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Full Length Research Paper

Alteration of outer membrane proteins, secreted proteins and virulence gene expression of *Salmonella enterica* serovar Typhimurium in response to long-term starvation

Rihab Lagha*, Ali Ellafi, Fethi Ben Abdallah, Nourhène Saidi and Amina Bakhrouf

Laboratoire d'Analyse, Traitement et Valorisation des Polluants de l'Environnement et des Produits. Faculté de Pharmacie Rue Avicenne. Monastir 5000, Tunisia.

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The foodborne pathogen *Salmonella enterica* serovar Typhimurium was subjected to starvation in seawater microcosms for three years to study modifications in its outer membrane and extracellular protein profiles. After incubation, outer membrane proteins and extracellular proteins profiles of stressed bacteria were found to be altered when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). These modifications were shown by the appearance of bands, as well as the level of expression of certain proteins. The expression levels of *sopE2*, *invA, sipA* and *sopB* virulence genes were also studied by reverse transcriptase polymerase chain reaction. Our findings showed that the expression level of *sopE2* was slightly decreased under starvation. Whereas the quantities of *sopB* mRNA were increased in the two starved strains S1 and S2. The expression of *sip*A was decreased in strain S1 after starvation, but was significantly increased in strain S2.

Key words: *Salmonella*, starvation, alteration, outer membrane protein, extracellular proteins, virulence gene expression.

INTRODUCTION

Salmonella is a facultative intracellular pathogen which, depending on the serotype and host, can cause diseases ranging from gastroenteritis to typhoid fever. For example, *Salmonella enterica* serovar Typhimurium, which initiates disease normally limited to gastroenteritis in humans, causes systemic disease in mice and has been used as an animal model of human typhoid fever. *Salmonella* infections are usually acquired by ingestion of contaminated food or water. In systemic (typhoid-like) disease, following ingestion, the bacteria survive the acid pH of the stomach, colonize the Peyer's patches of the intestine, and penetrate the gut barrier via M cells (specialized epithelial cells). From there, they disseminate to the local mesenteric lymph nodes and then to the spleen and liver via phagocytic cells (Jones and Falkow, 1996; Richter-Dahlfors et al., 1997).

Salmonella is exposed to a number of stressful environmental factors during its life cycle and the ways in which it responds to different stresses are correspondingly complex (Rychlik and Barrow, 2005). When exposed to seawater, enteric bacteria are challenged by a combination of hostile conditions threatening their viability. These include biotic (competition, predation; Barcina et al., 1991; Rozen and Belkin, 2001) and abiotic factors (pH, salinity, radiation, oxidative stress, nutrients deficiency, hydrostatic pressure and temperature) (Trousselier et al., 1998; Rozen and Belkin, 2001). Out of the different environmental factors combining to form seawater stress, the most prominent in the induction of several groups of genes was nutrient limitation (Rozen et al., 2002). In order to survive, become pathogenic and cause illness, bacteria must sense these changes and then respond with appropriate alterations in gene expression and protein activity (Boor, 2006).

*Corresponding author. E-mail: rihablagha@yahoo.fr. Tel: + 216 73 466 244. Fax: + 216 73 461 830.

Outer membrane proteins (OMPs) play an important role in the physiology of Gram-negative bacteria, by allowing small hydrophilic molecules to pass through a channel (Nikaido, 1994). Their expression was preferential according to the environmental growth conditions (Puente et al., 1991; Contreras et al., 1995). Several studies have shown that when bacteria are transferred to a new environment, the synthesis of their OMPs changes (Provenzano et al., 2001; Wibbenmeyer et al., 2002). Arockiasamy and Krishnaswamy (1999) demonstrated that in *S. typhi* strains OmpC showed greater expression under both low and high osmolarity. More and more similar regulatory mechanisms have been found in other bacteria, Wu et al. (2006) indicated that OmpW and OmpV are required for environmental salt regulation in *Photobacterium damsela*. In addition, Xu et al. (2005) demonstrated that the OMP profile of *Vibrio alginolyticus* is altered at different sodium concentrations.

Gram-negative bacteria secrete a wide range of proteins whose functions include biogenesis of organelles, nutrient acquisition, virulence, efflux of toxins, and injection of virulence factors into host cells (Thanassi and Hultgren, 2000). According to Secades and Guijarro (1999) environmental stress could play an important role in the induction or repression of enzymes by specific compounds. Production of extracellular proteases has been shown to be sensitive to repression by different carbohydrate and nitrogen sources (Haulon et al., 1982). Furthermore, Bajaj et al. (1996) reported that environmental signals, For example oxygen concentration, osmolarity, and the growth state of the bacteria, influence the expression of the secretion of invasion-associated proteins. Moreover, stress conditions can control gene expression by inducing changes in DNA topology which can provide an overlap between responses to different environmental stimuli (Dorman, 1991). In addition, Cheng et al. (2007) showed differential gene expression when *Shigella flexneri* cells were grown in pH 4.5 or 8.6. Ono et al. (2005) reported that a temperature shift from 25 to 37°C resulted in 11.9% of the genes changing in expression.

The aim of this work was to study the response of *S. enterica* serovar Typhimurium strains to starvation conditions in seawater microcosm during a period of three years. Extracellular proteins and outer membrane protein profiles of starved cells were analysed by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE). To study the expression level of virulence genes, reverse transcriptase polymerase chain reaction (RT-PCR) was used.

MATERIALS AND METHODS

Bacterial strains and growth conditions

S. enterica serovar Typhimurium ATCC 14028s (S1) and *S. enterica* serovar Typhimurium LT2 DT104 (S2) were used in this study. All strains were maintained at -80°C in Luria-Bertani (LB) broth supplemented with glycerol (15%, vol/vol). For the experiments, the cells were grown at 37°C in Tryptic soy broth (Pronadisa, Spain, TSB) for 24 h.

Natural seawater (100 ml) was filtered through membranes (pore size 0.22 µm; Millipore, Bedford, MA, U.S.A.) and autoclaved (121°C /20 min) in 250 ml Erlenmeyer flasks. *S. enterica* serovar Typhimurium cells were washed three times by centrifugation (16,000 × *g* for 10 min at 20°C) with autoclaved seawater and then suspended in 10 ml autoclaved seawater. The microcosms (100 ml) were inoculated with these suspensions (10^9 colony-forming units /ml) and then incubated at room temperature for 3 years under static conditions.

Outer membrane protein extraction

OMPs of *S. enterica* serovar Typhimurium were prepared according to the method described previously (Sabri et al., 2000). Briefly, bacterial cells were harvested by centrifugation at 4,000 × *g* for 20 min at 4°C. The cells were then washed three times in 40 ml of sterile saline solution (0.9% NaCl) and resuspended in 5 ml of sterile saline solution. Cells were disrupted by intermittent sonic oscillation. Supernatant was collected and further centrifuged at 100,000 × g for 40 min at 4°C. The pellet was resuspended in 10 ml of 2% (w/v) sodium lauryl sarcosinate (Sigma, St. Louis, MO, U.S.A.) and incubated at room temperature for 1 h, followed by centrifugation at 100,000 g for 40 min at 4°C. The resulting pellet was resuspended in 200 µl of sterile saline solution. The concentration of the OMPs in the final preparation was determined using the Bradford assay (Bradford, 1976).

Extracellular protein extraction

Extracellular proteins of *S. enterica* serovar Typhimurium were prepared according to the method described previously (Kaniga et al., 1995). Briefly, the cells were grown at 37°C in tryptic soy broth for 24 h. Bacterial cells were removed from cultures by centrifugation at 7000 × g for 20 min and subsequent filtration through a 0.45 µm pore-size filter. Proteins from the cell-free culture supernatants were then precipitated by addition of 10% (vol/vol) trichloroacetic acid and recovered by centrifugation at 7000 × g for 20 min. Pellets were resuspended in 4 ml of phosphate-buffered saline (PBS), and proteins were precipitated by addition of 20 ml of cold acetone. After centrifugation at 7000 × g for 20 min, the pellets were washed once with cold acetone, dried, and resuspended in 25 µl of PBS. The concentration of the secreted proteins in the final preparation was determined using the Bradford assay.

SDS-PAGE

OMPs and extracellular proteins (20 µg) were analysed by SDS-PAGE (Laemmli, 1970) with 15% acrylamide in the separating gel and 5% acrylamide in the stacking gel. After separation, the proteins were visualized according to standard procedures by staining with Coomassie Brilliant Blue G250 (Sigma, Chemical Co., St Louis, MO, USA) and molecular weights were determined by means of molecular weight markers (High-Range Rainbow; Amersham, Little Chalfont, Buckinghamshire, UK).

RNA extraction and RT-PCR

To study the expression level of *S. enterica* serovar Typhimurium *sopE2, invA, sopB* and *sipA* genes before and after stress, a semi-quantitative RT-PCR method was used. RNA from control and stressed cells was extracted by the SV total RNA isolation

Table 1. PCR primers selected for this study.

Oligonucleotide sequence	Amplification region (pb)	References
SopE2		
5'- TCC GGC CTA TGC TCG TCA G- 3' 5'- CTC GCG GAA GCA ATG AGG G -3'	234	(NCBI, AF217274)
SopB		
5'-CAA CCG TTC TGG GTA AAC AAG AC-3' 5'-AGG ATT GAG CTC CTC TGG CGA T-3	1348	Rahman (2006)
sipA		
5'- GTA GGA CGG GAA GCC CGG C -3' 5'- CGC TGC ATG TGC AAG CCA TCA - 3'	1324	(NCBI, NC003197)
invA		
5'-TAT CGC CAC GTT CGG GCA A 3' 5'-TCG CAC CGT CAA AGG AAC C 3'	275	Nayak et al. (2004)

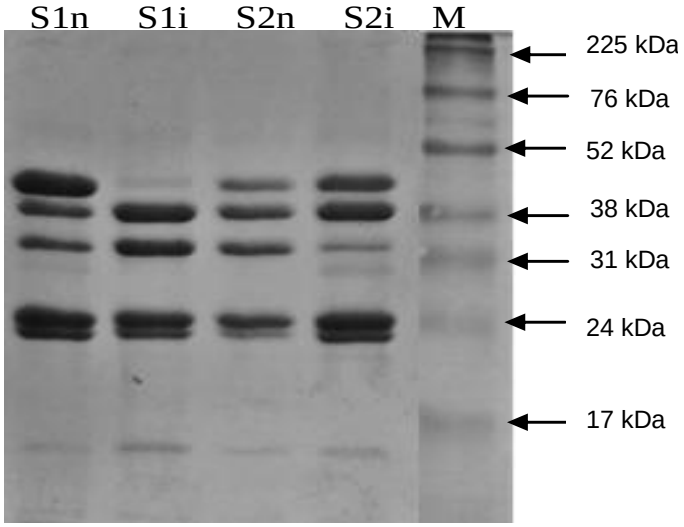

Figure 1. Outer membrane proteins of *Salmonella* cells exposed to starvation for three years in seawater. M: High-Range Rainbow (Amersham, Little Chalfont, Buckinghamshire, UK); S1: *Salmonella* Typhimurium ATCC 14028s. S2: *Salmonella* Typhimurium LT2 DT104. n: denotes strain before incubation in seawater; i: denotes strain incubated for three years in seawater microcosm.

system (Promega) according to the manufacturer's instructions. RNA was quantified by an Ultraspec spectrophotometer (Ultraspec 2100 pro; Amersham Bio-Sciences Europe GmbH, France). The primers for selected virulence genes are listed in Table 1.

RT-PCR was performed in triplicate using SuperScriptTM One-Step RT-PCR with platinum® Taq kit according to the manufacturer's recommendations (Invitrogen, Carlsbad, CA, U.S.A.). For cDNA synthesis, 100 ng of RNA served as template. RT-PCR (25 µl reaction volume) was performed as follows: 50°C for 30 min; 94°C for 5 min; 35 cycles at 94°C for 1 min; annealing at 57°C for 1 min for *sopE2* and *invA*; 72°C for 1 min; and a final extension at 72°C for 10 min. The annealing temperature for the *sipA* gene was 61°C and for *sopB* gene was 63°C. RT-PCR products (5 µl) were analyzed on a 1% agarose gel stained with ethidium bromide (0.5 mg/ml) at 90 V for 1 h and viewed under ultraviolet transillumination. Quantitative analysis of DNA bands was performed in triplicate using imaging software (Gene Tools, Sygene, UK).

Statistical analysis was performed using the S.P.S.S. 13.0 statistics package for Windows. Friedman test, followed by the Wilcoxon signed ranks test were used to examine differences in the expression levels of virulence genes before and after stress. P-values of < 0.05 were considered as significant.

RESULTS

Protein analysis

OMPs of *Salmonella* strains displayed different profiles before and after starvation. Before incubation in seawater, *S. enterica* serovar Typhimurium S1 and S2 had the same OMP profile. Six clear bands were detected in each profile at 14, 22, 24, 35, 38, 43 kDa (Figure 1).

After incubation in seawater, the OMP pattern of strain S1 showed significantly higher levels of proteins corresponding to molecular weights of approximately 14, 35 and 38 kDa of the strain S1 (Figure 1, lane S1i), whereas the 43 kDa protein became less abundant.

In comparison, bands at approximately 22, 24, 38 and 43 kDa increased in starved strain S2. In addition to these modifications, a 27 kDa protein appeared and the expression of a 35 kDa protein was reduced in strain S2 after long-term incubation.

Proteins secreted by the tested strains of *S. enterica* serovar Typhimurium were also analyzed by SDS-PAGE

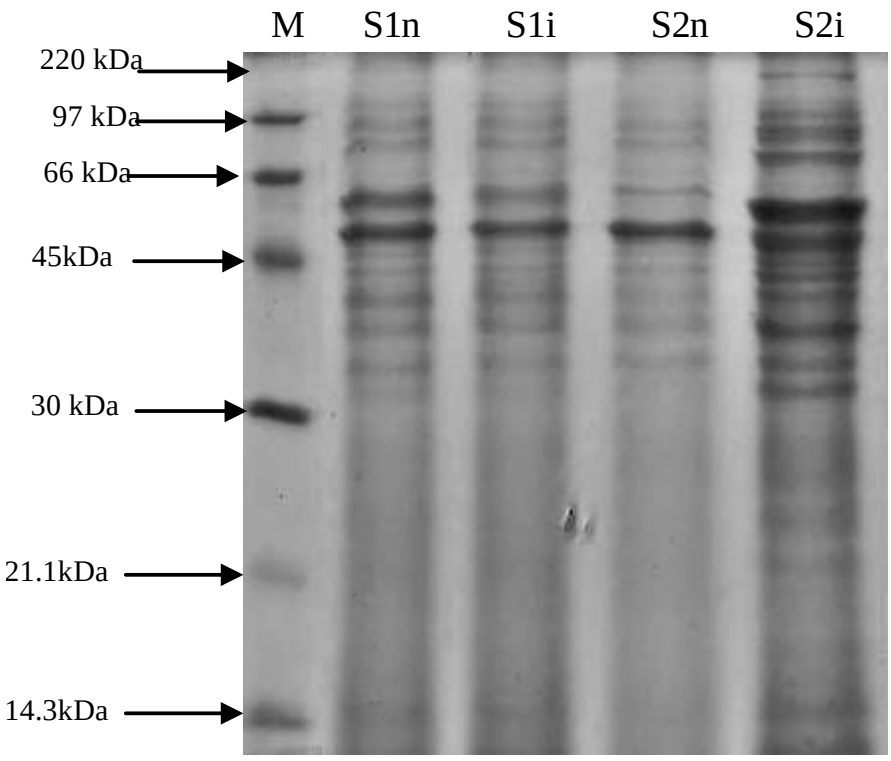

Figure 2. Extracellular proteins of *Salmonella* cells exposed to starvation for three years in seawater. M: High-Range Rainbow (Amersham, Little Chalfont, Buckinghamshire, UK); S1: *Salmonella* Typhimurium ATCC 14028s. S2: *Salmonella* Typhimurium LT2 DT104. n: denotes strain before incubation in seawater; i:denotes strain incubated for three years in seawater microcosms.

(Figure 2). Before their incubation in seawater, both strains secreted a significant number of proteins in the extracellular medium. After incubation in seawater, *S. enterica* serovar Typhimurium S1 retained the same extracellular protein profile, with proteins ranging in size from 30 to 97 kDa. For *S. enterica* serovar Typhimurium S2, the secretion of proteins was significantly altered after incubation in sea water (Figure 2, lane S2i). The expression of bands corresponding to proteins of 49, 52 and 62 kDa was significantly increased. Interestingly, bands corresponding to molecular weights of 21, 33, 66, 80, 97 and approximately 220 kDa appeared after stress.

Virulence genes expression

The expression levels of virulence genes of *Salmonella* cells, which encode the type III secretion systems, were analysed by semi-quantitative RT-PCR before and after incubation during 3 years in a seawater microcosm. Our results are shown in Figure 3. All the selected genes (*sopE, invA, sipA* and *sopB*) were expressed. After incubation in seawater, we observed the same expression level of *sopE2* gene in starved and control strain S1, whereas the expression of this gene was decreased slightly in strain S2. The quantity of *inv*A mRNA remained stable after starvation in all tested strains. For *sipA*, there was a significant decrease in expression in strain S1. In contrast, the quantity of mRNA encoding this protein remained stable in strain S2 after starvation. In addition, the expression level of the *sopB* gene was stable in both strains, with a slight increase in starved strain S2.

DISCUSSION

S. enterica serovar Typhimurium is able to survive for a long time (3 years) in seawater. All organisms respond to environmental stress by modifying the rate of synthesis of certain proteins. In this study, long-term starvation and/or osmotic stress induced several alterations in OMP patterns of the foodborne pathogen *S. enterica* serovar Typhimurium. These alterations were shown by the disappearance of bands on SDS-PAGE, as well as by variations in the mRNA expression of some proteins. These modifications are probably due to nutrient deficiency in seawater (Ben Abdallah et al., 2009). Because of its location and components, the cytoplasmic membrane has been traditionally suggested to sense

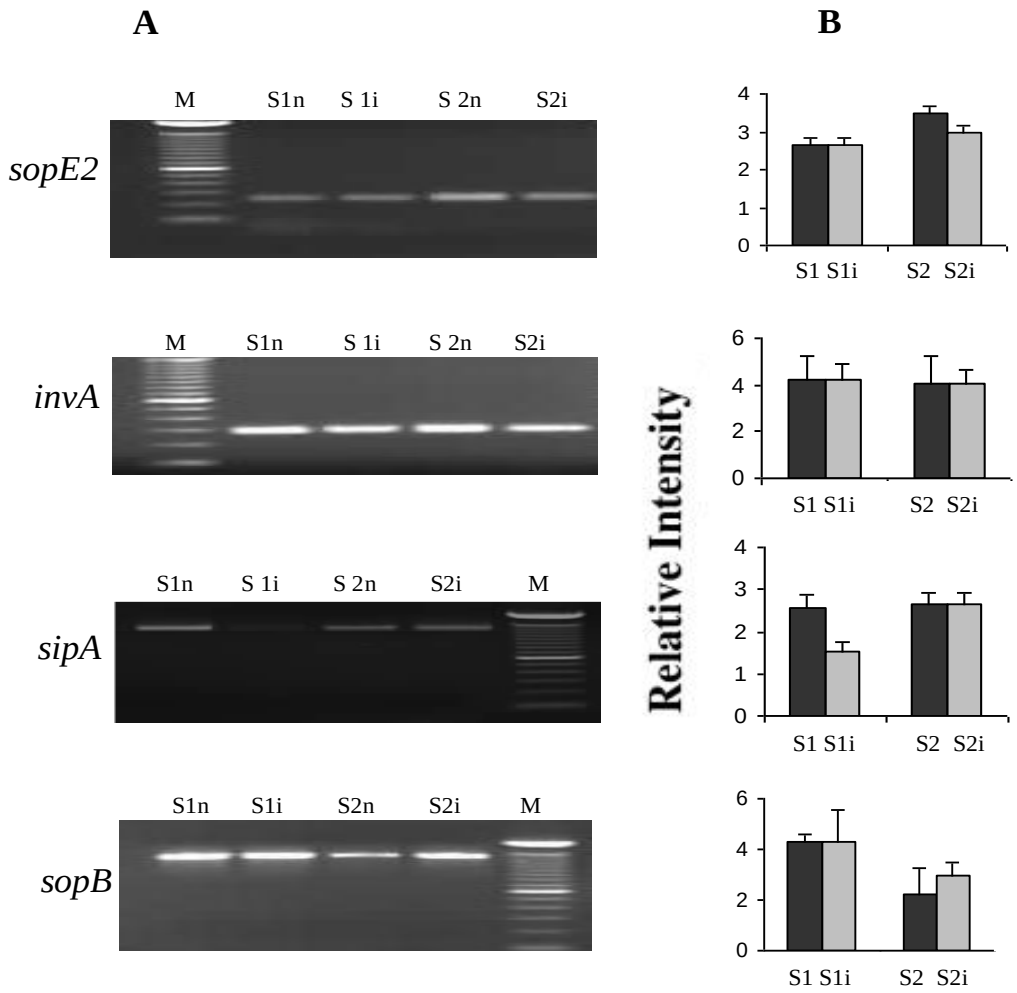

Figure 3. Virulence genes expression of *Salmonella* exposed to starvation for three years in seawater. M: 100 bp DNA ladder (Promega); S1: *Salmonella* Typhimurium LT2 DT104; S2: *Salmonella* Typhimurium ATCC 14028s. n: strain before incubation in seawater; i: strain incubated for three years in seawater microcosm; A: mRNA electrophoresed in 1% agarose gels; B: relative expression of virulence genes. $P < 0.05$.

environmental changes through certain proteins that expand into the periplasm in order to allow bacteria to adapt with stress (Neidhardt, 2002). As in all bacterial proteins, the OMPs are made in the cytoplasm, and their synthesis is often highly regulated in response to growth, nutrient and environmental conditions (Delihas and Forst, 2001; Nikaido, 2003).

Recently, it has been shown that long-term starvation induced several alterations in OMP profiles of the marine food-borne pathogens *V. alginolyticus* and *Vibrio parahaemolyticus* (Ben Abdallah et al., 2010). Changes in the expression of OMPs in response to osmotic stress have been reported in *Escherichia coli* (Nikaido and Vaara, 1987). Furthermore, a transition to acid pH environments also leads to dramatic changes in outer membrane protein synthesis in *Salmonella* (Foster et al., 1994). In addition, acidic pH induced the expression of new proteins on the surface of *Yersinia pestis* (Feodorova and Devdariani, 2001). Another study has shown that a rise in temperature may induce significant changes in the OMP expression of *Escherichia coli* (Molloy et al., 2000). In the present study, the alterations observed in the OMP profiles of starved *Salmonella* strains may indicate the existence of certain modifications in resistance toward some antibiotics. Indeed, Dupont et al. (2007) reported that the expression of *omp*X, encoding an outer membrane protein, is increased during early exposure to drugs or environmental stresses. At the same time, the level of OmpF porin expression is noticeably affected. Because of the role of these proteins in membrane permeability, these data suggest that OmpF and OmpX are involved in the control of the penetration of antibiotics such as β-lactams and fluoroquinolones through the *E. coli* and *Enterobacter aerogenes* outer

membrane. Thus, OMPs represent important virulence factors and play essential roles in bacterial adaptation by allowing the bacteria to inhabit several different, and often hostile, environments (Lin et al., 2002).

Bacteria produce various extracellular products (Hasegawa et al., 2008). These proteins include cytolysins, lipases, siderophores, exopolysaccharides and proteases. These proteases are mainly involved in providing peptide nutrients for the micro-organism. However, the production of bacterial proteases could contribute to the pathogenesis of infections, and therefore they could be considered virulence factors (Secades and Guijarro, 1999). Alterations in the extracellular protein profiles under starvation condition may thus reflect the stability of these virulence factors in *Salmonella*. In addition, the appearance of new proteins in stressed *Salmonella* cells may be due to starvation, making the bacteria able to change nutrient pathways. The paucity of food in seawater can also lead to the loss of some features, either by repression of the specific enzymes or following modifications at the level of the bacterial wall. The effect of environmental conditions on the production of extracellular proteolytic enzymes could play an important role in the induction or repression of the enzyme by specific compounds (Secades and Guijarro, 1999). Production of extracellular proteases has been shown to be sensitive to repression by different carbohydrate and nitrogen sources (Haulon et al., 1982). Catabolic enzymes responded to both carbon and nitrogen control in enteric bacteria (Goldberg et al., 1976). In the bacteria *Aeromonas hydrophila* (O'Reilly and Day, 1983), *Aeromonas salmonicida* (Dalhe, 1971), and *Pseudomonas aeruginosa* (Jensen et al., 1980), protease production is influenced by carbon and nitrogen sources. Additionally, temperature can influence protease production, as occurs in *A. hydrophila* (O'Reilly and Day, 1983).

Adaptation of *Salmonella* to the host milieu involves sensing of environmental changes and subsequent co-ordinated expression of virulence genes. In this work, the relative expressions of *sopE2*, *invA*, *sipA*, and *sopB* virulence genes in starved *S. enterica* serovar Typhimurium cells were investigated. Our study showed instability in the expression of several *Salmonella* Pathogenicity Island (SPI-1) genes after starvation. This is in good agreement with a previous report that environmental conditions such as oxygen, osmolarity, pH and Mg^{2+} deprivation, in addition to growth state, are conditions known to affect the expression of SPI-1 or SPI-2 genes (Bajaj et al., 1996; Deiwick et al., 1998; Lee and Falkow, 1990; Ernst et al., 1990; Deiwick et al., 1999). Under these stress conditions, bacteria modulate their gene expression (Asakura et al., 2006; Ben Abdallah et al., 2009). This modulation is essential for *in vivo* survival since strains lacking this ability due to a mutation in the *toxR* gene, the product of which is involved in signal-dependent virulence gene expression, do not efficiently colonize human volunteers (Herrington et al., 1988). However, contrary to Leclerc et al. (1998), as starvation can repress *invG* and *prgH* genes in *Salmonella*, it can also enhance the expression of their genes, such as *sopB.*

In summary, nutrient deficiency and/or osmotic stress in seawater cause alterations in the synthetic functions of *S. enterica* serovar Typhimurium cells, manifested by modifications in the OMP and extracellular protein profiles. In addition, the expression levels of some virulence genes are also altered. This may reflect the virulence state of starved bacteria.

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