THE EFFECTS OF HEAT AND OSMOTIC STRESS ON *VIBRIO PARAHAEMOLYTICUS* SURVIVAL RATES, CELLULAR MORPHOLOGY AND TDH PRODUCTION

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ABSTRACT : *Vibrio parahaemolyticus* is an emerging enteric pathogen often associated with the consumption of improperly cooked seafood. The major virulence factor of this pathogen is represented by the termostable direct hemolysin (TDH). When entering the host organism, the vibrion has to face a series of environmental changes (such as temperature and osmolarity) which determine various cellular responses among which the heat shock response has an important role.

The purpose of this study was to analyze the survival profiles, the morphological changes and the TDH production in *V. parahaemolyticus* strains grown under sub-lethal heat and osmotic stress.

Our study has demonstrated the thermolotolerance in *V. parahaemolyticus* strains and the absence of cross-protection between the osmotic and heat stress. We have also observed that the osmotic stress induced important changes to the bacterial cell morphology. The production of the thermostable direct hemolysin, the major virulence factor in *V. parahaemolyticus*, was increased in cells remained viable after thermal inactivation at 47°C, these results demonstrating the risk of virulence conservation in insufficiently heat treated food.

Key words: *Vibrio parahaemolyticus*, heat shock proteins, heat shock response, TDH

INTRODUCTION

Unlike commensal microorganisms, pathogenic bacteria have a life cycle which involves free living in the external environment and another one inside the host organism. The characteristic parameters of these two states (host vs. external environment) exert a series of different demands and presurre on the bacterial cell (Chowdhury et al., 1990).

Vibio parahaemolyticus is an estuarine bacterium which produces gastroenteritis, plague infections and in rare cases septicaemia, the main source of infection being represented by contaminated seafood (Nair et al., 2007). Based on the growth on Wagatsuma agar (culture media with mammal red blood cells), *Vibrio parahaemolyticus* was divided into 2 types one haemolytic-Kanagawa positive (KP) and one Kanagawa negative (KN). The cause of the hemolysis is represented by the presence of a *thermostable direct hemolysin* (TDH) and in some cases by TRH (*thermostable direct hemolysin related hemolysin*) or both hemolysins (Nishibuchi et al., 1992; Yeung and Boor, 2004).

When exposed to starvation, oxidative stress and variations of temperature and osmolarity, *Vibrio parahaemolyticus* enters a specific type of phase in which the cells are viable but not culturable (*viable but non culturable*-VBNC) (Bates and Oliver, 2004).

The VNBC state has also been documented in many species such as *Vibrio cholerae, Pseudomonas fluorescens*, *Salmonella typhimurium*, *Vibrio vulnificus* (Sahu et al., 1994; Weichart et al., 1992; Wolf and Oliver, 2002). During the VNBC state, the cells suffer some biochemical changes such as a lower rate of replication, transcription and protein synthesis. Furthermore, in the VNBC state the lipidic profile of these bacteria is altered in order to maintain the fluidity of the cellular membrane during the temperature shock; there have also been made some correlations with changes in the cellular morphology (Linder and Oliver, 1989; Falcioni et al., 2008). Flow cytometry studies have shown that *V.parahaemolyticus* enters the VNBC state after 69 days of starvation at 4°C. The studies regarding the modifications affecting the nucleic acids have revealed that the percentage of LNA (*low nucleic acids*) increases proportionally with the intensity of starvation and heat shock (Falcioni et al., 2008). Temperature stress in bacteria induces the generation of a heat shock response which involves the expression of a set of very conserved proteins called heat shock proteins (HSPs) (Lindquist and Craig, 2008). HSPs are commonly grouped into families based on their molecular weight: HSP10 (~ 10 kDa) or GroES-homologue proteins , HSP60 (~ 60 kDa) or GroEL-homologue proteins ,HSP40 (~ 40 kDa) or DnaJ-homologue proteins, HSP70 (~ 70 kDa) or DnaK-homologue proteins, HSP90 or HptG-homologue proteins (~ 90 kDa) and Clp ATP-dependent proteases (HSP100).

Studies have shown that these well conserved proteins have a role in bacterial virulence either as virulence factors or as agents which affect pathogenesis indirectly by regulating virulence gene expression (Gopha and Ron, 2003).

All in all, it is important to understand the modifications underlying the survival of *V. parahaemolyticus* under different stress conditions and to raise a conclusion regarding the influence of the stress response on bacterial pathogenicity.

The main object of the present study were the investigation of cell morphological changes and survival profiles of Kanagawa –negative and Kanagawa –positive *Vibrio parahaemolyticus* strains submitted to different stress conditions and also to establish the role of these stress conditions on the production of the major virulence factor of this pathogen (TDH).

MATERIAL AND METHODS

Bacterial strains and cultivation

Two strains of *V. parahaemolyticus*, one haemolytic, Kanagawa-positive (KP), isolated from acute diarrhea and one non-haemolytic, Kanagawa-negative (KN) from Danube Delta estuarine water, were used in our study. The strains were kindly provided by the National reference Center for Cholera and other vibrios from the National Institute for Development in Microbiology and Immunology. These strains were stocked in culture broth with 10% glycerol at –80°C. The strains were recovered from preserving medium on TCBS (thioglycolate citrate bile salts) and checked for their biochemical profile using the conventional biochemical tests: TSI (triple sugar agar), oxidase, nitrate reduction, motility, growth at 37°C, D-glucose fermentation (with/without gas production assessed by Durham tube), growth at different NaCl concentrations (0.5%, 1%, 3%, 6%, 8%, 10%), ornithine-decarboxylase, lysine-decarboxylase, arginin-dehydrolase, lactose, sucrose, manitol, mannose, maltose, arabinose, dulcitol, adonitol, inositol, sorbitol, galactose, Na citrate Simmons, indole production, methyl red and Voges Proskauer (on Clark medium) reactions and phenylalanine-desaminase.

The microbial strains were inoculated in liquid nutrient broth and respectively nutrient broth supplemented with 8% NaCl (osmotic shock) and incubated at 37°C and respectively at 42°C for 24 hrs with shaking at 160 rpm.

Bacterial growth was determined by measuring the absorbance at 600 nm and by plate count method on nutrient agar with 3% NaCl. Logarithmically grown cells at 37°C were heat shocked at 42°C and thereafter all variants were subjected to thermal inactivation at 47°C in water for 30 min.

Cell cultivability before and after exposure to heat and osmotic stress was checked by spotting ten-fold dilutions on TCBS medium and viable cell counts assay after 24 incubation at 37 °C.

The morphological examination was performed on Gram-stained smears obtained from liquid cultures submitted to normal and stress conditions.

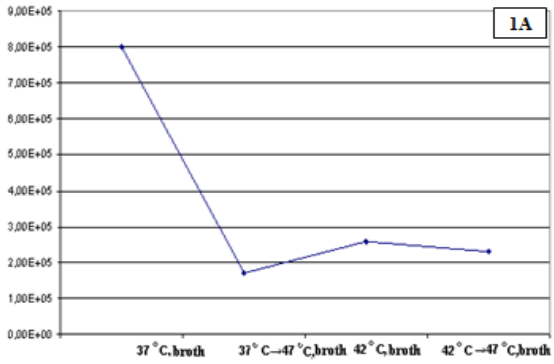
The plate hemolysis was evidenced by streaking the tested strains on Wagatsuma blood agar plates containing 5% (vol/vol) rabbit blood with a calibrated plastic loop. After incubation at 37°C for 24h, the clear areas (total lysis of the red blood cells) around the culture strip were registered as positive reactions.

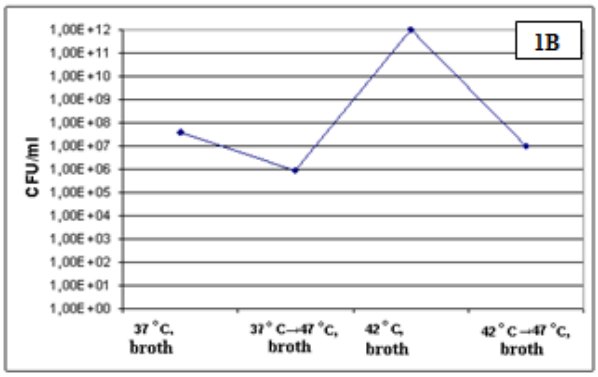
Preparation of genomic DNA and detection of the TDH gene

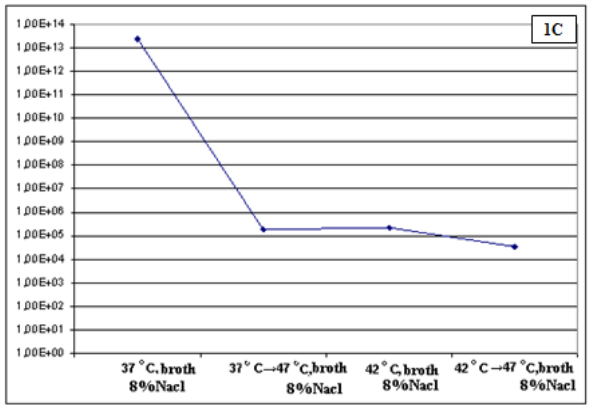
i)DNA extraction. Bacteria were cultured in tryptic soy broth, supplemented with 3% NaCl and incubated in a rotary shaking incubator at 37°C, 160 rpm for 16 h. Bacterial cells were collected by centrifugation. Genomic DNA of *V. parahaemolyticus* was prepared by thermal lysis.
ii) Detection of TDH gene. The TDH gene, a major virulence factor for *V.parahaemolyticus* was detected by PCR. Oligonucleotide primers (forward: 5'CGCAACAAAGCCTCATAGAG3' and reverse: 5'ACAATATCTCATCAGAACCGG3') were synthesized and DNA amplification was performed in a Palm cycler. The PCR reaction was carried out following the next steps: the reaction mixture was incubated in the thermal cycler at 95 °C for 5 min followed by 35 cycles, each of them as follows: 95 °C for 1 min, 53 °C for 50s, 72 °C for 50s. The PCR reaction also included an additional step at 72 °C for 5 min. The amplification product obtained had a molecular weight of 270bp and was highlighted on an 1% agarose gel.

RESULTS AND DISCUSSION

Our results showed that the cultivation of the *V. parahaemolyticus* microbial strains in sub-lethal temperature (42 ºC) and high salinity (8% NaCl) conditions enhanced their thermotolerance, probably by the induction of heat-shock proteins. When the heat shocked or un-shocked control cells were subjected to thermal inactivation at 47°C, a significantly higher survival rate was observed in the heat shocked cells than that of un-shocked cells (Figure 1A and 1B). It is to be noticed that the viability rates after the thermal inactivation at 47 ºC of the bacterial cultures heat shocked at 42°C are higher than those obtained after thermal inactivation of the 37 ºC cultured cells, despite the significantly higher number of viable cells (Figure 1A and 1B).
In the case of *Vibrio parahaemolyticus* non haemolytic strain the multiplication rate was also higher at 42 °C than at 37 °C degrees (Figures 1A and 1D), demonstrating that in the case of this strain the cultivation in sub-lethal thermal conditions is correlated with a positive regulation of cell cycle and multiplication rate.







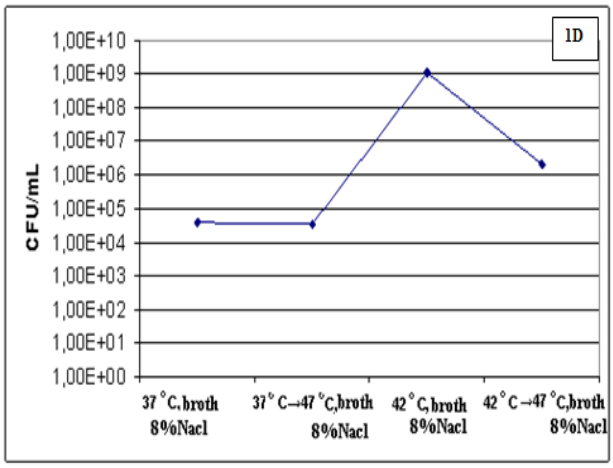


Figure 1A. The survival rates of *Vibrio parahaemolyticus* hemolytic strain (CFU/ml) cultivated at 37 and respectively 42°C and thereafter inactivated at 47 °C. **Figure 1B.** The survival rates of *Vibrio parahaemolyticus non*-hemolytic strain (CFU/ml) cultivated at 37 and respectively 42°C and thereafter inactivated at 47 °C. **Figure 1C.** The survival rates of *Vibrio parahaemolyticus*hemolytic strain (CFU/ml) cultivated 37°C and 42 ° C respectively at high concentrations of NaCl and after inactivation at 47 °. **Figure 1D.** The survival rates of *Vibrio parahaemolyticus non*-hemolytic strain (CFU/ml) cultivated 37°C and 42 ° C respectively at high concentrations of NaCl and after inactivation at 47 °

In our study, the *Vibrio parahaemolyticus* KP strain submitted to osmotic shock (37 ºC, 8% NaCl) showed a much more drastic reduction of viable cells after thermal inactivation that the cells concomitantly submitted to heat and osmotic shock (42 ºC, 8% NaCl) (Figure 1C), demonstrating that the growth at 8% NaCl is not inducing a cross protective heat response. However, an opposite behavior was exhibited by the KN strain, for which the osmotic stress alone proved to be protective against the thermal inactivation (Figure 1D).

Interestingly the osmotic stress induced important changes to the cell morphology, as demonstrated by the occurrence of fusiform, spiraled forms with low affinity for the Gram staining which completely replaced the normal Gram-negative thin rods morphology with frequent palisade grouping observed for the strain incubated at 37º C (Figure. 2, 3).

In exchange, the heat shock has not significantly influenced the cell morphology, except the decrease of palisade grouping with the predominance of isolated rods (Figure 3).

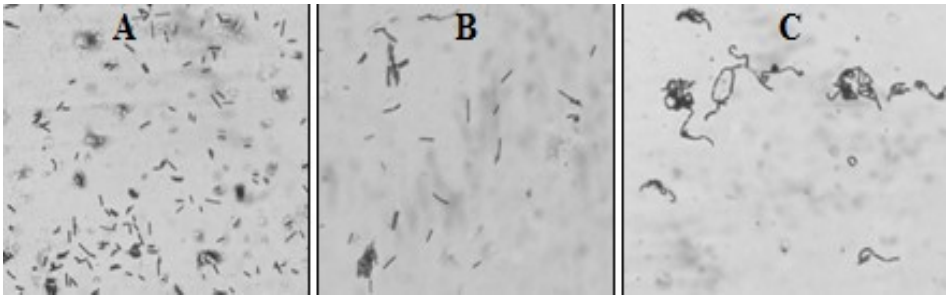


Figure 2. The morphological aspect of *Vibrio parahaemolyticus* KP strain cultivated at 37ºC (A), at 42 ºC (B), and respectively at 42º C and 8% NaCl (C).

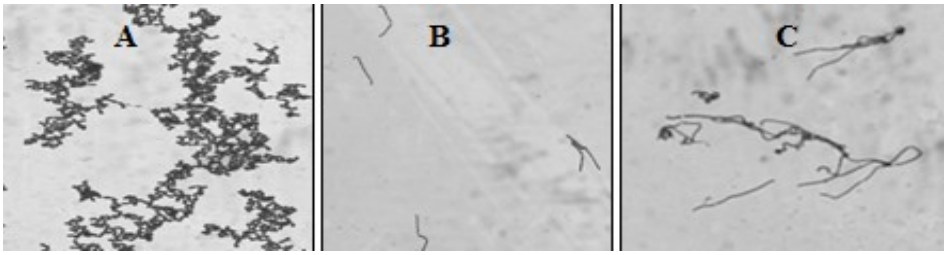


Figure 3. The morphological aspect of *Vibrio parahaemolyticus* KN strain cultivated at 37ºC (A), at 42 ºC (B) and respectively at 42º C and 8% NaCl (C).

The TDH gene was evidenced by PCR in the KP + strain isolated from a diarrheal case, while being absent in the KN strain. The production of the hemolysins by *Vibrio parahaemolyticus* was not influenced by the heat and osmotic stress conditions. However, the cells remained viable after thermal inactivation at 47ºC exhibited a more intensive hemolytic activity on blood agar (Figure 5), and even the non-haemolytic *Vibrio parahaemolyticus* strain (encoded with 2 in Figure 5) became slightly hemolytic after thermal inactivation.

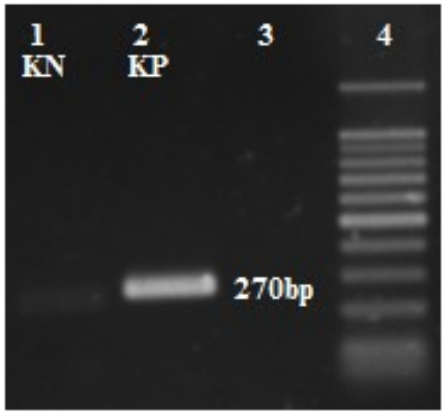


Figure 4.PCR amplification of the *tdh* gene(270 bp);1-KN strain; 2-KP strain, 3-negative control, 4-100bp alellic ladder

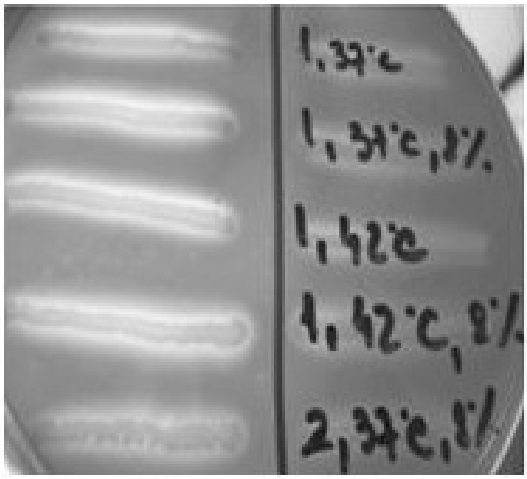


Figure5.Aspect of hemolytic ability of *Vibrio parahaemolyticus* strains (KP and KN) cultivated in different heat and osmotic stress conditions (right - NT) and thereafter submitted to thermal inactivation at 47°C (left - T).

DISCUSSION

The present study has successfully demonstrated the heat shock response at 42°C in *V.parahaemolyticus.* Increase of heat resistance by short-term exposure to heat shock condition has also been demonstrated in other enteropathogenic bacteria, such as *Yersinia enterocolitica* (Shenoy and Murano, 1996) and *Escherichia coli* (Chowdhury et al., 1990).

According to previous investigations, in many micro-organisms, sub-lethal exposure to a stress (e.g. starvation of marine vibrios) can confer resistance to a lethal exposure to the same stress (adaptive response) or to other stresses (cross-protection response) (Miyamoto et al., 1969; Neihardt et al., 1984, Nilsson et al., 1991).

As reviewed by Oliver (1993), high salt concentrations represent a significant stress to enteric bacteria, and salt concentrations found in seawater readily render these bacteria non-cultivable (Preyer and Oliver, 1993; Ray, 1999). Osmotic stress has some physiological features in common with starvation including the induction of some starvation proteins by osmotic shock in *E. coli* (Jenkins et al., 1990).

This prompted us to investigate the effect of osmotic stress on heat resistance, but an increased thermotolerance in osmotically-stressed cell could not be demonstrated. Similar results were reported by other authors for *Ps. fluorescens,* the osmotically-stressed *Ps. fluorescens* showing no protection against a subsequent heat challenge (Jørgensen and Knøchel, 2008).

Most clinical isolates of *V. parahamolyticus* (over 95% of the strains) are hemolytic on Wagatsuma agar (KP+) and produce a thermostable direct hemolysin (TDH) which is the major virulence factor in this pathogen. In exchange, the majority of isolates from environmental samples are Kanagawa negative (Takeda, 1993).

A very interesting finding accounting for the role of the heat-stress response in the increase of pathogenic potential is the increase of haemolytic activity on Wagatsuma culture medium of cells remained viable after heat inactivation at 37°C, this aspect being not observed in the cells heat shocked at 42°C or cross shocked by osmotic stress. Our results come into agreement with other studies demonstrating that that heat shock causes the injury of cell membrane of *V. parahaemolyticus* that lead to the enhanced leakage of protein and nucleic acid materials from the stressed cells [20], therefore suggesting that heat shock may stimulate the expression of *tdh* gene, which encodes TDH of *V. parahaemolyticus*, thus enhances the synthesis of TDH by test organism (Wong et al., 2002). Data obtained from the present study could be of significant importance with regard to food safety since heating regimes existent in the food industry could potentially induce the virulence increase of remained viable cells.

CONCLUSION

Our study has demonstrated the thermolotolerance in *V. parahaemolyticus* strains and the absence of cross-protection between the osmotic and heat stress. The osmotic stress has determined changes in the cellular morphology of the bacterial strains analyzed.
The production of the thermostable direct hemolysin, the major virulence factor in *V. parahaemolyticus*, was increased in cells remained viable after thermal inactivation at 47ºC, these results demonstrating the risk of virulence conservation in insufficiently heat treated food.

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