Full Length Research Paper

The combined effects of starvation and pH on the virulence of *Shigella sonnei* ATCC25931

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Shigella sonnei encounter numerous different stresses during their growth, survival and infection. In this study, the effect of stress response to pH and starvation was investigated. We studied the survival, adhesion and the morphology of *Shigella* after its incubation in several pH. Our results show that after 2 h of incubation, the rate of cell survival was proportional to the decrease of pH of the medium, but we also noted that the reduction of the cells viability is significant for the normal cells compared to starved cells. In addition to that, the results proved the evidence that the pH can influence significantly the hydrophobicity of *S. sonnei* (normal and starved) and their capacity to produce biofilm on Congo red agar and on polystyrene microplate wells. The atomic force micrographs showed a reduction of the cells size after stress.

Key words: *Shigella sonnei*, starvation, pH, survival, adhesion, morphology.

INTRODUCTION

Shigella is a member of the Enterobacteriaceae family and causes shigellosis characterized by bacillary dysentery (mucoid bloody stool). *Shigella* is commonly found in water contaminated with human feces and fecal–oral route is the primary mode of transmission. It is responsible for a worldwide health problem; however, it is a serious concern in developing countries. It is estimated that *Shigella* spp. account for 1.1 million deaths and 165 million cases of dysentery annually worldwide. Shigellosis has a high morbidity and mortality rate in children less than 5 years of age. Malnourished children are highly susceptible and *Shigella* infection further promotes impaired nutrition, recurring infection and retarded growth. Antibiotic resistant strains are continuously emerging thus treatment regiments become very difficult against shigellosis.

The environmental adaptation plays an important role for pathogens to survive in natural environment or invade hosts. When microorganisms encounter nutrient deprivation or starvation conditions, they respond to them by arresting all metabolic activity and growth. They can then carry out starvation-induced activities such as production of enzymes. This includes many degradative enzymes such as proteases, lipases, and substrate capturing enzymes such as glutamine synthetase, and alkaline phosphatase (Kjelleberg et al., 1987; Matin et al., 1989). During starvation, many bacterial species show increased resistance to a number of environmental stresses such as low pH and heat and oxidative stress (Hartke et al., 1994; Watson et al., 1998).

The enteric bacteria exhibit a stress response to sublethal environmental stresses (Ryu and Buchat, 1998; Leenanon and Drake, 2001). These stress responses can result in resistance to a variety of environmental or processing parameters (Abee and Wouters, 1999; Law, 2000). The nature and the intensity of the stress response may vary between bacteria (Leenanon and Drake, 2001). So, cross-protection is referred to as the ability of one stress condition to provide protection against other stresses. Microbial adaptation responses to one stress can lead to enhanced resistance to a different stress (Cheville et al., 1996).

Starvation conferred less acid tolerance to *Escherichia coli* O157:H7 than did stationary phase (Cheville et al., 1996). However, starved or stationary phase cells showed significantly more acid tolerance than acid-adapted (growth

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at pH 5) mid and late-log phase cells (Arnold and Kaspar, 1995; Cheville et al., 1996). The aim of this work was to study the effect of stress response to pH and cross-protection: pH and starvation. Slime production was realized on Congo red agar. *Shigella sonnei* capacity to biofilm formation was tested. The morphology of cells was examined by atomic force microscopy.

MATERIALS AND METHODS

Bacterial strains

Shigella strain: *S. sonnei* was obtained from Monastir hospital, Tunisia and maintained at -80°C in Luria-Bertani broth (LB) supplemented with glycerol (15%, vol/vol). For the starved *Shigella* was obtained after incubation in seawater during eight months. For the experiments, the cells were grown at 37°C in tryptic soy broth (TSB, (Difco)) for 24 h. TSB microcosmes (100 ml) was achieved to pH 3.5, 4.5, 5.5, 6.5 and 7.3 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 20°C) with TSB and then suspended in 10 ml of autoclaved TSB. The microcosms (100 ml) were inoculated with these suspensions (approximately 10^9 CFU/ml) and then incubated at 37°C.

Enumeration techniques

Plate counts of culturable cells were determined by the drop plate method (Hoben and Somasegaran, 1982), using Tryptic soy agar (TSA; Difco). Microcosms were sampled daily during the 30, 60, 120 min. Time zero (inoculation time) and subsequent samples were taken for plate counts. The plates were incubated at 37°C, and the number of colonies was counted after 24 and 48 h.

Phenotypic characterization of slime-producing bacteria

Qualitative detection of biofilm formation by tested strains was studied by culturing the strains on Congo red agar (CRA) plates as described previously (Freeman et al., 1989). *Shigella* strains were inoculated onto the surface of CRA plates, made by mixing 0.8 g Congo red with 36 g saccharose (Sigma) in 1 L of brain heart infusion agar, and were incubated for 24 h at 30°C under aerobic conditions and followed overnight at room temperature (Ellafi et al., 2009). Slime producing bacteria appeared as black colonies, whereas non-slime producers remained non pigmented (Subashkumar et al., 2006).

Cell surface hydrophobicity

Hydrophobicity was measured by the hexadecane partitioning method of Loosdrec et al. (1987). Bacterial cells grown overnight in tryptic soy broth were washed with PBS and resuspended in 4 ml of PBS, and the absorbance (DO_{540}) was determined. One milliliter of hexadecane was added to each cell suspension and equilibrated for 10 min. Each suspension was reincubated at 37°C for 30 min. The aqueous layer was removed and aerated to remove all traces of hexadecane, and absorbance (DO_{540}) was measured against a hexadecane-extracted PBS blank. The hydrophobicity index was expressed as the ratio of absorbance of the hexadecane-extracted sample to absorbance of the sample before extraction.

Adherence assay to measure biofilm production by *Shigella sonnei* cells under stress

Biofilm production by *Shigella sonnei* strains grown in TSB with different conditions was determined using a semi-quantitative adherence assay on 96-well tissue culture plates (Nunc, Roskilde, Denmark) as described previously (Chaieb et al., 2007) with some modifications. An overnight culture grown in TSB (Biorad) at 37°C was diluted to 1:100 in TSB supplement with 2% (wt/vol) glucose and different pH as reported elsewhere (Rachid et al., 2000). A total of 200 µl of these cell suspensions was transferred in a U-bottomed 96-well microtiter plate. Each strain was tested in triplicate. Wells with sterile TSB alone was served as controls.

After their incubation for 24 h at 37°C, the culture was removed and the plates were washed three times with 200 µl of phosphate-buffered saline (7 mM Na_2HPO_4, 3 mM NaH_2PO_4 and 130 mM NaCl at pH 7.4) to remove non adherent cells and dried in an inverted position. Adherent cells were fixed with 95% ethanol and were stained with 100 µl of 1% (wt/vol) crystal violet (Merck) for 5 min. Then, unbound crystal violet was removed and the wells were washed three times with 300 µl of sterile distilled water. The water was then cleared and the microtiter plate was air dried for 3 with 4 h. The optical density (OD) of each well was measured at 570 nm using an automated Multiskan reader (GIO. De Vitae, Rome, Italy). Biofilm formation was categorized as highly positive ($OD_{570} \geq 1$), low-grade positive ($0.1 \leq OD_{570} < 1$), or negative ($OD_{570} < 0.1$) (Chaieb et al., 2007)

Determination of morphological changes by AFM

In order to visualize any morphological changes in the stressed cells, *Shigella* cells were examined, in triplicate, by atomic force microscopy (AFM) (Nanoscope IIIA, Digital Instrument, VEECO). For the experiments the cells were collected, washed three times with 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).

Molecular identification of stressed bacteria

Bacteria were cultured on TSA for 24 h at 37°C. One colony was cultured in TSB for 24 h at 37°C, and 1.5 ml was centrifuged. The deoxyribonucleic acid (DNA) was extracted by boiling for 5 min and centrifugation at 13,000 rpm for 8 min. The supernatant was used for amplification by PCR with *Shigella* primers of the target *IpaH* gene. Polymerase chain reaction (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 *IpaH* forward primer 5′-GTTCCTTGACCGCCTTTCCGATAC-3′ (25 pM) and *IpaH* reverse primer 5′-CATTTC CTTCACGGCAGTGGA-3′ (25 pM) (Hartman et al., 1990), 1 U of GO *Taq* DNA polymerase (Promega, USA).

Amplification was conducted in the Thermocycler PTC 100 (BioRad). The reaction mixtures were heated at 94°C for 5 min and were subjected to 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 1 h and visualized under ultraviolet transillumination. The amplification products were photographed and their sizes determined with a 100-bp molecular size marker (Promega, France).

Statistical analysis

Statistical analysis was performed using the S.P.S.S. 13.0 statistics

package for Windows. The differences in the degree of adhesion assay were examined by the Friedman test, followed by the Wilcoxon signed ranks test. P-values of < 0.05 were considered as significant.

RESULTS

Survival *Shigella* with several pHs

We investigated the viability of the normal and starved *S. sonnei* in TSB with several pH (Table 1). After 2 h of incubation in the different pH, we noted a significantly decrease of the cell numbers. The cell remained constant in TSB at pH 7.3. Populations of cells inoculated in to TSB acidified to pH 3.5 rapidly decreased after 2 h of incubation, while a more gradual decrease was observed at pH 4.5 and 5.5. Populations of cells remained nearly constant at pH 6.5. These plots reveal that survival was proportional increased with an increase in pH. We also noted that the reduction of the cells viability is significant for the normal cells compared to starved cells. Indeed, the starved cells in seawater exhibited higher resistance to acid stress.

Phenotypic determination of slime production of *Shigella* sonnei under several pHs

Production of slime by all investigated strains was assessed by culture on CRA plates. Normal *S. sonnei* cells formed colonies with a red centre and a lighter outer zone and the starved cells in seawater developed colonies with black center and red contour. After 24 h of their incubation in TSB at different pH, all the strains developed colonies with black center and red contour (Figure 1).

Effect of stress on the surface hydrophobicity

The decrease of pH had a significant effect on the surface hydrophobicity of *S. sonnei* (Table 2). *S. sonnei* showed a significant increase (P<0.05) in surface hydrophobicity when subjected to diminution of pH. There was a 15 to 52% increase in the hydrophobicity for all the strains. This increase is important for the starved cells in seawater compared with the normal cells.

Biofilm formation by *Shigella* under several pHs

Biofilm formation of *S. sonnei* strains was evaluated in 96 wells plate with TSB at different pH (3.5, 4.5, 5.5, 6.5 and 7.3). The results of the OD_{570} presented in the Table 2 showed that the strain did not show any biofilm formation in the normal condition (OD_{570}<0.1). After incubation in several pH some normal or starved strain are able to form biofilm: were considered as low-grade positive ($0.1 \leq OD_{570} < 1$).

Morphological changes of *Shigella* under several pHs

The cell morphology under several pHs was examined by AFM (Figure 2). The control *S. sonnei* cells, whose length is about 3.4 µm have a normal rod shape with a smooth surface (Figure 2a). Whereas the cells starved in seawater present two coccoid shape forms whose length is less than 1 and 0.4 µm. After 24 h of incubation in the different medium we have noted a decrease of the cell size at 5.5, 4.5 and 3.5 for the normal strain. The size of cells is at 5.5 and 4.5 (Figure 2b); while to 3.5 it is lower to 1 µm. (Figure 2c). For the starved cells, the size is between 1 and 0.4 µm at different medium (Figure 2c).

Molecular confirmation of stressed strains

We used the technique of PCR to identify the stressed strains. After amplification of ipaH gene by PCR, we confirmed the identity of the investigated *S. sonnei* (Figure 3).

DISCUSSION

Bacterial cells have the ability to sense and respond to changes in their external environment (Rosen et al., 2001). The ability of bacteria to sense and respond effectively to changes in the environment is crucial for their survival (Ben Abdallah et al., 2009). Enteric bacteria such as *Shigella* are found in many diverse and extreme environments, and as a result it has developed responses to combat these adverse conditions (Foster and Spector, 1995). The present study showed that the survival of normal or starved *Shigella sonnei* is greatly influenced by the pH. We also noted that the reduction of the cells viability is significant for the normal cells compared to starved cells. Indeed, the starved cells in seawater exhibited higher resistance to acid stress.

During starvation, many bacterial species show increased resistance to a number of environmental stresses such as low pH and heat and oxidative stress (Hartke et al., 1994; Watson et al., 1998). The significant decrease of *Shigella* cells numbers at the beginning of the experiment may be a result of the death bacterial cells caused by the decrease of pH. These results are in accordance with those reported by Zaika and Phillips (2005), who demonstrated that the survival of *Shigella* is affected by the diminution of pH.

The persistence of *S. sonnei* under starvation and/or pH stress induced modifications in cell properties. Indeed, before their treatment, the normal *S. sonnei* cells formed colonies with a red center and a lighter outer zone and the starved cells in seawater developed colonies with black center and red contour. After 24 h of their incubation in TSB at different pH, all the strains developed colonies with black center and red contour. This state is

Table 1. Effect at pH on the survival of *Shigella sonnei* incubated at 37°C.

Percent of salt	Time (h)	Strains	
		Log_{10} CFU/ml ± Standard deviation	
		S	Si
3.5	0	9 ± 0.35	9 ± 0.28
	1	5.12 ± 0.5	5.43 ± 0.21
	2	5.033 ± 0.62	5.32 ± 0.16
4.5	0	9 ± 0.11	9 ± 0.14
	1	6.13 ± 0.77	6.4 ± 0.23
	2	6.28 ± 0.82	6.3 ± 0.51
5.5	0	9 ± 0.25	9 ± 0.27
	1	7.22 ± 0.11	7.81 ± 0.70
	2	6.3 ± 0.35	6.41 ± 0.15
6.5	0	9 ± 0.8	9 ± 0.78
	1	7.6 ± 0.65	7.7 ± 0.71
	2	7.3 ± 0.33	7.9 ± 0.91
7.3	0	9 ± 0.65	9 ± 0.2
	1	8.51 ± 0.35	8.16 ± 0.9
	2	8.7 ± 0.71	8.5 ± 0.6

S: strain before incubation in seawater; Si: strain incubated during eight month in seawater microcosms at room temperature.

considered a variable phenotype (Ellafi et al., 2009). This main difference between the normal and stressed strains is that there can be a state of passage toward the biofilm formation in case of stress elongation. According to Adam et al. (2002) the biofilm is a protective system under environment stress conditions (physical, chemical treatment).

Bacterial colonization of solid surfaces has been described as a basic and natural bacterial strategy in a wide variety of environments (Kjelleberg et al., 1983; Jana et al., 2000). Our observation showed that the low pH can influence significantly the capacity of *S. sonnei* cells to adhere to polystyrene and their hydrophobicity. This increase is important for the starved cells compared with the normal cells. Starvation is known to alter bacterial surface characteristics such as hydrophobicity, charge and irreversible attachment, which are essential factors in biofilm formation (Brown et al., 1977). According to Allan et al. (2002), *Citrobacter* sp biofilm formation was affected signifcantly by nutrient limitation.

Various studies suggest that fimbrial expression is associated with biofilm formation; fimbriae are known to be involved in attachment (Low et al., 1996) and biofilm development (Pratt and Kolter, 1998) and are likely to be involved in the mechanism of biofilm formation in the present case. Previous studies have shown that regulation of fimbrial production is affected by carbon source,

nitrogen source, pH and temperature (Xie et al., 1997). As a consequence, this modification caused a general increase in virulence and resistance against stress (Givskov et al., 1994). Both acid-shocked and -adapted cells showed cross-protection against heat or other stresses (Ryu and Beuchat, 1998).

The AFM revealed reduction in the *S. sonnei* cells size caused by pH stress. After 24 h of incubation in the different medium we noted a decrease of the cell size at 5.5, 4.5 and 3.5 for the normal strain. The size of cells is at 5.5 and 4.5 (Figure 2b); while to 3.5 it is lower to 1 μm (Figure 2c). For the starved cells, the size is between 1 and 0.4 μm at different medium (Figure 2c). The reduction of cell size and the increase of the adhesion property are probably linked. Indeed, Bower et al. (1996) observed that spores adhere more quickly than vegetative cells to food contact surfaces due to higher hydrophobicity of their cell surfaces. 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.

The reduction of the bacteria size, such as *Shigella,* during the stress is a strategy of survival (Jiang and Chai, 1996). In addition, the signification difference between normal and starved cells is caused by cross-protection.

These results are in agreement with those reported by
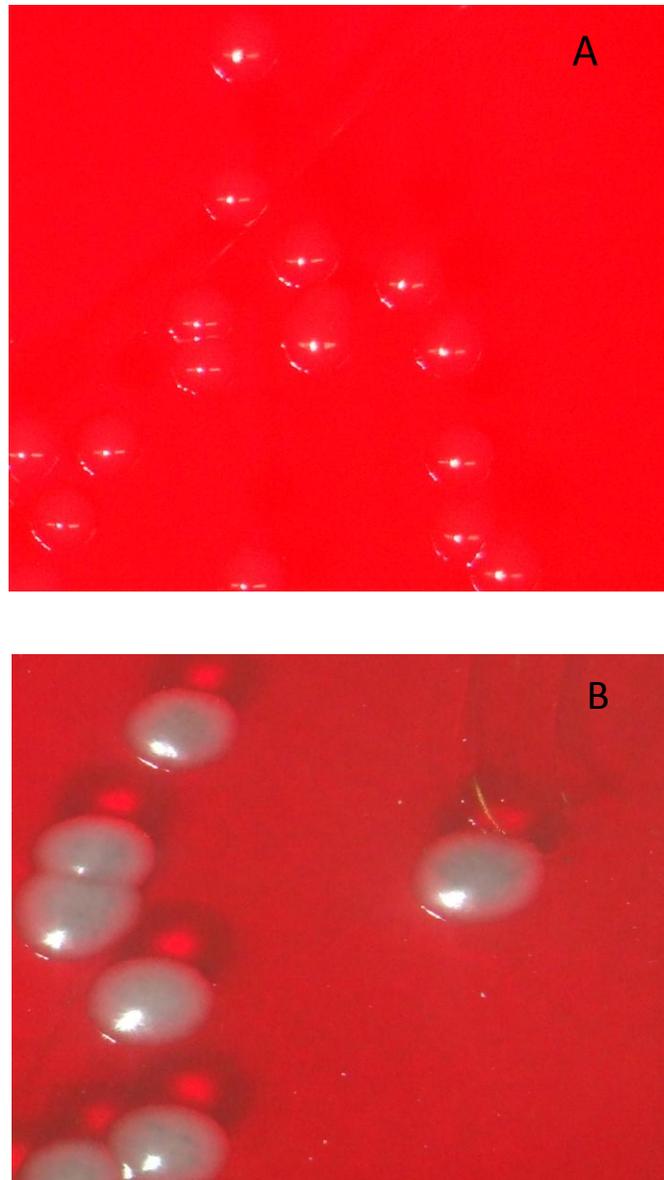
Figure 1. Detection of slime production by *S. sonnei* (a) before and (b) after incubation in several pH and starvation in seawater microcosms.

Table 2. Effect of pH on surface hydrophobocity and biofilm formation by *Shigella sonnei.*

Strain	pH									
	7.2		6.5		5.5		4.5		3.5	
	HI	Sd[1]	HI	Sd[1]	HI	Sd[1]	HI	Sd[1]	HI	Sd[1]
S1	15.2	0.53	16.83*	0.24	19.92*	0.2	39.25*	0.82	29.8*	0.66
S2	27.9	0.65	32.2*	0.13	50.9*	0.15	52.06*	0.15	23.89*	0.17
	DO_{570nm}	Sd[1]	DO_{570nm}	Sd[1]	DO_{570nm}	Sd[1]	DO_{570nm}	Sd[1]	DO_{570nm}	Sd[1]
S1	0.092	0.007	0.172*	0.022	0.195*	0.044	0.363*	0.22	0.225*	0.025
S2	0.189	0.033	0.214*	0.27	0.247*	0.37	0.308*	0.033	0.206*	0.17

[1]:Standard deviation. *:P<0.05.
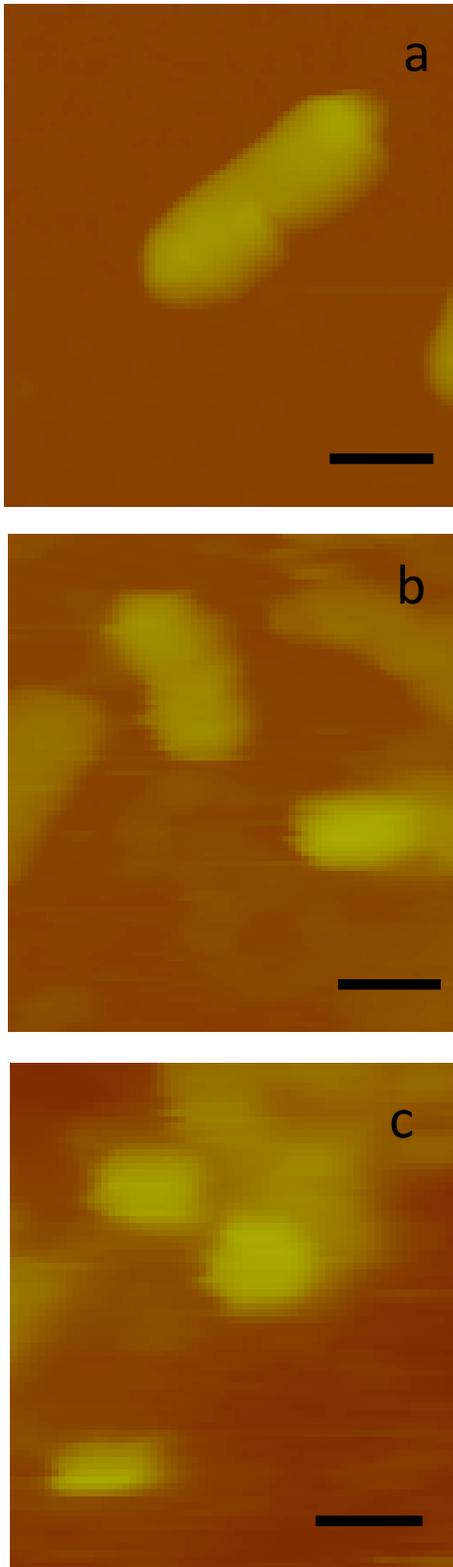
Figure 2. Atomic force micrographs of stressed S. sonnei cells. (a) control and cells stressed at pH: 6.5 ; (b) cells stressed at pH: 5.5 and 4.5; (c) cells starved and stressed in different pH and stressed at pH: 3.5. Bars: 1 µm.
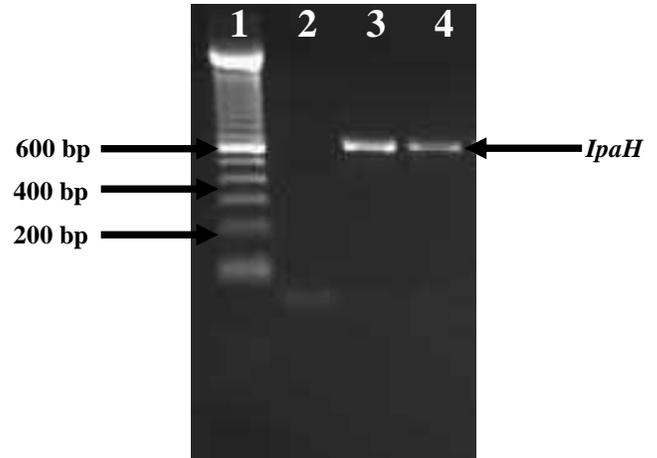
Figure 3. Agarose gel electrophoresis of polymerase chain reaction (PCR) amplification of *ipaH* gene. Lane 1, 100 bp DNA molecular size marker; lanes 2 to 4, PCR amplicons obtained with DNA amplification of *Shigella* strains. Lane 2, negative control; lane 3, S; lane 4, Si. **S:** strain before incubation in seawater; **Si:** strain incubated during eight month in seawater microcosms at room temperature.

Kalchayanand et al. (2004), which showed that hydro-static pressure and bacteriocin mixture induce change in the morphology of *Escherichia coli* O157:H7 and *Salmonella typhimurium*. The stress response of *S. sonnei* is complex, robust, and versatile. Bacterial stress adaptation of *Shigella* and the potential for stress-associated enhanced virulence need to be addressed in more detail to prevent potential risk of disease. An increased understanding of mechanisms and regulation of the stress adaptation will provide information for pathogenic control, increase effective design of novel control methods.

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