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Full Length Research Paper

Virulence gene expression, proteins secreted and morphological alterations of *Vibrio parahaemolyticus* and *Vibrio alginolyticus* in response to long-term starvation in seawater

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In this study, we incubated *Vibrio parahaemolyticus* and *Vibrio alginolyticus* (marine food-borne pathogens bacteria) in seawater for 8 months to study their morphologic, proteomic and genetic responses to starvation. The atomic force micrographs of stressed strains showed a reduction of the cells size and an evolution to two coccoid-shape forms whose length is less than 0.4 m and between 0.5 and 1 m. Extracellular protein patterns and gelatinase profiles of stressed bacteria were also altered. Indeed, these modifications were manifested by the appearance and/or disappearance of bands as well as in the level of expression of certain proteins. In addition, we also searched for the presence of eight *Vibrio cholerae* virulence genes: *toxR*, *toxS, toxRS, ctxA*, *zot*, *ace*, *toxT*, and Virulence Pathogenicity Island (VPI) in the genome of investigated strains. The expression level of VPI gene studied by reverse transcriptase polymerase chain reaction was decreased, whereas the mRNA quantities of *toxR, toxS,* and *ace* in starved *Vibrio* remained stable.

Key words: *Vibrio*, seawater, alterations, morphology, proteins secreted, virulence gene expression, RT-PCR.

INTRODUCTION

Bacterial cells can 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., 2009a). In general, micro-organisms do not respond to nutrient deprivation or starvation by simply arresting all metabolic activities and stopping growth. Instead, they carry out starvation-induced activities that may include the production of degradative enzymes, such as proteases and lipases, and substrate-capturing enzymes, such as glutamine synthetase and alkaline phosphatase (Siegele and Kolter, 1992; Kjelleberg et al., 1987). Marine *Vibrio* species are known to produce various extracellular products, some of which are known pathogenicity factors (Hasegawa et al., 2009). These toxic proteins include cytolysins, proteases, lipases, siderophores, exopolysaccharides, and effectors delivered via Type III secretion systems (Hasegawa et al., 2008). Secades and Guijarro (1999) reported that stress environmental conditions could play an important role in the induction or repression of the enzyme by specific compounds. Production of extracellular proteases has been shown to be sensitive to repression by different carbohydrate and nitrogen sources (Haulon et al., 1982). Faced with starvation conditions in seawater, *V. alginolyticus* cells have been shown to undergo strong structural and metabolic modifications under laboratory conditions (Ben Kahla et al., 2006). Srinivasan and Kjelleberg (1998) found that in conditions

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of nutrient limitation and starvation in natural environments, the vibrios response is not simply an arrest of all metabolic activity. It can be argued that the life cycle of bacteria broadly consists of two major phases. The transition between these two phases involves dramatic changes in gene expression, physiology and morphology.

Bacterial populations under nutritional stress react in order to adapt cell metabolism and physiology to stressful conditions. As a consequence, a general increase in virulence and resistance against stress and chemotherapic as well as antibiotic agents is typically observed (Givskov et al., 1994). One of the most frequently observed behaviours in the nutrient starvation response of Gram-negative bacteria is the size reduction and cell morphology conversion from rod to coccoid shape (Ben Abdallah et al., 2007; Kjelleberg et al., 1993).

Vibrio modulates its virulence gene expression in response to stress conditions (Peterson and Mekalanos, 1988; Ben Abdallah et al., 2009a). Gonzalez-Escalona et al. (2006) demonstrated that incubation temperature significantly affects the gene expression profile of *V. cholerae*. These authors compared the transcription of some target genes in the VBNC (induced at 4°C) and starvation (15°C) states, and showed that key enzymes for cellular metabolism, such as *tuf*, which is needed for protein synthesis, and *relA* or *rpoS*, which are stress response genes, were detected at higher levels in bacteria that had entered the VBNC state.

The aim of this work was to study the adaptive response of *Vibrio alginolyticus* and *Vibrio parahaemolyticus* strains under starvation conditions in seawater microcosm for eight months. The morphological modification was observed using atomic force microscopy (AFM). In order to study the extracellular protein profiles of starved cells, sodium dodecyl sulphate (SDS) - polyacrylamide gel electrophoresis (PAGE) was used. Gelatinase activity was determined by zymography. To study the expression level of virulence genes, Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) was used.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Six *Vibrio* strains were used in this study including three reference strains: *V. alginolyticus* ATCC 33787 (S1), *V. alginolyticus* ATCC 17749 (S2), *V. parahaemolyticus* ATCC 17802 (S5) and *V. parahaemolyticus* strain (S6), isolated from the Calich Estuary (Alghero, Italy). In addition, two pathogenic *V. alginolyticus* strains (S3 and S4) isolated respectively from the internal organs of aquacultured diseased gilthead sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*) in a Tunisian aquaculture farm, were included in this work (Ben Abdallah et al., 2009b). For the experiments, the cells were grown at 30°C in tryptic soy broth 1% NaCl (TSB 1%) for 24 h. Natural seawater (100 ml) was filtered through membranes (pore size, 0.22 m; Millipore Corp., Bedford, Mass.) and autoclaved (115°C for 15 min) in 250 ml Erlenmeyer flasks. *Vibrio* cells were washed three times (13,000 rpm for 10 min at 20°C) with autoclaved seawater and then suspended in 10 ml of autoclaved seawater. The microcosms (100 ml) were inoculated with these suspensions (10^9 CFU/ml) and then incubated at room temperature (22 to 25°C) for eight months.

Determination of morphological changes by AFM

In order to visualize any morphological changes in the starved strains, *V. alginolyticus* and *V. parahaemolyticus* cells were examined in triplicate, by AFM (Nanoscope IIIA, Digital Instrument, VEECO). For the experiments, the cells were collected, washed three times with phosphate-buffered saline (PBS), and centrifuged. The final pellet was resuspended in PBS, placed on a round microscope cover slide and was simply dried in air according to the method previously described (Braga and Ricci, 1998).

Analysis of *Vibrio* culture supernatant proteins

Extracellular proteins of *V. alginolyticus* and *V. parahaemolyticus* 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 30°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 0.45 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 l of PBS. Proteins secreted (2 g) 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).

Zymogarphy

Gelatinase profile of *V. alginolyticus* and *V. parahaemolyticus* before and after starvation in seawater were resolved in 15% sodium dodecyl sulfate (SDS)-polyacrylamide gels containing 1% of gelatin (Sigma) under non-reducing conditions. Samples were not boiled. The proteins were electrophoresed under standard conditions. After electrophoresis, SDS was removed from the gels by immersion in a solution containing 2.5% Triton X-100 (Sigma), three times, and then the gels were incubated overnight at room temperature in 50 mM Tris-HCl (pH 7.5) with and without $CaCl_2$ (1 mM) and $ZnCl_2$ (0.001 mM). Protease activity was visualized by staining the gels 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).

PCR detection of *Vibrio cholerae* virulence genes in *V. alginolyticus* and *V. parahaemolyticus*

Bacteria were cultured on TSA 1% for 24 h at 30°C. One colony was cultured in TSB 1% during 24 h at 28°C; 1.5 ml was

Table 1. PCR primers selected for this study (Sechi et al., 2000).

Oligonucleotide sequence	Amplification region (bp)
*tox*RS toxR0, ATGAGTCATATTGGTACTTAAATT 1397 toxS2, AACAGTACCGTAGAACCGTGA	
*tox*S toxS1, CCACTGGCGGACAAAATAACC 640 toxS2, AACAGTACCGTAGAACCGTGA	
*tox*R toxR1, TTTGTTTGGCGTGAGCAAGGTTTT 595 toxR2, GGTTATTTTGTCCGCCAGTGG	
VPI VPI1, GCAATTTAGGGGCGCGACGT VPI2, CCGCTCTTTCTTGATCTGGTAG	680
toxT toxT1, TTGCTTGGTTAGTTATGAGAT 581 toxT2, TTGCAAACCCAGACTGATAT	
ace ace1, GCTTATGATGGACACCCTTTA 284 ace2, TTTGCCCTGCGAGCGTTAAAC	
zot zot1, ACGTCTCAGACATCAGTATCGAGTT 198 zot2, ATTTGGTCGCAGAGGATAGGCCT	
ctxA ctx2, CGGGCAGATTCTAGACCTCCTG 563 ctx3, CGATGATCTTGGAGCATTCCCAC	

centrifuged. The DNA was extracted using a Wizard Genomic purification Kit (Promega, Madison, WI) according to the manufacturer's instructions. The primers of *V. cholerae* virulence genes used in this study are listed in Table 1.

PCR were performed in 25 l containing: 50 ng of extracted DNA, 5 l green Go *Taq* buffer (5×), 0.25 l of each deoxynucleoside triphosphates (10 mM), 0.5 l $MgCl_2$ (50 mM), 1 l of each primer (25 pM) and 1U of GO *Taq* DNA polymerase (Promega, USA). Reaction mixtures were incubated for 5 min at 94°C, followed by 35 cycles at 94°C for 45 s, annealing at 52°C for 45 s for *toxS*, *toxR* and VPI, 72°C for 1 min and a final extension at 72°C for 10 min. The annealing temperature for the detection of the *toxRS* and *toxT* genes was 58°C whereas for *ctxA*, *ace* and *zot,* the temperature was 60°C. PCR products (5 l) were analysed on 1% agarose gels stained with ethidium bromide (0.5 mg/ ml) at 90V for 1 h and visualized under ultraviolet transillumination. All PCR positive strains, indicated the presence of virulence genes which weas confirmed by repeating the PCR three times.

RT-PCR for virulence gene expression

In order to study the level of expression of *V. alginolyticus* and *V. parahaemolyticus* virulence genes before and after starvation, semi-quantitative RT-PCR method was used. RNA from control and starved cells was extracted by SV total RNA isolation system (Promega, Madison, WI) according to the manufacturer's instructions. RNA was quantified by Ultraspec spectrophotometer (Ultraspec 2100 pro; Amersham Biosciences Europe GmbH, France). RT-PCR was performed in triplicate, using SuperScript™ One-Step RT- PCR with platinum® *Taq* kit according to the manufacturer's recommendations (Invitrogen, Carlsbad, CA). For cDNA synthesis, 100 ng of RNA served as template. RT-PCR (25 l reaction volume) was performed as follows: 50°C for 30 min; 94°C for 2 min; and 35 cycles at 94°C for 45 s, annealing at 52°C for 45 s for *toxS*, *toxR* and VPI, 72°C for 1 min and a final extension at 72°C for 10 min. The annealing temperature for *ace* gene was 60°C. RT-PCR products (5 l) were analysed on 1% agarose gel stained with

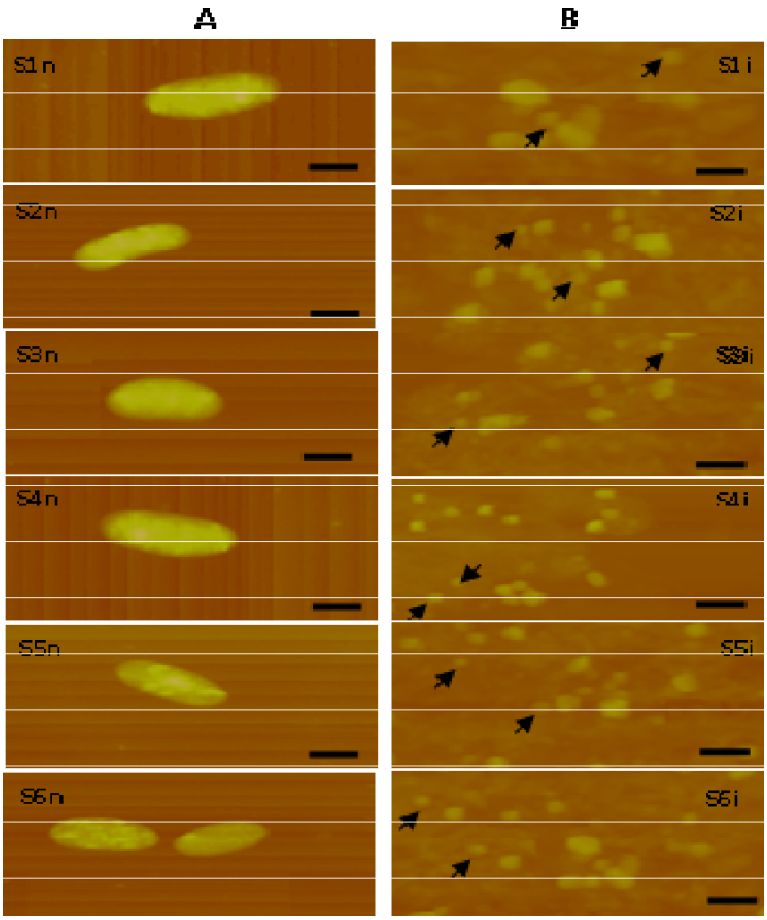

Figure 1. Atomic force micrographs of starved *Vibrio alginolyticus* and *Vibrio parahaemolyticus* cells. (A) Control; (B) Cells obtained after 8 months of incubation in seawater; S1, S2, S3 and S4: *Vibrio alginolyticus* strains; S5 and S6: *Vibrio parahaemolyticus* strains. n :strain before incubation in seawater; i :strain incubated for eight months in seawater microcosms. Bars: 1 µm; arrows indicate cells whose length is less than 0.4 µm.

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 100 bp molecular size marker (Promega, Madison, WI). Quantitative analysis of DNA bands was performed with imaging software (Gene Tools; Sygene, UK).

Molecular confirmation of starved cells

To confirm the starved V. alginolyticus and V. parahaemolyticus strains after their incubation for eight months in seawater microcosms, polymerase chain reaction (PCR) of the target collagenase gene was used. The primer pairs used in this study were: VA-F 5'- CGAGTACAGTCACTTGAAAGCC-3' and VA-R 5'-CACAACAGAACTCGCGTTACC-3' for V. alginolyticus producing a 737-bp long fragment and VP-F 5-GAAAGTTGAACATCATCAGCACGA-3' and VP-R 5'-GGTCAGAATCAAACGCCG-3', for V. parahaemolyticus which amplify a 271-bp region (Di Pinto et al., 2006). DNA was extracted by using a Wizard Genomic purification Kit (Promega) according to the manufactures instructions. PCR were performed in 25 L containing 50 ng of extracted DNA, 5 L Taq buffer (5×), 0.25 L dNTPs (10 mM), 0.5 L MgCl2 (50 mM) , 1 L of each primer (25 pM), 1U of Taq DNA polymerase (Promega). The PCR mixtures were subjected to thermal cycling. The cycle conditions were as follow: An initial incubation at 94°C for 5 min was followed by 35 cycles of denaturation at 94°C for 90 s, annealing at 62°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 L) were analysed on 1% agarose gel stained with ethidium bromide (0.5 mg/mL) at 90V for one hour and their sizes were determined using the 100 bp DNA Ladder (Promega).

RESULTS

Morphological alteration of stressed strains

Alterations in cell morphology due to starvation stress in seawater were examined by AFM (Figure 1). The control

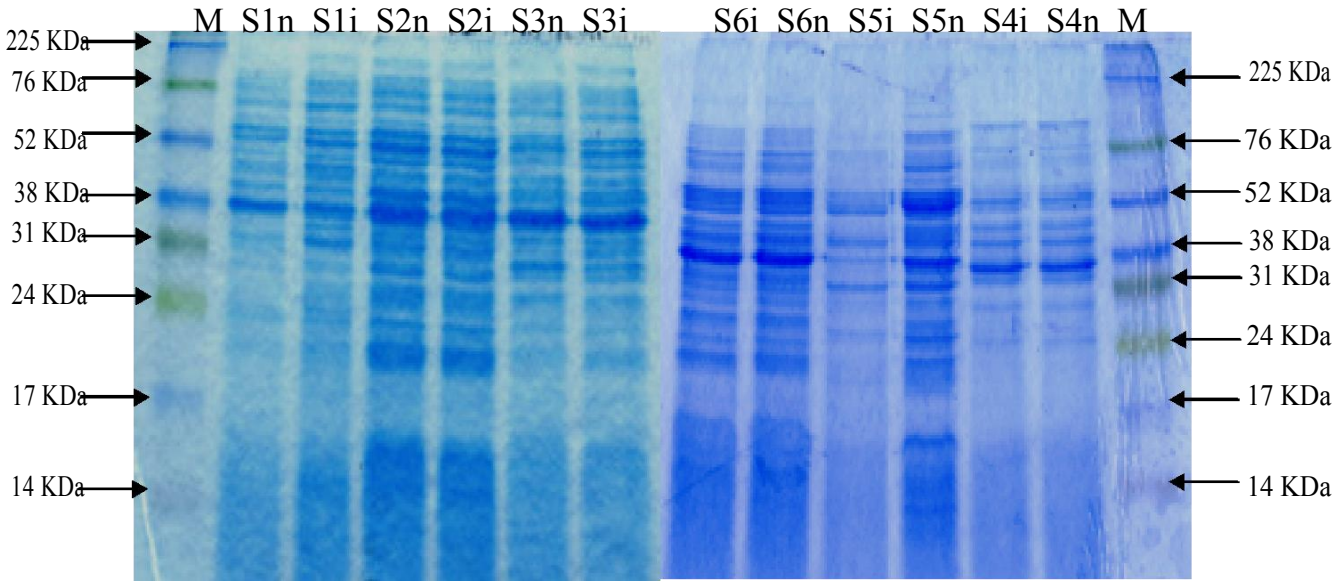

Figure 2. Extracellular proteins of *V. alginolyticus* and *V. parahaemolyticus* cells exposed to starvation for eight months in seawater. M: High-Range Rainbow (Amersham, Little Chalfont, Buckinghamshire, UK); S1, S2, S3 and S4: *V. alginolyticus* strains; S5 and S6: *V. parahaemolyticus* strains. n : strain before incubation in seawater; i :strain incubated for eight months in seawater microcosms.

V. alginolyticus and *V. parahaemolyticus* cells, whose lengths were between 2 and 2.5 m, has a rod shape (Figure 1a). Meanwhile, after eight months of their incubation in seawater microcosms, the cells obtained present two coccoid shape forms (Figure 1b) whose length is less than 0.4 m and between 0.5 m and 1 m.

Extracellular proteins analyses

Proteins secreted by *V. alginolyticus* and *V. parahaemolyticus* tested strains, were analyzed by SDS-PAGE (Figure 2). Before their incubation in seawater, we noted that all the *Vibrio* strains secreted a significant number of proteins in the extracellular medium. Among them, two major proteins were secreted by all the strains corresponding to molecular weights of 38 and 52 kDa approximately. After 8 months of incubation in seawater, we noticed that *V. alginolyticus* ATCC 17749 (S2), *V. alginolyticus* (S3), *V. alginolyticus* (S4) and *V. parahaemolyticus* (S6), isolated from the Calich Estuary (Alghero), preserved their initial extracellular protein profiles. While for *V. alginolyticus* ATCC 33787 (S1), we observed the appearance of two bands corresponding to molecular weights of 100 and 35 kDa approximately and a 31 kDa protein became less abundant after starvation. The major alterations in the extracellular protein profile were observed in *V. parahaemolyticus* ATCC 17802 (S5), which respond to nutrient limitation by the disappearance of bands corresponding to molecular weights 15, 20, 25, 30, 60 and 80 kDa approximately. In addition, the expression level of 31, 38, 45 and 52 kDa proteins was reduced.

Zymogarphy

Among the extracellular proteins secreted by *V. alginolyticus* and *V. parahaemolyticus* cells, one protease, gelatinase, was selected to study its expression level after starvation by zymography (Figure 3). Firstly, we noted that *Vibrio* tested strains do not secrete a metalloprotease, since similar results were founded with and without $CaCl_2$ and $ZnCl_2$. Our results showed the presence of three gelatinase corresponding to molecular weights of 76, 70 and 52 kDa for the strains S1, S2, S3, S4 and S6. For *V. parahaemolyticus* ATCC 17802 (S5), two 76 and 70 kDa proteins were founded. We also noted the existence of other proteases corresponding to molecular weights of 40 kDa for the strain S1; 31 kDa for the strain S4 and; 38 kDa for the strains S1 and S3. After 8 months of incubation in seawater, we observed that *V. alginolyticus* ATCC 17749 (S2), *V. alginolyticus* S4 and *V. parahaemolyticus* S6 conserved their initial profiles. For *V. alginolyticus* ATCC 33787 S1, we noticed the disappearance of a 40 kDa band and the abundance of a 38 kDa protein were reduced. Similar results have been found with *V. alginolyticus* S3 isolated from the internal organs of aquacultured-diseased gilthead sea bream, which respond to nutrient limitation by the abundance of 38 and 52 kDa proteins. For *V. parahaemolyticus* ATCC 17802 (S5), our results showed the disappearance of its two initial gelatinase and the appearance of new 30 and 35 kDa proteins after starvation.

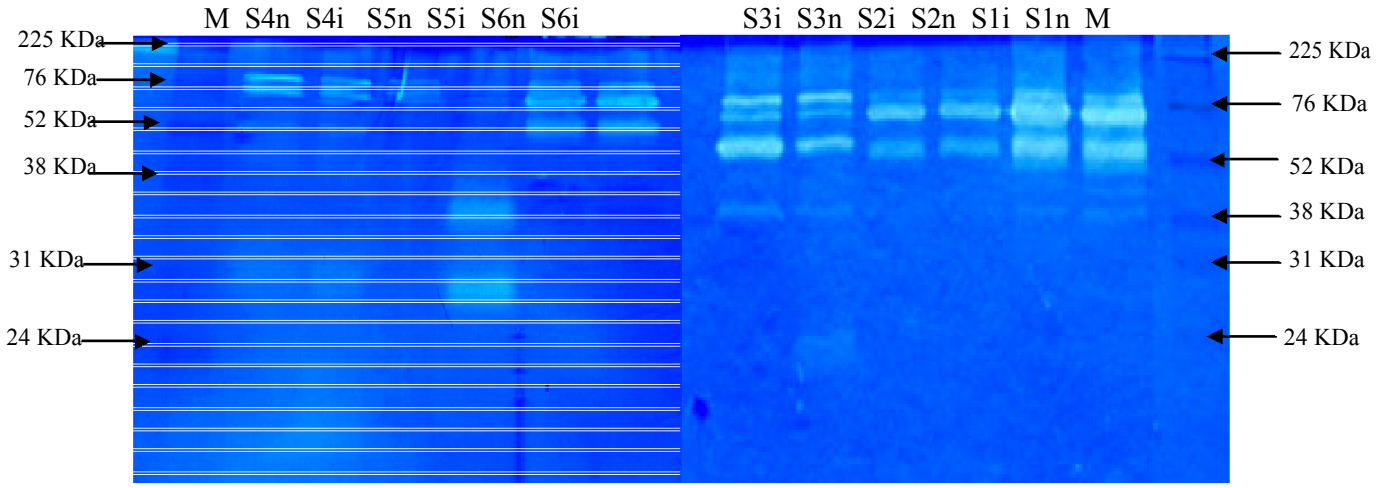

Figure 3. Gelatinase zymogram of *Vibrio alginolyticus* and *Vibrio parahaemolyticus* cells exposed to starvation for eight months in seawater. M: High-Range Rainbow (Amersham, Little Chalfont, Buckinghamshire, UK); S1, S2, S3 and S4: *Vibrio alginolyticus* strains; S5 and S6: *Vibrio parahaemolyticus* strains. n :strain before incubation in seawater; i :strain incubated for eight months in seawater microcosms.

Table 2. PCR detection of *Vibrio cholerae* virulence genes in *Vibrio alginolyticus* and *Vibrio parahaemolyticus* strains.

	Gene							
Strain	***toxRS***	***ToxS***	***toxR***	**VPI**	***toxT***	***ace***	***zot***	***ctxA***
S1: *V. alginolyticus* ATCC 33787	-	+	+	-	-	-	-	-
S2: *V. alginolyticus* ATCC 17749	-	-	-	-	-	-	-	-
S3: *V. alginolyticus* (*Sparus aurata*)	-	+	+	+	-	-	-	-
S4: *V. alginolyticus* (*Dicentrarchus labrax*)	-	-	-	-	-	+	-	-
S5: *V. parahaemolyticus* ATCC 17802	-	+	+	-	-	-	-	-
S6: *V. parahaemolyticus* (Calich estuary)	-	+	+	-	-	-	-	-

Detection of *Vibrio cholerae* virulence genes in *V. alginolyticus* and *V. parahaemolyticus*

The results of PCR amplification of the eight *V. cholerae* virulence genes in investigated *Vibrio* strains showed that *V. alginolyticus* (S1 and S3) and *V. parahaemolyticus* (S5 and S6) were positive for *toxR* and *toxS* genes. In addition, *V. alginolyticus* S3 and S4 were positive for VPI and *ace* genes respectively (Table 2).

Virulence genes expression

Virulence gene expression of *V. alginolyticus* and *V. parahaemolyticus* cells before and after starvation were analysed by semi-quantitative RT -PCR. We have noted that all the detected genes were expressed (Figure 4). After 8 months of incubation in seawater, we observed a decrease in the expression level of VPI gene in starved *V. alginolyticus* S3 isolated from the internal organs of aquacultured diseased gilthead sea bream. Furthermore, the mRNA quantities of *toxR toxS* and *ace* genes remained stable after starvation in all vibrios tested strains.

Molecular confirmation of starved *Vibrio*

After eight months of incubation in seawater microcosms, all tested strains of *V. alginolyticus* and *V. parahaemolyticus* were confirmed by PCR. Furthermore, the amplification of the collagenase gene was positive in control cells as well as in the starved *Vibrio* strains (Figure 5).

DISCUSSION

The results developed in the present work show that

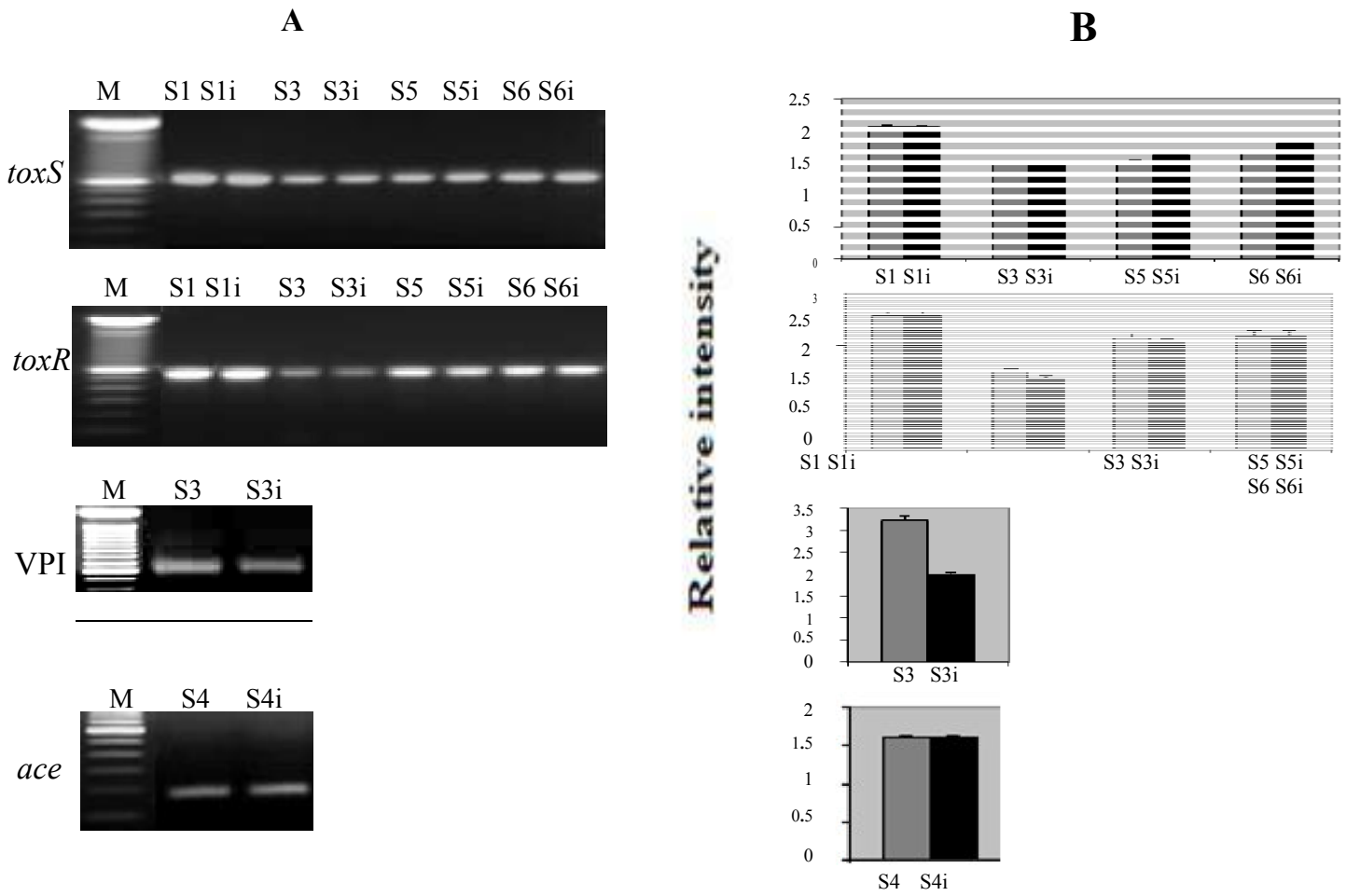

Figure 4. Virulence genes expression of *Vibrio alginolyticus* and *Vibrio parahaemolyticus* cells exposed to starvation for eight months in seawater. M: 100 bp DNA ladder (Promega); S1 and S3: *Vibrio alginolyticus* strains; S5 and S6: *Vibrio parahaemolyticus* strains. n: strain before incubation in seawater; i: strain incubated for eight months in seawater microcosm; A: 1% agarose gels analysis; B: relative expression of virulence genes.

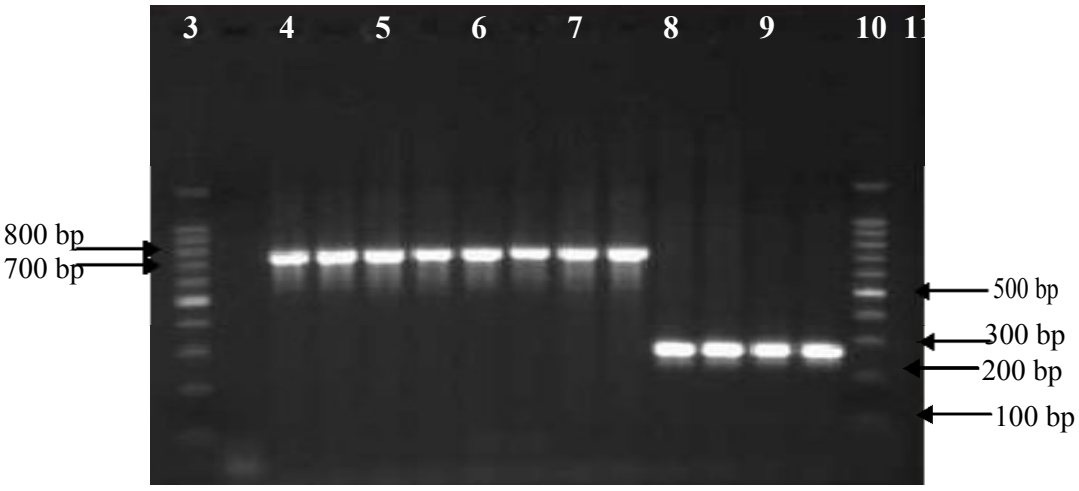

Figure 5. Electrophoretic profile of PCR products from *V. alginolyticus* and *V. parahaemolyticus* strains studied. M: 100 bp molecular size markers (Promega), Lane 1: negative control, Lanes 2, 3, 4, 5, 10 and 11: S1, S2, S3 S4, S5 and S6 respectively before incubation in seawater: Lanes 6, 7, 8, 9, 12 and 13: S1, S2, S3 S4, S5 and S6 respectively after incubation in seawater microcosms.

marine bacteria, such as *V. alginolyticus* and *V. parahaemolyticus*, are able to adapt and survive under starvation conditions in seawater. Many marine bacteria, especially *Vibrio* spp., can survive for a long time under starvation by sequential changes in cell physiology and gradual changes in morphology (Jiang and Chai, 1996). The long survival of *V. alginolyticus* and *V. parahaemolyticus* tested strains in seawater microcosm during 8 months, was accompanied by many morphological and metabolic modifications, among which, the reduction of cell sizes and their evolution to coccoid-shapes whose lengths were less than 0.4 m and between 0.5 m and 1 m. The evolution towards this state can make the bacterium survive for a long period under starvation conditions. The reduction in the bacteria size during stress is a survival strategy to minimize the needs of the cell for nutrients (Jiang and Chai, 1996) . This reduction in cell size is a result of cytoplasmic contraction as well as the reduction of the bacterial periplasm volume (Huisman et al., 1996). The metabolic activity of bacterial cells in a food deficiency state can reach 0.05% compared to the activity measured in an exponential phase (Kolter and Siegele, 1993). It has been proposed that under limited exogenous nutrient conditions, members of *Vibrionaceae* and *Pseudomonadaceae* can undergo a number of morphological and physiological changes to adapt to starvation conditions (Roszak and Colwell, 1987). Starvation- survival has been defined as ‘‘the process of survival in the absence of energy yielding substrates’’ (Morita, 1982) . Baker et al. (1983) reported that the exposure of *V. cholerae* to nutrient deprivation caused:

(i) the evolution of the cells to coccoid shape and the loss of over 90% of their original volume in 30 days, (ii) an increase in cell number, (iii) the loss of small granules and inclusion bodies, (iv) the loss of the distinct three-layered integrity of the outer membrane, peptidoglycan and the inner membrane to retain only remnants of those structures, (v) the compression of the nuclear region into the cell center surrounded by a denser cytoplasm and (vi) the formation of extended or convoluted structures from the cell wall which are pulled away from the cell membrane.

It has been suggested that these responses reflect the existence of various kinds of strategies to enhance survival under conditions of exogenous nutrient deprivation.

Our study showed alterations in total proteins secreted and in gelatinase patterns of starved *V. alginolyticus* and *V. parahaemolyticus* cells after 8 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). Secreted proteins play a major role in the virulence of bacteria (Sandra et al., 2000). According to Dorman et al. (2001), the *Shigella flexneri* secretion proteins depend on a complicated regulon of virulence genes whose expression is controlled by a multiplicity of environmental signals. Recently, Hao Gong et al. (2009) showed that high osmolarity is one of the environmental stresses that increase the level of *PrgI* and *SipB* protein expression in *Salmonella*. These two proteins enable the bacteria to enter epithelial cells and induce cytotoxicity in macrophages.

Marine *Vibrio* produces various extracellular products (Hasegawa et al., 2008). These proteins include cytolysins, lipases, siderophores, exopolysaccharides, and proteases such as gelatinase. These proteases are mainly involved in providing peptide nutrients for the micro-organism. 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). Thereby, alterations observed in extracellular protein profiles and gelatinase patterns under starvation condition can reflect on the stability of these virulence factors. In addition, these new proteases appearing in stressed *V. alginolyticus* and *V. parahaemolyticus* cells are due to starvation, which makes the bacteria able to change nutrient pathways. 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 and nitrogen control in enteric bacteria (Goldberg et al., 1976) . In the bacteria *Aeromonas hydrophila* (O’Reilly and Day, 1983), *Aeromonas salmonicida* (Dalhe, 1971), and *Pseudomonas aeruginosa* (Jensen et al., 1980) protease production is influenced by carbon and nitrogen sources. Additionally, temperature can influence protease production, as occurs in *A. hydrophila* (O’Reilly and Day, 1983).

Many vibrios are pathogenic for humans and/or marine vertebrates and invertebrates, with the virulence mechanisms reflecting the presence of enterotoxin, haemolysin, cytotoxin, and various enzymes such as protease, lipase (Zhang and Austin, 2005). Among them, in this work, the relative expressions of *toxR*, *toxS*, VPI

and *ace* virulence genes in starved *V. alginolyticus* and *V. parahaemolyticus* cells were investigated. Our study showed instability in the expression level of VPI gene after starvation. According to Peterson and Mekalanos (1988), *Vibrio* modulates its virulence genes expression in response to stress conditions. This modulation is essential for *in vivo* survival since strains lacking this ability due to a mutation in the *toxR* gene, of which the product is involved in signal-dependent virulence gene expressions and are deficient in intestinal colonization of human volunteers (Herrington et al., 1988). The gene expression instability of vibrios under environmental stress conditions has also been demonstrated (Asakura et al., 2006). Gonzalez-Escalona et al. (2006) presented an interesting finding that incubation temperature significantly affects the gene expression profile of *Vibrio cholerae*. These authors compared the transcription of some target genes in the VBNC (induced at 4°C) and starvation (15°C) states, and showed that key enzymes for cellular metabolism, such as *tuf*, which is needed for protein synthesis, and *relA* or *rpoS*, which are stress response genes, were detected at higher levels in bacteria that had entered the VBNC state.

Conclusion

The consequences of adaptation of *V. alginolyticus* and *V. parahaemolyticus* to nutrient deficiency in seawater are multiple. In addition to the modifications of the morphological characters by the appearance of the coccoid shape form, the variations also affect extracellular proteins and gelatinase patterns. The expression level of virulence genes was also altered. This can reflect on the genetic instability and virulence state of stressed bacteria. These variations can pose a serious problem for the characterization of these strains isolated from the environment. For this reason, it is necessary to find more adequate methods for the identification of bacteria. Under these conditions, molecular biology methods become more important for the identification and characterization of water environmental bacteria than traditional methods of culture. The target collagenase gene proved useful for the identification of *V. alginolyticus and V. parahaemolyticus* originating from the water environment and our results confirm that this molecular biology method is also valid for the identification of strains stressed for a long period in seawater microcosms.

REFERENCES

Asakura H, Ishiwa A, Arakawa E, Makino S, Okada Y, Yamamoto S, Igimi S (2006). Gene expression profile of *Vibrio cholerae* in the cold stress-induced viable but non-culturable state. Environ. Microbiol., 9: 869-879.

Baker RM, Singleton FL, Hood MA (1983). Effects of nutrient deprivation on *Vibrio cholerae*. Appl. Enviro. Microbiol., 46: 930-940.

Ben Abdallah F, Ayed A, Bakhrouf A, Kallel H (2009a). Alterations of outer membrane proteins and virulence genes expression in gamma-irradiated *Vibrio parahaemolyticus* and *Vibrio alginolyticus*. Foodborne pathog. Dis., 6: 1171-1176.

Ben Abdallah F, Chaieb K, Kallel H, Bakhrouf A (2009b). RT-PCR assays for in vivo expression of *Vibrio alginolyticus* virulence genes in cultured gilthead *Dicentrarchus labrax* and *Sparus aurata*. Ann. Microbiol., 59: 1-5.

Ben Abdallah F, Chaieb K, Snoussi M, Bakhrouf A, Gaddour K (2007). Phenotypic variations and molecular identification of *Salmonella enterica* serovar Typhimurium cells under starvation in seawater. Curr. Microbiol., 55: 485-491.

Ben Kahla NA, Besbes A, Chaieb K, Bakhrouf A (2007). Survival of *Vibrio alginolyticus* in seawater and retention of virulence of its starved cells. Mar. Environ. Res., 64: 469-478.

Braga PC, Ricci D (1998). Atomic force microscopy: application to investigation of *Escherichia coli* morphology before and after exposure to cefodizime. Antimicrob. Agents. Chemother., 42: 18-22.

Dalhe HK (1971). Regulation of the proteinase production in two strains of *Aeromonas*. Acta. Pathol. Microbiol. Scand. Sect. B., 79: 739-746.

Di Pinto A, Ciccarese G, Fontanarosa M, Terio V, Tantillo G (2006) Detection of *Vibrio alginolyticus* and *Vibrio parahaemolyticus* in shellfish samples using collagenase-targeted multiplex-PCR. J. Food. Safety., 26: 150-159.

Dorman CJ, McKenna SS, Beloin S (2001). Regulation of virulence gene expression in *Shigella flexneri*, a facultative intracellular pathogen. Int. J. Med. Microbiol., 291: 89-96.

Givskov M, Eberl L, Moller S, Poulsen LK, Molin S (1994). Responses to nutrient starvation in *Pseudomonas putida* KT2442: analysis of general cross-protection, cell shape, and macromolecular content. J. Bacteriol., 176: 7-14.

Goldberg RB, Bloom FR, Magasanik B (1976). Regulation of histidinase synthesis in intergenic hybrids of enteric bacteria. J. Bacteriol., 127: 114-119.

Gong H, Su J, Bai Y, Miao L, Kim K, Yang Y, Liu F, Lu S (2009). Characterization of the expression of *Salmonella* Type III secretion system factor PrgI, SipA, SipB, SopE2, SpaO, and SptP in cultures and in mice. BMC. Microbiol., 9: 73-87.

Gonzalez-Escalona N, Fey A, Hofle MG, Espejo RT, Guzman C (2006). Quantitative reverse transcription polymerase chain reaction analysis of *Vibrio cholerae* cells entering the viable but nonculturable state and starvation in response to cold shock. Environ. Microbiol., 8: 658-666.

Hasegawa H, Lind, EJ, Boin MA, Hase CC (2008). The Extracellular metalloprotease of *Vibrio tubiashi*i is a major virulence factor for pacific oyster (*Crassostrea gigas*) larvae. Appl. Enviro. Microbiol., 74: 4101-4110.

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.

Herrington DA, Hall RH, Losonsky GA, Mekalanos JJ, Taylor RK, Levine MM (1988). Toxin, toxincoregulated pilus and the *toxR* regulon are essential for *V. cholerae* pathogenesis in human volunteers. J. Exp. Med., 168: 1487-1492.

Huisman GW, Siegele DA, Zambrano MM, Kolter R (1996). Morphological and physiological changes during stationary phase in *Escherichia coli* and *Salmonella*. In: Neidhardt, F.C, Curtiss, R., Gross, C.A., Ingraham, J.L., Lin, E.E.C. et al. (eds) Cellular and molecular biology. Am. Soc. Microbiol., Washington, pp. 1672–1682.

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, ChainT (1996). Survival of *Vibrio parahaemolyticus* at low temperatures under starvation conditions and subsequent resuscitation of viable, nonculturable cells. Appl. Environ. Microb., 62: 1300-1305.

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.

Kjelleberg S, Flärdh K, Moriarty DJW (1993). Growth limitation and

starvation of bacteria. In: Ford T (ed) Aquatic microbiology: an ecological approach. Blackwell, Oxford, pp. 289-320.

Kjelleberg S, Hermannson M, Marden P, Jones GW (1987). The transient phase between growth and nongrowth of heterotrophic bacteria, with emphasis on the marine environment. Annu. Rev. Microbiol., 41: 25-49.

Kolter R, Siegele DA, Tormo A (1993). The stationary phase of bacterial life cycle. Annu. Rev. Microbiol., 47: 855-874.

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.

Morita RY (1982). Starvation survival of hetetrophs in the marine environment. Adv. Microbiol. Ecol., 6: 171-198.

O'Reilly T, Day F (1983). Effects of culture conditions on protease production by *Aeromonas hydrophila*. Appl. Environ. Microbiol., 45: 1132-1135.

Peterson KM, Mekalanos JJ (1988). Characterization of the *Vibrio cholerae* ToxR regulon: identification of novel genes involved in intestinal colonization. Infect. Immun., 56: 2822-2829.

Rosen R, Buttner K, Schmid R, Hecker M, Ron EZ (2001). Stress-induced proteins of *Agrobacterium tumefaciens*. FEMS. Microbiol. Ecol., 35: 277-285.

Roszak DB, Colwell RR (1987). Survival strategies of bacteria in the natural environment. Microbiol. Rev., 51: 365-379.

Sandra LM, Leigh AK, Finlay BB (2002). *Salmonella enterica* serovar Typhimurium effector SigD/SopB is membrane-associated and ubiquitinated inside host cells. Cell. Microbiol., 4: 435-446.

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.

Sechi LA, Dupre I, Deriu A, Fadda G, Zanetti S (2000). Distribution of *Vibrio cholerae* virulence genes among different *Vibrio* species isolated in Sardinia, Italy. J. Appl. Microbiol., 88: 475-481.

Siegele DA, Kolter R (1992). Life after log. J. Bacteriol., 174: 345-348.

Srinivasan S, Kjellberg S (1998). Cycles of famine and feast: the starvation and outgrowth strategies of a marine *Vibrio*. J. Biosci., 23: 501-511.

Zhang XH, Austin B (2005). Haemolysins in *Vibrio* species. J. Appl. Microbiol., 98: 1011-1019.