Motility, biofilm formation, apoptotic effect and virulence gene expression of atypical \emph{Salmonella} Typhimurium outside and inside Caco-2 cells

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1. Introduction

\emph{Salmonella enterica} is an important pathogenic bacterium that is responsible for foodborne diseases from self-limiting gastroenteritis to fatal systemic infections throughout the world \cite{1}. The fecal-oral route typically transmits this pathogen, and infections is associated with the ingestion of contaminated food or water \cite{2}. The occurrence and persistence of such enteric bacteria in aquatic ecosystems represent a serious risk to human health \cite{3}. During its life cycle, \emph{Salmonella} has to face numerous stresses in the natural environment and through an infected host \cite{4,5}. Interestingly, pathogenic bacteria discharged into marine environments are exposed to several stress cues, including temperature, solar radiations, pressure, high salinity, and depletion of micronutrients, with survival and proliferation often depending on the ability to regulate gene expression, morphology and physiology \cite{6}. On the other hand, after evacuating an intracellular environment, bacteria usually encounter several challenges, which are part of the host defence against infections \cite{7}. Pathogenic bacteria then must manage extreme acid conditions within the stomach, elevated concentrations of bile salts, increased osmolarity, changing nutrient/ion availability, low oxygen tension conditions and competition with the host’s microbiota in the intestine \cite{8}. Consequently, as other pathogenic bacteria associated with food and waterborne infections, \emph{Salmonella} has evolved different mechanisms to modulate phenotypic and genotypic expression as a function of their changing environmental \cite{9}. These modifications increase the chances of microorganisms to survive and grow under stressful conditions, both inside and outside the host. For example, \emph{Salmonella} has a complex regulatory system, which mediates their response to cope with the challenges. The alternative sigma factor RpoS is among the global regulators in this microorganism, which play a critical role in responses to changing conditions including starvation and low pH and for \emph{Salmonella} virulence \cite{10}. RpoE sigma factor also is known to be involved in response to nutrient deprivation, oxidative stress, and is important during survival, replication inside macrophages and \emph{Salmonella} virulence \emph{in vivo} \cite{11}.

In order to establish a successful infection in the broad-host-range, \emph{Salmonella} utilize a wide variety of virulence factors, such as flagella, host colonization factors (fimbriae, adhesins/invasins), many secreted toxins and biofilm-related proteins \cite{12}. Herein, motility and flagella are considered an important virulence factors contributing to...
gastrointestinal disease caused by this pathogen [13]. Previously, it has been demonstrated that flagellate S. Typhimurium mutants can attach to, but are defective in entering, cultured intestinal epithelial cells [14]. In addition to the flagella system, which is also considered a type III secretion systems (T3SS), S. enterica encodes two virulence-related T3SS, T3SS1 and T3SS2, on Salmonella pathogenicity island 1 (SPI1) and Salmonella pathogenicity island 2 (SPI2), respectively [15,16,17]. SPI1 is involved in bacterial invasion into epithelial cells [18], whereas SPI2 is responsible for intracellular pathogenesis and has a crucial role for systemic S. enterica infections [19]. The SPI-1 TTSS of this pathogen delivers at least 13 effector proteins, across the inner and outer membranes of the bacterial cell. Some of these effectors, including SopA, SopB (SigD), SopD, SopE1, SopE2, SspH1, and SliP, which are scattered around the S. Typhimurium chromosome [20]. It is interesting to note that, upon colonization, Salmonella operate a transcriptional machinery controlled by the master SPI1 regulator HilA [21].

Infections due to Salmonella species represent a major health problem worldwide and especially in developing countries [22,23]. Therefore, understanding how these microorganisms withstand and adapt to several environmental cues, both outside and inside the host, is extremely important. Until now, many questions remain unanswered about the link between preadaptation of pathogenic bacteria in harsh conditions such encountered in seawater and the appearance of persistent strains causing severe infection.

As a result of our previous study that demonstrated the appearance of atypical cells of Salmonella with highest virulence and resistance to antibiotics after 12 months in seawater microcosm [24], we further investigated the extent of biofilm formation and motility in stressed Salmonella enterica serovar Typhimurium ATCC LT2 DT104 during 1 year in seawater microcosm. Moreover, adhesion, invasion and apoptotic activity to Caco-2 epithelial cells were determined during infection with normal and stressed Salmonella under differing NaCl conditions. Additionally, the transcriptional profile of several virulence related genes including sopABDEZ and hilA was studied to find out whether seawater preadaptation could affect S. Typhimurium virulence gene expression outside and inside the host cells.

2. Materials and methods

2.1. Salmonella strain and growth conditions

Salmonella enterica serovar Typhimurium ATCC LT2 DT104 was used as the reference strain in this study. The strain was cultured in Luria–Bertani (LB) broth and agar at 37 °C.

2.2. Preparation of stressed bacteria

Filtered–autoclaved seawater samples were prepared by collecting natural seawater from the Tunisian coast of Mahdia (salinity 4%, pH 8), passing it through a 0.22 μm Millipore filter (Bedford,MA) and autoclaving the filtrate at 121 °C for 20 min. The bacterial cultures were grown overnight at 37 °C in Luria Bertani (LB) broth, centrifuged at 13,000 rpm for 15 min and then suspended in 100-ml filtered sterilized seawater to a concentration of 10^8 CFU/ml and then incubated at room temperature under static conditions in 250-ml Erlenmeyer flasks. These stress treatments were given for 12 months in triplicate. One microcosm without bacteria served as negative control [24,25].

2.3. Molecular confirmation of atypical S. Typhimurium

The confirmation of S. Typhimurium incubated under stressful conditions in seawater microcosms was determined by polymerase chain reaction (PCR) according to the method described previously [26]. Bacteria were first cultured on Luria–Bertani agar medium for 24 h at 37 °C. One colony was then cultured in Luria–Bertani broth for 24 h at 37 °C and 1 ml of the culture was centrifuged. The DNA was extracted with an EZ-10 Spin Column Bacterial DNA Mini-Preps Kit (Bio Basic, Canada, INC) in accordance with the manufacturer's protocols. All genomic DNA preparations were quantified by A260/280 spectrophotometry and diluted to 200 ng/μl before use. PCR was performed in a 25-μl reaction volume containing 50 ng of extracted DNA, 5 μl 5 × PCR buffer with 1.5 mM MgCl₂, 200 μM of each deoxynucleotide triphosphates (dNTPs; JenaBioscience, Jena, Germany), 1 μM of each 16S rRNA forward (Salm16S-F) 5′-CCGGGAGGAAAGGTTGTTG-3′ and 16S rRNA reverse (Salm16S-R) 5′-GAGCCGGGAGATTCATCAC-3′ and 1U of GO TagDNA polymerase (Biotype B&M Labs, Madrid, Spain). Amplification was conducted in a MultiGene gradient thermal cycler (Labnet International, Edison, NJ). The reaction mixtures were heated at 94 °C for 2 min and then subjected to 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and elongation at 72 °C for 1 min, followed by a 10-min final extension period at 72 °C. The PCR products (5 μl) were analyzed by electrophoresis (1% agarose gel, 100 V, 30 min), and the products stained with ethidium bromide (0.5 mg/ml) and visualized under ultraviolet transillumination. The amplification products were photographed and their sizes determined by comparison with a 100-bp molecular size marker.

2.4. Motility assay

Swimming and swarming motilities were evaluated in our study to investigate the motility of normal and stressed S. Typhimurium in environments with different NaCl concentrations. Semi solid swarm plates containing (25 g of Luria-Bertani per liter, 0.5 g of glucose per liter, and 0.5% agar). Swim plates were prepared by supplementing (10 g of tryptone per liter, 5 g of NaCl per liter, 2.5 g of glucose per liter, and 0.3% agar) [27]. The swimming and swimming zones were measured after 18, 36, 54 and 72 h incubation periods at 37 °C.

2.5. Biofilm quantification

2.5.1. Total bacteria in biofilm

The ability of bacteria to adhere and form biofilms on solid surfaces was performed in 96-well polystyrene flat-bottom microtiter plates (Orange scientific) as described previously [28], with some modifications. The normal and stressed S. Typhimurium cultures were grown in Luria Bertani (LB) broth without NaCl (Formedium) prepared with various NaCl concentrations (0, 0.3, 0.6, and 1 M) until mid-log phase. A quantity of 20 μl of overnight bacterial culture was added into each well. Each condition was tested in triplicate. Negative control wells contained broth only. After inoculation, plates were incubated at 37 °C for 18, 36, 54 and 72 h. The content of the plate was then poured off and planktonic cells were removed by rinsing with distilled water. The remaining attached bacteria were fixed with 200 μl of methanol per well. After 10 min incubation, the methanol was removed and plates were emptied and air dried. The microplates were stained with 200 μl per well of 1% crystal violet for10 min. Excess stain was removed by washing three-times with sterile distilled water and wells were dried at room temperature. Finally, 200 μl of 33% (v/v) glacial acetic acid were added to each well and bound dye was solubilized from the adherent cells. Biofilm formation was quantified by measuring absorbance of the solution at 570 nm using an automated Multiskan FC reader (Thermo Scientific).

2.5.2. Bacterial viability assays

The spread plate technique was used for the enumeration of viable cells in biofilms. Wells were washed three times with phosphate-buffered saline (PBS) and sonicated (VWR, Radnor, PA) for 5 min, 45 kHz, 240 W in order to disrupt the polysaccharide matrix and homogenize the bacteria. After sonication, a 100 μl of each sample was serially diluted and plated in triplicate on Petri dishes containing TSA medium. Plates were incubated at 37 °C for 24 h, and then the CFU were counted.
for each plated dilution. The results were expressed in log CFU/cm² [29].

2.6. Caco-2 cells culture

The human intestinal epithelial cell line, Caco-2 (ECACC 86010202), was grown in Eagle’s Minimal Essential Medium (EMEM) supplemented with 10% foetal bovine serum (FCS, Gibco), 100 mM nonessential amino acids, and 2 mM l-glutamine (Gibco) 2% penicillin/streptomycin and transferrin (Invitrogen, Merelbeke, Belgium) at 10% CO₂. Caco-2 cells were subcultured every 3 days.

2.6.1. In vitro bacterial adhesion and invasion assay

The adhesive and invasive capacities of normal and stressed S. Typhimurium ATCC LT2 DT104 to Caco-2 cells were performed as described previously [24]. Briefly, Caco-2 monolayers of 80% confluence were sub-cultured and placed into 24-multiwell plates at a concentration of 10⁵ cells per well. Salmonella cells were recovered by centrifugation at 6000 rpm for 15 min, washed twice with sterile phosphate-buffered saline (PBS, pH 7.3) and suspended at a concentration of 10⁶ CFU (colony forming units) ml⁻¹ in EMEM medium. Cells were infected at an MOI of 30 for 1 h at 37 °C in 5% CO₂ atmophere. Following incubation, the unattached bacteria were removed by washing the cell cultures three times with PBS and lysed in 1% Triton X-100 at 37 °C for 5 min in order to release the bacteria. The suspensions were serially diluted and 100 μl of each dilution was plated on solid media. The plates were incubated for 24 h at 37 °C. Numbers of bacterial suspension prepared as described for the adhesion assay.

The adhesion was expressed as the percentage of the number of adhered cell associated bacteria were calculated as total adhering bacterial CFU. The adhesion was expressed as the number of adhered bacteria to total bacteria used for the experiment. In the invasion assay, the bacterial suspension prepared as described for the adhesion assay was applied on Caco-2 cells and incubated at 37 °C for 1 h. The infected cells were washed twice with PBS and incubated in fresh EMEM medium supplemented with 20% PBS and 150 μg/ml gentamicin at 37 °C for 1 h in order to kill extracellular bacteria which had not invaded the cells. Serial dilutions of the lysates were plated onto agar to quantify the intracellular bacteria. The invasion was expressed as the percentage of the number of invaded bacteria to total number of cell-associated Salmonella.

2.6.2. Apoptotic studies

To assess the potential of stressed and control S. Typhimurium to cause apoptosis in Caco-2 cells, the flow cytometric analysis using Fluorescein diacetate (FDA)/PI staining was assessed.

Caco-2 cells were collected by centrifugation at 6000 rpm for 10 min and washed twice with cold PBS. Approximately 5 × 10⁵ cells were resuspended in 500 μl of PBS/0.2% BSA containing 10 nM of FDA (Sigma; F7378; 0.05 g/4 ml DMSO) (from a 5 mM stock in DMSO) for 30 min at room temperature. The cells were then cooled and 10 μl of IP (400 μg/ml PBS) were added. Finally, after 30 min at 37 °C, the stained cells were analyzed with a Coulter Epics Altra flow cytometer [30].

2.7. Bacterial RNA extraction and gene expression

In order to distinguish between sopABDE2 and hilA gene expression in stressed Salmonella under different NaCl conditions and during infection, bacterial RNA was first extracted from overnight cultures in LB (without NaCl, Formedium) prepared with various NaCl concentrations (0, 0.3, 0.6, and 1 M) using SV total RNA isolation system in accordance with the manufacturer’s protocols (Promega). Upon infection, bacterial RNA was extracted from Caco-2 cells as previously described [31]. For each extraction of Salmonella RNA, a total of 10⁶ Caco-2 cells (ECACC 86010202) was seeded in 6-well cell culture plates (Costar; 120 wells in total) and infected with normal and stressed S. Typhimurium at a multiplicity of infection (MOI) of 100:1 (bacteria:cells) [31-34]. After 1 h of infection, extracellular bacteria were killed with 50 g/mL gentamicin. Incubations were continued for 2 h. Then, infected Caco-2 cells were lysed on ice for 30 min in 0.1% SDS, 1% acidic phenol, 1% ethanol in water. The phenol-ethanol mixture acted to stabilize all bacterial RNA [35]. Bacterial RNA was isolated from infected Caco-2 cells and pooled. Pellets were collected by centrifugation, and RNA was prepared using the Promega SV total RNA purification kit. Bacterial RNA was purified further by phenol–chloroform extraction. Size chromatography of RNA

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**Table 1**

Primers used for real-time PCR.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gene abbreviation</th>
<th>Gene ID</th>
<th>Primer Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>E3 ubiquitin ligase</td>
<td>sopA</td>
<td>1253587</td>
<td>F: 5′-TTGCCACCAGTTGCCGACC-3′</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: 5′-TTCACGGCTTGATCTG-3′</td>
</tr>
<tr>
<td>Inositol phosphatase</td>
<td>sopB</td>
<td>1252609</td>
<td>F: 5′-ACACGGCTGGGAAACCCAT-3′</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: 5′-ATGAGAGGAAAGACCCAT-3′</td>
</tr>
<tr>
<td>Secreted effector protein sigD</td>
<td>sopD</td>
<td>125468</td>
<td>F: 5′-AAAGAACATGCTGGAAG-3′</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: 5′-AAAGAACATGCTGGAAG-3′</td>
</tr>
<tr>
<td>Invasion-associated secreted effector protein</td>
<td>sopE2</td>
<td>1253374</td>
<td>F: 5′-ATCAGTTGGAAGACGCCTCTG-3′</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: 5′-ATGCGCTGAGCTGATGAGG-3′</td>
</tr>
<tr>
<td>Transcriptional regulator HilA</td>
<td>hilA</td>
<td>1254399</td>
<td>F: 5′-CATTACGAAGACGGCCGG-3′</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: 5′-AGCGGGTTGAGGTCATTCA-3′</td>
</tr>
</tbody>
</table>
was done with an Agilent 2100 Bioanalyser. The extracted total RNA from each condition was then treated with DNase I (Promega) to remove genomic DNA contamination. Size chromatography of RNA was done with an Agilent 2100 Bioanalyser. Complementary DNA (cDNA) was synthesized from 1 μg of total RNA using the SuperScript III reverse transcriptase (Invitrogen) with an oligo-dT18 primer. The expression of the selected genes was analyzed by real-time PCR, which was performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures (containing 10 μl of 2xSYBR Green supermix, 5 μl of primers (0.6 μM each) and 5 μl of cDNA template) were incubated for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C, and finally 15 s at 95 °C, 1 min at 60 °C and 15 s at 95 °C. As control gene the 16S rRNA was analyzed. The primers used in the present study are shown in Table 1. In all cases, each PCR was performed with triplicate samples.

2.8. Statistical analysis

The results are expressed as mean ± SEM. Data were statistically analyzed by one-way analysis of variance (ANOVA) to determine differences among groups and Tukey test as a post-hoc. All the statistical analysis was conducted using Statistical Package for Social Science (SPSS for Windows; v19.0, USA) and differences were considered statistically significant when p < 0.05.

3. Results

3.1. Molecular confirmation of stressed strain

The resulting PCR product of 16S rRNA gene of Salmonella (178 pb) confirm the identity of the investigated S. Typhimurium strain incubated for 12 months in seawater microcosms (Fig. 1).

3.2. Motility

In both strains, we observed an increase of swimming and swarming motility at all measured time points (Fig. 2, Fig. 3). The swarming motility exhibited by normal S. Typhimurium decrease with increasing NaCl concentrations. In contrast, the cell swar of stressed strain increased significantly when the NaCl concentrations were increased (from 9.44 to 11.15 mm after 72 h of incubation in LB 1 M NaCl). However, no significant variations were observed in stressed S. Typhimurium swimming in the presence of high salt concentrations (Fig. 3C).

3.3. Biofilm formation on polystyrene microplate under different NaCl concentrations

The adherence of normal and stressed S. Typhimurium was
Fig. 3. Swarming (A, B) and Swimming (C) motilities of normal and stressed S. Typhimurium under different NaCl concentrations. Cells were inoculated with a sterile toothpick. Plates were incubated at 37 °C, and pictures were taken after 54 h using Gel Doc Syngene (Ing).

Fig. 4. Biofilm formation at 18, 36, 54 and 72 h post inoculation of the non stressed strain of S. Typhimurium LT2 DT104 and its derivative strain recovered after 12 months of incubation in seawater microcosm under different NaCl concentrations. (A, B) Biofilm formation was assessed by staining the attached bacteria with crystal violet and measuring the OD values at 570 nm (CV570). (C, D) Quantitative biofilm formation using viable bacteria counting method. Different letters on the bar are significantly different at P < 0.05 between the different NaCl concentrations at the same time. Significant differences between normal and stressed bacteria in the same condition were denoted by asterisks.
determined in LB broth prepared with different concentrations (0, 0.3, 0.6, and 1 M) of NaCl during 18, 36, 54 and 72 h of incubation at 37 °C (Fig. 4A, B). The data showed that the ability to form biofilms was significantly increased in starved strain compared to the wild-type strain at high NaCl concentrations (0.6 and 1 M) (P < 0.05).

Interestingly, the numbers of viable adhered cells of normal S. Typhimurium in 1 M were decreased from 8.81 to 5.1 and 9.54 to 4.01 log CFU/cm², respectively, after 54 and 72 h of incubation (Fig. 4C), whereas starved bacteria showed high numbers of viable adhered cells at different NaCl concentrations (Fig. 4D).

3.4. The impact of different NaCl concentrations on S. Typhimurium adhesion and invasion

The adhesion and invasion abilities of normal and stressed S. Typhimurium to Caco-2 cells were evaluated under different NaCl concentrations (Fig. 5). Adhesion of stressed bacterium was significantly increased in a high NaCl environment compared to normal or low NaCl conditions giving mean values of 1.94% and 6.85%, respectively, in LB prepared with 0 and 0.6 M NaCl (Fig. 5A). However, the ability of normal S. Typhimurium to adhere to Caco-2 cells was inversely regulated by the NaCl concentration (Fig. 5A).

The invasive ability of the stressed strain was significantly increased (P < 0.05) in high salt conditions, corresponding to the adhesion ability (Fig. 5B).

3.5. Apoptotic cell death analysis

The double FDA/PI staining flow cytometry assay was used to discriminate apoptotic cells from live cells. Caco-2 cells treated with normal and stressed S. Typhimurium grown at various salt concentrations showed apoptotic cells (FDA/PI) at 6, 12 and 24 h (Fig. 6). Interestingly, when bacteria grown in LB containing 0.3 M NaCl that mimic the salt concentration in the intestine, the number of apoptotic cells was found to be significantly higher (P < 0.05) when the Caco-2 cells were treated with stressed S. Typhimurium (20.1% ± 2.52% at 12 h).

3.6. Gene expression of stressed S. Typhimurium in response to salt stress and upon infection of Caco-2 cells

In the present study, expression levels of four SPI-1 virulence genes (sopA, sopB, sopD, sopE2 and hilA) of normal and stressed Salmonella at different salt stress conditions and upon infection of Caco-2 cells were investigated by qRT-PCR (Fig. 7).

Gene expression analysis found significant difference between bacterial samples grown in LB medium and S. Typhimurium grown in Caco-2 cells. Interestingly, we found that expression of most selected genes was greatly increased in stressed bacteria cultured on human intestinal epithelial cell line. When stressed S. Typhimurium was grown at an osmolarity (0.3 M NaCl) equivalent to that of human intestine, expression of hilA gene was significantly overexpressed compared to nonstressed S. Typhimurium. Under hypersomotic conditions (1 M NaCl), the expression level of sopA gene was significantly upregulated in stressed strain. Infection of Caco-2 cells with stressed S.
Typhimurium significantly regulated expression of sopB, sopD, sopE2 and hilA genes. Here, we found that growth conditions can affect the expression of invasion-related genes and therefore the ability of Salmonella to adapt to the intracellular environment.

4. Discussion

The enteric pathogen Salmonella enterica encounters various unscheduled changes during its life cycle in the environment and in the host during infection [36]. The pathogen then modulates its gene expression and trigger appropriate responses that allow survival and propagation upon exposure to these above stresses [37,38]. In many bacterial species, biofilm formation can be considered a stress response mechanism. It has long been known that hostile conditions, including temperature, iron and pH, starvation, oxygen tension and osmolarity have important effects on biofilm formation [39]. In our study, we showed that the preadaptation of Salmonella to challenges in seawater microcosms influences its ability to form biofilms in the presence of different NaCl concentrations. Several studies have underlined the correlation between the persistence of Salmonella strains and their ability to form biofilms in both host and non-host environments. Vestby et al. [40] have demonstrated that the persistent strains clearly were better biofilm-producers than the non-persistent strains. Thus, biofilm formation is an efficient mechanism that makes bacteria resistant to stressful conditions, antibiotics disinfectants and the host immune system, consequently providing a fitness advantage for the spread of Salmonella and its persistent infections in patients. Biofilm formation has been demonstrated in host cancer tissue, on cultured epithelial cells and on gallstones [41]. In addition to biofilm forming, motility is also correlated with virulence in pathogens [42]. Herein, we investigated how sequential increase of NaCl concentrations affect swimming and swimming motilities of normal and stressed S. enterica. These results demonstrated that unlike swimming, swimming motility of stressed S. Typhimurium increased significantly when the NaCl concentrations were increased. This allowed us to conclude that preadaptation to high osmolarity in seawater microcosms alter the influence of such parameter on Salmonella motility. Several reports investigated the effect of various stressful conditions on the motility and chemotaxis of pathogenic bacteria. In E. coli, it has been shown that even modest increases in extracellular NaCl (400–500 mOsM/L) arrest swimming and impair the flIC regulatory element [43,44]. Similarly, both acidification and alkalization of the culture medium restricted bacterial motility. This is in accordance with reports on P. aeruginosa, where a 500 millimolar NaCl in an environmental isolate abolished motility and diminished flagellar gene synthesis [45]. The relationship between biofilm formation and motility tends to be complex because both processes might involve similar components at specific conditions and certain stages [46]. For instance, Yeung et al. [47] reported that at least 18 transcriptional regulators are involved in the regulation of biofilm formation and motility such as swarming. Many of these transcriptional regulators were also shown to have inverse effects on biofilm formation and swarming motility For enteric bacteria like Salmonella, decision-making between rapidly colonizing a surface and biofilm formation is central to bacterial survival among various stress conditions. Several reports showed that switching between motility and sessility aids the pathogen to overcome harsh environmental conditions by increasing the efficiency of nutrient acquisition, escaping from toxic substances and accessing to favorable colonization sites [48]. Flagella and motility are required early in biofilm formation for locating and initiating attachment of bacteria to an appropriate surface [49]. Some pathogenic strains require motility early in biofilm formation. By comparing multiple strains of E. coli in flow cells, motility and biofilm formation were found to be correlated such that strains with the most robust biofilm formation also displayed the most vigorous motility as planktonic cells [50]. However, motility is also involved in the release of bacteria from mature biofilms [51]. Furthermore, some mutants with enhanced motility are associated with decreased biofilm formation suggesting that the maintenance of motility may destabilize nascent multicellular aggregates [49]. Swarming is one of the types of bacterial motilities through which bacterial cells move around to aid systemic infection and biofilm formation [52]. Shrouf et al. [52] demonstrated that P. aeruginosa must be able to integrate several important cell functions early in biofilm formation, namely, swarming motility and matrix production. Swarming is the fastest known bacterial mode of surface translocation and enables the rapid colonization of a nutrient-rich environment and host tissues [53]. It has been particularly well studied with respect to its relationship to biofilm formation [54]. Nevertheless, bacteria might select between motility, such as swarming, and biofilm formation at certain stages [46]. The ability of Salmonella to survive in a wide range of extreme conditions in the nonhost environment, which likely play a role in increased its fitness in the host environment, draws the attention of several researchers. In fact, it remains unclear how the bacteria adapt, escape immune responses and overcome body defenses. In this regard, we compared the impact of different NaCl concentrations on the adhesive and invasive capacity of normal and stressed S. Typhimurium. Our findings show that the preincubation in extreme environments specifically in seawater enhance the capacity of bacteria to grow at high salt content and improve its performance to adhere to and invade intestinal epithelial cells in vitro.

As a pathogenic gram-negative bacterium, Salmonella uses various strategies to withstand host defenses, and one of the major common strategies used during the course of infection is to induce programmed cell death, apoptosis, in eukaryotic cells [55]. Little is known about the induction of apoptosis under widely contrasting host environments including the high osmolarity encountered by the pathogen in the intestine. In the current study, the flow cytometric analysis using Fluorescein diacetate (FDA)/PI staining demonstrated the characteristic changes of apoptosis. A significant increase in the number of apoptotic cells was observed following the infection of intestinal epithelial cells.
with the pathogen grown under stressful conditions in seawater. Likewise, Chanana et al. [56,57] have examined the induction of apoptosis under the different host environments, including the iron limitation and oxidative stress experienced by pathogens in the macrophage milieu.

In an effort to understand the effect of various stressors on pathogenic bacteria discharged into marine waters, we compared the virulence genes expression of stressed \textit{S. Typhimurium} in response to salt conditions encountered in the extracellular environment in LB broth and after epithelial cell exposure. The studied SPI-1 genes (\textit{sopA}, \textit{sopB}, \textit{sopD}, \textit{sopE} and \textit{hilA}) had significantly different expression levels in bacteria grown in LB medium and within Caco-2 cells. Compared to gene expression inside Caco-2 cells, expression levels of \textit{sopB}, \textit{sopE} and \textit{hilA} in stressed strain, which grown in LB supplemented with 0.3 M NaCl at an osmolarity equivalent to that of human intestine, was significantly more overexpressed. The lumen of the distal ileum is a low-oxygen and high-osmolarity environment, and approximate osmolality (Osm = moles per kilogram) of human colonic contents is known to be 0.3 to 0.6 Osm [58,59]. It has been believed that bacterial invasion of the intestine is related to expression of pathogenic genes altered in response to high-osmolarity of the environment [60].

Generally, virulent genes are thought to be repressed or shut down in bacteria growing outside the host [61]. In the external environment, pathogens need to express certain sets of gene which make them competent enough to survive and overcome stressful situations. Many studies have shown that other sublethal stress conditions such as thermal stress contribute to the down-regulation of SPI-1 invasion genes [62]. Virulence factors are repressed under such conditions and metabolic genes are activated [63]. While, in the intracellular environment, pathogens need to express specific genes which help them to succeed in invading and surviving the host [64]. Previous research studies revealed that high osmolarity in the intestine lumen of host induce the expression of some SPI-1 genes [65]. It has been demonstrated that expression of SPI-1 genes was optimal under conditions of high osmolarity during late-log phase growth, and promoted by HilA and SirA in \textit{S. enterica} serovar Typhimurium [66,67]. Moreover, the expression of SPI1 genes and the secretion of T3SS1 effectors have been shown to be strongly influenced by several environmental signals including bacterial growth state and oxygen concentration [68]. Ibarra et al. [69] have reported that aerobically grown late-exponential-phase bacteria were more invasive and this was associated with a greater
frequency of SPI1-induced. In fact, the crucial role of SPI1 in the invasion was previously demonstrated because mutations in this region reduce the ability of S. Typhimurium to invade epithelial cells in vitro [70].

In conclusion, this study suggests that environmental challenges induced changes in motility influence the ability of persistent Salmonella to cause human infection. This may elucidate why invasion by the pathogen occurs mainly in distal portion of the ileum of the small intestine. It is also suggests that bacterial infection of the intestine is by the pathogen occurs mainly in distal portion of the ileum of the small Salmonella [10]. P.C. Loewen, R. Hengge-Aronis, The role of the sigma factor sigma (KatF) in E. coli. Microbiol. 48 (1994) 53–68.

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