Effects of Heat Shock on the Thermotolerance, Protein Composition, and Toxin Production in *Vibrio parahaemolyticus*

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Running Title: Heat Shock of *V. parahaemolyticus*

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ABSTRACT

*Vibrio parahaemolyticus*, an important seafood associated enteropathogen, usually encounters different adverse conditions in its native or food processing environment and these stresses may affect its survival and thus change the risk of this pathogen in food hygiene. In this study, we investigated the thermotolerance of *V. parahaemolyticus* under sublethal heat shock and characterized this response by examining the change in protein profiles and toxin production. Logarithmically grown cells heat shocked at 42°C for 30 min were more resistant to thermal inactivation at 47°C than the unshocked cells. After heat shock of the 25°C culture, twenty four species of proteins were induced while thirteen species were inhibited as analyzed by polyacrylamide gel electrophoresis. DnaJ-, GroEL- and GroES-like proteins, with a molecular size of 47, 62, and 12 kDa, respectively, were detected by immunoblotting with antibodies raised against the *Escherichia coli* proteins. GroEL-like protein was produced in substantial amount during the heat shock of one to eight hours and presented in the periplasmic and extracellular fractions, while DnaJ- and GroES-like proteins mostly presented in the total cellular fraction. DnaK-like protein was not detected, nevertheless, the presence of dnaK-like genetic element was revealed by Southern blot. Production of thermostable direct hemolysin, the major virulence factor in this pathogen, was enhanced in the cells heat shocked at 42°C, but not at the 37°C.

Keywords: *Vibrio parahaemolyticus*, heat shock proteins, TDH, GroEL
INTRODUCTION

Vibrio parahaemolyticus, a halophilic Gram-negative enteropathogenic bacterium, is an important food-borne pathogen in Taiwan, Japan and other Asian Pacific countries. High incidence of this pathogen undoubtedly originates from the frequent consumption of marine foods in these countries. Clinical manifestations have included diarrhea, abdominal cramps, nausea, vomiting, headache, fever, and chills, with incubation periods ranging from 4 to 96 hours (5;14). Most clinical isolates are hemolytic on Wagatsuma agar (Kanagawa-positive, KP+) and produce a thermostable direct hemolysin (TDH) which is the major virulence factor in this pathogen (33).

Sublethal heat shock treatment of Salmonella typhimurium and Listeria monocytogenes enhanced its thermotolerance (2). Increase of heat resistance by short-term exposure to heat shock condition has also been demonstrated in other enteropathogenic bacteria, such as Yersinia enterocolitica (32) and Escherichia coli (21). In fact, sublethal exposure of bacteria to various stresses also enhances their survival under the corresponding adverse condition and also cross-protect against other stresses. Under these stresses, bacteria generally produce several stress proteins (also known as heat shock proteins, hsp), such as the hsp60 family (GroEL, GroES) and hsp70 family (DnaK, DnaJ, GrpE). These stress proteins act as chaperones in restoring normal function of inactivated proteins (1).

According to previous investigations, starvation of marine vibrios generally induce stress responses which cross-protect against other stresses (29). Related studies indicated that nutrition starvation in V. parahaemolyticus not only induced cross-protection against heat, osmotic, or hydrogen peroxide challenges (19), but also enhanced its survival at low temperature (13). Our previous study confirmed the adaptive acid tolerance response (ATR) in V.
parahaemolyticus (38). That study also demonstrated that acid adapted cells cross-protected against the challenge of low salinity and thermal inactivation. In natural habitat, *V. parahaemolyticus* and other marine vibrios typically live at 20-25°C during the summer and shift to an even lower temperature during the winter. However, in food processing environment, *V. parahaemolyticus* may be heat shocked as recontamination of cooked product before it cools with drip from raw seafood, or in food subjected to mild pasteurization process used to control other pathogens. Improper thermal stress may enhance its risk in food, however, the heat shock phenomenon has not been investigated in detail in *V. parahaemolyticus*. In this study, we examine the sub-lethal heat shock phenomenon in this pathogen and identify various proteins which are regulated during heat shock treatment by 1-dimensional (1-D) and two-dimensional (2-D) polyacrylamide gel electrophoresis (PAGE) and immunoblotting. Presence of groEL- and dnaK-like gene elements was also identified by polymerase chain reaction (PCR) and Southern blotting.

**MATERIALS AND METHODS**

**Bacterial strain and cultivation.** *V. parahaemolyticus* ST550, a serotype O4:K13 and KP+ clinical strain originated from Japan, was used in this study. This strain was stocked in culture broth with 10% glycerol at −80°C. It was cultured in Luria-Bertani Medium (LB, Difco Laboratories, Detroit, Mich.)-3% NaCl (pH 7.5) at 37°C. Bacteria growth was determined by measuring the absorbance at 600 nm and by plate count method on Luria-Bertani agar (LA)-3% NaCl. *V. cholerae* 569B and *E. coli* strain 10405 furnished by I.M. Lawrence, cultured in Luria-Bertani Agar Medium (Difco), were also used as reference in this study.

**Heat shock conditions.** For the determination of thermotolerance of the
heat shocked cells, fifty milliliter of LB-3% NaCl medium (pH 7.5), in 250 ml Erlenmeyer flask, was inoculated with 0.1 ml of overnight V. parahaemolyticus ST550 culture and incubated at 25°C with shaking at 160 rpm. At the mid-exponential phase (3 h), 50 ml culture in 250-ml Erlenmeyer flask was shifted to heat shock temperature at 42°C in a water bath for 30 min. The heat shocked culture was subjected to thermal inactivation at 47°C in another water bath. At different intervals, the survivors were counted after serial dilution in LB-3% NaCl, plated on LA-3% NaCl, and incubated at 37°C for 16 h.

**Examination of heat shock proteins by gel electrophoresis.** Labeling and analysis of stress proteins by gel electrophoresis in V. parahaemolyticus has been described previously (38). To analyze the protein profiles during heat shock period, fifty milliliters of modified M9 (MM9) medium supplemented with twenty amino acids, 0.2 mg/ml each, except methionine and cysteine (4), was inoculated with 0.1 ml of the inoculum and cultured at 25°C or 37°C for 3 h. The culture was then shifted to 42°C and the labeling mixture (Pro-mix with specific activity >1000 Ci/mmol and contained 70% L-[35S]-methionine and 30% L-[35S]-cysteine, Amersham International, Buckinghamshire, England, U.K.) was added at different intervals. While preparing for one-dimensional (1-D) analysis, 15 μCi/ml labeling mixture was added, and the culture was incubated for 7 min. While preparing for two-dimensional (2-D) analysis, 34 μCi/ml labeling mixture was added and incubated for 15 min.

After isotope labeling, the bacterial cultures were immediately stored in an ice bath to stop reactions; cells were collected by centrifugation at 3,000xg for 20 min. Next, samples subjected to 1-D electrophoresis were lysed in a buffer solution which contained the following contents: per 100 ml, 10 ml of TBS
buffer (0.41M Tris, 0.4 M boric acid, 1% sodium dodecyl sulfate [SDS], pH 8.64), 5 g of glucose, 185 mg of EDTA, 5 ml of 2-mercaptoethanol, 1.9 g of SDS, and 5 ml of glycerol (8). Samples subjected to 2-D electrophoresis were then lysed in another lysis solution containing, per 25 ml, 13.5g of urea, 0.5ml of Triton X-100, 0.5 ml of 2-mercaptoethanol, 0.5ml of Pharmalyte 3-10, and 35 mg of phenylmethylsulfonyl fluoride. A homogenous SDS 12.5% polyacrylamide gel (ExcelGel SDS, Pharmacia Biotech, Uppsala, Sweden) was then used in 1-D electrophoresis. In 2-D electrophoresis, Immobiline DryStrip (pH 4-7) and ExcelGel SDS 12.5% polyacrylamide gel were used. Next, the protein samples were resolved by 1-D and 2-D gel electrophoresis according to the procedures of the supplier (Pharmacia). Prestained SDS-PAGE broad range standards (Bio-Rad Laboratories., Hercules, Calif.) were used. Autoradiography was performed using BioMax MR Film (Eastman Kodak Co., Rochester, N.Y.) and the image was analyzed by Stratascan 7000 1-D and 2-D densitometry (Stratagene, La Jolla, Calif.).

**Preparation of different heat shocked cellular fractions.** Production of heat shock proteins in the total cellular, extracellular and periplasmic fractions of *V. parahaemolyticus* was examined by immunoblot procedures. The total cellular fraction was obtained by lysing the cell pellet in the same buffer as described in the 1-D PAGE procedure. The extracellular fraction was obtained by centrifugation of the culture and the supernatant was concentrated 100-fold by ultrafiltration (Amicon Corp., Lexington, Mass.). The periplasmic fraction was obtained by following the method of Callahan et al. (3). Briefly 40 ml of bacterial culture was pelleted by centrifugation, resuspended in 4 ml of 50 mM Tris.HCl buffer (pH 8.8) at 4 °C and to which 0.8 ml of chloroform was added and incubated at room temperature for 10 min. Eight milliliters of the Tris.HCl buffer was added to this mixture, mixed, centrifuged at 16,000xg for 20 min and the supernatant was collected. The contamination of cytoplasmic
proteins into the periplasmic and extracellular fractions was monitored by
determining the presence of cytoplasmic glucose-6-phosphate dehydrogenase
using a spectrophotometric enzyme assay kit (Sigma Co., St. Louis, MO) (34).

Protein concentration in the samples was determined by Coomassie blue dye binding method (7).

**Analysis of heat shock proteins by immunoblotting.** The heat shocked cellular fractions were analyzed by 1-D PAGE as described earlier. The proteins on the gel were electrically transferred to nitrocellulose membrane and hybridized separately with different antibodies raised against different heat shock proteins of *E. coli* following the methods of Koga et al. (18). Antibodies used were rabbit anti-GroEL and anti-GroES (Sigma), anti-DnaJ polyclonal, anti-DnaK monoclonal, and anti-GrpE polyclonal antibodies (StressGen Biotechnologies Corp., Victoria, B.C., Canada). For immunoblot analysis, prestained molecular weight markers were used, consisting of triosephosphate isomerase (26.6 kDa), lactic dehydrogenase (36.5 kDa), fumarase (48.5 kDa), pyruvate kinase (58 kDa), fructose-6-phosphate kinase (84 kDa), β-galactosidase (116 kDa), and α2-macroglobulin (180 kDa) (Sigma).

**Determination of TDH production.** Production of TDH in the supernatants of the heat shocked or control cultures of *V. parahaemolyticus* in LB-3% NaCl was determined using a reverse phase latex agglutination kit, following the manufacturer’s procedures (Denka Seiken, Tokyo, Japan).

**Preparation of genomic DNA.** Bacteria were cultured in tryptic soy broth, supplemented with 0 or 3% NaCl for *V. cholerae* or *V. parahaemolyticus*, respectively, 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. cholerae* and *V. parahaemolyticus* was prepared by following the method of Sambrook et al. (31), suspended in TE buffer (10 mM Tris hydrochloride buffer, 1 mM EDTA, pH 7.5), and was stored at -20 °C until required.

**Detection of groEL- and dnaK- like gene elements.** The groEL-like gene element was detected by PCR and followed by Southern blotting. Oligonucleotide primers (5’-GATCCATATGGCGCTAAAGACGTAAAA TTCGG-3’, 5’-CTAGGTCGACTTACATCATGCC GCCCATGCCAC-3’) were synthesized according to the groEL gene of *Salmonella typhi* (24). DNA amplification was carried out in Dynazyme buffer (Finnzymes Oy, Espoo, Finland) and 200 μM (each) dATP, dCTP, dGTP, and dTTP, 1 μM each primers, 2 U DyNAZyme II thermostable DNA polymerase (Finnzymes), and 100 ng template DNA in a final volume of 100 μl. Amplification was performed in a thermal cycler, Personal cycler 20 (Biometra biomedizinische analytik Gmbh, Gottingen, Germany). The reaction mixture was incubated in the thermal cycler at 95 °C for 5 min. Then, thermostable DNA polymerase was added and amplification was carried out for 30 cycles, each of which went as follows: 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1.5 min, and finally, an additional 72 °C for 10 min. The amplified mixtures were resolved in 1% agarose gel and transferred to nylon membrane (Boehringer Gmbh, Mannheim, Germany) and hybridized with groEL gene probe. The groEL gene probe was obtained by amplifying the *E. coli* genomic DNA with the specific primers, eluted, and labeled with non-radioactive digoxigenin (DIG) following the procedure provided by the supplier (Boehringer Mannheim). Hybridization analysis was performed according to the manufacturer’s recommendations at 55 °C (Boehringer Mannheim). In the PCR, lambda DNA/BstE digest (New England BioLabs, Inc., Beverly, Mass.) or the DIG-labeled lambda DNA/HindIII digest (Boehringer Mannheim) was
used as molecular weight marker.

For the detection of the dnaK-like gene element in *V. parahaemolyticus*, the genomic DNA was digested with *Hind*III, resolved by 1% agarose gel electrophoresis and Southern blotted with a specific gene probe. This dnaK gene probe was generated by PCR using template DNA of *V. cholerae* and specific oligonucleotide primers (5′-GTGTTGACCACCG ACTAG GATAACG -3′, 5′-TCTCCATCCCCACATTAGGGG -3′). These primers were synthesized according to the dnaK gene nucleotide sequence of *V. cholerae* from GeneBank database (Accession Y14237, submitted by Chakrabarti,S. and Chowdbury,R., 1997). Conditions used in PCR and Southern blot hybridization were similar to those for the detection of groEL-like element, except that the annealing temperature was 50°C.

**Statistics analysis.** The survival and the TDH production data were a mean average of at least triplicate determinations. The data were analyzed using an SPSSPC computer program with t-test and analysis of variance (ANOVA) procedure.

**RESULTS**

**Heat shock response and thermotolerance.** This study has successfully demonstrated the heat shock response at 42°C in *V. parahaemolyticus*. The heat shocked or unshocked control cells were subjected to thermal inactivation at 47°C. Notably, a significantly higher survival rate was observed in the heat shocked cells than that of unshocked cells. D_{47°C} values (the time to cause a 90% reduction in the count of viable cells at the designated temperature) determined for the heat shocked or the unshocked cells were 3.33 and 2.03 min, respectively (Fig. 1).
Production of heat shock proteins. This study further analyzed and compared the proteins profiles of the pathogen originally cultured at 25°C or 37°C and heat shocked at 42°C by 1-D and 2-D PAGE. When the culture was shifted from 25°C to 42°C, with a temperature gradient of 17°C, fourteen species of proteins were rapidly enhanced with 12.2, 47.6, 62.3, 65.6, 80.5, 88.2 and 96.9 kDa proteins in substantial quantity. Proteins 30.1, 36.9, 60.0, 82.1, 86.5 and 91.7 kDa were significantly inhibited (Fig. 2A). When the culture was shifted from 37°C to 42°C, with only a 5°C temperature gradient, fewer proteins were significantly enhanced or inhibited than the heat shock of culture originally grown at 25°C. Only 47.6, 62.3, and 65.6 kDa proteins were in high quantity (Fig. 2B). Also, a longer heat shock time was deemed necessary for inducing some of these proteins. For instance, proteins 62.3 and 65.6 kDa were highly induced only after incubating at 42°C for 10-20 min (Fig. 2B). Meanwhile, these two proteins were at high quantity at the first 10 min heat shock of the 25°C culture (Fig. 2A).

The 2-D PAGE revealed more detail protein patterns than the 1-D SDS PAGE. After heat shock of the 25°C culture at 42°C, twenty four species of proteins were induced while thirteen species became inhibited (Fig. 3A). Coordinates of these proteins also refer to those of the ATR proteins previously published (38). Proteins with coordinates 21 x 89, 37 x 14, 42 x 14, 48 x 116, 57 x 24, 59 x 24, 65 x 105, 73 x 98, 80 x 100, and 80 x 107 were significantly enhanced (Fig. 3B). When the V. parahaemolyticus was cultured at 37°C and subjected to heat shock at 42°C, fewer proteins were regulated as compared to the heat shock of the 25°C culture, with ten species of proteins enhanced and five species inhibited (data not shown). Nevertheless, all of the regulated proteins in the heat shock treatment of the 37°C culture were also found in the heat shock treatment of the 25°C culture. Notably, proteins
substantially enhanced in the heat shock of the 25°C culture were also markedly enhanced in the heat shock of 37°C culture (Fig. 3B).

Heat shock or other environmental stresses usually induces several universal stress proteins in both eukaryotic and prokaryotic organisms (27;39). In this study, these heat shock proteins in *V. parahaemolyticus* were identified by immunoblotting using antibodies raised against heat shock proteins of *E. coli*. GroEL-like (62 kDa), GroES-like (12 kDa) and DnaJ-like (47 kDa) proteins were identified while DnaK- and GrpE-like proteins were not observed in this pathogen (Fig. 4). GroES-like proteins were detected only in the total cellular and the periplasmic fractions of the heat shocked cells. DnaJ-like protein was detected only in the total cellular fraction of the heat shocked cells, but not existed in the periplasmic and extracellular fractions. Detectable amount of GroEL-like protein was found in the unstressed cells and it was produced in much higher quantity than GroES-like or DnaJ-like proteins, and could be detected in periplasmic, extracellular as well as total cellular fractions of the heat shocked cells (Fig. 4).

Quantity of GroEL-like protein in the total cellular fraction of *V. parahaemolyticus* was determined at different heat shocked intervals. Maximum amount of GroEL-like protein was found at the first hour and remained at high quantity during the heat shock for one to eight hours (Fig. 5).

**Presence of groEL- and dnaK- like gene elements.** By using specific primers for the *groEL* gene of *S. typhi*, a 1.7 kb fragment was amplified in *E. coli* and *V. parahaemolyticus* (Fig. 6A). The amplified fragments from both bacteria were highly homologous. When the *E. coli* amplified fragment was used as probe in Southern hybridization, strong hybridization signal was observed in *V. parahaemolyticus* amplified fragment (Fig. 6B) and vice versa.
The PCR primers designed for the *dnak* sequence of *V. cholerae* successfully and correctly amplified a 1,090 bp fragment in *V. cholerae*, however, the amplification failed in *V. parahaemolyticus*. When this *V. cholerae* amplified fragment was eluted and used as a hybridization probe in Southern blot analysis, positive bands were observed in both species, 3,100 bp fragment in *V. cholerae* and 2,500 bp fragment in *V. parahaemolyticus* (Fig. 6C).

**Production of TDH under heat shock.** Bacterial growth of the control at 25°C or of the heat shocked group at 37 or 42°C was not significantly different from each other (Fig. 7A). Titer of TDH in the culture at 25°C or in the heat shocked culture at 37°C for one to three hours were identical, while the titer was significantly enhanced when the culture was heat shocked at 42°C (Fig. 7B).

**DISCUSSION**

It has been demonstrated that heat shock treatment enhances therмотolerance in several food-borne pathogenic bacteria in both culture medium and food (2;32). This study has demonstrated the thermotolerance in the sublethally heat shocked *V. parahaemolyticus* and characterized the response in terms of protein production. In our previous report, mild acid treatment enhanced the survival of this pathogen in acid and also cross-protected against other stresses such as low salinity and heat shock (38). Cross-protection is a generally recognized phenomenon in the heat shocked or other stressed enteric pathogens (15). Although the survival of the heat shocked *V. parahaemolyticus* cells under other stresses has not been examined, cross-protection may probably exist and increase the risk of this pathogen in
Production of heat shock proteins has been analyzed in other Vibrio species. In V. cholerae, a total of 24 species of heat shock proteins were identified by 1-D PAGE (15), while in another study, sixteen species were indentified (30). Difference in the number of heat shock proteins in the same species may be due to use of different bacterial strains in their studies (15) or due to the use of different methods (15). In this study, 14 species of enhanced heat shock proteins were identified by 1-D PAGE in V. parahaemolyticus, while twenty four were observed by 2-D PAGE (Fig. 2, 3) which is a method of better resolution. Many of these proteins as reported in this study had molecular masses similar to those reported in V. cholerae and E. coli (Fig. 2) (15).

Several universal stress or heat shock proteins have been characterized (27;39). These heat shock proteins usually function in teams, for example, the hsp70 family (DnaK, DnaJ and GrpE) and the hsp60 family (GroEL and GroES) (26;37). In the hsp70 family, DnaK acts as a molecular chaperone which recognizes the folding polypeptide as an extended chain and cooperates with DnaJ in stabilizing protein (20). DnaJ and GrpE jointly stimulate ATPase activity of DnaK (22). These three heat shock proteins also involve in protein secretion (37). GroEL (hsp60) is also an molecular chaperone that facilitates folding of monomeric proteins and assembly of oligomeric protein complexes. Presence of GroES (hsp10) stimulates the function of GroEL (27). Although these heat shock proteins are essential for the survival of eukaryotic or prokaryotic organisms under stresses, not all the members of the same family could be identified in some bacteria by immunological method. In the heat shock of V. cholerae, proteins only homologues of GroEL and DnaK were detected with molecular masses of 60 and 68 kDa, respectively (15). In
another \textit{Vibrio} species, \textit{V. harveyi}, heat shock response was demonstrated by shifting the 30°C culture to 39°C, and DnaK (70 kDa), DnaJ (41 kDa), GroEL (58 kDa) and GroES (14.5 kDa) were identified (17). DnaK is generally detected in substantial amount in the heat shock of \textit{E. coli} or many other species (6; 21). Nevertheless, Dnak could not be detected in several bacteria by immunoblot method, such as Porphyromonas, Bacteroides, Prevotella, or Psuobacterium species (36). In this study, GroEL-like (62 kDa), DnaJ-like (47 kDa), and GroES-like proteins (12 kDa) were identified in \textit{V. parahaemolyticus} (Fig. 4). Koga et al. also previously reported the presence of GroEL-like proteins in \textit{V. parahaemolyticus} and six other \textit{Vibrio} species by the immunoblotting procedure (18). In this study, DnaK- and GrpE-like proteins were not detected in \textit{V. parahaemolyticus}. Due to the key role of DnaK in the function of hsp70 family and its production in some \textit{Vibrio} species (15; 17), it is reasonable to speculate that DnaK-like protein is present in \textit{V. parahaemolyticus}. In addition, the presence of dnaK-like genetic fragment was identified in this pathogen by Southern blot procedure (Fig. 6C). The reasons why some of the heat shock proteins were not detected may be due to the transient properties of some of these stress proteins including DnaK (10; 16). These proteins, if present, may also be immunologically not so close to the homologous proteins of \textit{E. coli} and therefore failed to cross-react with the antibodies raised against their counterparts in \textit{E. coli}. Further biochemical or molecular studies are needed to clarify the structure and function of these two major heat shock protein families.

GroEL is a universal stress protein and usually higher amount is detected in stressed cells than in unstressed cells (12). GroEL-like protein is of special interest in \textit{V. parahaemolyticus}. It was produced in substantial amount in this pathogen under heat shock and remained at high quantity through the heat shock period of one to eight hours and could be found in the periplasmic,
extracellular and total cellular fractions (Fig. 4, 5). Contamination of cytoplasmic proteins into the periplasmic or extracellular fractions was not likely the source of GroEL-like protein in these fractions, since such contamination was negligible as no housekeeping cytoplasmic enzyme (glucose-6-phosphate dehydrogenas) was detected in this process. Selective secretion may be involved in the relocation of this GroEL-like proteins in _V. parahaemolyticus_. The occurrence of the GroEL-like proteins in extracellular, periplasmic or outer-membrane fractions have been demonstrated in several bacteria, for example, _Hyalicobacter pylori_ (9), _Haemophilus ducreyi_ (11), and _Actinobacillus actinomycetemcomitans_ (12). Selective secretion of GroEL-like protein into extracellular fraction was also demonstrated in _H. pylori_ (35).

Few comparisons have been made on the protein productions in heat shock and ATR in enteropathogenic bacteria. In this study, we could compared the heat shock protein profile reported in this paper with the ATR proteins previously reported for this pathogen (38). Protein profiles in the heat shock response of _V. parahaemolyticus_ apparently differed from those of ATR. The 2-D PAGE revealed that these two stresses regulated seventy five species of proteins. More proteins were inhibited in ATR than heat shock response (38). Only a few of these regulated proteins appeared concurrently in both stresses. As analyzed by 1-D SDS PAGE, three species of proteins became substantially enhanced in both stresses, namely, 47.6, 88.2 and 96.9 kDa (38). In the 2-D PAGE analysis, only four species of proteins were enhanced in both stresses with coordinates namely, 21 x 89, 30 x 88, 48 x 116 and 80 x 107, with the last two proteins significantly enhanced in both stresses. GroEL-like (62 kDa) and DnaJ-like proteins (47 kDa) were detected in both ATR and heat shocked _V. parahaemolyticus_, however, no substantial amount of GroEL-like protein occurred in ATR, as demonstrated by the protein profile
analysis (38). These data show that the ATR and heat shock response in \textit{V. parahaemolyticus} regulates the production of many different proteins and GroEL-like proteins may have different role in these two stress processes.

Beside enhancing bacterial survival, heat shock response may also regulate the pathogenesis of pathogenic bacteria. Thus, presence of substantial amount of GroEL-like protein in \textit{V. parahaemolyticus} may play certain role in its pathogenesis. Role of GroEL-like protein in the pathogenesis has been demonstrated in some pathogenic bacteria. The GroEL protein of \textit{H. ducreyi} adheres to the cultured eukaryotic cells and inhibits the attachment of this bacteria to HEp-2 cells, indicating possible involvement of this heat shock protein in the pathogenesis of this bacterium (11). The GroEL protein isolated from \textit{A. actinomycetemcomitans} is strongly cytotoxic for the periodontal ligament epithelial cells at 4 μg/ml (12).

Environmental signals generally controls the expression of virulence factors in bacteria (25). In \textit{V. cholerae} the expression of virulence factors is regulated by the ToxR which mediates the effect of heat shock and other environmental stresses (28). The \textit{toxRS} operon also occurs in \textit{V. parahaemolyticus} that mediates environmentally induced regulation of the \textit{tdh} gene (23). In addition to the heat shock proteins discussed, we also confirmed that heat shock at 42°C significantly enhanced the production of TDH in \textit{V. parahaemolyticus}. However, the heat shock at 37°C, the body temperature of host, could not significantly enhanced the production of TDH in this pathogen (Fig. 7) suggesting that such heat shock response may not play a key role in the pathogen. Nevertheless, enhanced production of TDH and thermotolerance by the sublethal heat shock may still raise the risk of the pathogen in food.

In conclusion, the production and cellular location of heat shock proteins were identified in \textit{V. parahaemolyticus} by gel electrophoresis and
immunoblotting method. DnaJ-, GroEL- and GroES-like proteins were detected. GroEL-like protein presented in the periplasmic and extracellular fractions, while DnaJ- and GroES-like proteins mostly presented in the total cellular fraction. Production of thermostable direct hemolysin was enhanced in the cells heat shocked at 42°C, but not at the 37°C.
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Figure Legends

Fig. 1. Thermal inactivation of the V. parahaemolyticus ST550 heat shocked at 42 °C for 30 min. The exponential phase culture was shifted to 42 °C for 30 min and challenged by 47 °C treatment. ○, control, no heat shock; ●, heat shocked at 42 °C for 30 min. Vertical bars represent standard errors.

Fig. 2. Analysis of protein profile during heat shock response of the 25 °C (A) and the 37 °C (B) culture of V. parahaemolyticus ST550 by 1-D PAGE. The culture was shifted to 42 °C and labeling mix was added at different heat shock intervals and labeled for 10 min. Protein profiles were analyzed by 1-D PAGE and autoradiography. Lane 1, control group, 25 °C or 37 °C culture labeled for 10 min. Lane 2, labeling mix was added at heat shock time 0 min; lane 3, at time 10 min; lane 4, at time 20 min; lane 5, at time 30 min. Numbers at the right indicate the molecular weight markers in kDa. Circles and arrows designate the proteins that decreased and increased, respectively.

Fig. 3. Analysis of protein profile during heat shock response of the 25 °C culture of V. parahaemolyticus ST550 by 2-D PAGE. (A) control group, cell cultured at 25 °C; (B) Cells were heat shocked at 42 °C. Labeling mix was added to the control group or the heat shock culture and labeled for 15 min. Protein profiles were analyzed by 2-D PAGE and autoradiography. All proteins identified to be regulated by the heat shock treatment in panel B were compared with the proteins in panel A and assigned coordinates based on that comparison (38). Circles and arrows represent proteins that decreased and increased,
respectively.

Fig. 4. Detection of heat shock proteins in *V. parahaemolyticus* by immunoblotting procedures using antibodies raised against (A) GroEL, (B) GroES, and (C) DnaJ proteins of *E. coli*. *V. parahaemolyticus* cultured at 25°C was heat shocked at 42 °C for one hour, and fractionated into different fractions, resolved by 1-D PAGE and immunoblotted with different antibodies. Lane E, *E. coli* positive control, heat shocked at 42 °C for one hour; lane 1 and 2, periplasmic fraction; lane 3 and 4, total cellular fraction; lane 5 and 6, 100-fold concentrated extracellular fraction. The odd lanes represent the heat shocked groups and the even lanes represent the non-heat shocked control groups. Concentration of the protein fractions was 50 µg/ml for the periplasmic fractions, 70 µg/ml for the total cellular fractions, and 20 µg/ml for the concentrated extracellular fractions. *E. coli* positive control for GroES has been performed and photo not presented.

Fig. 5. Production of GroEL-like protein in the heat shock response of *V. parahaemolyticus*. The bacteria were cultured at 25°C and heat shocked at 42 °C for different intervals. Total cellular protein sample was resolved by 1-D PAGE and immunoblotted with anti-GroEL antibody. Concentration of the total cellular fractions were 70 µg/ml. Odd lanes represent heat shocked groups; lane 1, heat shocked for 1 h; lane 3, for 2 h; lane 5, for 4 h; lane 7, for 8 h. Even lanes represent control groups; lane 2, at 25 °C for 1 h; lane 4, for 2 h; lane 6, for 4 h; lane 8, for 8 h.

Fig. 6. Detection of the *groEL*-like and *dnaK*-like genetic element by PCR
and Southern blot. Panel A, PCR of the E. coli genomic DNA (lane 1 to 4) and V. parahaemolyticus genomic DNA (lane 5 to 8) by groEL-specific primers. M, molecular weight marker, lambda DNA/BstE digest. Panel B, the PCR amplified fragments hybridized with probe for groEL element. Lane 1-3, E. coli; lane 4-6, V. parahaemolyticus. Panel C, Southern blot of the HindIII digested V. cholerae genomic DNA (lane 1) and V. parahaemolyticus genomic DNA (lane 2 and 3) with a probe generated for the dnaK gene of V. cholerae. M, molecular weight marker, DIG-labeled lambda DNA/HindIII digest.

Fig. 7. Production of thermostable direct hemolysin in the heat shock response of V. parahaemolyticus. Panel A, bacterial growth; Panel B, TDH titer. V. parahaemolyticus was cultured at 25°C and heat shocked at 37°C (□) or 42°C (△) for different intervals, and the control continued to be cultured at 25°C (○). Vertical bars symbolize standard errors.
Fig. 2.
Fig. 4.
Fig. 5.
Fig. 6.