Response of Heat-Shocked *Vibrio parahaemolyticus* to Subsequent Physical and Chemical Stresses

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**ABSTRACT**

*Vibrio parahaemolyticus* foodborne strains 405, 556, and 690 and a *V. parahaemolyticus* chopping board isolate were heat shocked at 42°C for 15, 30, or 45 min. Heat shock, regardless of heating periods tested, caused an increased demand for NaCl during recovery from heat injury. Further study with strain 690 and the chopping board isolate also revealed that heat shock generally increased the survival of the test organism during subsequent exposure to 47°C, 20 ppm H2O2, and 8% ethanol and reduced the tolerance of the test organism to low temperatures (5 and −18°C). The extent of the heat shock response of *V. parahaemolyticus* varied with strain and the duration of treatment. Furthermore, heat shock treatments in the present study caused the leakage of nucleic acids from *V. parahaemolyticus* cells. This effect was most pronounced with cells heat shocked at 42°C for 45 min.

*Vibrio parahaemolyticus* is a gram-negative facultative anaerobic and moderately halophilic foodborne pathogen that causes gastrointestinal disease (3, 6). It was first isolated in 1950 during a food-poisoning outbreak in Japan, which involved 272 individuals and caused 20 deaths (10). This pathogen is widely distributed in natural aquatic environments around the world (6) and is widely recognized as a foodborne pathogen. Illness resulting from the consumption of food contaminated with *V. parahaemolyticus* has also been documented in various parts of the world (6). Although seafood is the main vehicle for *V. parahaemolyticus* food poisoning, cross-contamination may make other foods vehicles for this organism (3). In areas where raw and semiprocessed seafoods are consumed as part of the daily diet, *V. parahaemolyticus* is a major cause of gastrointestinal illness. In Japan, this pathogen accounts for 40 to 60% of the foodborne illness (13). In Taiwan, among the 86 outbreaks of bacterial food poisoning that occurred in 2001, 52 were confirmed to have *V. parahaemolyticus* as the causative agent (1). Thus, adequate control measures taken to prevent the contamination and proliferation of *V. parahaemolyticus* in food have received the attention of regulatory agencies, the food industry, and consumers.

Foods are often subjected to heat treatment during processing or by the consumer, and these treatments control or inactivate spoilage and pathogenic microorganisms. For example, mild heat treatment at ≥47°C has been proposed as an processing aid to reduce the numbers of *V. vulnificus* in raw oysters (7). Heat shock response occurs in microorganisms when they are subjected to sublethal exposure to heat a few degrees Centigrade above their growth temperature. These heat-shocked cells may develop resistance to subsequent heat treatment or other stresses (5, 9, 19, 23). Because pathogen control measures are usually based on studies with the non–heat-shocked microorganisms, these measures may not be sufficient to ensure the safety of processed food. Therefore, the possibility of a heat shock response should be considered when developing microbial control treatments (20).

The sublethal heat shock response of food pathogens such as *Salmonella*, *Listeria monocytogenes*, and *E. coli* O157:H7 has been extensively studied. However, information concerning the heat shock response of *V. parahaemolyticus* is limited. The purpose of this study was to investigate the survival of *V. parahaemolyticus* cells exposed to various environmental stresses after the cells had been subjected to various heat shock treatments. The effect of sodium chloride on the recovery of heat-shocked *V. parahaemolyticus* cells also was examined.

**MATERIALS AND METHODS**

**Microorganism.** In the present study, *V. parahaemolyticus* strains 405, 556, and 690 and a *V. parahaemolyticus* chopping board isolate were used as the test organisms. *V. parahaemolyticus* strains 405, 556, and 690 (Prof. H. C. Wong, Department of Microbiology, Soochow University, Taipei) were originally isolated from patients. The chopping board isolate (National Laboratories of Foods and Drugs, Department of Health, Executive Yuan, Taipei) was also associated with a foodborne outbreak.

After two successive transfers of the test organism in tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, Md.) supplemented with 3% NaCl (Nacalai, Tesque, Kyoto, Japan) at 37°C for 4 h, a 0.1-ml aliquot of the properly diluted culture was inoculated into 50.0 ml of TSB-NaCl and incubated at 37°C for 4 h to the late log phase when the population reached approximately 10⁸ CFU/ml. This culture served as the inoculum for the preparation of heat-shocked cells and the controls (no heat shock).

**Heat shock treatment.** To prepare the heat-shocked cells of *V. parahaemolyticus*, a 10.0-ml aliquot (log 10⁹ cells/ml) of the
TABLE 1. Recovery of heat-shocked and non-heat-shocked V. parahaemolyticus cells on TSA with different amounts of added NaCl

<table>
<thead>
<tr>
<th>V. parahaemolyticus</th>
<th>NaCl (%) in TSA</th>
<th>Non-heat shocked (control)</th>
<th>15 min</th>
<th>30 min</th>
<th>45 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain 690</td>
<td>0.25</td>
<td>8.90 ± 0.10 A a</td>
<td>8.72 ± 0.14 B b</td>
<td>8.62 ± 0.18 n b</td>
<td>8.54 ± 0.06 n b</td>
</tr>
<tr>
<td></td>
<td>3.00</td>
<td>9.00 ± 0.08 A a</td>
<td>8.84 ± 0.07 AB b</td>
<td>8.76 ± 0.07 A b</td>
<td>8.72 ± 0.09 A b</td>
</tr>
<tr>
<td></td>
<td>5.50</td>
<td>8.95 ± 0.11 A a</td>
<td>8.88 ± 0.11 A a</td>
<td>8.78 ± 0.14 A a</td>
<td>8.71 ± 0.10 A a</td>
</tr>
<tr>
<td>Chopping board isolate</td>
<td>0.25</td>
<td>8.76 ± 0.20 A a</td>
<td>7.53 ± 0.64 B b</td>
<td>7.83 ± 0.33 n b</td>
<td>8.04 ± 0.22 n b</td>
</tr>
<tr>
<td></td>
<td>3.00</td>
<td>8.84 ± 0.02 A a</td>
<td>8.69 ± 0.03 A a</td>
<td>8.65 ± 0.04 A a</td>
<td>8.59 ± 0.04 A a</td>
</tr>
<tr>
<td></td>
<td>5.50</td>
<td>8.85 ± 0.13 A a</td>
<td>8.59 ± 0.16 A a</td>
<td>8.41 ± 0.20 A a</td>
<td>8.35 ± 0.21 A a</td>
</tr>
<tr>
<td>Strain 405</td>
<td>0.25</td>
<td>8.78 ± 0.15 A a</td>
<td>7.59 ± 0.06 B c</td>
<td>7.65 ± 0.05 n c</td>
<td>7.98 ± 0.17 n b</td>
</tr>
<tr>
<td></td>
<td>5.50</td>
<td>8.80 ± 0.10 A a</td>
<td>8.53 ± 0.04 A b</td>
<td>8.40 ± 0.11 A b</td>
<td>8.19 ± 0.14 A c</td>
</tr>
<tr>
<td>Strain 556</td>
<td>0.25</td>
<td>8.89 ± 0.11 A a</td>
<td>8.09 ± 0.23 B b</td>
<td>8.12 ± 0.21 n b</td>
<td>8.16 ± 0.10 n b</td>
</tr>
<tr>
<td></td>
<td>5.50</td>
<td>8.98 ± 0.09 A a</td>
<td>8.75 ± 0.12 A b</td>
<td>8.63 ± 0.14 A b</td>
<td>8.48 ± 0.11 A b</td>
</tr>
</tbody>
</table>

Values in the same column of the same strain with different letters (A, B) are significantly different (P < 0.05). Values in the same row with different lowercase letters (a, b, c) are significantly different (P < 0.05).

A late-exponential-growth-phase culture of V. parahaemolyticus strain 690 and the chopping board isolate grown in TSB-NaCl was first centrifuged at 10,000 × g for 10 min. The pellet was resuspended in PBS-NaCl and subjected to heat shock as described. Samples were taken at various heating intervals and centrifuged at 20,000 × g for 12 min. The supernatant was then examined for leakage of nucleic acid by measuring the absorbance at 260 nm with a spectrophotometer. Nonheated cells were examined similarly for the leakage of nucleic acid.

Susceptibility studies. To determine the effect of heat shock on the survival of V. parahaemolyticus at 47°C, a 10.0-ml aliquot of heat-shocked or control cells was inoculated into 190 ml of PBS-NaCl (pretempered at 47°C) with an initial population of 10⁶ CFU/ml. Cells were then incubated at 42°C for 15, 30, or 45 min. After heat shock, the cell suspension was immediately used for determining the sodium chloride demand during cell recovery and the thermal tolerance and for other susceptibility studies.

Nucleic acid leakage study. A late-exponential-growth-phase culture of V. parahaemolyticus strain 690 and the chopping board isolate grown in TSB-NaCl was first centrifuged at 10,000 × g for 10 min. The pellet was resuspended in PBS-NaCl and subjected to heat shock as described. Samples were taken at various heating intervals and centrifuged at 20,000 × g for 12 min. The supernatant was then examined for leakage of nucleic acid by measuring the absorbance at 260 nm with a spectrophotometer. Nonheated cells were examined similarly for the leakage of nucleic acid.

Enumeration of V. parahaemolyticus. V. parahaemolyticus samples were prepared for enumeration by serial dilution in PBS-NaCl. Viable counts were determined by surface plating (0.1 ml) on tryptic soy agar (TSA) with 3% NaCl except when studying the effect of NaCl on the recovery of heat-shocked cells, when 0.25, 3.00, or 5.50% NaCl was added to TSA. Colonies were counted after 18 h of incubation at 37°C.

Statistical analysis. The means and standard deviations were calculated from the data obtained from triplicate trials. These data were compared using Duncan’s multiple range test (25).

RESULTS AND DISCUSSION

Effect of NaCl on recovery of heat-shocked V. parahaemolyticus cells. Various strains of V. parahaemolyticus were subjected to heat shock at 42°C for 15, 30, or 45 min. Data showing recovery of cells on TSA containing various amounts of NaCl are presented in Table 1. Depending on the amounts of NaCl in the recovery medium and the strain of V. parahaemolyticus, heat-shocked cell suspensions had viable populations similar to or smaller than those of control cells. For example, when plated with TSA + 0.25% NaCl and TSA + 3.00%, the heat-shocked V. parahaemolyticus strain 690 exhibited a viable population of 8.54 to 8.65 x 10⁸ CFU/ml, which was significantly smaller (P < 0.05) than that of the control (8.90 to 9.00 log CFU/ml). The viable population of the heat-shocked cells (8.72 to 8.84 log CFU/ml) recovered on TSA + 5.5% NaCl was not different (P > 0.05) from that of the control. However, after heat shock treatment, viable counts of both strains 405 and 556 were less than those of the control, regardless of the NaCl content of the TSA.

Regardless of strain, the viable count for control cells of V. parahaemolyticus detected with TSA containing various amounts of added NaCl were all similar (P > 0.05).
TABLE 2. Effect of heat shock on the leakage from V. parahaemolyticus cells of materials absorbing at 260 nm (nucleic acids)

<table>
<thead>
<tr>
<th>V. parahaemolyticus&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Control</th>
<th>15 min</th>
<th>30 min</th>
<th>45 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain 690</td>
<td>0.143 ± 0.037&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.143 ± 0.022&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.186 ± 0.040&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>0.232 ± 0.056&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>Chopping board isolate</td>
<td>0.105 ± 0.022&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.128 ± 0.031&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>0.145 ± 0.037&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>0.166 ± 0.030&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Supernatant of centrifuged cell suspension was examined.

<sup>b</sup> Values in the same row with different letters are significantly different (P < 0.05).

In general, recovery of heat-shocked V. parahaemolyticus cells, regardless of the duration of heat shock treatment, was higher on TSA with ≥3.0% NaCl than on TSA with 0.25% NaCl. Koga and Takumi (16) reported that after heat shock at 42°C for 30 min V. parahaemolyticus cells exhibited increased resistance to low osmotic pressure (13 mM NaCl), whereas their resistance to high osmotic pressure (2.5 M NaCl) decreased. However, Emswiler et al. (8) found that when V. parahaemolyticus was heat shocked at 41°C for 30 min, the plate counts obtained using TSA with 0.25% NaCl were nearly 99.9% higher than those obtained using TSA with 5.5% NaCl.

The increased recovery of heat-shocked V. parahaemolyticus on TSA with ≥3.0% NaCl as observed in the present study differs from the findings of Emswiler et al. (8). This discrepancy may be attributed to the differences in the strain of V. parahaemolyticus and the heat shock treatment conditions. Thermal stress under the conditions tested in the present study might cause death or injury of some V. parahaemolyticus cells (12) that are unable to recover on the plating medium used and thus could have led to the observed smaller viable population of heat-shocked V. parahaemolyticus compared with controls. Furthermore, an altered increased demand for NaCl was noted for the recovery of the heat-shocked V. parahaemolyticus cells. Increased recovery of viable cells was associated with an increased amount of NaCl in the plating medium.

Effect of heat shock on cell leakage. Table 2 shows the effect of heat shock treatment on the leakage of materials with absorbance at 260 nm (nucleic acids) from cells of V. parahaemolyticus strain 690 and of the chopping board isolate. Leakage of intracellular components such as proteins, amino acids, enzymes, and nucleic acids is evidence of membrane damage. Such damage is commonly associated with heat-injured cells and implies an alternation of the metabolic mechanism (8, 24). Heinis et al. (12) reported that heating at 45°C for 8 min resulted in the thermal injury of V. parahaemolyticus but caused no cell leakage of nucleic acids (11). Probably because of the longer period of heat shock treatment in the present study than that in the previous study (11), a higher absorbance at 260 nm (more leakage of nucleic acids) was observed in supernatants collected from the suspension of cells subjected to heat shock treatment compared with cells without heat shock treatment. In addition, the absorbance at 260 nm increased upon extending the heat shock treatment to 42°C. Statistical analysis also revealed that the absorbance at 260 nm of V. parahaemolyticus cells subjected to heat shock for 45 min, regardless of strain, was significantly higher (P < 0.05) than that of the controls. These results demonstrated that membrane damage and the leakage of nucleic acids from cells occurred when V. parahaemolyticus was subjected to heat shock under the test condition, possibly resulting in the reduced viable count of V. parahaemolyticus after heat shock treatments (Table 1).

Effect of heat shock on the thermal tolerance of V. parahaemolyticus at 47°C. Data on survival of V. parahaemolyticus cells at 47°C are presented in Figure 1. Survival of both control and heat-shocked cells decreased upon extending the treatment time. After 20 min of treatment at 47°C, the percent survival of the chopping board isolate control cells was reduced to 18.6% (Fig. 1B). The D<sub>47°C</sub> of this organism, roughly estimated to be 45 to 50 min, was similar to that reported by Beuchat and Worthington (4). Strain 690 was less thermal resistant than the chopping board isolate, with a percent survival of only 0.3% after 20 min of exposure at 47°C (Fig. 1A). Regardless of the duration of heat shock treatment, heat shock increased the thermal tolerance of both strain 690 and the chopping board isolate. The increased thermal tolerance of V. parahaemolyticus observed after heat shock was similar to that reported for Salmonella and Listeria (5, 9, 19). In addition, a trend toward significant increase in thermal tolerance upon extending the duration of heat shock treatment was observed with strain 690 (Fig. 1A).

Effect of heat shock on the survival of V. parahaemolyticus at low temperature. Figure 2 shows the effect of various heat shock treatments on the survival of V. parahaemolyticus strain 690 and the chopping board isolate when incubated at 5°C, which is reported to be the minimal temperature for growth (3). Survival of both strains, regardless of heat shock treatment, decreased upon extending the exposure time at 5°C (Fig. 2). However, the heat-shocked cells showed reduced survival compared with their controls. In general, survival of V. parahaemolyticus held at 5°C decreased with increased severity of heat shock treatment and increased storage time. After 8 days of exposure to 5°C, survival of the 42°C for 30 min and 42°C for 45 min heat-shocked cells of strain 690 were similar (P > 0.05), whereas survival was lower (P < 0.05) with controls and the 42°C for 15 min heat-shocked cells (Fig. 2A). However, the 42°C for 45 min heat-shocked chopping board
isolate cells exhibited the greatest survival reduction down to 8.8%, which is less ($P < 0.05$) than the control and other heat-shocked cells (Fig. 2B).

Heat shock treatment also caused *V. parahaemolyticus* to become more susceptible to storage at $-18^\circ C$ ($P < 0.05$) when compared with the control (Fig. 3). As the duration of heat shock treatment increased, survival of the treated cells decreased. A more marked reduction in survival was observed with both control and heat-shocked cells exposed to $-18^\circ C$ (Fig. 3) compared with $5^\circ C$ (Fig. 2). For example, control and the $42^\circ C$ for 45 min heat-shocked cells of strain 690 exhibited survival of 3.2 and 0.1%, respectively, after 4 days of exposure to $-18^\circ C$ (Fig. 3A), whereas greater survival, 38 and 17%, respectively, was noted after 8 days of exposure to $5^\circ C$ (Fig. 2A). Detrimental effects due to the formation of ice crystals and the concentration effect that occurs during exposure to $-18^\circ C$ (22) may account for the decreased survival of *V. parahaemolyticus* at $-18^\circ C$.

Effect of heat shock on the susceptibility of *V. parahaemolyticus* to ethanol. Effect of heat shock on the survival of *V. parahaemolyticus* when exposed to 8% ethanol is presented in Figure 4. Ethanol is a commonly used food preservative (26). High concentrations of alcohol solubilize lipids and denature proteins, leading to membrane destruction and injury or death of microorganisms (14). Survival of heat-shocked and control cells of all tested strains of *V. parahaemolyticus* decreased with extended exposure to 8% ethanol (Fig. 4). Heat shock treatments decreased the susceptibility of *V. parahaemolyticus* to ethanol, a result that is consistent with data obtained with *L. monocytogenes* (17, 21). During the entire exposure period, survival of the heat-shocked cells was in general higher than that of the respective control cells. This phenomenon was more marked with the chopping board isolate (Fig. 4B). In addition, as the duration of heat shock at $42^\circ C$ increased, percent survival of the ethanol-exposed cells increased. Among the various heat shock treatments tested, heat shock at $42^\circ C$ for 45 min was the most effective method of protect the cells from ethanol damage. Both control and heat-shocked cells of strain 690 (Fig. 4A) were less susceptible to ethanol than were cells of the chopping board isolate (Fig. 4B).

Effect of heat shock on the susceptibility of *V. parahaemolyticus* to $H_2O_2$. Hydrogen peroxide is a strong oxidizing agent used in food industry to inactivate microor-
organisms (2, 15). Susceptibility of microorganisms to H$_2$O$_2$ as influenced by environmental stresses has been reported (17, 18, 21). Lou and Yousef (21) found that resistance of L. monocytogenes to 0.1% H$_2$O$_2$ increased after adaptation to acid, ethanol, H$_2$O$_2$, heat, or NaCl. Lin (18) indicated that the influence of heat shocking on the survival of L. monocytogenes exposed to H$_2$O$_2$ varied with the conditions of the heat shock treatment and the bacterial strain. Lin (17) observed that cold shock treatment made V. parahaemolyticus more susceptible to H$_2$O$_2$. Koga and Takumi (16) observed that heat shock at 42°C for 30 min did not change the sensitivity of V. parahaemolyticus to 200 µM H$_2$O$_2$.

Heat shock treatment employed in the present study increased ($P < 0.05$) the resistance of V. parahaemolyticus to 20 ppm H$_2$O$_2$ (Fig. 5). Extending the duration of heat shocking at 42°C in general increased the resistance of the heat-shocked cells to H$_2$O$_2$ damage. The chopping board isolate exhibited greater resistance to H$_2$O$_2$ than did strain 690. The chopping board isolate also exhibited a higher survival percentage than did strain 690 subjected to a similar heat shock treatment and H$_2$O$_2$ challenge.

The increased resistance of the heat-shocked V. parahaemolyticus to H$_2$O$_2$ as observed in the present study is contradictory to the findings of Koga and Takumi (16). These discrepancies may be attributed to the differences in H$_2$O$_2$ challenge conditions and strains tested. Instead of examining the survival of the test organism in the presence of 20 ppm H$_2$O$_2$ as was performed in the present study, a stress condition with a high concentration of H$_2$O$_2$ (200 µM) was employed. This adverse condition may have been too extreme to illustrate the heat shock response of the test organism.

Based on data obtained from the present study, we conclude that susceptibility of V. parahaemolyticus to stresses may be altered by sublethal heat shock. In general, heat shock at 42°C increased the survival of the tested V. parahaemolyticus strains exposed to 47°C, H$_2$O$_2$ (20 ppm), and ethanol (8%) but decreased their survival at 5 and −18°C. In addition, heat shock treatments in general increased the amount of NaCl needed for recovery of V. parahaemolyticus. The extent of the heat shock response of V. parahaemolyticus varied with strains and the duration of heat shock treatment. Data obtained from the present study is of value because related information concerning V. parahaemolyticus is limited. These results indicate that the heat shock response of V. parahaemolyticus should be taken into ac-
Effect of heat shock on the survival of *Vibrio parahaemolyticus* exposed to 20 ppm H$_2$O$_2$. ○, cells without heat shock; ●, cells heat shocked at 42°C for 15 min; ▲, cells heat shocked at 42°C for 30 min; ■, cells heat shocked at 42°C for 45 min.

FIGURE 5. Effect of heat shock on the survival of *Vibrio parahaemolyticus* exposed to 20 ppm H$_2$O$_2$. ○, cells without heat shock; ●, cells heat shocked at 42°C for 15 min; ▲, cells heat shocked at 42°C for 30 min; ■, cells heat shocked at 42°C for 45 min.

ack count to improve accuracy of quality control and risk assessment measures employed by the food industry.

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REFERENCES