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Research Note

Protein Expression in *Vibrio parahaemolyticus* 690 Subjected to Sublethal Heat and Ethanol Shock Treatments

MING-LUN CHIANG,[1,2] WEI-LI HO,[3] ROCH-CHUI YU,[1] AND CHENG-CHUN CHOU[1]*

[1]*Graduate Institute of Food Science and Technology, National Taiwan University, Taipei, Taiwan;* [2]*Department of Tourism and Hospitality, Kainan University, Taoyuan, Taiwan; and* [3]*Department of Hotel & Restaurant Management, Ching Kuo Institute of Management and Health, Keelung, Taiwan*

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ABSTRACT

Cells of *Vibrio parahaemolyticus* 690 were subjected either to heat shock at 42°C for 45 min or to ethanol shock in the presence of 5% ethanol for 60 min. The protein profiles of the unstressed and stressed *V. parahaemolyticus* cells were compared. Additionally, the induction of DnaK- and GroEL-like proteins in the unstressed and stressed cells of *V. parahaemolyticus* was also examined. Analysis with one-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) indicated that three proteins with molecular masses of 93, 77, and 58 kDa were induced by both heat shock and ethanol shock. The protein patterns revealed by two-dimensional electrophoresis were more detailed than those revealed by one-dimensional SDS-PAGE. It was found that heat shock and ethanol shock affected the expression of a total of 28 proteins. Among them, four proteins with molecular masses of 94, 32.1, 26.7, and 25.7 kDa were enhanced by both heat shock and ethanol shock. Furthermore, immunoblot analysis showed the presence of a GroEL-like protein with a molecular mass of 61 kDa in the test organism, with the heat-shocked and ethanol-shocked cells producing a GroEL-like protein in a larger quantity than the unstressed cells. However, DnaK-like protein was not detectable in either the unstressed or the stressed cells.

In their environments, microorganisms may encounter many gradual or sudden environmental stresses, such as nutrition starvation, temperature changes, and other stressful conditions. They have to evolve complex regulatory networks for adaptation to repair damage and to survive during these stresses. One characteristic of such regulatory networks is the induction of stress proteins *(35).* Heat shock response has been suggested as one of these adaptive responses with its induction of heat shock proteins (Hsps) *(4, 8).* Hsps produced by various bacteria after exposure to sublethal heat treatments have been observed *(26, 28, 37).* However, ethanol shock has also been reported to induce stress proteins of a profile similar to that induced by heat shock *(6, 28).* Sahu et al. *(32)* found that four proteins were increased in *Vibrio cholerae* by heat shock (42°C, 10 min) and ethanol (6%, 20 min) treatments. Boutibonnes et al. *(6)* reported that ethanol and heat treatments elicited a similar response in *Enterococcus faecalis,* although there was a weaker induction of proteins with ethanol shock than with heat shock. Furthermore, other studies have shown that several stress proteins are induced in various bacteria including *Leuconostoc oeni* and *Bacillus* spp. after heat shock and ethanol stress *(3, 16, 28).*

DnaK, one of the well-characterized Hsps, was reported to act as a molecular chaperone that recognizes the folding polypeptide as an extended chain in stabilizing protein *(23)* and was also involved in protein secretion *(36).* GroEL, another well-characterized Hsp, is also a molecular chaperone that facilitates the folding of monomeric proteins and the assembly of oligomeric protein complex *(27).* Elevated levels of these proteins are believed to be correlated with an increased tolerance to thermal and other forms of stress *(1, 24).*

Vibrio parahaemolyticus is a foodborne pathogen that causes gastroenteritis associated with the consumption of contaminated seafood or foods cross-contaminated with this pathogen *(5).* It is one of the major causes of foodborne illness in Taiwan and Japan, where people consume raw and semiprocessed seafood as part of the daily diet *(2, 17).* We have previously noted that sublethal heat shock and ethanol shock treatments increased the survival of *V. parahaemolyticus* with exposure to 47°C, 20 ppm of H_2O_2, and 8% ethanol *(10, 11, 13).* Furthermore, we also found that heat shock and ethanol shock resulted in cell surface damage, change of fatty acid composition, and a reduced level of superoxide dismutase as well as catalase capacity in *V. parahaemolyticus (13, 14).* In addition, the stressed *V. parahaemolyticus* was observed to show an enhanced synthesis of thermostable direct hemolysin (*12*).

In this study, our objective was to examine and compare the protein profiles of *V. parahaemolyticus* cells subjected to heat shock and ethanol shock by one-dimensional (1-D) sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and 2-D electrophoresis. In addition, the induction of DnaK- and GroEL-like proteins in the un-

* Author for correspondence. Tel: 886-2-33664111; Fax: 886-2-2362-0849; E-mail: fstcchou@ntu.edu.tw.

stressed and stressed cells of *V. parahaemolyticus* was examined by immunoblotting.

MATERIALS AND METHODS

Microorganism strain. *V. parahaemolyticus* 690, a clinical strain capable of producing thermostable direct hemolysin was used as the test organism in the present study. It was obtained from Professor H. C. Wong (Department of Microbiology, Soochow University, Taipei, Taiwan).

Heat shock and ethanol shock treatments. Procedures described by Chang et al. *(10)* and Chiang et al. *(11),* respectively, were followed to prepare the heat-shocked and ethanol-shocked cells of *V. parahaemolyticus.* Briefly, cells of the test organism were suspended in 0.1 M phosphate buffer (pH 7) supplemented with 3% NaCl (phosphate-buffered saline [PBS]–3% NaCl), which had been preheated at 42° C in a shaking bath (model 903, Hotech Co., Taipei, Taiwan) at 150 rpm for 45 min to prepare the heat-shocked cells. To prepare the ethanol-shocked cells, the washed cells of *V. parahaemolyticus* were resuspended in 50 ml of PBS–3% NaCl containing 5% (vol/vol) ethanol and were held at room temperature for 60 min. The control cells were prepared by resuspending in PBS–3% NaCl at room temperature and were not subjected to stress conditions.

Preparation of cell extract. Cells of *V. parahaemolyticus* were collected by centrifugation and were suspended in 50 mM phosphate buffer (pH 7) containing 0.1 mM EDTA. The cell suspension was then sonicated (cooled on ice between treatments) with a sonicator (model 3000, Misonix, Farmingdale, N.Y.) and centrifuged at 10,000 × *g* for 20 min. The supernatant extracted from cells served as the cellular proteins. The protein content of the cell extract was determined using the colorimetric method of Bradford *(7),* with bovine serum albumin (Sigma, St. Louis, Mo.) as the standard.

1-D SDS-PAGE. 1-D SDS-PAGE was performed according to the method of Laemmli *(22)* using a separating gel of 10% acrylamide. The cell extract was mixed with an equal volume of sample buffer (250 mM Tris, 4 mM EDTA-2Na, 4% SDS, 10% β-mercaptoethanol), and a small amount of bromophenol blue was added, after which it was heated in a water bath at 100°C for 5 min. After cooling to room temperature, the same quantity of extracted protein (10 μg) was loaded onto a gel and separated with a minivertical electrophoresis unit (model SE260, Hoefer, San Francisco, Calif.) at 150 V for 1.5 h.

2-D electrophoresis. 2-D electrophoresis was performed essentially following the method described by Santoni et al. *(33).* Proteins were separated in the first dimension by isoelectric focusing (IEF) followed by SDS-PAGE in 12.5% polyacrylamide gels. Briefly, a cell extract was first precipitated with an acetone solution containing 10% trichloroacetic acid and 0.07% β-mercaptoethanol for 1 h at −20°C. The protein was centrifuged at 3,000 × *g* for 10 min and washed twice with cold acetone containing 0.07% β-mercaptoethanol, and then the protein pellet was solubilized in IEF sample buffer (6 M urea, 2 M thiourea, 0.5% Triton X-100, 100 mM dithiothreitol, 0.5% Bio-Rad IPG buffer, pH 4 to 7). Immobilized pH gradient strips (13 cm, pH 4 to 7) (Amersham Pharmacia Biotech, Uppsala, Sweden) were used to prepare the first dimension with an application of 250 μl of the protein sample (125 μg). IEF was carried out using Ettan IPGphor IEF system (Amersham Pharmacia Biotech) with a total of 18,000 V·h for 16 h. Before the second dimension, the strips were equilibrated with buffer (50 mM Tris, 6 M urea, 2% SDS, 30% glycerol, 0.01% bromophenol blue) containing 100 mM dithiothreitol for 15 min and then equilibrated with the same buffer containing 100 mM iodoacetamine for 15 min. The second dimension was performed in a vertical electrophoresis unit (model SE600, Hoefer) on SDS-PAGE gels (12.5%) of 13 by 13 cm at 200 V for 4 h.

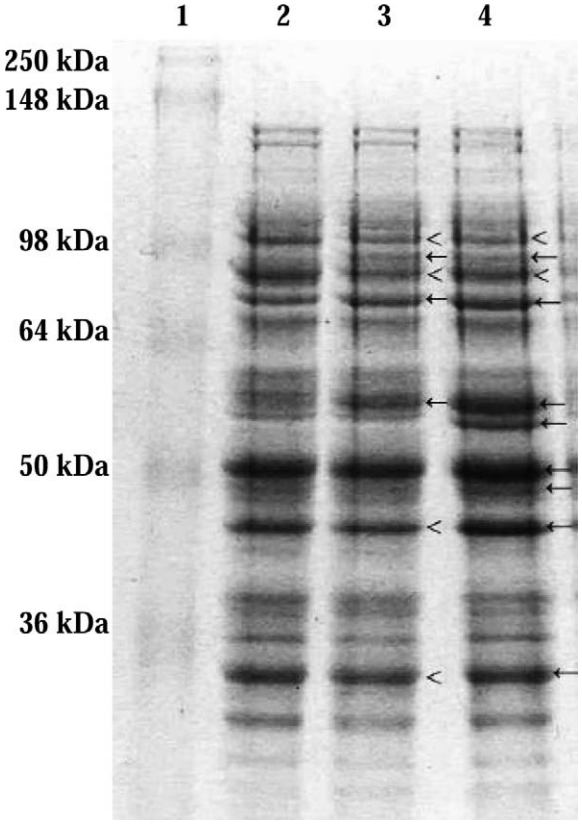

FIGURE 1. *One-dimensional SDS-PAGE (10%) pattern of cellular proteins from* V. parahaemolyticus. *Lane 1, marker; lane 2, control cells; lane 3, ethanol-shocked cells; lane 4, heat-shocked cells.* ←, *increased proteins;* <, *decreased proteins.*

Gel staining and image analysis. A prestained protein standard (Invitrogen, Carlsbad, Calif.) was used to run concurrently as a size marker. The gels were stained with colloidal Coomassie blue (2 mM Coomassie Brilliant R-250, 45% methanol, 10% acetic acid) for 1 h and destained with a solution containing 20% methanol and 10% acetic acid. The images of 1-D and 2-D gels were scanned and analyzed using EZ-1D (EZlab, Taipei, Taiwan) and ImageMaster 2D (Amersham Pharmacia Biotech) softwares, respectively. All gel bands or spots were detected and quantified automatically.

Immunoblotting. The immunoblotting experiment was performed according to the method described by Burnette *(9).* Protein gels were transferred electrophoretically onto a blotting membrane (0.45-μm pore size; Immobilon-P, Millipore, Billerica, Mass.). The blotting membranes were subsequently incubated with 6 M urea in PBS solution containing 0.05% Tween 20 (6 M urea–PBST) for 30 min at room temperature with gentle shaking. The membranes were then incubated with anti-DnaK (mouse monoclonal antibody) (Stressgen Bioreagents, Ann Arbor, Mich.) and anti-GroEL (rabbit polyclonal antibody) (Stressgen Bioreagents) diluted 1:1,000 in gelatin-NET (0.25% gelatin, 0.15 M NaCl, 5 mM EDTA, 0.05% Tween 20, 50 mM Tris, pH 8) for 1.5 h. The unbound antibodies were removed by washing three times with PBST, and the membranes were then incubated for 1 h with horse-

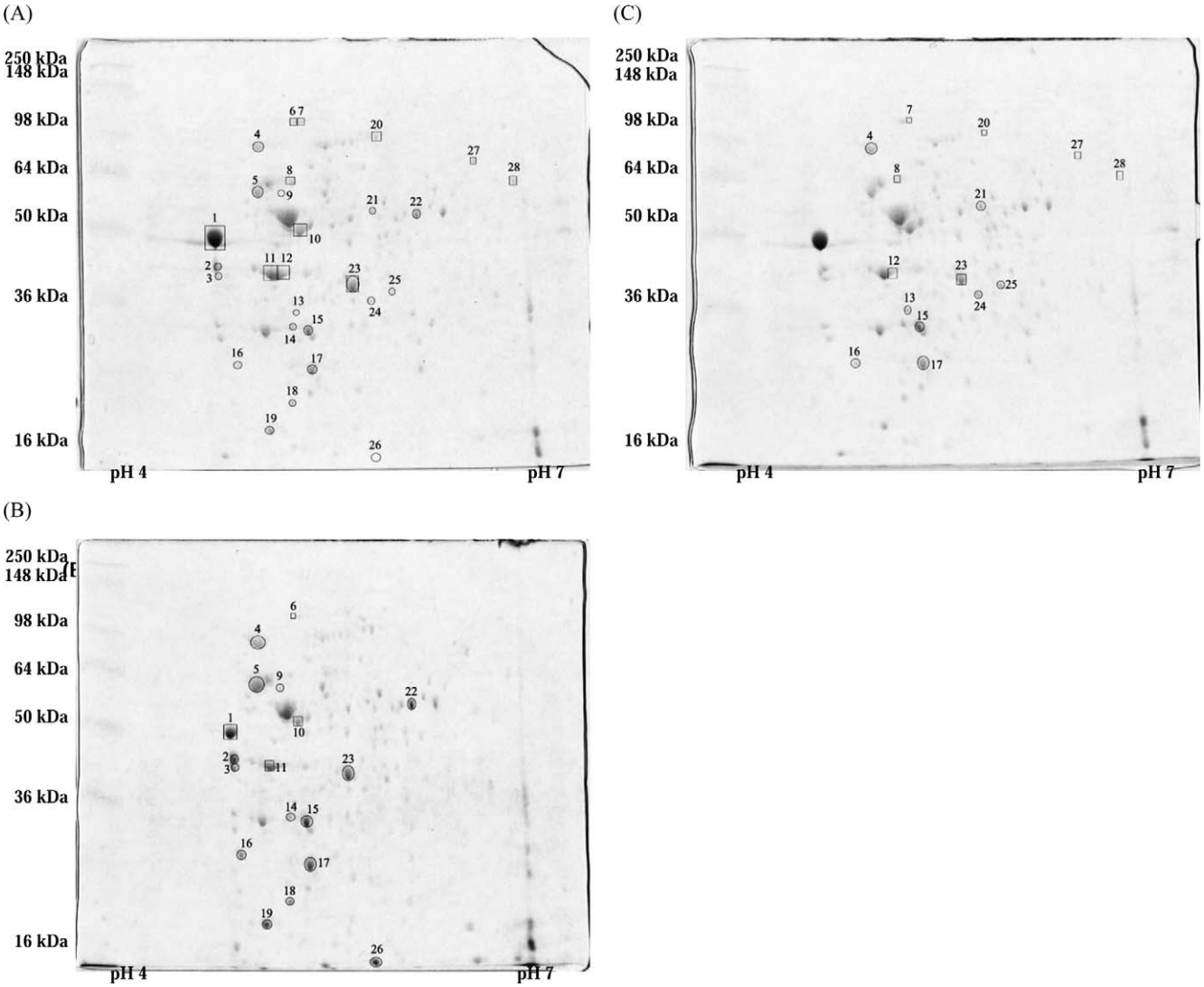

FIGURE. 2. *Protein profiles of* V. parahaemolyticus *by two-dimensional electrophoresis. (A) Control cells; (B) heat-shocked cells; (C) ethanol-shocked cells. First dimension: IEF with pH 4 to 7. Second dimension: 12.5% SDS-PAGE. Protein spots marked on the gels were relatively different in quantity (≥10%). Circles and squares represent proteins that, compared with control, were increased and decreased, respectively.*

radish peroxidase–conjugated anti-mouse immunoglobulin G diluted 1:10,000 and anti-rabbit immunoglobulin G diluted 1:5,000 in gelatin-NET, respectively. After washing the membranes three times with PBST, the immune complexes were detected with the DAB peroxidase substrate system (Amresco, Solon, Ohio). Positive homologous controls consisting of *E. coli* DnaK and GroEL proteins (Stressgen Bioreagents) were also used to ensure antibody detection.

RESULTS AND DISCUSSION

Heat-shocked and ethanol-shocked proteins analyzed with 1-D SDS-PAGE. With image analysis, eight protein bands with molecular masses of 93, 77, 58, 56, 51, 49, 46, and 30 kDa, showing an increased expression level of 1.10 to 3.38, were noted in the heat-shocked cells of *V. parahaemolyticus* (Fig. 1). These proteins were compared to the respective protein expression in the control, which was set as 1.0. In contrast, proteins with molecular masses of 100 and 86 kDa showed a reduced level of 0.67 and 0.89, respectively.

Image analysis also revealed that three species of proteins with molecular mass of 93, 77, and 58 kDa in cells of ethanol-shocked *V. parahaemolyticus* were enhanced 1.24- to 1.46-fold compared with those in the control cells. On the other hand, the expression of 100-, 86-, 46-, and 30-kDa proteins in the ethanol-shocked cells was reduced 0.50- to 0.83-fold.

Heat-shocked and ethanol-shocked proteins analyzed with 2-D electrophoresis. In the present study, proteins were separated by IEF in a linear pH 4 to 7 gradient in the first dimension, followed by SDS-PAGE in the second dimension. As shown in Figure 2, the protein patterns revealed by 2-D gel electrophoresis were more detailed than those revealed by 1-D SDS-PAGE (Fig. 1). Comparing those proteins detected before and after heat shock treatment, four categories of proteins, suggested by Phan-Thanh and Gormon *(29),* were applied to the analysis: (i) those which appeared only after heat shock, (ii) those that had an increased level after heat shock, (iii) those which disappeared after heat shock, and (iv) those that had a decreased

TABLE 1. *Expression of protein in* V. parahaemolyticus *after heat shock and ethanol shock analyzed by 2-D electrophoresis*

Protein expression	Spot no.	Molecular mass (kDa)	pI[a]	Expression level after[b]: No treatment (control)	Heat shock	Ethanol shock
Repressed	1	47.9	4.61	1	0.24	—[c]
	6	107.9	5.17	1	0.25	—
	7	111.0	5.21	1	—	0.56
	8	63.4	5.12	1	—	0.48
	10	51.5	5.19	1	0.57	—
	11	41.2	4.98	1	0.65	—
	12	41.4	5.00	1	—	0.34
	20	102.5	5.80	1	—	0.60
	23	40.2	5.61	1	—	0.64
	27	81.7	6.55	1	—	0.22
	28	65.8	6.87	1	—	0.47
Enhanced	2	43.7	4.63	1	2.48	—
	3	41.0	4.62	1	4.21	—
	4	94.0	4.88	1	2.30	1.28
	5	59.7	4.87	1	2.92	—
	9[d]	59.6	5.03	1	1.51	—
	13	35.6	5.18	1	—	1.11
	14	33.3	5.13	1	2.03	—
	15	32.1	5.27	1	3.08	1.94
	16	26.7	4.74	1	4.74	1.80
	17	25.7	5.30	1	2.85	1.51
	18	22.3	5.13	1	1.39	—
	19	19.7	4.94	1	2.87	—
	21	54.5	5.77	1	—	1.82
	22	52.9	6.12	1	1.94	—
	23	40.2	5.61	1	2.20	—
	24	36.4	5.75	1	—	1.63
	25	38.8	5.93	1	—	1.33
	26	16.9	5.84	1	5.59	—

[a] Based on migration in a 2-D gel.

[b] The level of each protein spot or background in control was set as 1, and the level of respective protein in the stressed cells was expressed as that relative to control.

[c] —, no induction or repressed protein showed a level of >0.9, while enhanced protein exhibited a level of <1.1 relative to the respective protein in the control cells.

[d] Not detected in the unstressed cells.

level after heat shock. The proteins included in the first two categories were called Hsps. In this study, one protein with a molecular mass of 59.6 kDa and a pI of 5.03 (spot no. 9) consistent with category (i) above was detected. Thirteen other species of heat shock proteins, consistent with category (ii), with molecular masses ranging from 16.9 to 94.0 kDa were observed (Fig. 2B). The increase in the level of protein expression ranged from 1.39 to 5.59 as shown in Table 1. The most strongly induced heat shock protein (spot no. 26) had a molecular mass of 16.9 kDa and a pI of 5.84. On the other hand, the level of some proteins appeared to decrease in response to heat shock. Four species of proteins (spot no. 1, 6, 10, and 11; Fig. 2B) had a reduced protein expression level ranging from 0.24 to 0.65 compared with those without heat shock. Worthy of mention here is that the molecular mass of proteins with spot no. 4, 5, 9, 15, and 22 came close to those detected with 1-D SDS-PAGE (Fig. 1). However, data obtained with 2-D gel electrophoresis more clearly demonstrated the enhanced expression level of these proteins (Fig. 2 and Table 1).

As shown in Figure 2C and Table 1, after ethanol shock in the presence of 5% ethanol, the expression of eight proteins with spot no. 4, 13, 15, 16, 17, 21, 24, and 25 were significantly enhanced, 1.11- to 1.94-fold, compared with that of the control while the expression of seven species of proteins (spot no. 7, 8, 12, 20, 23, 27, and 28; Fig. 2C) was reduced 0.22- to 0.64-fold in cells of the ethanol-shocked *V. parahaemolyticus* (Table 1). Using 2-D electrophoresis, Rince et al. *(31)* reported that ethanol stress altered the expression of 20 proteins in cells of *E. faecalis.*

Analysis with 2-D gel electrophoresis revealed that both heat shock and ethanol shock affected the expression of a total of 28 proteins in *V. parahaemolyticus.* Among them, the expression of four proteins (spot no. 4, 15, 16, and 17) were enhanced by both heat shock and ethanol shock. Other proteins either induced or inhibited in the heat-shocked cells were not identical to those observed in the ethanol-shocked cells.

Characterization of DnaK- and GroEL-like proteins. Enhanced expression of DnaK- and GroEL-like pro-

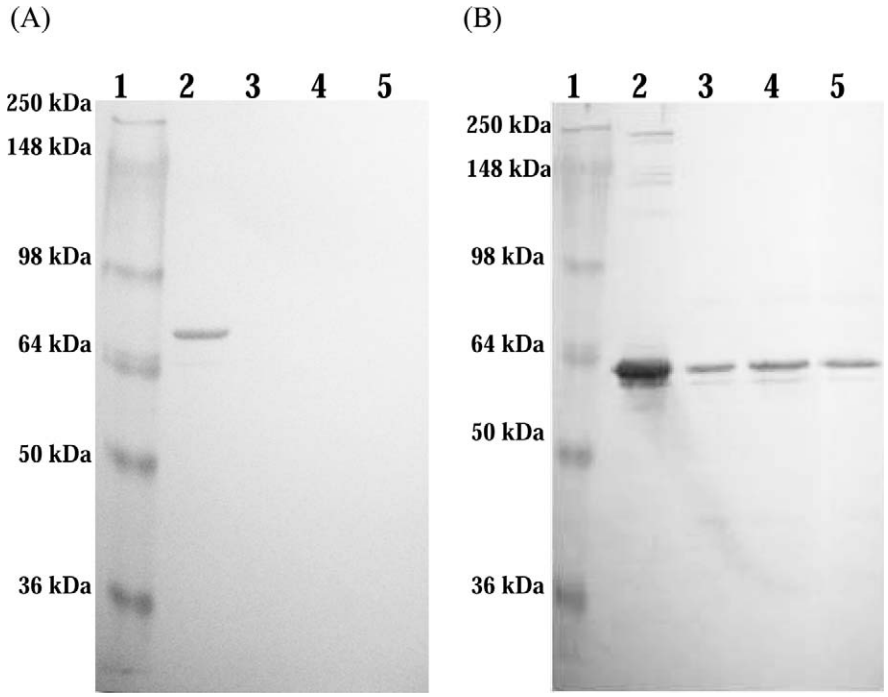

FIGURE. 3. *Immunoblotting analysis of the homologues of* E. coli *DnaK (A) and GroEL (B) proteins in* V. parahaemolyticus. *Lane 1, marker; lane 2,* E. coli*–positive control; lane 3, control cells; lane 4, heat-shocked cells; lane 5, ethanol-shocked cells.*

teins in various microbial cells subjected to heat shock and other stresses has been noted *(28, 30, 37).* In the present study, two proteins with molecular masses of 77 and 58 kDa (Fig. 1), which showed enhancement in both heat-shocked and ethanol-shocked cells of *V. parahaemolyticus,* with molecular masses similar to those of *E. coli* DnaK and GroEL proteins, respectively, seemed to be their homologues. Therefore, immunoblotting using antibodies prepared against *E. coli* DnaK and GroEL proteins was performed to identify whether or not the presence of these proteins was native to the unstressed cells or was induced in cells subjected to heat shock or ethanol shock treatment. Despite the detection of DnaK-like protein in the heat-shocked and other stressed cells of various microorganisms *(15, 18),* DnaK-like protein was not detected in either the unstressed or stressed cells of *V. parahaemolyticus* (Fig. 3A). The failure to find DnaK may be due to the transient properties of this stress protein *(19).* A similar failure to detect DnaK by the immunoblotting method in several bacteria including *Porphyromonas, Bacteroides, Prevotella,* and *Vibrio* species has previously been reported *(34, 37).* However, the 61-kDa protein was found to react strongly with *E. coli* GroEL antibody (Fig. 3B). The GroEL antiserum also immunoprecipitated a slightly faster migrating protein species in addition to the 61-kDa protein. A similar observation was reported by Koga et al. *(21).* They suggested that this protein might represent a degradation product or a modified form of the GroEL protein. The presence of bands with reasonable intensity corresponding to the protein in the control lane indicated that this protein is synthesized constitutively in *V. parahaemolyticus* 690. This finding is consistent with that reported by Wong et al. *(37).* Furthermore, it was also noted that the heat-shocked and ethanol-shocked cells enhanced the expression of GroEL-like protein 1.64- and 1.44-fold, respectively, when compared with that of the unstressed cells of *V. parahaemolyticus.*

When immunoblotting analysis was performed with 2-D gel electrophoresis, GroEL-like protein was also detected, while DnaK-like protein was not detectable in the stressed and unstressed cells of *V. parahaemolyticus* 690 (data not shown). The enhanced GroEL-like protein production in the heat-shocked and ethanol-shocked cells of the test organism was about 2.90- and 1.57-fold, respectively, greater than that in the unstressed cells.

The GroEL-like protein in *V. parahaemolyticus* 690 showed an apparent molecular mass of 61 kDa with a pI of 4.8 to 4.9, which is neither the same as that (62 kDa) for *V. parahaemolyticus* ST 550 *(37)* nor exactly identical to the reported value of 62.88 kDa for the *E. coli* GroEL based on the sequence data *(25).* The discrepancy may be due to differences between the true molecular mass based on sequence data and the apparent one as measured by SDS-PAGE *(18).* Similar to that observed in the present study, the enhanced expression of GroEL-like protein with a molecular mass of 58 kDa in the heat-shocked cells of *Vibrio* species was also noted by Klein et al. *(20)* and Koga et al. *(21).* Hsp has been reported to enhance the survival of bacteria under the corresponding adverse condition and to also provide cross-protection against other types of stress *(1, 24).* Thus, the induction of GroEL-like protein detected in the present study may lead to the enhanced survival of the heat-shocked and ethanol-shocked *V. parahaemolyticus* 690 with exposure to 47°C, 20 ppm of H_2O_2, and 8% ethanol *(10, 11, 13).*

This study demonstrates that heat shock and ethanol shock treatments affect the expression of protein in cells of *V. parahaemolyticus* 690. Based on molecular mass and isoelectric point, it was found that their effects on protein expression were not exactly similar. The protein induction

by heat shock was, generally, more intense than that by ethanol shock. Heat shock and ethanol shock were both found to induce the synthesis of Hsps. At least one GroEL-like protein, with an apparent molecular mass of ca. 61 kDa, was detected. The induction of this Hsp might relate to the enhanced tolerance of the heat-shocked and ethanol-shocked *V. parahaemolyticus* to some of the subsequent environmental stresses observed *(10, 11, 13).* On the other hand, identification of the stress proteins induced by heat shock and ethanol shock by matrix-assisted laser desorption ionization–time of flight mass spectrometry merits further investigation, so that these stress proteins can be characterized.

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