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Full Length Research Paper

Outer membrane proteins and morphological alterations of *Shigella spp*. under starvation in seawater

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In this study, we incubated four strains of *Shigella* in seawater microcosms (at room temperature and at 4°C) for eight months and we studied the alteration of their morphologic and outer membranes proteins. The starved cells showed an evolution to the filterable minicells state capable to pass membrane pore size 0.45 µm. In addition, the atomic force micrographs showed a reduction of the cells size and an evolution to coccoid-shapes. Outer membrane proteins patterns of stressed bacteria did not changed too much and these modifications were manifested by the appearance of one new band.

Key words: *Shigella*, seawater, starvation, morphological, outer membrane proteins.

INTRODUCTION

Bacillary dysentery is an acute inflammatory bowel disease caused by the enteroinvasive bacteria *Shigella*, leading to a condition known as shigellosis (Hale, 1998). *Shigella* are Gram-negative bacteria that have the ability to invade the colonic and rectal epithelium in humans, causing the acute mucosal inflammation that characterizes the disease. *Shigella* is a highly contagious microorganism because as few as 10 to 100 bacteria can cause the disease in adult volunteers. After oral contamination, bacteria pass through the stomach and the small intestine before reaching the colon where they invade the mucosa, initiating the acute destructive rectocolitis that causes the dysenteric symptoms: fever, intestinal cramps, and emission of mucopurulent and bloody stools. Many factors such as the 220 kbp plasmid, invasive plasmid antigens, antioxidant enzymes, lipopolysaccharides, and outer membrane proteins (OMPs) contribute to its virulence (Raja et al., 2008).

Enteric bacteria, disseminated in marine environment, are submitted to multiple physicochemical stresses: high osmolarity, low temperature, nutrient starvation and solar light irradiation (Ellafi et al., 1992). The ability of enteric bacteria to adapt to fluctuations in the ambient osmolarity is of fundamental importance for their survival (Bakhrouf et al*.,* 1994). To survive prolonged periods of starvation, many bacteria have developed starvation-survival strategies enabling them to persist in the environment until conditions become favorable for growth. One of the most frequently observed behaviours in the nutrient starvation response of Gram negative bacteria is the size reduction and cell morphology conversion from rod to coccoid shape (Kjelleberg et al., 1993). Indeed, the starvation conditions caused dramatic changes in gene expression, physiology and morphology. The starvation survival of *Escherichia coli, Vibrio* spp. and *Salmonella typhimurium* is dependent on differential protein synthesis (Nystrom et al., 1990; Sepector and Cubitt, 1992; Morton and Oliver, 1994). Bulk protein synthesis is sharply reduced during starvation and is associated with the lower expression of proteins necessary for exponential growth (Reeve et al., 1984; Nystrom et al., 1990). During the early hours of starvation, novel proteins whose continued activity and production is required for long-term survival are synthesized (Reeve et al., 1984a; Morton and Oliver, 1994). There is also evidence that the degradation of existing proteins may be a major source of amino acids utilized for protein synthesis during

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starvation (Reeve et al., 1984b).

Additionally, outer membrane proteins (OMPs) play a key role in the adaptation to changes of external environments, due to their location at the outmost area of the cell (Xu et al., 2005).

The mechanisms of molecular responses of the bacteria to signals coming from the external environment are complex and depend, among others, on two-component regulatory systems (Albright et al., 1989; Barrett and Hoch, 1998). The regulatory system *EnvZ*/*OmpR* participates in the bacterial response to changes in the osmolarity of the external environment (Aiba et al., 1989). It has best been studied in *E. coli*, but is also found in such other pathogens as *Salmonella* or *Shigella*. The function of the regulatory protein *OmpR* of *E. coli* includes both the positive and negative regulation of the transcription of porin proteins *OmpF* and *OmpC* (Forst et al., 1989). Under different osmolarities, *OmpF* and *OmpC* are the most studied models of regulation in *E. coli*. *OmpF* is highly expressed at low osmolarity and suppressed at high osmolarity, while *OmpC* is totally the opposite (Cai and Inouye, 2002). More and more similar regulations have been found in other bacteria, such as major outer membrane protein and *Omp50* in *Campylobacter jejuni* (Dedieu et al., 2002), and *OmpK35* and *OmpK36* in *Klebsiella pneumoniae* (Hernandez-Alles et al., 1999). Furthermore, Wu et al. (2006) indicated that *OmpW* and *OmpV* are required for environmental salt regulation in *Photobacterium damsela*, in which *OmpW* and *OmpV*, respectively, elevate and reduce the ability in salinity tolerance.

The aim of this work was to evaluate the morphologic change using Atomic force microscopy (AFM) of *Shigella* to starvation conditions in seawater. Appearance of filterable minicells was determined through membranes pore size 0.45 and 0.22 µm.

In order to study the OMPs profiles of starved cells, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was used.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Four *Shigella* strains were used in this study: two *Shigella sonnei* (S1+ S2), *Shigella boydii* (S3) and *Shigella flexneri (*S4). All strains were provided from the Monastir hospital in Tunisia and maintained at -80°C in Luria-Bertani broth (LB) supplemented with glycerol (15%, v/v). For the experiments, the cells were grown at 37°C in tryptic soy broth (TSB, (Difco)) for 24 h. Natural seawater (100 ml) from the Tunisian coast of Monastir (salinity 4%, pH 8) was filtered through membranes (pore size, 0.22 µm; Millipore Corp., Bedford, Mass.) and autoclaved (115°C for 15 min) in 100 ml Erlenmeyer flasks. *Shigella* cells were washed three times by centrifugation (13000 rpm for 10 min at 29°C) with autoclaved seawater and then suspended in 10 ml of autoclaved seawater (Ellafi et al., 2009). The microcosms (100 ml) were inoculated with these suspensions (approximately 10^9 cfu/ml) and then incubated in a static state at room temperature (22 to 25°C) and at 4°C.

Production of filterable minicells

In order to detect the filterable minicells produced by all starved *Shigella* strains after its incubation for eight months in seawater, 1 ml of each microcosm was filtered through membranes pore size 0.45 and 0.22 µm (Millipore Corp., Bedford, Mass.).

100 µl of each filtrate was plated on TSB prepared with seawater and incubated for 24 h at 37°C.

Determination of morphological changes by AFM

In order to visualize any morphological changes in the starved strains, *Shigella* cells were examined, in triplicate, by AFM (Nanoscope IIIA, Digital Instrument, VEECO).

For the experiments, the cells were collected, washed three times with phosphate-buffered saline (PBS), and centrifuged. The final pellet was resuspended in PBS, placed on a round microscope cover slide and was simply dried in air according to the method previously described (Braga and Ricci, 1998; Ben Abdallah et al., 2007).

OMP extraction

OMPs of *Shigella* strains, before and after incubation in seawater, were prepared according to the method described previously (Sabri et al., 2000). Briefly, the bacterial cells were harvested by centrifugation at 4000 g for 15 min at 4°C. The cells were then washed three times in 40 ml of sterile saline water (0.9% NaCl) and resuspended in 5 ml sterile saline water. Cells were disrupted by intermittent sonic oscillation. Unbroken cells and cellular debris were removed by centrifugation at 5000 g for 20 min. Supernatant was collected and further centrifuged at 100,000 g for 40 min at 48°C. The pellet was resuspended in 10 ml of 2% (w/v) sodium lauryl sarcosinate (Sigma, St. Louis, MO) and incubated at room temperature for 1 h, followed by centrifugation at 100,000 g for 40 min at 48°C. The resulting pellet was resuspended in 200 ml sterile saline water and stored at -20°C. The concentration of the OMPs in the final preparation was determined using the Bradford kit (Sigma).

SDS-PAGE

OMPs (1 mg) were analyzed by SDS-PAGE (Laemmli, 1970) with 15% acrylamide in the separating gel and 5% in the stacking gel. After separation, the proteins were viewed according to standard procedures by staining with Coomassie brilliant blue G250 (Sigma) and their molecular weights determined using commercial markers (High-Range Rainbow; Amersham, Little Chalfont, Buckinghamshire, UK).

Molecular identification of stressed bacteria

Aiming to confirm the starved *Shigella* strains incubated during one month in seawater microcosms, polymerase chain reaction (PCR) of the gene *ipaH* was used. The primer pairs used in this study were: 5'-GTT CCT TGA CCG CCT TTC CGA TAC-3' and 5'-CAT TTC CTT CAC GGC AGT GGA-3' (Hartman et al., 1990). Chromosomal DNA was extracted by using a direct lysis method. PCR were performed in 25 µl containing 50 ng of extracted DNA, 5 µl green Go *Taq* buffer (5×), 0.25 µl dNTPs (10 mM), 0.5 µl $MgCl_2$ (50 mM) , 1 µl of each primer (25 pM), 1 U of GO *Taq* DNA polymerase (Promega, USA). The PCR mixtures were subjected to thermal cycling. The cycle conditions were as follow:

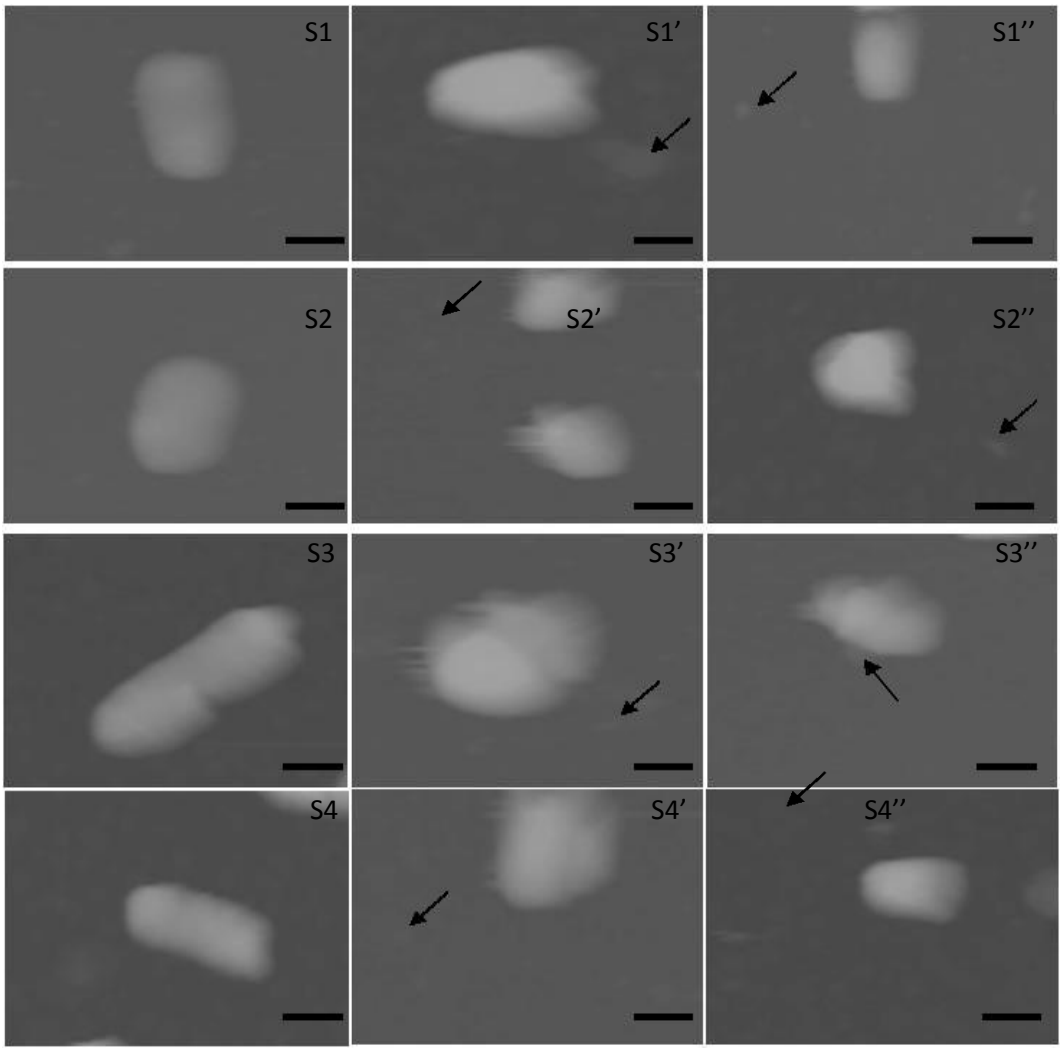

Figure 1. Atomic force micrographs of *Shigella* cells exposed to starvation for eight months in seawater. Sn : strain before incubation in seawater; Sn' :strain incubated during eight months in seawater microcosms at room temperature. Sn'':strain incubated during eight months in seawater at 4°C; arrows indicate the cells of size less than 0,4 µm; bars:1 µm.

An initial incubation at 94°C for 5 min was followed by 35 cycles of denaturation at 94°C for 90 s, annealing at 57°C for 30 s and elongation at 72°C for 90 s, followed by 10 min of final extension period at 72°C. PCR products (5 µl) were analysed on 1% agarose gels stained with ethidium bromide (0.5 mg/ml) at 90 V for one hour and visualized under ultraviolet transillumination. The amplification products were photographed and their sizes were determined with 100 bp molecular size marker (Promega, France).

RESULTS

Appearance of filterable minicells

Before their incubation, all the *Shigella* strains are unable to cross membranes pore size 0.45 and 0.22 µm. After one eight of starvation in seawater, all the studied strains produced a filterable minicells through membranes pore size 0.45 µm. Indeed, the filterable *Shigella* minicells formed colonies on TSA prepared with seawater after 48 h of incubation at 37°C and confirmed their identity by amplification of *ipaH* gene.

Furthermore, no strain can be able to pass membranes pore size 0.22 µm after this period of stress.

Morphological alteration of stressed strains

Alterations in cell morphology due to starvation stress in seawater were examined by AFM (Figure 1). The control *Shigella* cells, whose length is between 4 and 3 µm, have

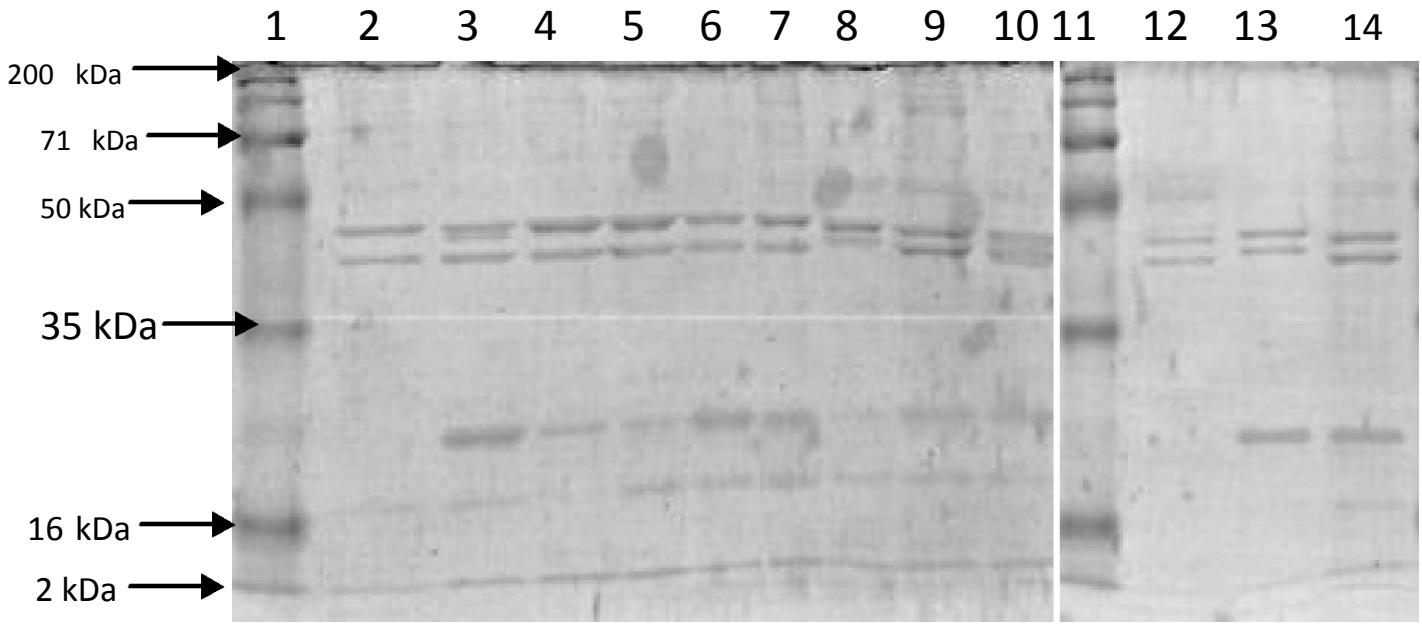

Figure 2 Outer membrane proteins of *Shigella* cells exposed to starvation for eight months in seawater. Lane 1 and lane 11, molecular weight marker; lane 2, S1; lane 3, S1'; lane 4, S1''; lane 5, S2; lane 6, S2'; lane 7, S2". lane 8, S3; lane 9, S3'; lane 10, S3''; lane 12, S4; lan e 13, S4'; lane 14, S4''. Sn : strain before incubation in seawater; Sn' :strain incubated during eight months in seawater microcosms at room temperature. Sn'':strain incubated during eight months in seawater at 4°C.

a rod shape (Sn). Whereas, after eight months of their incubation in seawater microcosms, the cells obtained present two coccoid shape forms (Sn' and Sn") whose length is less than 1 and 0.4 µm.

OMP analysis

OMPs of *Shigella* strains before and after incubation in seawater were analyzed by SDS-PAGE (Figure 2). Before their incubation in seawater, we noted that all the *Shigella* cells present two or three majors proteins in their profiles. After 8 months of incubation in seawater, we have noticed these cells preserved their initial OMPs and their expression was stable. In addition, we have observed the appearance or the level expression of one band corresponding to molecular weights of ⊃ 26 kDa approximately after starvation at room temperature and at 4°C for all the strains. These modifications are probably due to nutrient deficiency in seawater.

Molecular confirmation of stressed cells

We used the technique of PCR to identify all tested strains of *Shigella* after eight months of incubation in seawater microcosms (Figure 3). After amplification of *ipaH* gene by PCR, we confirmed the identity of the investigated *Shigella* strains.

DISCUSSION

The results, developed in this study, showed that *Shigella* is able to adapt and survive under starvation conditions. After eight months of incubation in seawater, we have noted an evolution of investigated *Shigella* cells to filterable minicells capable to pass membrane pore size 0.45 µm but not 0.22 µm. Similar results have been reported in *Salmonella paratyphi B* (Bakhrouf-Ben Fedhila et al., 1990), *Vibrio* (Ben abdallah et al., 2009) and *Pseudomonas aeruginosa* (Bakhrouf et al., 1989) after their incubation in seawater. In addition, *Shigella* decreased in size and became coccoid in morphology scanning electron microscopy analysis, which was in agreement with the results reported by other authors (Chaiyanan et al., 2001).

According to Morita (1993), several bacteria such as *Shigella* can survive for a long period under stressing environmental conditions owing to gradual changes in cellular physiology and morphology. When nutrient conditions of aquatic habitats become unfavourable, both sessile and planktonic bacterial cells are sharply reduced in size to form very small (±0.3 µm), spherical ultramicrobacteria (also termed ultramicrocells) by a process that is now well documented as starvation-survival (Kjelleberg, 1993; Morita, 1997). As a consequence of forming ultramicrobacteria, the surface/ volume ratio becomes larger, which allows nutrients to be sequestered more efficiently in low-nutrient

Figure 3. Agarose gel electrophoresis of polymerase chain reaction (PCR) amplification of ipaH gene. Lane 1, 100 bp DNA molecular size marker; lanes 2 to 14, PCR amplicons obtained with DNA amplification of Shigella strains. Lane 2, negative control; lane 3, S1; lane 4, S1'; lane 5, S1''; lane 6, S2; lane 7, S2'; lane 8, S2". lane 9, S3; lane 10, S3'; lane 11, S3'' ; lane 12, S4; lane 13, S4'; lane 14, S4". Sn : strain before incubation in seawater; Sn' :strain incubated during eight months in seawater microcosms at room temperature. Sn'':strain incubated during eight months in seawater at 4°C.

environments. The concept of starvation-survival is fundamental to the evolutionary point of view. In order to provide a pragmatic approach to this concept, a definition has been provided by Morita (1997):

"Starvation-survival is a physiological state resulting from an insufficient amount of nutrients, especially energy, to permit growth (cell size increase) and/or reproduction."

To confront nutrient limitation, bacteria may develop defence mechanisms to enhance their ability to survive periods of starvation. Some differentiating bacteria respond to starvation by a marked alteration in their ultrastructure, producing spores or cysts.

Outer membrane profiles of *Shigella* starved eight months in seawater does not show a very large variation from the normal cells. After incubation, we have noticed these cells preserved their initial OMPs and their expression was stable. In addition, we have observed the appearance or the level expression of one band corresponding to molecular weights of ⊃ 26 kDa approximately after starvation at room temperature and at 4°C for all the strains. These modifications are probably due to nutrient deficiency in seawater. Indeed, it is clear now that changes in the environment induce several alterations in bacterial function and protein expression.

After the beginning of an adverse effect, such as starvation, the synthetic functions of cells became inhibited and cells division is interrupted. *E. coli* was shown to synthesize specific sets of proteins in response to individual stress conditions involved in seawater stress, such as carbon, phosphate, or nitrogen starvation (Groat et al., 1986), osmotic stress (Botsford, 1990; Clark and Parker, 1984), cold shock (Jones et al., 1987), elevated hydrostatic pressure (Welchet al., 1993), and oxidative stress (Walkup and Kogoma, 1989). Starvation was shown to induce cross protection against osmotic stress (Jenkins et al., 1990) and H_2O_2 (Jenkins et al., 1988), attributed in both cases to proteins induced under both conditions. The altered osmolarity of the culturing medium caused changes in the OMP patterns of *Vibro alginolyticus* (Xu et al., 2005) and *Vibro parahaemolyticus* (Xu et al., 2004). The composition of the culturing medium may have significant eVects on protein expression. Furthermore, Yoshida et al. (2002) revealed changes in concentrations of *OmpR* and *OmpP*, while Contreras et al. (1995) demonstrated an increase in the *OmpC* and a decrease in the *OmpF* expression of *E. coli*. The effects of magnesium limitation on *OMP* expression in *P. aeruginosa* (Shand et al., 1988) and *Salmonella* strains (Hwang et al., 2002) have been also observed. *OMPs*, whose production is often regulated by environmental cues, play important roles in bacterial pathogenesis by enhancing the adaptability of bacterial pathogens to various environments. Thus, *OMPs* represent important virulence factors and play essential roles in bacterial adaptation to host niches, which are usually hostile to invading pathogens (Lin et al., 2002; Ben Abdallah et al., 2009). The various observed in the *OMP* profiles of stressed strains reflect the stability of these virulence factors under stress conditions.

Conclusion

The consequences of adaptation of *Shigella* to starvation in seawater are multiple: change in the morphological characters and the *OMP* profiles. These variations can pose a serious problem during the characterization of these strains isolated from the environment.

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