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Survival of *Vibrio parahaemolyticus* under environmental stresses as influenced by growth phase and pre-adaptation treatment

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ABSTRACT

In this study, the susceptibility of *Vibrio parahaemolyticus* in different growth phases after exposure to lethal stresses including 47 °C and 8% ethanol was first investigated. The effect of a culture's growth phase on both the heat and ethanol shock response of *V. parahaemolyticus* was then examined. It was found that cells of *V. parahaemolyticus* in the mid-exponential phase, regardless of adaptation, were most susceptible to environmental stresses, while cells in the stationary phase were least susceptible to the lethal stresses examined. Adaptation with heat shock at 42 °C for 45 min or ethanol shock with 5% ethanol for 60 min induced an increased resistance of *V. parahaemolyticus* to subsequent lethal stresses at 47 °C and 8% ethanol. While the adaptation treatments resulted in a reduced resistance of the test organism to pH 4.4 and 20% NaCl. Generally, the extent of changes in the resistance of *V. parahaemolyticus* to lethal stresses between the adapted and control cells was found to be growth phase dependent. Compared with the respective control cells, the adapted late-exponential phase cells exhibited the greatest extent of change, while the adapted stationary phase cells showed the least change in their resistance to the lethal stresses examined.

1. Introduction

Vibrio parahaemolyticus is a gram-negative and moderately halophilic bacterium naturally found in marine environments (Beuchat, 1982). It is frequently found in a variety of seafood and recognized as the leading cause of food-borne illness (Liston, 1990; Chai and Pace, 2001). The major clinical symptoms of human gastroenteritis caused by *V. parahaemolyticus* are characterized by diarrhea, headache, vomiting, nausea, abdominal cramps and low fever (Su and Liu, 2007). Food poisoning outbreaks associated with *V. parahaemolyticus* have been reported throughout the world, especially occurring in areas like Taiwan and Japan, where people often consume raw and semi-processed seafood in their daily diet (Honda and Iida, 1993; Anonymous, 2006).

It has been found that stress responses occur in cells of microorganisms that have previously been exposed to sub-lethal adaptation treatments (Lou and Yousef, 1996, 1997; Browne and Dowds, 2001, 2002; Periago et al., 2002a,b; Wong et al., 2002). These responses are affected by a number of factors including different strains and species of the test organisms, pre-adaptation treatments, variation in lethal stress challenges, and differentiation in the cultures' growth phase (Mackey and Derrick, 1990; Jørgensen et al., 1996, 1999; Cheng et al., 2003; Lin and Chou, 2004; Lin et al., 2004).

Growth phase is among the most important factors which may influence the extent of induced stress response in microorganisms (Jørgensen et al., 1999; McMahon et al., 2000; De Angelis et al., 2004; Yeung and Boor, 2004). Jørgensen et al. (1999) reported that the magnitude of heat shock induced thermotolerance in *Listeria monocytogenes* was lower in stationary cultures compared with exponentially growing cultures. McMahon et al. (2000) indicated that the thermal resistance of *Yersinia enterocolitica* and *L. monocytogenes* was dependent on cell growth phase. They also reported that the magnitude of change in heat resistance between heat-shocked and non-heat-shocked cells for exponential cultures was greater than that for stationary cultures. Moreover, De Angelis et al. (2004) observed that the extent of increased heat resistance of *Lactobacillus plantarum* was greater with mid-exponential phase cells than stationary phase cells after heat adaptation at 42 °C for 1 h. Finally, Yeung and Boor (2004) reported that survival rates at pH 3.6 for exponential phase cells of *V. parahaemolyticus* that had been previously exposed to a sub-lethal acidic condition (pH 5.5) were enhanced when compared with that of nonacid-adapted cells. In contrast, acid adaptation impaired the survival of stationary phase cells at pH 3.6.

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Previously, a series of studies have been conducted in our laboratory to explore the responses of *V. parahaemolyticus* in late-exponential phase to sub-lethal heat and ethanol shock treatments. We noted that heat shock and ethanol shock increased the survival rate of *V. parahaemolyticus* with exposure to 47 °C, 8% ethanol, and 20 ppm H_2O_2 (Chang et al., 2004; Chiang et al., 2006, 2008a). Heat shock and ethanol shock resulted in cell-surface damage and the change of fatty acid composition in *V. parahaemolyticus* (Chiang et al., 2005, 2006, 2008a). Furthermore, we also found that heat shock and ethanol shock affected the expression of proteins, thermostable direct hemolysin (TDH), superoxide dismutase and catalase by *V. parahaemolyticus* (Chiang and Chou, 2008; Chiang et al., 2008b).

In the present study, we further investigated the survival of *V. parahaemolyticus* in different growth phases after exposure to various stresses. Specifically, the effect of culture growth phase on the induction of heat shock and ethanol shock responses of *V. parahaemolyticus* was examined.

2. Materials and methods

2.1. Microorganism and growth medium

V. parahaemolyticus 690, a clinical strain capable of producing thermostable direct hemolysin (TDH), was used as the test organism in the present study. It was obtained from Professor H. C. Wong (Dept. of Microbiology, Soochow University, Taipei, Taiwan). The test organism was first activated by two successive transfers in Tryptic Soy Broth (Difco, Sparks, MD, USA) supplemented with 3% NaCl (Nacalai, Tesque, Kyoto, Japan) (TSB-3% NaCl) at 37 °C for 4 h. This activated culture served as the inoculum.

2.2. Measurement of heat and ethanol susceptibilities during the growth cycle

To measure heat and ethanol susceptibilities of *V. parahaemolyticus* during the growth cycle, one 0.1 ml aliquot of the properly diluted activated culture was inoculated into 50 ml of TSB-3% NaCl in a 100-ml Erlenmeyer flask yielding an initial population of ca. 10^5 cfu/ml that was then incubated at 37 °C for 6 h. During the various incubation periods, samples of cultures were taken periodically to determine the viable population and their susceptibilities at 47 °C and in presence of 8% ethanol.

In addition, according to the full growth curve in the above susceptibility test, the cultures incubated at 37 °C for 2.5, 4 and 5.5 h were prepared as the mid-exponential, late-exponential and stationary phase cultures, respectively. These three phase cultures were then subjected to adaptation treatments for obtaining the heat-shocked, ethanol-shocked cells (adapted cells) and control cells (non-adapted cells).

2.3. Procedure of heat and ethanol adaptation treatments

To perform the adaptation treatments, the different growth phase cultures of *V. parahaemolyticus* were first harvested by centrifugation (3000 × g, 10 min). The harvested cells were washed twice with PBS-3% NaCl. They were then resuspended in PBS-3% NaCl and submerged in a circulating water bath at 42 °C with shaking (150 rpm) for 45 min to prepare the heat-shocked cells. To prepare the ethanol-shocked cells, the previously washed cells of *V. parahaemolyticus* were resuspended in PBS-3% NaCl containing 5% (v/v) ethanol and held at room temperature for 60 min. The control cells were prepared by resuspending the washed cells in PBS-3% NaCl at room temperature for the same period of time as did the adapted cells.

2.4. Study of stress challenges

Cells taken during growth cycle, adapted and non-adapted cells of test organism were exposed to lethal stress challenges and their survivals were determined.

To determine the thermal tolerance and survival in the presence of ethanol, cells of *V. parahaemolyticus* were inoculated into PBS-3% NaCl which was pre-heated at 47 °C or one containing 8% (v/v) ethanol at room temperature for 30 min at an initial population of ca 10^6 cfu/ml.

To investigate the susceptibility of test organism to acidic condition and high NaCl content cells were inoculated into PBS-3% NaCl, which was adjusted to a pH of 4.4 with 1 N HCl solution or PBS containing 20%NaCl for a period of 3 h of initial population of 10^6 cfu/ml. At the end of exposure period, the viability of cells was determined. Samples were serially diluted in PBS-3% NaCl solution and viable counts made by pour plating (1 ml) on TSA-3% NaCl. Colonies were counted after 18 h of incubation at 37 °C.

2.5. Statistical analysis

The mean value and standard deviation were calculated from the data obtained from the three separate experiments. These data were compared using Duncan's multiple range test (SAS, 2001).

3. Results and discussion

3.1. Susceptibility of V. parahaemolyticus to 47 °C and 8% ethanol during the growth cycle

It was noted that the cells of *V. parahaemolyticus* with 0 h of cultivation had a survival rate of 8.7% after a sudden exposure to 47 °C for 30 min (Fig. 1A). As the culture continued to grow, the susceptibility of *V. parahaemolyticus* cells to heat exposure at 47 °C increased until a maximum value was observed in mid-exponential phase with the lowest survival rate of only 0.03% occurring after 2.5 h of cultivation. However, heat resistance of *V. parahaemolyticus* cells returned as the cultivation further proceeded. The cells obtained after 6 h of cultivation, in stationary phase, exhibited a survival rate of 5.9%, which was about 200-fold that of cells in mid-exponential phase after exposure to 47 °C for 30 min.

Similar to that observed on the susceptibility to heat exposure at 47 °C (Fig. 1A), cells of *V. parahaemolyticus* in mid-exponential phase (2.5 h-culture) were most susceptible to ethanol, with the lowest survival rate being only 20.4%, while cells in stationary phase, obtained after 6 h of cultivation, exhibited a survival rate of 75.2% (Fig. 1B). This physiological state-dependent susceptibility of *V. parahaemolyticus* to the lethal stresses observed is consistent with that reported by Lorca and de Valdez (1999). Previously, Hansen and Riemann (1963) reported that changes in the osmotic barrier during cell division may make the actively growing organism more sensitive to heat than stationary cells. The high mortality rate of mid-exponential phase cells of *V. parahaemolyticus* after exposure to lethal heat and ethanol challenges observed is in accordance with those reported for *L. monocytogenes*, *Escherichia coli* O157: H7 and *Bacillus cereus* (Davis et al., 1996; O'Driscoll et al., 1996; Jordan et al., 1999; Browne and Dowds, 2001, 2002). This may be attributed to the increased sensitivity of the cells to environmental stresses during cell division (Lorca and de Valdez, 1999).

3.2. Survival of heat-adapted and non-adapted V. parahaemolyticus in different growth phases after exposure to various lethal challenges

Table 1 shows the survival of non-adapted and heat-adapted cells in various growth phases after lethal challenge at 47 °C, 8%