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ORIGINAL ARTICLE

Impact of long-term starvation on adhesion to and biofilm formation on stainless steel 316 L and gold surfaces of *Salmonella enterica* serovar Typhimurium

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Abstract The objective of this study was to evaluate the effect of 3-year starvation in seawater microcosms on adhesion to and biofilm formation of two *Salmonella enterica* serovar Typhimurium strains on model stainless steel 316 L and gold surfaces. The bacteria were characterized in terms of morphological alteration, electrophoretic mobility, and affinity to various solvent interfaces. Scanning electron micrographs showed the appearance of coccoid and elongated cells after starvation. All stressed cells were characterized by a hyperflagellation, a significant increase in the global surface charge, and a conservation of their hydrophilic character. Epifluorescence microscopy highlighted an increase in the levels of adhered cells to stainless steel and gold surfaces after starvation stress. Confocal laser scanning microscopy produced evidence of variability between the three-dimensional biofilm architectures of the control and stressed cells on gold compared to stainless steel. The results obtained so far led us to hypothesize that the pervasiveness of nutrient deficiency in natural environments may generate new adaptation strategies for long-term starved *Salmonella* Typhimurium and probably create protection against other types of stress. The stress adaptation mechanisms identified in this study may induce a genetic instability and change virulence state of starved bacteria. This fundamental study provides information which may aid in the development of sanitation programs for effective pathogen removal in the food industry or from medical devices. The task is certainly complex given that several concomitant physicochemical parameters affect the adhesion to and biofilm formation on model surfaces of stressed bacteria.

Keywords *Salmonella* Typhimurium · Starvation stress · Bacterial morphology · Adhesion · Biofilm · Stainless steel · Gold

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Introduction

Microbial adhesion to solid surfaces and the subsequent biofilm formation are major concerns in food, biotechnology, medical, marine, and other industrial situations. In the food industry, adhesion of pathogenic or spoilage microorganisms to equipment materials and biofilm development lead to lowered shelf-life of products and transmission of diseases (Carpentier and Cerf 1993; Dunne 2002). In addition, bacterial adhesion and biofilms play a pivotal role in healthcare-associated infections, particularly those related to implanted medical devices such as intravascular catheters, urinary catheters, and orthopedic implants (Francolini and Donelli 2010). Salmonellosis is often suspected to be of nosocomial origin when an infection is identified after animals, such as horses, have been hospitalized for 72 h or longer or when the serotype and antimicrobial susceptibility pattern match those of a serotype previously identified as causing nosocomial infection (Hartmann et al. 1996; Tillotson et al. 1997). Consequences of outbreaks of nosocomial *Salmonella* infections can be

severe, resulting in human infections, equine fatalities (Schott et al. 2001), disruption of hospital routine (Hird et al. 1984), and the potentially devastating effects of lawsuits.

Salmonella is problematic due to its ubiquitous distribution in nature and its tolerance to various stresses. Enteric bacteria, such as *Salmonella*, disseminated in marine environments, are challenged by a combination of hostile conditions threatening their viability (Rozen and Belkin 2001). Of the different environmental factors combining to form seawater stress, the most prominent in the induction of several groups of genes was nutrient limitation or starvation (Rozen and Belkin 2001). Starvation may affect many characteristics and factors of the bacteria, such as cell shape (Kim and Fogler 1999), cell viability (Nelson et al. 1997), resistance to environmental stresses (Nelson et al. 1997; Pichereau et al. 2000), cell surface hydrophobicity (Sanin et al. 2003), and cell adhesion (van Schie and Fletcher 1999; Ellafi et al. 2009). Cell surface charges also exert great influence on cell adhesion, and this characteristic may also be modified during nutrient starvation (van Schie and Fletcher 1999). Starvation can result in an elongation of bacteria (Steinberger et al. 2002), although other stresses may also stimulate elongation. In liquid culture, cells enlarge in response to elevated temperatures (Cooper 1991), swell with osmotic upshock (Galinski 1995), and often elongate with antibiotics (Umbreit 1976). Another strategy for bacteria to survive extreme stress is to tightly adhere to surfaces by forming biofilms, a well-known survival mechanism (Busscher and van der Mei 2012). This phenomenon occurs on virtually all natural and synthetic surfaces (Fletcher 1994; Hall-Stoodley et al. 2004). It is generally recognized that bacterial adhesion is the first step in biofilm formation; however, the phenomenon of bacterial adhesion is a complex process involving the physicochemical properties of all three phases involved, namely (1) adhering bacteria, (2) material surface, and (3) the suspending liquid medium (Merritt and An 2000). The initial step in bacteria fixation is mainly governed by interplay of Lifshitz-van der Waals, electrostatic, and Lewis acid–base interactions between the bacterial surface and the solid material (van Loosdrecht et al. 1987; Poortinga et al. 2002). The rate and extent of attachment of bacterial cells to a surface is influenced, amongst others, by cell surface hydrophobicity, presence of flagella, pili and adhesins, and production of extracytoplasmic polymeric substances (O'Toole and Kolter 1998; Espinosa-Urgel et al. 2000). The developmental process of biofilms involves both cell–surface and cell–cell interactions which determine their structure, function, and composition (Karunakaran et al. 2011; Wong and O'Toole 2011). Such interactions are affected by the chemical and physical environment to which the bacterial cells and the surface are exposed, and take place in the context of an intricate regulatory network (Karatan and Watnick 2009). The integration of these influences ultimately determines the pattern of behavior of a given bacterium with respect to biofilm development (Goller and Romeo 2008). Of relevance to this work, Stepanović et al. 2004 showed an increase in biofilm formation when *Salmonella* was incubated in low nutrient conditions. The interest in investigating *Salmonella* behavior is driven by the fact that it is an international food-borne pathogen disseminated widely in seawater that regularly causes large outbreaks of food poisoning.

Despite the achievements in the understanding of the effects of external stresses or stimuli on physicochemical properties and behaviors of bacteria such as *Salmonella*, the study of starvation in seawater has seldom been reported (Bakhrouf et al. 1990; Ben Abdallah et al. 2007a), hence the motivation for this fundamental study. Thereby, we focused in the present work on the effect of 3-year starvation condition in seawater microcosms on the adhesion ability and biofilm formation on stainless steel and gold surfaces of two *Salmonella enterica* serovar Typhimurium strains. The objectives of the investigation were the following:

(i) to study the morphologic alteration of stressed cells using scanning electron micrographs,
(ii) to investigate the electrophoretic mobility and affinity of bacteria to various solvent interfaces,
(iii) to interrogate the ability of bacterial cells to adhere to stainless steel and to gold surfaces, two metallic substrates of industrial and medical importance, by means of epifluorescence microscopy, and finally,
(iv) to study the three-dimensional biofilm structure on tested materials by using confocal laser scanning microscopy.

Materials and methods

Bacterial strains and growth conditions

Salmonella Typhimurium ATCC 14028 s (S1) and *Salmonella* Typhimurium LT2 DT104 (S2), provided from French Food Safety Agency, were used in this study. These two species are part of *S. enterica* subspecies I, which colonizes mammals and birds and causes 99 % of *Salmonella* infections in humans. All strains were maintained at –80 °C in Luria-Bertani (LB) broth supplemented with glycerol (15 %, v/v). For the experiments, the cells were grown at 37 °C in Tryptic soy broth (TSB; Pronadisa, Spain) for 24 h. The microcosms, natural seawater (100 mL) were filtered through membranes (pore size 0.22 μm; Millipore, Bedford, MA, USA) and autoclaved (121 °C /20 min) in 250-mL Erlenmeyer flasks. *Salmonella* Typhimurium cells were washed three times by centrifugation (13,000 rpm for 10 min at 20 °C) with autoclaved seawater and then suspended in 10 mL autoclaved seawater. The microcosms (100 mL) were inoculated with these suspensions

(10^9 CFU/mL) and then incubated at room temperature for 3 years under static conditions. Three microcosms were used for each strain and one microcosm without bacteria served as negative control. All the experiments were performed, in triplicate, with stressed and non stressed bacteria.

Molecular confirmation of stressed bacteria

To confirm the starved cells of *Salmonella* Typhimurium incubated during 3 years in seawater microcosms, polymerase chain reaction (PCR) was used according to the method previously described (Lagha et al. 2012). Bacteria were cultured on trypticase soy agar (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 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 *Salmonella* primers. PCR was 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 MgCl2 (50 mM), 1 μL of each *SipA* forward 5'-GTAG GACGGGAAGCCCGGC-3'and *SipA* reverse 5'-CGCTGC ATGTGCAAGCCATCA-3' (25 pM), ATPase 1 U of *GO Taq* DNA polymerase (Promega, USA). Amplification was conducted in the Thermocycler PTC 100 (Bio-Rad). The reaction mixtures were heated at 94 °C for 5 min and then subjected to 35 cycles of denaturation at 94 °C for 1 min, annealing at 61 °C for 1 min, and elongation at 72 °C for 1 min, followed by 10 min of final extension period at 72 °C. PCR products (5 μL) were analyzed on 1 % agarose gel 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 were determined with a 100 bp molecular size marker (Promega, France).

Scanning electron microscopy (SEM) analysis

In order to visualize the morphological alterations of starved *Salmonella*, bacterial cells were fixed with 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) and left at room temperature (RT) for 1 h. The glutaraldehyde was then removed and the substrates were rinsed three times by immersing them for 10 min in 0.1 M sodium cacodylate buffer (pH 7.4) at RT. Dehydration was performed through an ascending series of ethanol concentrations (50, 70, 90, and 2× 100 %) for 10 min for each concentration.

Samples were critical point dried at 75 bar and 37 °C with liquid CO^2 as the transition fluid, then depressurized slowly (400 cm^3/min) in Quorum Technologies K850 device (Elexience, France). The samples were then mounted on an aluminum platform and sputter-coated in Ar with Au-Pd (30 nm) in Polaron SC 7640 device at 10 mA and 0.8 kV 200 s. The substrates were observed in FE-SEM Hitachi S4500 (Hitachi, Japan) (sample holder tilted at 45°) with low detector, at 2 kV and 15 mm WD. The SEM is part of the MIMA2 microscopy platform (http://voxel.jouy.inra.fr/mima2).

Physicochemical characterization of bacteria cell surface

Electrophoretic mobility

Electrophoretic mobility (EM) was measured according to the method described previously (Giaouris et al. 2009). Briefly, bacteria were suspended in NaCl 1.5×10^{-3} mol/L (pH 7). Electrophoretic mobility was measured using a 5 V/m electric field using a Laser Zetaphoremeter (CAD Instruments, France). The results were based on an automated video analysis of approximately 200 particles for each measurement.

Solvent-bacteria interactions

In order to study solvent-bacteria interactions, we employed the microbial adhesion to solvents method (MATS) developed by Bellon-Fontaine et al. (1996). This is a partitioning method based on a comparison between microbial cell affinity with four solvents: chloroform, hexadecane, ethyl acetate, and decane (all Sigma). Experimentally, 2.4 ml of a suspension containing approximately 10^8 cells in NaCl 1.5×10^{-1} mol/ L was vortex-mixed for 60 s with 0.4 mL of the solvent under investigation. The mixture was allowed to stand for 15 min to ensure complete separation of the two phases before a sample (1 mL) was carefully collected from the aqueous phase and the optical density measured at 400 nm. The percentage of cells dispersed in each solvent was subsequently calculated using the equation: % affinity=100 x [1- (A1/A0)], where A0 is the optical density of the bacterial suspension measured at 400 nm before mixing, and A1 is the absorbance after mixing.

Bacterial adhesion

Solid surface preparation

Samples of stainless steel AISI 316 (Goodfellow, UK) were soaked for 15 min in a 2 % (v/v) of a commercial RBS 35 detergent (Société des Traitements Chimiques de Surface, France), and rinsed five times for 5 min in demineralized hot water and then five times for 5 min in demineralized water.

Samples of gold (SSens, Netherlands) were ultrasonically rinsed with acetone, water, and ethanol, to remove the organic residues on the surface, and dried in a stream of argon, then treated in an UV surface decontamination system (PSD-UV; Novascan).

Microbial adhesion tests

Solid surfaces were immersed in 25 mL of bacterial suspension (10^7 CFU/L) and adhesion assays were performed by

sedimentation for 3 h at 37 °C. To remove non-adherent bacteria, the surfaces were rinsed five times in NaCl 1.5× 10^{-1} mol/L.

Determination of total adhered cells

To control the bacterial surface coverage on gold and stainless steel, epifluorescence microscopy measurements were performed employing blue excitation filter "I3" with a bandpass of 450–490 nm. The adhering bacteria were stained with the nucleic acid dye acridin orange (0.01 % in water) for 15 min in the dark. The dye solution was washed and replaced by pure water before mounting the sample under a Leica DM2 microscope equipped with a C 5060 WZ digital camera (Olympus, France).

Viable adhering cells

To recover the sessile cells, the solid surfaces were placed in a tube containing 6 mL of NaCl 1.5× 10^{-1} mol/L and all of the adhered bacterial cells were detached in a sonication bath (47 MHz; Branson 1510, France) for 2 min. The tubes were vortexed for 30 s before the microbial counts were performed. After preparation of serial dilutions, the bacterial counts were determined by plating on TSA (tryptic soy agar; Biomérieux, France) incubated at 37 °C for 24 h.

Biofilm formation and confocal laser scanning microscopy (CLSM) image acquisition and analysis

After biofilm formation for 24 h at 37 °C, the plate was mounted on the motorized stage of a Leica SP2 AOBS Confocal laser scanning microscope (LEICA Microsystems, France) at the MIMA2 microscopy platform (http://voxel.jouy.inra.fr/mima2) for image acquisition.

All biofilms were scanned at 400 Hz using a ×40 with a 0.8 N.A. (Leica HCX Apo) water immersion objective lens with a 488-nm argon laser set at 25 % intensity. Emitted fluorescence was recorded within the range 500–600 nm in order to visualize Syto9 fluorescence. Three stacks of horizontal plane images (512× 512 pixels) corresponding to 119×119 μm) with a z-step of 1 μm were acquired for each biofilm at different areas in the well. Two independent experiments were performed for each strain. Three-dimensional projections of biofilm structures were reconstructed using the Easy 3D function of the IMARIS 7.0 software (Bitplane, Switzerland).

Results

Molecular confirmation of stressed strains

We used the PCR technique to identify the stressed strains. After amplification of *SipA* gene by PCR, we confirmed the identity of the investigated *Salmonella* Typhimurium strains incubated for 3 years in seawater microcosms (Fig. 1).

SEM observations

Scanning electron micrographs showed that control *Salmonella* Typhimurium S1 and S2 presented a normal rod shape with a cell length of approximately 2.5 μm (Fig. 2). We also noted the presence of structures resembling flagella. These filaments were seen protruding from the bacteria, apparently forming physical bridges between them. In addition to the flagella-like filaments, the high magnification shows the presence of thin fibrillar structures connecting bacteria to the surface (Fig. 2. S1, c).

After starvation, the rod shape was generally preserved for stressed *Salmonella* Typhimurium ATCC 14028 s (S1i) and stressed *Salmonella* Typhimurium LT2 DT104 (S2i). In contrast, we noted the appearance of coccoid, intermediate, and elongate forms.

The coccoid and intermediate cell length ranged between 0.6 and 1 μm. In addition, the elongated cells presented an average size approximately 7.5 μm (Fig. 2d, k). It is worth noting that the starved cells (S1i and S2i) became hyperflagellated, but this hyperflagellation was more marked in S2i than S1i. Further, we observed two highly cohesive cell clusters in S2i linked together by flagella-like filaments (Fig. 2, S2i, l).

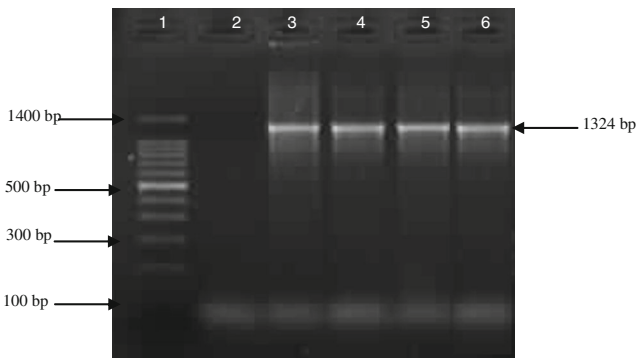

Fig. 1 Agarose gel electrophoresis of polymerase chain reaction (PCR) amplification of *SipA* gene. *Lane 1* 100-bp DNA molecular size marker; *lane 2* negative control; *lanes 3*, *4* S1 and S2, respectively, before incubation; *lanes 5*, *6* S1 and S2, respectively, after incubation in seawater microcosm

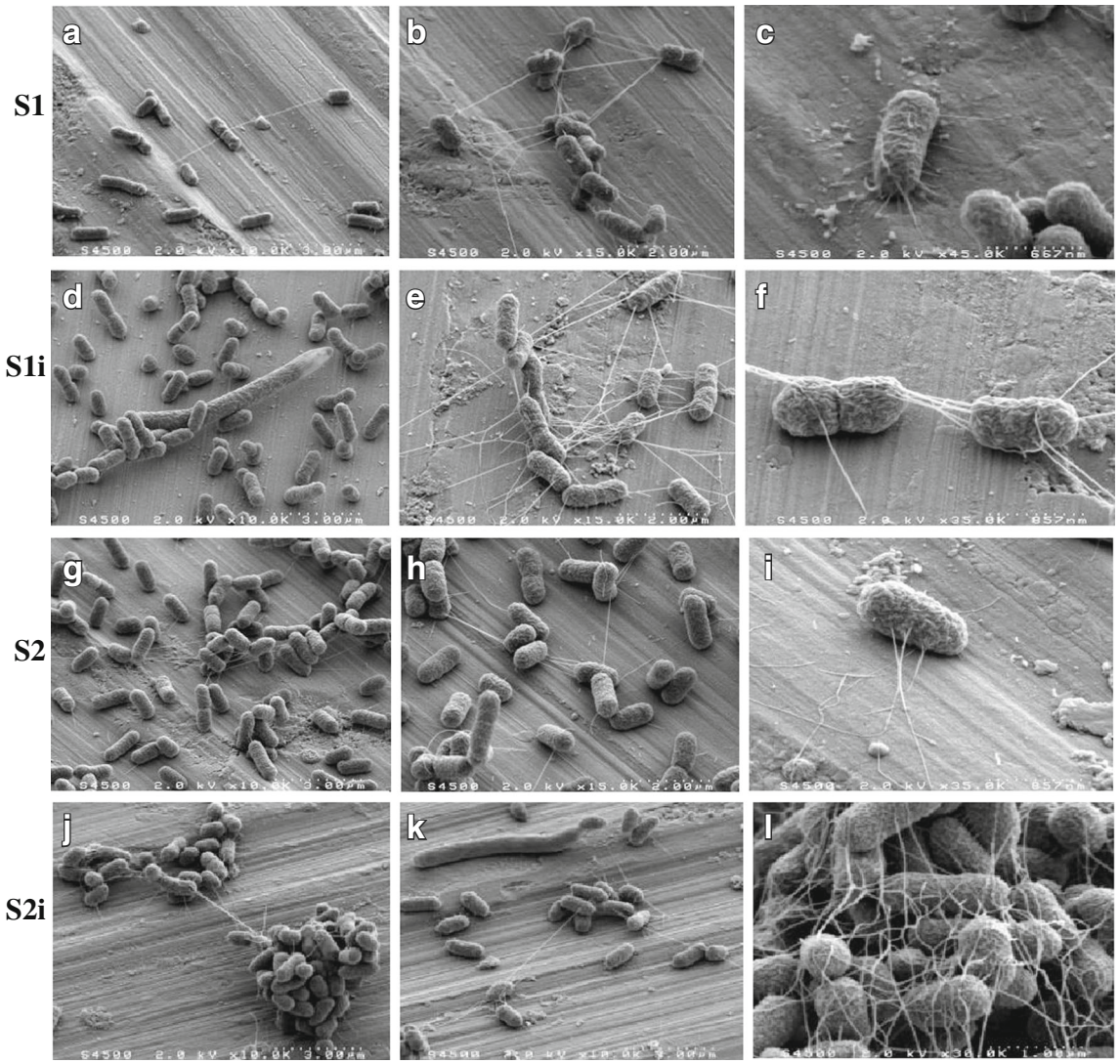

Fig. 2 Scanning electron micrographs of planktonic starved *Salmonella* Typhimurium cells, *S1 Salmonella* Typhimurium ATCC 14028 s; *S2 Salmonella* Typhimurium LT2 DT104; *i* strain incubated for 3 years in seawater microcosms

Physicochemical properties of *Salmonella* strains

The results of electrophoretic mobility of *Salmonella* Typhimurium strains before and after starvation are detailed in Table 1. All strains appeared to be electronegative at the studied pH value. Although this property remained stable for S1 and S1i, it nevertheless evolved for S2 and S2i. Thus, the global surface charge of the stressed cells increased significantly compared to the unstressed control bacteria ($p<0.05$).

Table 2 presents the percentage affinity to solvents of *Salmonella* Typhimurium strain determined using MATS method (Bellon-Fontaine et al. 1996). All strains appeared to be hydrophilic (weak affinity with hexadecane and decane). *Salmonella* Typhimurium adhered preferentially to chloroform and diethyl ether when compared to the apolar solvents, indicating the predominance of basic and acidic properties. However, a higher basic character was observed in S2i (82.6 % affinity to chloroform) and this strain was considered strongly hydrophilic.

Table 1 Electrophoretic mobility of *S. enterica* serovar Typhimurium strain at pH 7

Strain	Homogeneity	Electrophoretic mobility (10^{-8} $m^2V^{-1}S^{-1}$) Peaks
S1	2 peaks	−0.17; −1.59
S1i	2 peaks	−0.11; −1.32
S2	1 peak	−0.27
S2i	2 peaks	−2.06;−3.77

S1 S. enterica serovar Typhimurium ATCC 14028 s; *S2 S. enterica* serovar Typhimurium LT2 DT104; *i* strain incubated for three years in seawater microcosms

Table 2 Percentage affinity to solvents of *S. enterica* serovar Typhimurium

Strain	Chl	Hx	Dc	EA
S1	31.7±1.5	5.0±0.2	3.9±0.3	18.3±0.1
S1i	19.8*±1.3	0.5*±0.3	0.5*±0.3	10.0*±0.1
S2	12.8±2.2	1.1±0.3	1.3±0.1	15.8±0.1
S2i	82.6*±3.1	0.5*±0.1	0.4*±0.1	28.5*±1.2

All values ±SD

S1 S. enterica serovar Typhimurium ATCC 14028 s; *S2 S. enterica* serovar Typhimurium LT2 DT104; *i* strain incubated for three years in seawater microcosms

Chl Chloroform; *Hx* Hexadecane; *Dc* Decane; *EA* Ethyl Acetate

*P <0.05

Adhesion of starved strains to stainless steel and gold surfaces

Epifluorescence microscopy images (Fig. 3) showed that both surfaces were readily colonized by all strains. The total number of attached cells to stainless steel and gold surfaces remained nearly constant for S1 and S1i. However, S2i cells had greater affinity for the two substrates than S2, with a higher number of attached cells.

Figure 4 present the number of viable adhered cells to stainless steel and gold. The levels of cells adhesion to stainless steel were particularly higher than those to gold surfaces. The starved cells S1i showed a significant increase in number of adhered cells compared to control S1 (43.7 10^5 and 31.3 10^5 CFU/cm^2, respectively). However, on gold surfaces a significant decrease in adhered cells was observed for S1i compared to S1 (2.5 10^5 and 7.6 10^5 CFU/cm^2). Furthermore, the levels of adhered cells for S2i on both surfaces ($p<0.05$) increased significantly.

Three-dimensional structure of biofilm

Figure 5 displays representative 24-h biofilm structures observed using CLSM for the four strains under test. The images corresponding to three-dimensional reconstructions obtained from confocal stack images by the IMARIS software, including virtual shadow projections on the right hand side of the figures. We found a marked variability in three-dimensional biofilm architecture between the control and stressed cells on gold and stainless steel materials. Indeed, S1 and S2 strains produced rough biofilms containing of variable thickness on stainless steel and gold, whereas S1i and S2i strains displayed a high degree of variability in terms of biofilm structure by forming flat and compact structures that completely covered materials surfaces compared to controls.

Discussion and conclusion

Salmonella Typhimurium encounters many diverse and extremely severe environments to which it develops responses to overcome these adverse conditions (Foster and Spector 1995). The results achieved in the present work show that *Salmonella* Typhimurium is able to adapt and survive under extremely stressful conditions. The persistence of *Salmonella* under starvation and stress induced modifications in the physicochemical surface characteristics. The results of electrophoretic mobility measurements of *Salmonella* Typhimurium strains showed a significant increase in the global surface charge of the stressed cells compared to unstressed control bacteria. Moreover, all strains appeared to be hydrophilic; in particular, S2i was considered strongly hydrophilic. As suggested by Neidhardt et al. (1994), it is the architecture and nature of the chemical groups present on the surface of bacterial cells which determine the physicochemical surface characteristics of micro-organisms. Therefore, the negligible affinity with apolar solvents exhibited by *Salmonella* strains may be due to the hydrophilic portion of the lipopolysaccharides present on the surface of Gram-negative bacteria. As for the negative surface charge of bacteria, this may be directly linked to the presence of ionized groups (phosphate, carboxylic, sulfate, amine) of macromolecules making up the outer membrane of Gram-negative bacteria (Rijnaarts et al. 1995). However, these properties may evolve, depending on the physical and chemical environment of the micro-organisms. Environmental conditions encountered by bacterial cells at the time of cell growth or/and at the time of attachment may also affect their adherence ability through modification of cell surface physicochemical properties (Briandet et al. 1999). Starvation is known to alter these bacterial surface characteristics, which are essential factors in biofilm formation (Kjelleberg and Hermansson 1984).

Generally, bacteria have evolved complex systems to maintain consistent cell morphologies. Nevertheless, in certain circumstances, bacteria alter this highly regulated process to transform into elongated and/or coccoid organisms. Based on the results reported above, it is clear that starvation is the main cause of cell elongation (or filamentation) and may be implicated in the hyper-flagellation of stressed cells. These results are in line with those reported by Steinberger et al. (2002) who found that *Pseudomonas aeruginosa* cell elongation was caused by nutrient deprivation. It was postulated that bacteria elongate to enhance their nutrient uptake by increasing the specific surface of the cells as a part of their adaptation process for starvation. Cell elongation was also reported to take place under non-permissive conditions, such as high growth temperature Bhatti et al. (1976), the treatment with certain

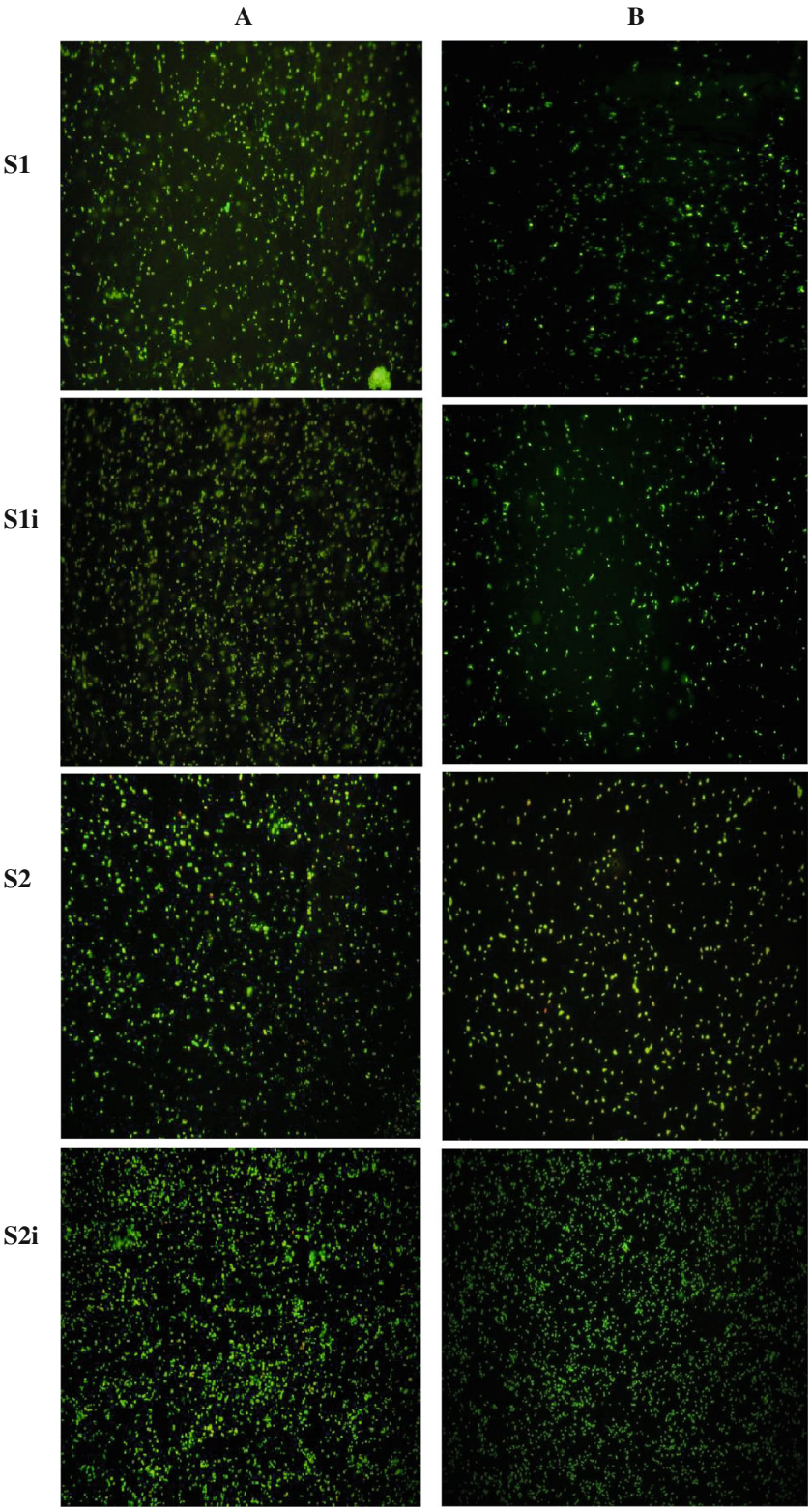

Fig. 3 Epifluorescent visualization of *Salmonella* cells attached to stainless steel (*A*) and gold surfaces (*B*); (magnification ×10); *S1 Salmonella* Typhimurium ATCC 14028 s; *S2 Salmonella* Typhimurium LT2 DT104; *i*: strain incubated for 3 years in seawater microcosms

antibiotics (Rolinson 1980), and UV irradiation (Burton and Holland 1983). It has been shown that elongation occurs when cell growth continues in the absence of cell division, and results in the formation of elongated organisms that have multiple chromosomal copies. Filament lengths are typically 10–50 times longer than their bacillary counterparts. There are many conditions that lead to bacterial elongation, including metabolic changes and DNA damage (Rothfield et al. 1999).

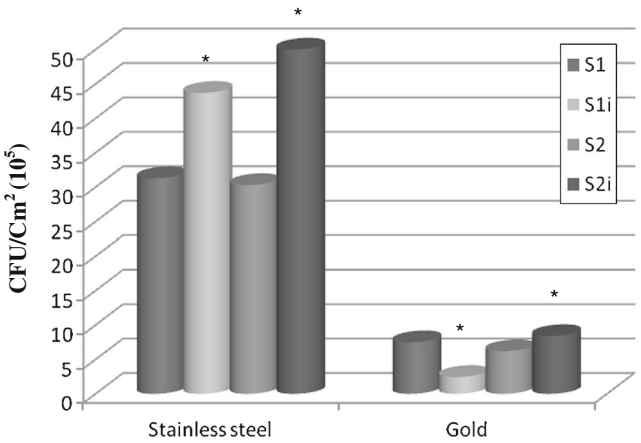

Fig. 4 The number of viable *Salmonella* Typhimurium cells attached to stainless steel and gold surfaces. *S1 Salmonella* Typhimurium ATCC 14028 s; *S2 Salmonella* Typhimurium LT2 DT104; *i* strain incubated for 3 years in seawater microcosms; *$p<0.05$

Elongation also results from the mutation and/or alteration of the stoichiometry of the cell-division components (Harry et al. 2006). However, bacterial growth under these highly stressful conditions was completely ceased and, furthermore, the level of cell elongation appeared to be significantly greater than was observed in the present study. Furthermore, it has been demonstrated that elongated *P. aeruginosa* cells are inclined to form highly cohesive clumps, which accounts for the robust biofilm formation under anaerobic condition (Yoon et al. 2011). Recently, Visvalingam and Holley (2013) have shown that elongated and filamentous cells of *Escherichia coli* under cold stress enhanced attachment during biofilm formation. Thus, cell elongation is likely to be a survival mechanism that is a direct response to lethal environments. Furthermore, we suggest that hyper-flagellation observed in starved cells can play an important role on adhesion and subsequent biofilm formation. Indeed, Byrne and Swanson (1998) reported that the intracellular bacterium *Legionella pneumophila* developed flagella in response to nutrient depletion, becoming motile and osmotically resistant in order to escape its spent host and disperse in the environment. Carey et al. (2009) have demonstrated that the flagellin (fliC), the major protein component of flagella, was found to play an important role in biofilm formation, and its synthesis was found to increase when *E. coli* O157:H7 was moved from 37 to 15 °C. However, other stressful conditions, such as high concentrations of salts, sugars, or alcohols, high temperature, both low and high pH, or conditions of blocked DNA replication, inhibit flagellum biosynthesis (Shin and Park 1995; Soutourina et al. 2002). In addition, our results have shown the reduction of the cells size and their evolution to coccoid-shapes. The evolution towards this state can help the bacterium to survive for a long period under starvation conditions (Ben Abdallah et al. 2007b). The reduction of the bacteria size, in the case of *Salmonella* Typhimurium, during the stress is a strategy of survival to minimize the needs of the cell for nutrients (Jiang and Chai 1996). In order to assess the impact of the physicochemical properties and cell surface modifications observed in this study, we investigated the adhesion and the biofilm formed on stainless steel and gold surfaces of starved and control cells. Our study demonstrates the different ability of control and starved cells to attach to stainless steel and gold surfaces. The levels of adhered cells to stainless steel were shown to be particularly higher than the levels of adhered cells to gold surfaces. These results are consistent with the findings of Steenackers et al. (2012) who found that *Salmonella* adhere to a greater extent to hydrophobic surfaces than to hydrophilic ones. Indeed, both glass and gold plates grafted with hydrophilic polymer brushes resisted non-specific *Salmonella* adhesion (Mrabet et al. 2009, 2011). Furthermore, the levels of adhered cells for S2i on both surfaces increased significantly ($p<0.05$).

This fact can be explained by the marked hyper-flagellation and the elongation of these starved cells. The biofilm-forming ability of *Salmonella* under starvation conditions, as evaluated in this study, appears to be related to all modifications induced by starvation. Indeed, starved cells produced flat and compact biofilm on both materials. Recent studies have shown that some *Salmonella* Typhimurium strains exhibited their highest biofilm-forming ability at stressful environmental conditions: pH 4.5, 6.0 % NaCl and 8 °C (Lianou and Koutsoumanis 2012). Sanders et al. (2008) reported the attachment of *Campylobacter jejuni* on stainless steel, in which biofilm formation was affected by a combination of temperature and nutrient availability.

In summary, the results of the present study demonstrated an extensive variation among *Salmonella* Typhimurium strains with regard to their physicochemical properties, surface structure, adhesion, and biofilm formation as a result of starvation stress. These findings lead us to hypothesize that the pervasiveness of nutrient deficiency in natural environments may generate new adaptation strategies for long-term starved *Salmonella* Typhimurium and probably create protection against other types of stress. The stress adaption mechanisms identified in this study may induce a genetic instability and change virulence state of starved bacteria. In addition, the results provide information on the behavior of attached starved cells to both surfaces used in food industry and medicals devices which may aid in the development of sanitation programs for effective pathogen removal.

This study on the adhesion of stressed bacteria to model gold and stainless steel 316 L surfaces paves the way to the investigation of adhesion to other

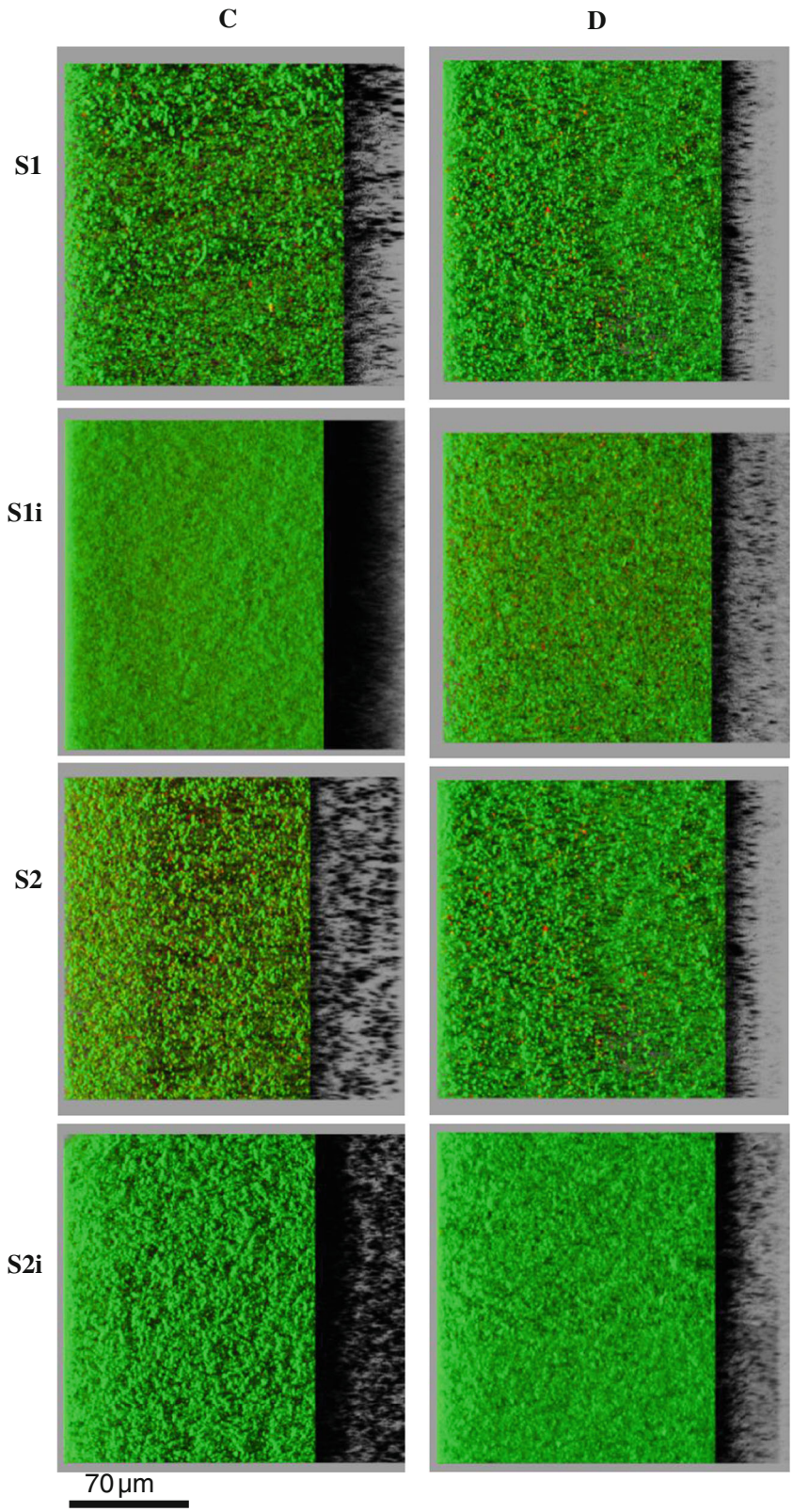

Fig. 5 3D projections of biofilm structure obtained from confocal z-stacks using IMARIS software. These images present an aerial view of biofilm structures obtained with the 4 *Salmonella* strains on stainless steel (*C*) and gold surfaces (*D*), with the shadow projection on the *right*. *S1 Salmonella* Typhimurium ATCC 14028 s; *S2 Salmonella* Typhimurium LT2 DT104; *i* strain incubated for 3 years in seawater microcosms

industrially important materials (plastic bags, cans and other food packaging, plastified metals, etc.) under the conditions of a variety of severe stresses (heat, cold, starvation, acidity, ionizing radiation, etc.).

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