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Full Length Research Paper

Biofilm production, adherence and hydrophobicity of starved *Shigella* in seawater

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In this work, we incubated four *Shigella* strains for one month at room temperature and at 4°C. Their adhesion capacity to KB cells, biofilm formation on microplate and surface hydrophobicity of starved cells were investigated. Our results showed that the stressed cells exhibited a remarkable increase in their hydrophobicity from 19 to 34 % and in their capacity to produce biofilm. Adherence assays to KB cells showed an increase in the number of adherent cells from 0.7 to 6.7% after one month of incubation in seawater. However, the percentage of invasion was decreased from 34 to 11% under stress condition.

Key words: *Shigella,* seawater, starvation, hydrophobicity, adhesion, invasion.

INTRODUCTION

Gram-negative, facultative intracellular anaerobes of the genus *Shigella*, the principal etiologic agents of bacillary dysentery or shigellosis, continue to pose a threat to public health. A retrospective, literature-based study on shigellosis published in 1999 suggested an estimated annual incidence of 164.7 and 1.1 million deaths worldwide. In developing countries, there are 163.2 million annual cases and 69% of all patients are children under the age of 5 years (Kotloff et al., 1999). The high mortality caused by *Shigella* is generally attributed to the lack of clean water, poor sanitation, malnutrition and the cost of antibiotic treatment. Propagation is held by faecal-oral direct humans. Drinking water contaminated food and soil by faeces containing *Shigella* represent another traditional source of contamination, called indirect. Since the environmental adaptation plays an important role for pathogens to survive in natural environment or invade hosts, it seems that identification of key genes related to stress resistance would contribute to understand the virulence mechanism of pathogens (Ben et al., 2007).

Contaminated water is regarded as an important vehicle for transmission of various entero-pathogens. Bacteria live in habitats of frequently changing conditions and have evolved very sophisticated responses to adapt to environmental changes. These responses lead frequently to the activation and/or repression of a number of genes, such as those responsible of the adhesion and biofilm formation, to adapt cell physiology or metabolism to new conditions. Indeed, microbial adhesion to surfaces is an important step in the infection process associated with prosthetic implants in medicine (Gristina et al., 1985; Albesa et al., 2004), in the degradation and/or pathogenicity of foods (Driessen et al., 1984), and, more generally, in the formation of biofilms (Carpentier and Cerf, 1993; Oliveira, 1992). Biofilms are more resistant to environmental stress such as dehydration and oxidative stress (Chang and Halverson, 2003; Dunne, 2002). In addition, biofilms showed high levels of tolerance to prolonged antibiotic therapy in human and veterinary infections (Hoyle and Costerton, 1991). This biofilm formation is subject to complex regulation that is influenced by a number of environmental factors, including osmolarity, glucose levels, anaerobiosis, temperature (Schlag et al., 2007; Shanks et al., 2008).

Regarding the scarcity of data, we considered that it would be worthwhile to undertake a laboratory based study to assess the starvation in seawater microcosms of

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four *Shigella* strains and investigated their adhesive and invasive properties.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Four *Shigella* species 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 20°C) with autoclaved seawater and then suspended in 10 ml of autoclaved seawater (Ben et al., 2007). 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 (Ellafi et al., 2009).

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 Phosphate-buffered saline (PBS) and resuspended in 4 ml of PBS, and the absorbance (OD_{540}) was determined. 1 ml 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 (OD_{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* cells

Biofilm production by *Shigella* strains, grown in TSB with different conditions (normal and starved), was determined using a semi-quantitative adherence assay on 96-well tissue culture plates (Nunc, Roskilde, Denmark) (Chaieb et al., 2007). After incubation for 24 h at 37°C, the absorbance at 570 nm (OD_{570}) was recorded as a measure of total growth. An overnight culture grown in TSB (Bio-Rad) at 37°C was diluted to 1:100 in TSB with 2% (wt/vol) glucose 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, were served as controls. The plates were incubated aerobically for 24 h at 37°C. Furthermore, the culture was removed and 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 bacteria 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 2 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 positive ($0.1 \leq OD_{570}$), or negative ($OD_{570} < 0.1$).

Culture cells adherence assays

Quantitative adherence assays were performed with human oral cavity epidermoid carcinoma (KB) cell line as described by Ellafi et al. (2009). KB cells were seeded at a concentration of 2×10^5 and grown overnight in minimal essential medium (MEM) with Earle's salts and 10% fetal bovine serum in 96-well microtiter plates at 37°C with 5% CO_2. Each *Shigella* strain was grown overnight in TSB at 37°C with shaking (150 rpm). The bacterial cells were washed three times by centrifugation at 6000×g for 15 min with MEM without serum and resuspended in the same medium. The number of bacteria in the suspension was adjusted to 10^8 UFC/ml. The monolayers of human cells were inoculated, for each tested strain, with 10^8 UFC/ml, and incubated at 37°C in 5% CO_2 for 60 min. Then, bacterial suspension was removed to exclude the unattached bacteria. The monolayers of KB cells were washed 3 times with Dulbecco/Vogt modified Eagle's minimal essential medium (DMEM), and 1 ml Triton X-100 in PBS was added for 5 min at room temperature to release the bacteria from the cells. The number of bacteria was estimated by plating serial dilutions. All experiments were performed in triplicate.

For the invasion experiments, nonadherent bacteria were removed by aspiration after 3 to 4 h of infection, the medium was replaced with fresh DMEM (Chatti et al., 2007). The number of bacteria was estimated by plating serial dilutions. All experiments were performed in triplicate.

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' (sense) and 5'-CAT TTC CTT CAC GGC AGT GGA-3' (antisense) (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 (10mM), 0.5 µl $MgCl_2$ (50 mM), 1 µl of each primer (25 pM), 1U of GO *Taq* DNA polymerase (Promega, USA). The PCR mixtures were subjected to thermal cycling. The cycle conditions were as follows: 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 trans-illumination. The amplicons were photographed and their sizes were determined with 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 hydrophobocity, adhesion and invasion assays were examined by the Friedman test, followed by the Wilcoxon signed ranks test. P-values of < 0.05 were considered as significant.

RESULTS

Effect of stress on the surface hydrophobicity

The starvation on the microcosm's seawater had a

Table 1. Effect of starvation in seawater microcosms on surface hydrophobocity and Biofilm formation by *Shigella* strains.

	Strains											
	S1	**S1'**	**S1''**	**S2**	**S2'**	**S2''**	**S3**	**S3'**	**S3''**	**S4**	**S4'**	**S4''**
HI*(average ± sd)*	24.3±2.5	34.7*±1.6	30*±2.5	15.2±2.4	27.9*±2.2	22.2*±2.4	11±2.1	23*±2.9	19*±2.1	27.3±3.5	36*±1.6	33.3*±3.5
OD_{595nm} ± *sd*	0.085±0.007	0.2*±0.012	0.11*±0.001	0.092±0.003	0.189*±0.054	0.112*±0.021	0.098±0.001	0.18*±0.035	0.115*±0.015	0.083±0.004	0.176*±0.031	0.113*±0.014

* P<0.05; Sn : strain before incubation in seawater; Sn' : strain incubated during one month in seawater microcosms at room temperature. Sn'' : strain incubated during one month in seawater at 4°C.

Table 2. Effect of starvation in seawater microcosms on the capacity of *Shigella* strains to adhere and invade KB cells.

	Strains											
	S1	**S1'**	**S1''**	**S2**	**S2'**	**S2''**	**S3**	**S3'**	**S3''**	**S4**	**S4'**	**S4''**
Adherence % (average ± sd)	0.8±0.07	5.36*±0.96	5.36*±0.15	1.03±0.04	4.81*±0.52	4.1*±0.02	0.7±0.02	6.71*±0.55	3.56*±0.48	1.1±0.01	1.88*±0.28	1.59*±0.7
Invasion % (average ± sd)	14±0.98	11.7*±0.46	12.6*±0.45	16±1.23	14.7*±0.52	15.5±2.5	33±4.56	30.6*±5.52	31.1*±4.8	35.7±2.53	31.8*±2.85	33.87*±2.7

* P<0.05; Sn : strain before incubation in seawater; Sn' : strain incubated during one month in seawater microcosms at room temperature. Sn'' : strain incubated during one month in seawater at 4°C.

significant effect on the surface hydrophobicity of *Shigella* strains (Table1). All the strains of *Shigella* showed a significant increase (P<0.05) in surface hydrophobicity when subjected to starvation. There was a 18 to 34.7% increase in the hydrophobicity for all the strains. This increase is important at room temperature compared with 4°C.

Biofilm formation by *Shigella* under starvation

Biofilm formation of four *Shigella* strains was studied in 96 wells plate with TSB before and after incubation in seawater microcosms. Our results showed that all the strains were biofilm-negative. After incubation in the seawater, the strains were biofilm-positive (Table 1). The biofilm formation is very important at room temperature compared with 4°C.

Adherence and invasion assays to KB cells

Adherence assays to culture cells was performed with KB line. Indeed, before their incubation, we have found that S1 (0.8%), S2 (1.03%), S3 (0.7%) and S4 (1.1%) are weakly adherent cells KB (Table 2). After one month of incubation in seawater, we have observed a significant increase (P<0.05) in this percentage for all the strains (0.7 to 6.7%). The adherence is very important at room temperature compared with 4°C. For the invasion assays, we have noted a significant decrease (P<0.05) in this percentage after starvation (Table 2).

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 *Shigella* strains (Figure 1).

DISCUSSION

Since the environmental adaptation plays an important role in pathogens to survive in natural environment or invade hosts, it seems that comprehension of capacity to stress resistance would contribute to understand the virulence mechanism of pathogens. In the present study, we investigated the effect of starvation in the capacity of *Shigella* to produce biofilm, adhere and invade.

Before their incubation, our results showed that all the strains were biofilm-negative. After incubation in the seawater, the strains were biofilm-positive. The biofilm formation is very important at room temperature compared with 4°C. The

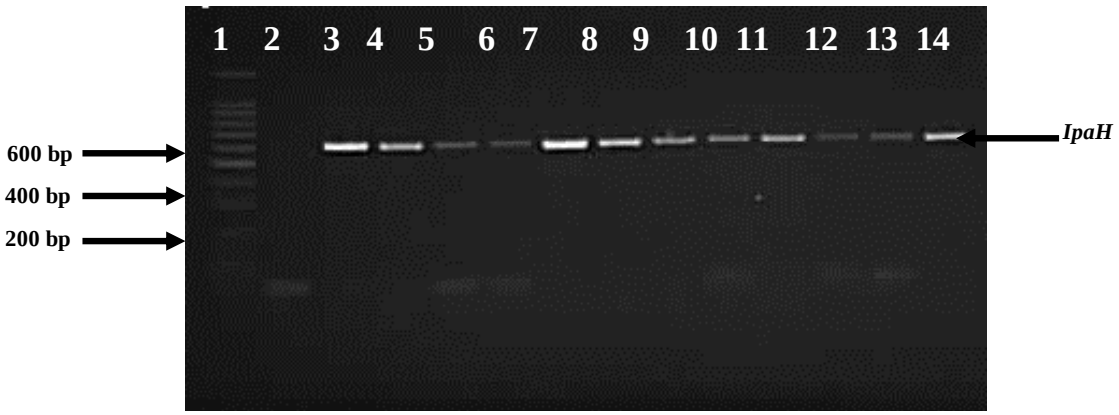

Figure 1. Agarose gel electrophoresis of polymerase chain reaction (PCR) amplification of *ipaH* gene. Lane 1, 100 bp DNA molecular size marker; lanes 2 to 14, PCR amplicons obtained with DNA amplification of *Shigella* strains. Lane 2, negative control; lane 3, S1; lane 4, S1'; lane 5, S1''; lane 6, 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 one month in seawater microcosms at room temperature. Sn'' : strain incubated during one month in seawater at 4°C.

biofilm is a protective system under environment stress conditions (Ellafi et al., 2009).

Our results showed that the values of the hydrophobicity and the adhesion to KB cells and polystyrene were increased significantly in stressed bacteria. The hydrophobicity and the adherence are very important at room temperature compared with 4°C. Surface hydrophobicity of micro-organisms is one of the virulence factors which contribute to the adherence of microorganisms to host tissue (Jann et al., 1981; Evans et al., 1979). Previous studies have shown a correlation between surface hydrophobicity and *in vitro* adherence to epithelial cells (Wadston et al., 1981; Gilbert et al., 1991; Polomar et al., 1995). This increase at the level adherence is probably owing to the effect of the temperature, the starvation and the osmolarity. Indeed, under a poor nutrient environment, bacteria increase the production of extracellular polysaccharides, which provide hydrophobic interactions and promote sorption onto the solid surface (Bengtsson, 1991). The surface properties of starved cells have been reported to be different from those of logarithmically growing cells, including becoming increasingly hydrophobic and more adhesive. This change is believed to enhance the chances of survival under adverse conditions. Hence, Bacteria have a natural tendency to adhere to surfaces as a survival mechanism. Bacterial colonization of solid surfaces has been described as a basic and natural bacterial stratagem in a wide variety of environments (Kjelleberg et al., 1983; Jana et al., 2000; Hunt et al., 2004).

For the invasion assays, we have noted reduction in the percentage of *Shigella* cells invading cells KB after their incubation in seawater. These modifications are probably due to nutrient deficiency in seawater. Indeed, it is now clear 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 cell division is interrupted (Ben et al., 2009). In addition, the starvation in the seawater reduced the superhelicity of DNA (Ben et al., 2007). Hence, these reductions in the invasion of line cells are the direct effect of a remarkable pertinent decrease in the expression of many genes and alterations of the membranes. These results are in agreement with the report of Linder and Oliver (1989), who demonstrated that *Vibrio vulnificus* lose its infectivity for mice under starvation in seawater.

In the present study, we observed that the starvation of *Shigella* could significantly influence the virulence factors of the bacterium. This in turn, may influence the pathogenesis of *Shigella* infections.

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