta\Delta CT$) method. The *sdiA* gene was used as the internal control. (**A**) 0 days; (**B**) 7 days e (**C**) 25 days; (■) Wild-type; (□) single mutant (Δ*rel*A) and (▩) double mutant (Δ*rel*AΔ*spo*T)

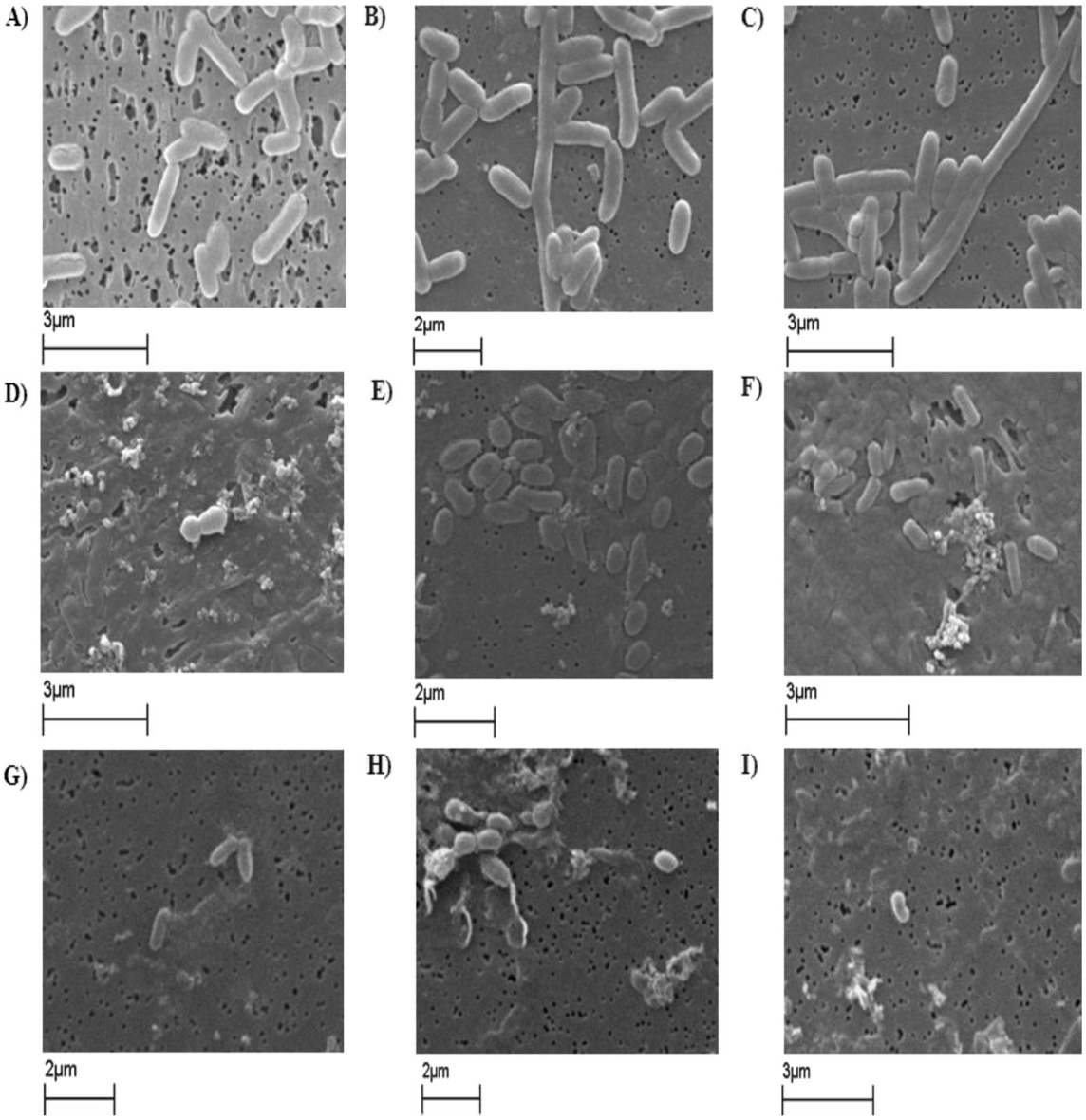

Fig. 3 Micrographs of *Salmonella* Enteritidis PT4 578 wild-type and mutant cells in the logarithmic growth phase (**A**, **B** and **C**), in the VBNC state after induction in BPS solution (**D**, **E** and **F**) and in BPS solution supplemented with 0.6 M NaCl (**G**, **H** and **I**) at 4 °C using scanning electron microscopy on polycarbonate membranes. 10,000× magnification. Wild-type (**A**, **D** and **G**); single mutant (Δ*rel*A) (**B**, **E** and **H**); double mutant (Δ*rel*AΔ*spo*T) (**C**, **F** and **I**)

(Fig. 3e and f). The decrease in cell volume observed by atomic force microscopy was approximately 71 % with non-stressed cells presenting a volume of approximately 0.63 μm^3 and VBNC cells presenting 0.18 μm^3 of volume, on average.

Cell division in Gram-negative bacteria involves coordinated constriction of the cytoplasmic membrane, the peptidoglycan layer and the outer membrane, as well as formation of a transverse wall of the peptidoglycan septum (Yu and Margolin 1999). Both hydrolysis and synthesis of the peptidoglycan formed during cell division are under the control of (p)ppGpp (Ramey and Ishiguro 1978). We propose that the change in the (p)ppGpp concentration through the disruption of the *relA* and/or *spoT* genes in the mutant strains of *Salmonella* Enteritidis PT4 affected the expression of genes responsible for cell septation and peptidoglycan formation. This may have contributed to the formation of filamentous cells during the logarithmic growth phase in LB broth (Fig. 3). Filamentation has been reported in *E. coli* cells lacking a functional *spoT* gene or *relA* and *spoT* genes (Xiao et al. 1991; Magnusson et al. 2007). In *E. coli*, (p)ppGpp synthesis is correlated with an increase in the concentration of the FtsZ protein, which is essential for cell septation (Powell and Court 1998). Thus, it is likely that mutations in the *spoT* gene or *relA* and *spoT* genes affect the production of the FtsZ protein

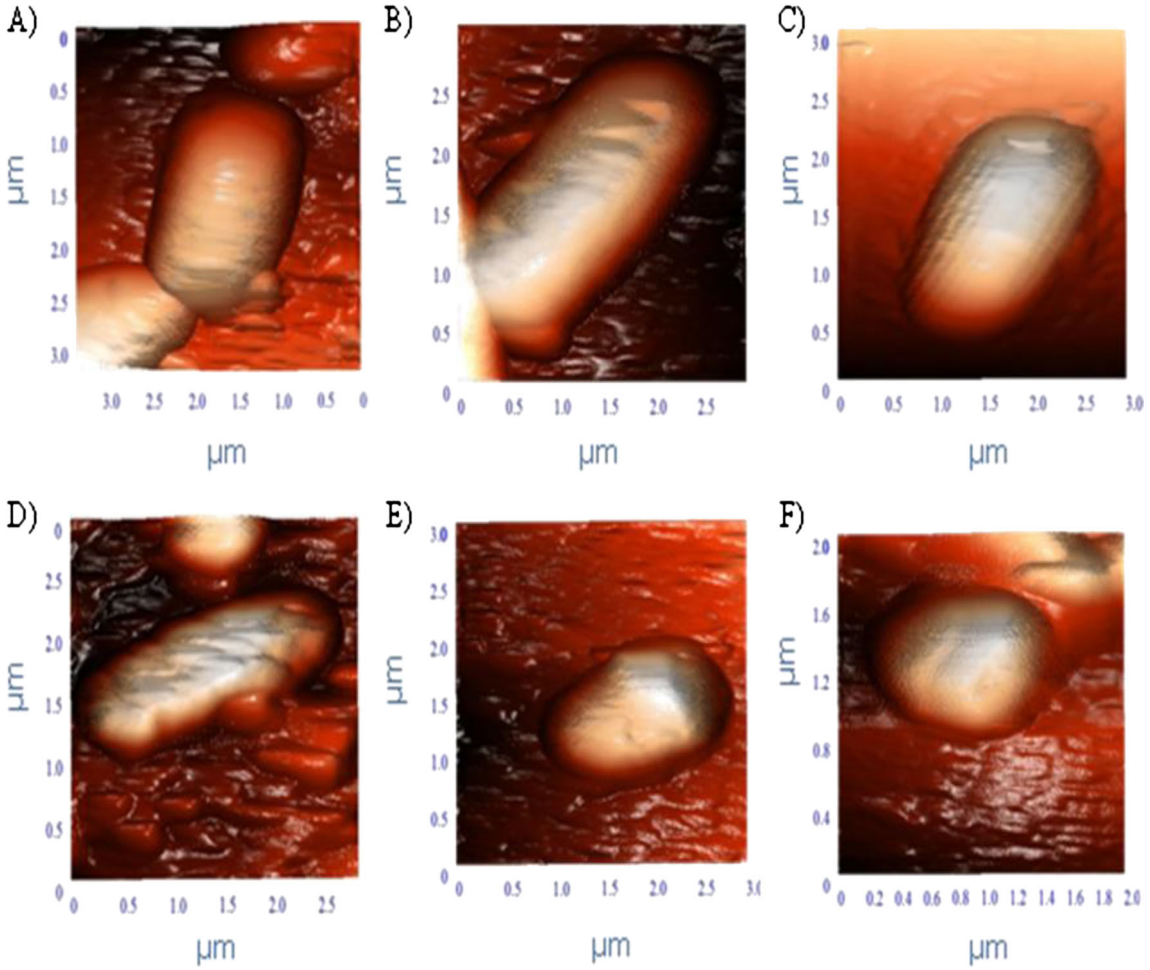

Fig. 4 3D images of cells of the *Salmonella* Enteritidis PT4 578 wild-type strain and mutants deficient in (p)ppGpp synthesis in the logarithmic growth phase (**A**, **B** and **C**) and in the VBNC state (**D**, **E** and **F**) after induction in BPS solution supplemented with 0.6 M NaCl at 4 °C using atomic force microscopy on glass slides coated with poly-L-lysine. Wild-type (**A** and **D**); single mutant (Δ*rel*A) (**B** and **E**); double mutant (Δ*rel*AΔ*spo*T) (**C** and **F**)

causing cell filamentation in *Salmonella* Enteritidis PT4. However, further studies are needed to clarify the role of (p)ppGpp in cell filamentation in this organism.

Morphological change and the reduction in cell size were probably responsible for the adaptation and survival of the strain in the VBNC state under stressful conditions. Cell size reduction and the transition to the coccoid form may be associated with a modification in the expression of the *mreB* gene (Fig. 2), a gene responsible for maintaining bacillary cell morphology. In *Salmonella*, the proteins MreB, MreC and MreD are determinants of cell morphology and are located in the *mre* operon (Doble et al. 2012).

The reduction in *mreB* expression during the period of nutritional and cold stress is most likely due to the morphological change that the cells exhibit under stressful conditions. Other microorganisms, such as *Vibrio parahaemolyticus*, *Helicobacter pylori* and *Bacillus subtilis*, also showed a reduction in *mreB* gene expression under conditions of nutrient limitation (Levin et al. 1992; Eymann et al. 2002; Thompson et al. 2003; Chiu et al. 2008).

In this study, we evaluated the ability of *Salmonella* Enteritidis PT4 cells deficient in (p)ppGpp synthesis to enter and remain in the VBNC state under conditions of osmotic, nutritional and cold stress. In addition, we quantified via RT-PCR *rpoS* gene expression, which regulates genes in response to stress, and *mreB* expression, which is related to the cytoskeleton. We analyzed the morphological changes of these cells by using scanning electron microscopy and atomic force microscopy. This study demonstrated that the mutant strains *relA* and *relA*/*spoT* of *Salmonella* Enteritidis PT4 578 entered the VBNC state in BPS solution in the presence or absence of NaCl, at 4 °C. Additionally, the expression of *rpoS* genes was increased in the double mutant, and *mreB* gene expression was reduced in both mutant strains after 25 days under stressful conditions. We report, for the first time, *mreB* gene expression in cells deficient in the synthesis of (p)ppGpp, indicating an

induction of this gene by this nucleotide. Morphological changes, such as the transition from the bacillary to the coccoid form and a reduction in cell size, were observed in cells in the VBNC state for the mutant and wild-type strains. Our study is the first to show that ppGpp is not essential for the entry and maintenance into the VBNC state in *Salmonella* Enteritidis. Further studies in strains deficient in (p)ppGpp synthesis in the VBNC state will be needed to further elucidate the mechanisms of resistance and survival of *Salmonella* under adverse conditions.

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