Full Length Research Paper

Variations in structural and plasmid profiles of starved *Shigella* in seawater

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In this study, we incubated three strains of *Shigella* (*Shigella sonnei* (S1), *Shigella boydii* (S2) and *Shigella flexneri* (S3)) in seawater microcosms (at room temperature and at 4°C) for eight months and we studied the modification of the proteins secreted, cytosolic and inner membrane profiles and their composition in fatty acids. Plasmid profiles were also investigated. Plasmids profiles analysis showed that *S. boydii* (S2) and *S. flexneri* (S3) did not lose any of their plasmids. In addition, fatty acids composition of *Shigella* under stress conditions was modified. Proteins secreted, cytosolic and inner membrane of stressed bacteria was changed and these modifications were manifested by the appearance and/or disappearance of bands.

Key words: *Shigella*, seawater, starvation, plasmids, proteins, fatty acids.

INTRODUCTION

In their natural environment, bacteria are constantly subjected to alternating between good growing conditions and conditions of stress or nutritional deficiencies which they must quickly adapt to survive. Indeed, enteric bacteria such as *Shigella*, disseminated in marine environment, are submitted to multiple physicochemical stresses: high osmolarity, low temperature, nutrient starvation (Moriarty and Bell 1993) and solar light irradiation (Arana et al. 1992). The ability of enteric bacteria to adapt to fluctuations in the ambient osmolarity is of fundamental importance for their survival (Nakamura et al. 1964; Bakhrouf et al. 1994; Ellafi et al. 2012).

In general, microorganisms do not respond to starvation by simply arresting all metabolic activities and stopping growth. Instead, they carry out starvation-induced activities that may include production of degradative enzymes, such as proteases and lipases,

and substrate-capturing enzymes, such as glutamine synthetase and alkaline phosphatase. In addition, nutrient-deprived bacteria may try to differentiate into a more resistant state to maintain viability for starvation (Siegele and Kolter, 1992). During nutrient deficiency, bacteria can survive for a long time by sequential changes in cell physiology and gradual changes in morphology (Morita, 1993).

The mechanisms involved in the bacterial tolerance are not fully understood and the majority of studies are focused on the role of stress proteins and the regulation of gene expression in response to environmental changes (Foster and Spector, 1995; Foster, 2000; Dodd and Aldsworth, 2002). However, a link between the membrane fatty acid composition and the bacterial resistance has also been found (Annous et al., 1999; Sampathkumar et al., 2004; Alvarez-Ordóñez et al.,

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2008). In general, these authors have shown that cells with a decreased concentration of unsaturated fatty acids or with an increased content of saturated fatty acids have a decreased membrane fluidity, which is linked to a higher heat resistance. Furthermore, Alvarez Ordonez et al. (2008) found that the formation of cyclic fatty acids plays an important role in protecting acid-adapted *Salmonella typhimurium* cells from heat inactivation.

The aim of this work was to study the responses elicited in *Shigella* spp. following their incubation in seawater for eight months, by analyzing their: (i) secreted proteins, (ii) cytosolic and inner membrane profiles, (iii) membrane fatty acid profiles, and (iv) plasmid profiles.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Three *Shigella* strains were used in this study: two *Shigella sonnei* (S1), *Shigella boydii* (S2) and *Shigella flexneri* (S3). All strains were provided from the Monastir hospital in Tunisia and maintained at -80°C in Luria-Bertani broth (LB) supplemented with glycerol (15%, vol/vol). 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 (Ellafi et al., 2009). The microcosms (100 ml) were inoculated with these suspensions (approximately 10⁹ CFU/ml) and then incubated in a static state at room temperature (22 to 25°C) and at 4°C.

Plasmid extraction and analysis

Isolation of plasmid DNA was done using plasmid mini preparation kit obtained from Biobasic, according to the manufacturer's instruction. Plasmids were detected by electrophoresis in 0.7% agarose gel containing 0.5 µg of ethidium bromide per milliliter and photographed with ultraviolet light illumination.

Analysis of fatty acids

To analyze the total cellular fatty acids, cells recovered from 10 ml of each cell suspension were pretreated following the MIDI protocols (Sasser, 1990). All reagents for saponification, methylation, extraction, and washing were dispensed with autopipets into this same tube, making the hands-on time minimal. Next, the final extracts were analyzed by gas chromatography (column: 30 m × 0.25 mm HP-Innowax; flame ionization detect temperature at 280°C; carrier gas N₂ at 1 ml/min; injector temperature 270°C; oven temperature programmed from 130 to 230°C) using a Hewlett-Packard HP 5890 capillary gas chromatograph linked to an HP Chemstation integrator. The identification of fatty acid methyl esters was performed by external standards (all purchased from Sigma Chemical Co.) submitted to the same processes of manipulation as the biological samples analyzed. The values of fatty acids are presented as area percentage of total fatty acids. Total saturated fatty acids (SFA), total unsaturated fatty acids (UFA) were used to determine the differences among membrane fatty acids of *Shigella* cells grown under the different conditions. The UFA/SFA ratio was used as an indirect indicator of the membrane

membrane fluidity. It has been previously reported that membranes with high UFA/SFA ratio show a high fluidity (Casadei et al., 2002).

Extracellular proteins extraction

Extracellular proteins of *Shigella* before and after incubation for eight months in seawater were prepared according to the method described previously (Kaniga et al., 1995). Briefly, the cells were grown at 37°C in tryptic soy broth prepared with seawater (100%) to an optical density at 600 nm of 0.5. Bacterial cells were removed from cultures by centrifugation at 7000×g for 20 min and subsequent filtration through a 0.22 µm pore-size filter. Proteins from the cell-free culture supernatants were then precipitated by addition of 10% (v/v) trichloroacetic acid and recovered by centrifugation at 7000×g for 20 min. Pellets were resuspended in 4 ml of phosphate-buffered saline (PBS), and proteins were precipitated again by addition of 20 ml of cold acetone. After centrifugation at 7000×g for 20 min, the pellets were washed once with cold acetone, dried, and resuspended in 25 µl of PBS.

Proteins secreted (2 µg) were analysed by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970) with 15% acrylamide in the separating gel and 5% acrylamide in the stacking gel. After separation, the proteins were visualized according to standard procedures by staining with Coomassie brilliant blue G250 (Sigma, Chemical Co., St Louis, MO, USA) and molecular weights were determined by means of commercial markers (High-Range Rainbow; Amersham, Little Chalfont, Buckinghamshire, UK).

Cytosol and inner membrane fractions

The cytosol fractions were isolated as described previously (Kumar et al., 2001). Overnight cultures were centrifuged for 5 min at 7000 g. Pellets were washed once with 20 mmol/L Tris, 10 mmol/L EDTA, pH 8 (TE), then resuspended in the same buffer. Bacteria were disrupted by sonication for 1 min, followed by a 2 min rest, then an additional 1 min sonication. Samples were centrifuged for 5 min at 7000 g to remove debris, and the resulting supernatant was centrifuged for 1 h at 60 000 g at 4°C. The clear supernatant was retained as cytosolic fraction. The pellet was resuspended in TE, and the protein concentration was estimated. Protein concentration was adjusted to 5 mg/mL and solubilized with sodium lauryl sarcosinate 1% w/v (final concentration) at 4°C for 1 h. Samples were centrifuged again for 1 h at 60 000 g at 4°C, and the supernatant was taken for analysis of inner membrane fraction. Protein concentrations of cytosol and membrane fractions were estimated and analysed by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970) with 15% acrylamide in the separating gel and 5% acrylamide in the stacking gel.

Statistical analysis

Statistical analysis was performed using the S.P.S.S. 13.0 statistics package for Windows. The differences in UFA/SFA ratio were examined by the Friedman test, followed by the Wilcoxon signed ranks test. P-values of < 0.05 were considered as significant.

RESULTS

Plasmid analysis

Plasmid profiles of investigated *Shigella* strains analyzed

Figure 1. Plasmid DNA profiles of *Shigella* cells exposed to starvation for eight months in seawater. M, λ DNA-*Hind*III molecular size marker (Promega); S_n, strain before incubation in seawater; S_n', strain incubated during eight months in seawater microcosms at room temperature. S_n'', strain incubated during eight months in seawater at 4°C.

Table 1. Fatty acids composition of *Shigella* exposed to starvation for eight months in seawater.

Strain	Fatty acid								
	C10 :0	C12 :0	C14 :0	C14 :1	C15 :0 iso	C15:0 antieso	C16:1n7	C16:1n5	C16 :0
S1	1.43	0.26	0.46	0.21	0.44	0.55	4.03	6.52	33.08
S1'	0.34	0.2	0.08	-	0.31	0.42	4.35	5.62	31.87
S1''	0.6	0.53	0.2	0.16	0.08	0.61	4.43	1.06	31.35
S2	1.86	0.43	0.14	0.15	0.06	0.62	4.44	5.62	34.8
S2'	1.03	0.49	0.29	0.04	0.06	0.91	4.79	5.82	23.07
S2''	1.53	0.4	0.12	0.12	0.44	0.45	4.82	6.23	34.82
S3	0.95	0.42	0.12	-	0.32	0.2	1.33	0.71	43.63
S3'	1.09	0.33	0.12	0.12	0.04	0.53	4.59	5.61	35.52
S3''	-	-	-	-	-	0.56	3.57	5.93	36.96

on 0.7% agarose gel showed that all *Shigella* harbored four to six plasmids, ranging in size from 3 to 23 kb before incubation. After eight months of incubation in seawater, we also observed that S1 conserved its plasmids. Furthermore, we noted that S2 and S3 lost its many original plasmids, which corresponds to 9, 7.5 and 4 kb sizes for S2 and 3kb for S3 (at 4°C), respectively, after this period of starvation (Figure 1).

Membrane fatty acids composition of *Shigella*

The membrane fatty acid composition of *Shigella* cultured under the different growth conditions (starved and normal cells) was determined using a chromatographic method (Tables 1 and 2). Twenty (20) fatty acids were found when cells were grown both under normal and stress conditions. The five main peaks were identified as palmitoleic acid (C16:1n7), n-hexadécanoïque (C16:

1n5), palmitic acid (C16:0), vaccenic acid (C18:1n7) and linoleic acid (C18:2n6). Their relative percentages were between 1.33 and 39.88%. fifteen fatty acids were also detected at lower relative concentrations: C10:0, C12:0, C14:0, C14:1, C15:0 iso, C15:0 antieso, C17:0, C17:0 iso, C17 antieso, C17:1, C18:0, C18:1n9, C18:3n6, C20:1n9 and C20:0. As expected, incubation of *Shigella* in seawater resulted in differences in membrane fatty acid composition. Indeed, we also observed a significant ($P < 0.05$) decrease in SFA accompanied by a significantly ($P < 0.05$) increase in the UFA. The UFA/SFA ratio observed for control cells (S1, S2 and S3) was higher (1.05; 1.51;1.43) than cells grown under stress conditions (Table 2).

Extracellular proteins analysis

After eight months of incubation in seawater, the

Table 1. Contd.

Strain	Fatty acid										
	C17:0	C17:0 iso	C17:0 antieso	C17:1	C18:0	C18:1n9	C18:1n7	C18:2n6	C18:3n6	C20:0	C20:1n9
S1	0.31	1.16	0.43	0.91	1.14	1.11	24.44	20.34	2.31	0.52	0.23
S1'	0.15	0.77	0.19	0.6	1.19	0.27	30.05	20.95	2.24	0.2	0.07
S1''	0.86	0.93	0.5	0.93	1.27	1.88	31.43	20.01	2.71	0.16	0.19
S2	0.53	0.88	0.46	0.66	0.71	3.24	19.06	24.23	1.47	0.53	-
S2'	0.45	1.33	0.94	1.97	3.33	2.35	31.34	14.92	5.72	0.55	0.5
S2''	0.57	0.9	0.41	0.84	0.67	3.53	20.97	21.06	1.51	0.37	0.15
S3	0.65	0.88	0.13	0.24	0.86	0.71	2.47	39.88	1.26	0.43	4.7
S3'	0.45	0.63	0.32	0.59	0.56	1.88	24.64	20.04	1.43	0.17	1.25
S3''	0.2	0.59	0.31	0.3	1.03	0.27	20.62	26.3	1.19	0.01	0.05

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. C10:0, Capric acid; C12:0, lauric acid; C14:0, myristic acid; C14:1, myristoleic acid; C15:0 iso, isopentadecylic acid; C15:0 antieso, antiesopentacyclic acid; C16:1n7, palmitoleic acid; C16:1n5, n-hexadécanoïque acid; C16:0, palmitic acid; C17:0, margaric acid; C17:0 iso, isomargaric acid; C17:0 antieso, antiesomargaric acid; C17:1, heptadecenoic acid; C18:0, stearic acid; C18:1n9, oleic acid; C18:1n7, vaccenic acid; C18:2n6, linoleic acid; C18:3n6, gamma-linolenic acid; C20:0, arachidic acid; C20:1n9, eicosenoic acid.

Table 2. Effect of starvation on the fatty acids composition *Shigella*.

Strain	Fatty acid		
	SFA	USF	USF/SFA
S1	39.78	60.1	1.51
S1'	35.72*	64.28*	1.79*
S1''	37.09*	62.8*	1.69*
S2	41.02	58.87	1.43
S2'	32.45*	67.45*	2.07*
S2''	40.68*	59.23*	1.45*
S3	48.59	51.3	1.05
S3'	39.76*	60.15*	1.51*
S3''	39.66*	58.23*	1.46*

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. SFA: Total saturated fatty acids; UFA: Total unsaturated fatty acids. *: $P < 0.05$.

pearance of two bands of size 22 and 57 kDa after stress. We also noted the appearance of new bands after incubation in seawater for S2: 80, 40 and 37kDa and S3: 65kDa. We also noted the disappearance of other bands of sizes 40 and 37 kDa for S2 and 71 and 16kDa for S3.

Cytosol and inner membrane analysis

The proteins were fractionated and subjected to SDS-PAGE. SDS-PAGE of the cytosolic and inner membrane is shown in Figures 3 and 4, respectively. Cytosolic fraction of *Shigella* grown in the starvation conditions showed significant difference. At the profile S1, we observed the appearance of two bands corresponding to molecular weights: 200 and 60kDa and about the disappearance of two bands of size 22 and 16 kDa after stress. We also noted the appearance of new bands after incubation in seawater for S2: 15 and 5kDa and S3: 64, 22 and 4kDa. We also noted the disappearance of other bands of sizes, 50 and 17 kDa for S2 and for S3: 29kDa. The inner membrane fractions of normal and starved cells of *Shigella* did not show a significant difference (Figure 4).

DISCUSSION

The results in the present work show that Gram-negative bacteria like *Shigella* are able to adapt and survive under starvation conditions. Many marine bacteria, especially *Shigella* spp., can survive for a long time for starvation by sequential changes in cell physiology and gradual changes in morphology (Jiang and Chai, 1996). Our results show the starved cells of *Shigella* lose many their plasmids, only for S1. These results are in agreement with the report of Gauthier et al. (1988), who demonstrated

extracellular proteins of *Shigella* strains were examined by SDS-PAGE (Figure 2). Before their incubation in seawater, we found that all strains of *Shigella* had almost the same profile. *Shigella* secretes a large number of proteins in the extracellular medium. After eight months of incubation in seawater, we noticed a remarkable difference between the profiles of non-incubated and incubated strains. Indeed, we observed a decreased expression for almost all proteins compared to the normal state. At the profile *S. sonnei* (S1), we observed the appearance of four bands corresponding to molecular weights: 80, 47, 30 and 25 kDa and about the disap-

Figure 2. Extracellular proteins of *Shigella* cells exposed to starvation for eight months in seawater. M, molecular weight marker; S_n, strain before incubation in seawater; S_n', strain incubated during eight months in seawater microcosms at room temperature. S_n'', strain incubated during eight months in seawater at 4°C.

Figure 3. Cytosolic proteins of *Shigella* cells exposed to starvation for eight months in seawater. M, Molecular weight marker; S_n, strain before incubation in seawater; S_n', strain incubated during eight months in seawater microcosms at room temperature. S_n'', strain incubated during eight months in seawater at 4°C.

that *Escherichia coli* lost plasmid pCS1, encoding synthesis CFAl, after nine days of incubation in seawater. Our results testify to the genetic instability of stressed bacteria. Indeed, some marine bacteria can lose plasmid DNA in seawater while gaining others. This can reflect on the stability of virulence factors of bacteria in marine environment, as well as the transformation of non-pathogenic strain to pathogenic strain and vice versa.

Total saturated fatty acids (SFA) and total unsaturated fatty acids (UFA) were used to determine the differences among membrane fatty acids of *Shigella* cells grown under the different conditions. The UFA/SFA ratio was used as an indirect indicator of the membrane fluidity. A significant ($P < 0.05$) decrease in the UFA/SFA ratio was

observed with all the serogroups. These changes in membrane fatty acid composition result in *Shigella* cells with decreased membrane fluidity. These results confirm those found by Ellafi et al. (2009) and Ali et al. (2011) on antibiotic resistance and morphological change after starvation in seawater. It has been previously reported that membranes with high UFA/SFA ratio show a high fluidity (Casadei et al., 2002). Various studies have indicated that growth conditions, such as the composition of the growth medium (Annous et al., 1999), the growth phase of the cells (Russell et al., 1995; Kadner, 1996; Casadei et al., 2002), the incubation temperature (Annous et al., 1999; Casadei et al., 2002; Kadner, 1996; Wang et al., 2005), and the pH value (Russell et al.,

Figure 4. Inner membrane proteins of *Shigella* cells exposed to starvation for eight months in seawater. M, Molecular weight marker; S_n, strain before incubation in seawater; S_n', strain incubated during eight months in seawater microcosms at room temperature. S_n'', strain incubated during eight months in seawater at 4°C.

1995; Brown et al., 1997; Sampathkumar et al., 2004; Wang et al., 2005) markedly affect the bacterial membrane fatty acid composition. However, little is known on the influence of these modifications in membrane composition on the bacterial resistance to subsequent stresses. One of the most important consequences of membrane fatty acid changes in microorganisms is to modulate the activity of intrinsic proteins that perform functions such as ion pumping and nutrient uptake (Russell and Fukunaga, 1990).

Our study shows alterations in total proteins secreted and cytosolic patterns of starved *Shigella* cells after eight months of incubation in seawater microcosm. These alterations were manifested by the appearance and/or disappearance of bands as well as in the level expression of certain proteins. 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. In parallel, the production of several proteins increases (Kustos et al., 2007). These proteins include cytolysins, lipases, siderophores, exopolysaccharides, and proteases such as caseinase and gelatinase. These proteases are mainly involved in providing peptide nutrients for the microorganism. However, the production of bacterial proteases could contribute to the pathogenesis of infections, and

therefore they could be considered virulence factors (Secades and Guijarro, 1999). The paucity of food in seawater can also lead to the loss of some features either by repression of the specific enzymes or following modifications at the level of the bacterial wall. The effect of environmental conditions on the production of extracellular proteolytic enzymes could play an important role in the induction or repression of the enzyme by specific compounds (Secades and Guijarro, 1999). Production of extracellular proteases has been shown to be sensitive to repression by different carbohydrate and nitrogen sources (Haulon et al., 1982). Catabolic enzymes responded to both carbon control and nitrogen control in enteric bacteria (Goldberg et al., 1976). In the bacteria *A. hydrophila* (O'Reilly and Day, 1983), *A. salmonicida* (Dalhe, 1971), and *P. aeruginosa* (Jensen et al., 1980) protease production is influenced by carbon and nitrogen sources. Additionally, the temperature can influence the protease production, as occurs in *A. hydrophila* (O'Reilly and Day, 1983).

In the present study, we observe that the starvation of *Shigella* could significantly influence the structural and plasmid profiles of the bacterium. This, in turn, may influence the pathogenesis of *Shigella* infections.

REFERENCES

Alvarez-Ordóñez a, Fernandez, a, Lopez, M, Arenas, R, Bernardo (2008). Modifications in membrane fatty acid composition of

- Salmonella typhimuri* in response to growth conditions and their effect on heat resistance. *Int. J. Food. Microbiol.* 123:212-219.
- Ali Ellafi, Fethi Ben Abdallah, Rihab Lagha, Amina Bakhrouf (2011). Outer membrane proteins and morphological alterations of *Shigella* spp. under starvation in seawater. *Afric. J. Microb. Res.* 5(8):904-909.
- Annous BA, Kozempel MF, Kurantz MJ (1999). Changes in membrane fatty acid composition of *Pediococcus* sp. strain NRRL B-2354 in response to growth conditions and its effect on thermal resistance. *Appl. Environ. Microbiol.* 65:2857-2862.
- Bakhrouf A, Zaafrane S, Mzoughi R, (1994). Phenotypic alteration of *Salmonella* isolated from healthy carriers in seawater. *Mar. Life.* 4:3-8.
- Brown JL, Ross T, McMeekin TA, Nichols PD (1997). Acid habituation of *Escherichia coli* and the potential role of cyclopropane fatty acids in low pH tolerance. *Int. J. Food. Microbiol.* 37:163-173.
- Casadei MA, Mañas P, Niven G, Needs E, Mackey BM (2002). Role of membrane fluidity in pressure resistance of *Escherichia coli* NCTC 8164. *Appl. Environ. Microbiol.* 68:5965-5972.
- Dalhe HK (1971). Regulation of the proteinase production in two strains of *Aeromonas*. *Acta. Pathol. Microbiol.* 79:739-746.
- Dodd CER, Aldsworth TG (2002). The importance of RpoS in the survival of bacteria through food processing. *Int. J. Food. Microbiol.* 74:189-194.
- Ellafi A, Ben Abdallah F, Lagha R, Bakhrouf A (2012). Biofilm production, adherence and hydrophobicity of starved *Shigella* in seawater. *Afric. J. Microb. Res.* 6:4355-4359.
- Ellafi A, Denden I, Ben Abdallah F, Souissi I, Bakhrouf A (2009). Survival and adhesion ability of *Shigella* spp. Strains after their incubation in seawater microcosms. *World. J. Microbiol. Biotechnol.* 25:1161-1168.
- Foster JW (2000). Microbial responses to acid stress. In: Storz, G, Hengge-Aronis, R. (Eds.), *Bacterial Stress Responses*. ASM Press, Washington, DC. pp. 99-115.
- Foster JW, Spector MP (1995). How *Salmonella* survive against the odds. *Annu. Rev. Microbiol.* 49:145-174.
- Gauthier MJ, Munro PM, Breittmayer VA (1988). Damage to surface colonisation factors of enteric adhesive *Escherichia coli* during starvation in seawater. *Microbios. Lett.* 38:37-45.
- Goldberg RB, Bloom FR, Magasanik B (1976). Regulation of histidinase synthesis in intergenic hybrids of enteric bacteria. *J. Bacteriol.* 127:114-119.
- Haulon GW, Hodges NA, Russell AD (1982). The influence of glucose, ammonium and magnesium availability on the production of protease and bacitracin by *Bacillus licheniformis*. *J. Gen. Microbiol.* 128:845-851.
- Jensen SE, Phillippe L, Teng TJ, Stemke GW, Campbell JN (1980). Purification and characterization of exocellular proteases produced by a clinical isolate and a laboratory strain of *Pseudomonas aeruginosa*. *Can. J. Microbiol.* 26:77-86.
- Jiang X, Chai TJ (1996). Survival of *Vibrio parahaemolyticus* at low temperatures under starvation conditions and subsequent resuscitation of viable nonculturable cells. *Appl. Environ. Microbiol.* 62:1300-1305.
- Kadner RJ (1996). Cytoplasmic membrane. In: Neidhasdt, F.C, Curtiss III, R, Ingraham, J.L, Lin, E.C.C, Low, K.B, Magasanik, B, Reznikoff, W.S, Riley, M, Schaechter, M, Umbarger, H.E. (Eds.), *Escherichia coli* and *Salmonella*: Cellular and Molecular Biology. ASM Press, Washington, DC. pp. 58-87.
- Kaniga K, Trollinger D, Gala'N JE (1995). Identification of two targets of the type III protein secretion system encoded by the *inv* and *spa* loci of *Salmonella typhimurium* that have homology to the *Shigella* *ipad* and *ipaa* proteins. *J. Bacteriol.* 177:7078-7085.
- Kumar SS, Sankaran K, Haigh R, Williams PH, Balakrishnan A (2001). Cytopathic effects of outer-membrane preparations of enteropathogenic *Escherichia coli* and co-expression of maltoporin with secretory virulence factor, EspB. *J. Med. Microbiol.* 50:602-612.
- Kustos, I, Kocsis B, Kilár F (2007). Bacterial outer membrane protein analysis by electrophoresis and microchip technology. *Exp. Rev. Proteomics.* 4:91-106.
- Laemmli UK (1970). Cleavage of structural proteins for the assembly of the head of bacteriophage T4. *Nature* 227:680-685.
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
- Morita RY (1993). Bioavailability of energy and the starvation state. In: Kjelleberg S (ed) *Starvation in Bacteria*. Plenum Press, New York, pp. 1-23.
- Nakamura M, Stone RL, Krubsack JE, Pauls FP (1964). Survival of *Shigella* in seawater. *Nature.* 203:213-214.
- O'Reilly T, Day F (1983). Effects of culture conditions on protease production 422 by *Aeromonas hydrophila*. *Appl. Environ. Microbiol.* 45:1132-1135.
- Russell NJ, Evans RI, ter Steg PF, Hellemons J, Verheul A, Abee T (1995). Membranes as a target for stress adaptation. *Int. J. Food. Microbiol.* 28:255-261.
- Russell NJ, Fukunaga N (1990). A comparison of thermal adaptation of membrane lipids in psychrophilic and thermophilic bacteria. *FEMS. Microbiol. Rev.* 75:171-182.
- Sampathkumar B, Khachatourians GG, Korber DR (2004). Treatment of *Salmonella enterica* serovar *Enteritidis* with a sublethal concentration of trisodium phosphate or alkaline pH induces thermotolerance. *Appl. Environ. Microbiol.* 70:4613-4620.
- Sasser M (1990). Identification of bacteria by gas chromatography of cellular fatty acids, 1990: 1-7. MIDI technical note 101. MIDI, Inc., Newark, Del. pp. 1-7.
- Secades P, Guijarro JA (1999). Purification and characterization of an extracellular protease from the fish pathogen *Yersinia ruckeri* and effect of culture conditions on production. *Appl. Environ. Microbiol.* 65:3969-3975.
- Siegele DA, Kolter R (1992). Life after log. *J. Bacteriol.* 174:345-348.
- Wang Y, Corrieu G, Beal C (2005). Fermentation pH and temperature influence the cryotolerance of *Lactobacillus acidophilus* RD758. *J. Dairy. Sci.* 88:21-29.