Alteration of outer membrane proteins, secreted proteins and virulence gene expression of \textit{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.

Accepted 31 May, 2012

The foodborne pathogen \textit{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 \textit{sopE2}, \textit{invA}, \textit{sipA} and \textit{sopB} virulence genes were also studied by reverse transcriptase polymerase chain reaction. Our findings showed that the expression level of \textit{sopE2} was slightly decreased under starvation. Whereas the quantities of \textit{sopB} mRNA were increased in the two starved strains S1 and S2. The expression of \textit{sipA} was decreased in strain S1 after starvation, but was significantly increased in strain S2.

Key words: \textit{Salmonella}, starvation, alteration, outer membrane protein, extracellular proteins, virulence gene expression.

INTRODUCTION

\textit{Salmonella} is a facultative intracellular pathogen which, depending on the serotype and host, can cause diseases ranging from gastroenteritis to typhoid fever. For example, \textit{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. \textit{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). \textit{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).
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 osmorality. 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 damselae. 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 the genes chalcing 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 osmorality. 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 damselae. 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 the genes chalcing 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 osmorality. 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 damselae. 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 the genes chalcing 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 osmorality. 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 damselae. 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 the genes chalcing 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 osmorality. 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 damselae. 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 the genes chalcing 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 osmorality. 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 damselae. 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 the genes chalcing 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 osmorality. 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 damselae. In addition, Xu et al. (2005) demonstrated that the OMP profile of Vibrio alginolyticus is altered at different sodium concentrations.
Table 1. PCR primers selected for this study.

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

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.

Figure 1. OMPs 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.

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 S1), 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.
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 seawater (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.

(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 seawater (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 *sopE*2 gene in starved and control strain S1, whereas the expression of this gene was decreased slightly in strain S2. The quantity of *invA* 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...
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 ompX, 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 cytolsins, 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 coordinated 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.

**REFERENCES**


