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Effect of Acid Adaptation on the Survival of Three *Vibrio parahaemolyticus* Strains under Simulated Gastric Condition and their Protein Expression Profiles

Ming-Lun Chiang, Hsi-Chia Chen, Chieh Wu, Yu-Ting Tseng, Ming-Ju Chen

***Abstract*—**In this study, three strains of *Vibrio parahaemolyticus* (690, BCRC 13023 and BCRC 13025) were subjected to acid adaptation at pH 5.5 for 90 min. The survival of acid-adapted and non-adapted *V. parahaemolyticus* strains under simulated gastric condition and their protein expression profiles were investigated. Results showed that acid adaptation increased the survival of the test *V. parahaemolyticus* strains after exposure to simulated gastric juice (pH 3). Additionally, acid adaptation also affected the protein expression in these *V. parahaemolyticus* strains. Nine proteins, identified as atpA, atpB, DnaK, GroEL, OmpU, enolase, fructose-bisphosphate aldolase, phosphoglycerate kinase and triosephosphate isomerase, were induced by acid adaptation in two or three of the test strains. These acid-adaptive proteins may play important regulatory roles in the acid tolerance response (ATR) of *V. parahaemolyticus.*

***Keywords*—**Acid adaptation, protein expression, simulated gastric juice, *Vibrio parahaemolyticus*

I. Introduction

ACIDIFICATION is a common method used in the food processing to inhibit the growth of spoilage and pathogenic microorganisms [1], [2]. Gastric acid is also the first defensive barrier to destroy foodborne pathogens in stomach [3], [4]. However, bacterial cells may survive and become adapted to these and other acid environments such as acid spraying and fermentation [5]-[7]. Acid tolerance response (ATR) has been reported in several foodborne pathogens, including *Escherichia coli* O157:H7, *Salmonella typimurium*, *Bacillus cereus* and *Listeria monocytogenes* [5], [8]-[10]. Bacteria could enhance the resistance to severe acidic conditions when they have previously exposed to a mild acidic treatment [11], [12]. The synthesis of some specific acid-adaptive proteins in acid-adapted cells could prevent cellular damage and promote bacterial survival in following extreme acid stress [2], [13]-[15].This phenomenon should be considered for microbial food safety risk assessments.

M. L. Chiang is with the Department of Tourism and Hospitality, Kainan University, Taoyuan, Taiwan (phone: 886-3-3412500 ext.6133; e-mail: mlchiang@mail.knu.edu.tw).

H. C. Chen is with the Food and Drug Administration, Department of Health, Executive Yuan, Taipei, Taiwan (e-mail: hcchen@fda.gov.tw).

C. Wu is with the Department of Animal Science and Technology, National Taiwan University, Taipei, Taiwan (e-mail: r00626023@ntu.edu.tw).

Y. T. Tseng is with the Department of Animal Science and Technology, National Taiwan University, Taipei, Taiwan (e-mail: r98626020@ntu.edu.tw).

M. J. Chen is with the Department of Animal Science and Technology, National Taiwan University, Taipei, Taiwan (e-mail: cmj@ntu.edu.tw).

V. parahaemolyticus is a moderately halophilic bacterium that is native to the marine environments throughout the world [16]. It is generally isolated from manifold seafoods and can cause acute gastroenteritis associated with consumption of contaminated seafood [17]-[19]. This pathogen has been recognized as a leading cause of foodborne illness in some Asian coastal regions, where people frequently eat raw or minimally processed seafood [20]-[23]. The acid tolerance of *V. parahaemolyticus* is an important factor for its ability to withstand acidic challenges in the food systems. The objective of this study was to examine the effect of acid adaptation on the survival of *V. parahaemolyticus* under simulated gastric condition. Additionally, the protein expression of acid-adapted *V. parahaemolyticus* was also investigated.

II. Materials And Methods

A. Microorganisms

Three *V. parahaemolyticus* strains were used as the test organisms. *V. parahaemolyticus* 690, originally isolated from clinical samples of gastroenteritis patients, was obtained from Professor H. C. Wong, Department of Microbiology, Soochow University (Taipei, Taiwan). *V. parahaemolyticus* BCRC 13023 and BCRC 13025, involved in food poisoning outbreaks in Taiwan, were purchased from Bioresource Collection and Research Center (BCRC), Food Industry Research and Development Institute (FIRDI) (Hsinchu, Taiwan). The test organisms were activated by two transfers in tryptic soy broth supplemented with 3% NaCl (TSB-3% NaCl) at 37˚C for 6 h, these activated cultures served as the inocula of experiment.

B. Acid Adaptation Treatment

To perform the acid adaptation treatment of *V. parahaemolyticus*, 50 mL of the activated culture was centrifuged (3,000 ×g, 10 min) and washed twice with phosphate-buffered saline containing 3% NaCl (PBS-3% NaCl, pH 7). The acid-adapted cells were prepared by suspending in 50 mL of acidified TSB-3% NaCl (pH 5.5) and held at 37˚C for 90 min. The non-adapted cells were prepared by suspending in TSB-3% NaCl without acidification and incubation.

C. Determination of Survival in Simulated Gastric Juice

To determine the survival of *V. parahaemolyticus* in simulated gastric juice (0.01% pepsin, 0.35% mucin, 0.85 g NaCl, pH 3), 1 mL of the acid-adapted or non-adapted cells was inoculated into 50 mL of simulated gastric juice at an initial population of ca. 10^7 CFU/mL. The viability of the test

organism was determined every 15 min during 1-h incubation at 37˚C. Samples of *V. parahaemolyticus* were serially diluted in PBS-3% NaCl and pour plated with tryptic soy agar supplemented with 3% NaCl (TSA-3% NaCl). The viable colonies were enumerated after 18 h of incubation at 37˚C.

D.Protein Extraction

V. parahaemolyticus cells were centrifuged at 3,000 × *g* for 10 min and suspended in 50 mM phosphate buffer (pH 7) containing 0.1 mM EDTA. The cell suspension was then broken with a sonicator and centrifuged at 10,000 × *g* for 20 min. The supernatant was precipitated with acetone solution containing 10% trichloroacetic acid and 0.07% β-mercaptoethanol for 1 h at -20˚C. The protein pellet was collected and washed twice with acetone containing 0.07% β-mercaptoethanol. The extracted protein was then solubilized in sample buffer (2 M thiourea, 6 M urea, 100 mM dithiothreitol, 0.5% Triton X-100, 0.5% Bio-Rad IPG buffer, pH 4 to 7).

E. Two-Dimensional Electrophoresis and Protein Identification

Protein samples were separated in the first dimension by isoelectric focusing (IEF) followed by SDS-PAGE in 12.5% polyacrylamide gels. Immobilized pH gradient strips (13 cm, pH 4 to 7) were used with an application of 125 μg of the protein sample. The strips were equilibrated with buffer (0.01% bromophenol blue, 30% glycerol, 2% SDS, 50 mM Tris, 6 M urea) containing 100 mM dithiothreitol for 15 min and then equilibrated with above buffer containing 100 mM iodoacetamine for 15 min. IEF was performed using Ettan IPGphor IEF system with a total of 18,000 V·h for 16 h. The second dimension was carried out in a vertical electrophoresis unit on SDS-PAGE gels (12.5%) of 13 by 13 cm at 200 V for 4 h. The gels were stained with colloidal Coomassie blue (2 mM Coomassie Brilliant R-250, 10% acetic acid, 45% methanol) for 1 h and then destained with a solution containing 20% methanol and 10% acetic acid. The images of gels were scanned and analyzed using ImageMaster 2D software. The protein identification was performed by Mass Solutions Technology Co. Ltd. (Taipei, Taiwan) as follows. Protein spots were excised and subjected to tryptic digestion. The samples of tryptic peptides were collected for LC/MS/MS analysis. Proteins were then identified by using MASCOT search engine v2.3 (Matrix Science, UK) and database was set to be SwissProt and NCBInr.

III. Experimental Results

A. Effect of Acid Adaptation on the Survival of V. parahaemolyticus under Simulated Gastric Condition

The survival of three *V. parahaemolyticus* strains under simulated gastric condition was presented in Fig. 1. The survival of non-adapted and acid-adapted *V. parahaemolyticus* strains decreased with the exposure time extended. After 60 min of exposure in simulated gastric juice (pH 3), the survival values of non-adapted strains 690, BCRC 13023 and BCRC 13025 were reduced to 12.7, 21.8 and 32.4%, respectively. *V. parahaemolyticus* BCRC 13025 exhibited the highest tolerance to simulated gastric juice, followed by BCRC 13025 and 690. However, after acid adaptation, the survival values of acid-adapted strains 690, BCRC 13023 and BCRC 13025 were 29.8, 33.6 and 46.8%, respectively. It was found that acid adaptation significantly ($p<0.05$) increased the tolerance of the test *V. parahaemolyticus* strains after exposure to simulated gastric juice (Fig. 1 a-c). Additionally, the extent of increased tolerance to simulated gastric condition varied with bacterial strains. These phenomena may be involved in the induction of acid-adaptive proteins in *V. parahaemolyticus* during acid adaptation.

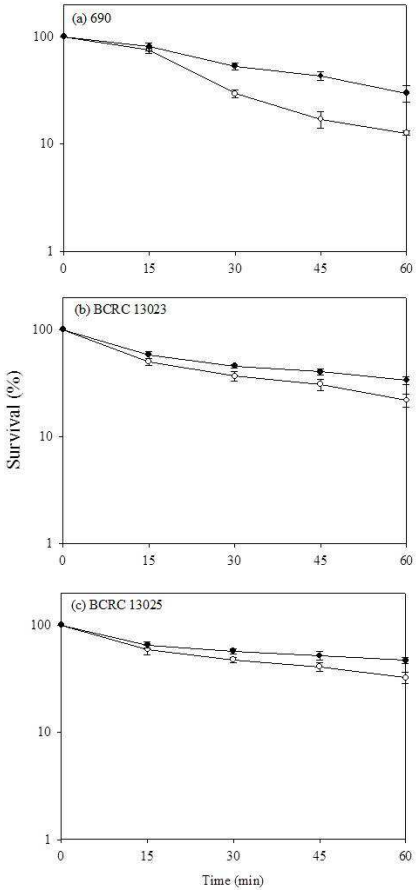

Fig. 1 Effect of acid adaptation on the survival of *V. parahaemolyticus* in simulated gastric juice (pH 3). (a) 690; (b) BCRC 13023; (c) BCRC 13025. ○, Non-adapted cells; ●, acid-adapted cells. The initial populations of non-adapted and acid-adapted cells were ca. 10^7CFU/mL. Surviving percentage was obtained by dividing the survival population by the initial population which corresponds to 100%. Data were expressed as mean ± standard deviations from the three separate experiments

B. Effect of Acid Adaptation on the Protein Expression of V. parahaemolyticus

The protein patterns of three *V. parahaemolyticus* strains revealed by two-dimensional electrophoresis were shown in Fig. 2. Results showed that 11 protein spots exhibited higher abundance levels and 10 protein spots had lower abundance levels in acid-adapted cells of *V. parahaemolyticus* 690 when

compared with non-adapted cells. (Fig. 2 a and b). After acid adaptation, the intensities of 16 spots were enhanced and the intensities of 4 spots were reduced in acid-adapted cells of *V. parahaemolyticus* BCRC 13023 (Fig. 2 c and d). Additionally, acid adaptation also increased the quantity of 18 proteins while decreased the quantity of 3 proteins in cells of *V. parahaemolyticus* BCRC 13025 (Fig. 2 e and f). Acid adaptation affected the expression of a total of 26 proteins in the test strains of *V. parahaemolyticus*. Among these proteins, it was worthy noted that the synthesis of 9 proteins (spot no. 1, 7, 10, 13, 14, 16, 17, 18 and 21) were jointly induced by acid adaptation in two or three of the test strains (TABLE I). One protein was identified as outer membrane protein U (OmpU). Two proteins belonged to the chaperone proteins (DnaK and GroEL). Two proteins (atpA and atpB) involved in energy metabolism. Four proteins, including triosephosphate isomerase (TPI), fructose-bisphosphate aldolase (FBA), phosphoglycerate kinase (PGK) and enolase (ENO), involved in carbohydrate metabolism (TABLE II). The induction of these acid-adaptive proteins may play important regulatory roles in acid tolerance response that allow *V. parahaemolyticus* to become more tolerant to external acid stress.

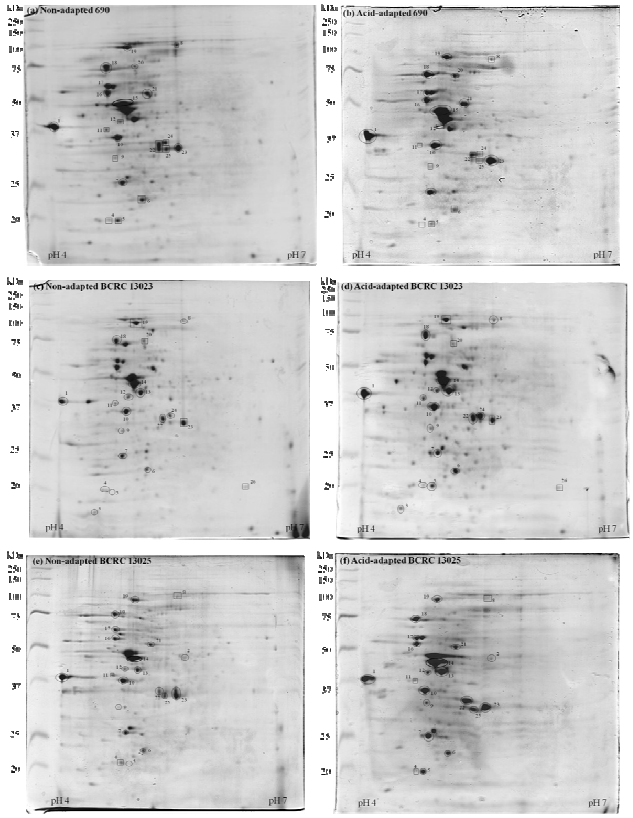

Fig. 2 Protein profiles of V. parahaemolyticus by two-dimensional electrophoresis. (a) Non-adapted 690; (b) acid-adapted 690; (c) non-adapted BCRC 13023; (d) acid-adapted BCRC 13023; (e) non-adapted BCRC 13025; (f) acid-adapted BCRC 13025. First dimension: IEF with pH 4 to 7. Second dimension: 12.5% SDS-PAGE. Protein spots marked on the gels were relatively different in quantity (>10%). Circles and squares on the gels represent increased and decreased proteins, respectively, compared with non-adapted groups.

TABLE I
PROTEIN EXPRESSION BETWEEN ACID-ADAPTED AND NON-ADAPTED *V. PARAHAEMOLYTICUS* STRAINS

Spot no.	Expression factor[a]		
	690	BCRC 13023	BCRC 13025
1	1.21±0.44 (+ [b])	1.25±0.05 (+)	1.15±0.09 (+)
2	#	#[d]	1.12±0.03 (+)
3	#	1.96±0.14 (+)	#
4	0.78±0.22 (- [c])	1.22±0.15 (+)	0.27±0.02 (-)
5	0.89±0.29 (-)	3.57±0.03 (+)	6.34±0.22 (+)
6	0.88±0.10 (-)	1.18±0.13 (+)	1.46±0.23 (+)
7	1.17±0.11 (+)	1.59±0.02 (+)	2.76±0.56 (+)
8	0.78±0.05 (-)	2.63±0.05 (+)	0.55±0.15 (-)
9	0.87±0.09 (-)	1.39±0.06 (+)	1.26±0.11 (+)
10	1.14±0.23 (+)	1.30±0.27 (+)	1.75±0.21 (+)
11	0.55±0.12 (-)	1.16±0.24 (+)	0.67±0.23 (-)
12	0.39±0.16 (-)	1.72±0.16 (+)	1.82±0.08 (+)
13	#	1.67±0.15 (+)	2.52±0.22 (+)
14	#	1.15±0.21 (+)	1.82±0.12 (+)
15	2.14±0.15 (+)	#	#
16	2.58±0.88 (+)	#	2.05±0.25 (+)
17	1.12±0.18 (+)	#	1.42±0.32 (+)
18	3.31±0.15 (+)	1.47±0.03 (+)	1.75±0.15 (+)
19	1.35±0.52 (+)	0.83±0.02 (-)	1.18±0.09 (+)
20	1.61±0.45 (+)	0.57±0.22 (-)	#
21	1.46±0.33 (+)	#	1.54±0.27 (+)
22	0.88±0.10 (-)	1.37±0.29 (+)	2.73±0.88 (+)
23	1.15±0.55 (+)	0.87±0.16 (-)	1.72±0.32 (+)
24	0.82±0.27 (-)	2.23±0.37 (+)	#
25	0.66±0.27 (-)	#	2.37±0.31 (+)
26	#	0.89±0.09 (-)	#

[a]Expression factor is the ratio of expression level of each protein in acid-adapted cells to expression level of respective protein in non-adapted cells. Values were expressed as mean ± standard deviations from three independent trials.

TABLE II
CHARACTERISTICS OF NINE ACID-INDUCED PROTEINS IN *V. PARAHAEMOLYTICUS*

Spot no.[a]	MW[b] (kDa)	pI[c]	Homologous protein
1	36.26	4.25	Outer membrane protein U (OmpU)
7	27.03	4.72	Triosephosphate isomerase
10	38.97	4.70	Fructose-bisphosphate aldolase
13	41.05	4.90	Phosphoglycerate kinase
14	45.59	4.84	Enolase
16	50.84	4.69	ATP synthase subunit beta (atpB)
17	57.59	4.68	60kDa chaperonin protein (GroEL)
18	69.07	4.69	Chaperone protein DnaK
21	56.72	5.10	ATP synthase subunit alpha (atpA)

[a]Spot numbers refer to the 2-D gels.
[b]Theoretical values of molecular weight.
[c]Theoretical values of isoelectric point.

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