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Growth Behaviors, Thermostable Direct Hemolysin Secretion and Fatty Acid Profiles of Acid-adapted and Non-adapted *Vibrio parahaemolyticus*

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***Abstract*—**Three strains of *Vibrio parahaemolyticus* (690, BCRC 13023 and BCRC 13025) implicated in food poisoning outbreaks in Taiwan were subjected to acid adaptation at pH 5.5 for 90 min. The growth behaviors of acid-adapted and non-adapted *V. parahaemolyticus* in the media supplemented with various nitrogen and carbon sources were investigated. The effects of acid adaptation on the thermostable direct hemolysin (TDH) secretion and fatty acid profiles of *V. parahaemolyticus* were also examined. Results showed that acid-adapted and non-adapted *V. parahaemolyticus* 690, BCRC 13023 and BCRC 13025 grew similarly in TSB-3% NaCl and basal media supplemented with various carbon and nitrogen sources during incubation period. Higher TDH secretion was noted with *V. parahaemolyticus* 690 among the three strains. However, acid-adapted strains produced less amounts of TDH than non-adapted strains when they were grown in TSB-3% NaCl. Additionally, acid adaptation increased the ratio of SFA/USFA in cells of *V. parahaemolyticus* strains.

Keywords*—Vibrio parahaemolyticus*, acid adaptation, thermostable direct hemolysin, fatty acid profile.

I. Introduction

ACIDIFICATION is often used to inhibit the growth of spoilage and pathogenic microorganisms in the food industry. Studies have demonstrated that mild acid-adaptation treatment induced acid tolerance response (ATR) and cross-protection against other environmental stresses in various Gram-positive and Gram-negative bacteria [1]-[4]. The induction of bacterial stress tolerance was reported to be involved in the synthesis of acid shock proteins and other cellular physiological mechanisms [5]-[7]. One of the major defense mechanisms to adapt to acid challenges is the alteration of membrane fatty acid composition [8], [9]. Additionally, some researchers have shown that the expression of virulence factors can be affected by acid stress in several pathogens [10]-[12]. This phenomenon of acid stress response may promote survival of bacteria and enhance their pathogenicity.

Vibrio parahaemolyticus is a marine bacterium commonly isolated from a variety of seafood. It can cause acute gastrointestinal symptoms in humans, including diarrhea, nausea, vomiting, abdominal cramps, headache, low fever and chills, primarily related to the consumption of contaminated seafood [13]-[15]. This pathogen has been recognized as a main cause of foodborne illness in many coastal countries. Thermostable direct hemolysin (TDH) is the major virulence factors of *V. parahaemolyticus*. This hemolysin has been shown to exhibit hemolytic, cardiotoxic, cytotoxic and enterotoxic activities [16], [17]. According to the published data from 1981 to 2012 in Taiwan, there were 1507 cases of food poisoning caused by *V. parahaemolyticus* and these cases accounted for 61.1% of the total bacterial foodborne outbreaks [18]. The Centers for Disease Control and Prevention (CDC) also reported that 4500 cases of *V. parahaemolyticus* infection occur each year in the United States [19].

Like other pathogenic microorganism, *V. parahaemolyticus* need to be tolerant to acid treatments used in food processing and preparation and pass through the gastric acid barrier to cause gastrointestinal disease. Therefore, the resistance of *V. parahaemolyticus* to acid stress is an important factor for its survival in food systems. Our previous studies have demonstrated that acid adaptation responses of *V. parahaemolyticus* can be affected by growth phase of cells, acid adaptation conditions, types of media and stress challenges, and varied among strains [20]-[22]. Additionally, differences in protein expression also observed between acid-adapted and non-adapted strains of *V. parahaemolyticus* [23]. In this study, we further investigated the effects of acid adaptation on the growth behaviors, thermostable direct hemolysin (TDH) secretion and fatty acid profiles in three strains of *V. parahaemolyticus*.

II. Materials and Methods

A. Bacterial Strains

Three strains of *V. parahaemolyticus* implicated in food poisoning outbreaks in Taiwan were used as test microorganisms. *V. parahaemolyticus* 690 was obtained from Department of Microbology, Soochow University (Taipei, Taiwan). *V. parahaemolyticus* BCRC 13023 and BCRC 13025 were purchased from Bioresource Collection and Research Center (BCRC), Food Industry Research and Development Institute (Hsinchu, Taiwan). Before each experiment, the test strains were inoculated (1% v/v) and subcultured three times in tryptic soy broth (Acumedia Manufactures, Inc., Lasing, MI, USA) supplemented with 3% NaCl (TSB-3% NaCl) at 37˚C for 4 h.

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B. Acid Adaptation Treatment

V. parahaemolyticus cultures were centrifuged (3000 × g, 10 min) and washed twice with phosphate-buffered saline containing 3% NaCl (PBS-3% NaCl, Na_2HPO_4/NaH_2PO_4, pH 7). To prepare acid-adapted *V. parahaemolyticus*, the washed cells were suspended in TSB-3% NaCl acidified to pH 5.5 with 10 N HCl and held at 37˚C for 90 min. Non-adapted *V. parahaemolyticus* was prepared by suspending in TSB-3% NaCl without acidification. Both acid-adapted and non-adapted *V. parahaemolyticus* were used in the following studies.

C. Growth Study with Various Nitrogen and Carbon Sources

The test nitrogen sources (BD Difco, Sparks, MD, USA) included tryptone, soytone, peptone, yeast extract, beef extract, malt extract, casamino acid and N-Z Amine A. Gucose, fructose, galactose, maltose, sucrose, lactose, mannitol and sorbitol were used as carbon sources (Sigma, St. Louis, MO, USA). 2% of each nitrogen source or 0.25% of each carbon source was added to a basal medium containing 3% NaCl and 0.25% K_2HPO_4 (pH 7). Acid-adapted and non-adapted *V. parahaemolyticus* were inoculated (1% v/v) in the media supplemented with various nitrogen and carbon sources and incubated at 37 °C for 6 h, respectively. After different periods of incubation, the growth of *V. parahaemolyticus* was measured by absorbance at 600 nm (Model Helios α, Spectronic Unicam, Cambridge, UK).

D. TDH Assay

Acid-adapted and non-adapted *V. parahaemolyticus* were inoculated (1% v/v) in TSB-3% NaCl and incubated at 37°C for 6, 12 and 18 h, respectively. After different incubation times, culture samples were taken to determine the growth and the corresponding TDH production. Cell density in cultures was measured by absorbance at 600 nm. Cell culture supernatants were collected and assayed for TDH content. TDH in cell-free supernatants was quantified by commercial KAP-RPLA kit (Denka Seiken Co., Tokyo, Japan) according to the manufacturer's procedure. The kit had a sensitivity of 1-2 ng/mL of TDH at the titer of 1:2.

E. Fatty Acid Analysis

Acid-adapted and non-adapted *V. parahaemolyticus* were centrifuged (3000 × g, 10 min) and washed twice with PBS-3% NaCl. Cellular fatty acid methylesters were extracted according to the method of [24] with slight modifications. Analysis was conducted by Intertek Testing Services Taiwan Ltd. (Taipei, Taiwan) using a gas chromatography (GC, 6890N, Agilent Technologies, Palo Alto, CA, USA). The commercial standards (Sigma) were used to determine the retention time and identity of fatty acids from *V. parahaemolyticus*.

F. Statistical Analysis

All triplicate experiments were executed independently. Data expressed as mean ± SE were analyzed by analysis of variance with SAS statistical software (SAS Institute, Cary, NC, USA), and the significant differences ($P < 0.05$) among data values were compared using Duncan's multiple range test.

III. Results and Discussion

A. Growth of Acid-Adapted and Non-Adapted V. parahaemolyticus in the Media Supplemented with Various Nitrogen and Carbon Sources

Some studies reported that stress treatment may cause changes of nutrient availability in bacterial cells [25], [26]. Various nitrogen and carbon sources were used to compare their effects on the growth of *V. parahaemolyticus* in this study. As shown in Fig. 1, the growth of *V. parahaemolyticus* varied with the nitrogen sources used in the basal medium during the incubation period. Regardless of acid adaptation, the three test strains of *V. parahaemolyticus* had the highest growth rate and cell density in the medium supplemented with tryptone, followed by soytone, yeast extract, beef extract, N-Z-Amine A and peptone. However, a partial or no growth was observed in the media supplemented with casamino acid and malt extract in all three strains. The growth patterns of non-adapted *V. parahaemolyticus* were similar to those of acid-adapted *V. parahaemolyticus* in the media supplemented with the same nitrogen source. Fig. 2 shows the growth of acid-adapted and non-adapted *V. parahaemolyticus* in the media supplemented with different carbon sources. After incubation for 6 h, *V. parahaemolyticus* exhibited higher growth rate and cell density in the media containing glucose, galactose and molasses, while lower numbers in the media containing lactose, sucrose and sorbitol were found among the carbon sources examined. The effects of these carbon sources on the growth of acid-adapted and non-adapted strains of *V. parahaemolyticus* were similar. Overall, tryptone and glucose were the most effective nitrogen and carbon sources for the growth of *V. parahaemolyticus*, respectively. Additionally, the condition of acid adaptation used in the present study did not affect the growth behaviors of *V. parahaemolyticus* in the media supplemented with various nitrogen and carbon sources.

B. Growth and TDH Secretion of Acid-Adapted and Non-Adapted V. parahaemolyticus in TSB-3% NaCl

As shown in Table I, acid-adapted and non-adapted *V. parahaemolyticus* grew similarly in TSB-3% NaCl during the incubation period. After 18 h of cultivation, the optical density at 600 nm between acid-adapted and non-adapted *V. parahaemolyticus* was not significantly different ($P > 0.05$) in the three test strains. Additionally, regardless of acid adaptation, the highest TDH secretion was observed in strain 690, followed by BCRC 13023 and BCRC 13025. However, acid-adapted *V. parahaemolyticus* produced less amounts of TDH than non-adapted *V. parahaemolyticus*. For example, non-adapted *V. parahaemolyticus* 690 had a higher content of TDH with a titer of 1:16, which was twofold that determined in acid-adapted cells at the end of incubation. Similar results were also noted in strains BCRC 13023 and BCRC 13025. These findings indicated that acid adaptation decreased the production of TDH by *V. parahaemolyticus*. Differences in levels of TDH secretion were also observed among strains. Our results are consistent with the findings of other studies, who reported that acid-adapted *E. coli* O157:H7 produced less verotoxin than did non-adapted cells [27]-[29]. The decreased toxin secretion may

be related to the alteration of membrane fluidity and permeability in cells of *V. parahaemolyticus*.

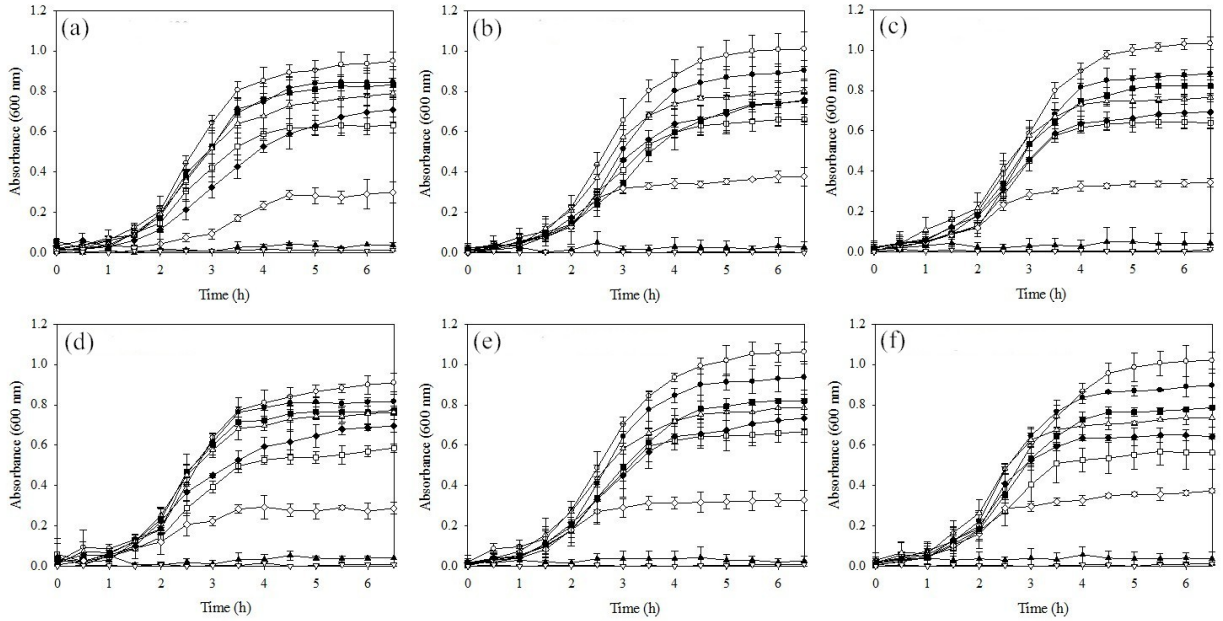

Fig. 1 Growth of *V. parahaemolyticus* strains in the media supplemented with various nitrogen sources. (a) Non-adapted 690; (b) non-adapted BCRC 13023; (c) non-adapted BCRC 13025; (d) acid-adapted 690; (e) acid-adapted BCRC 13023; (f) acid-adapted BCRC 13025. ○, Tryptone; ●, soytone; □, peptone; ■, yeast extract; ∆, beef extract; ▲, malt extract; ◇, casamino acid; ◆, N-Z-Amine A; ▽, basal medium contains 0.25% glucose, 3% NaCl and 0.25% K_2HPO_4 (pH 7)

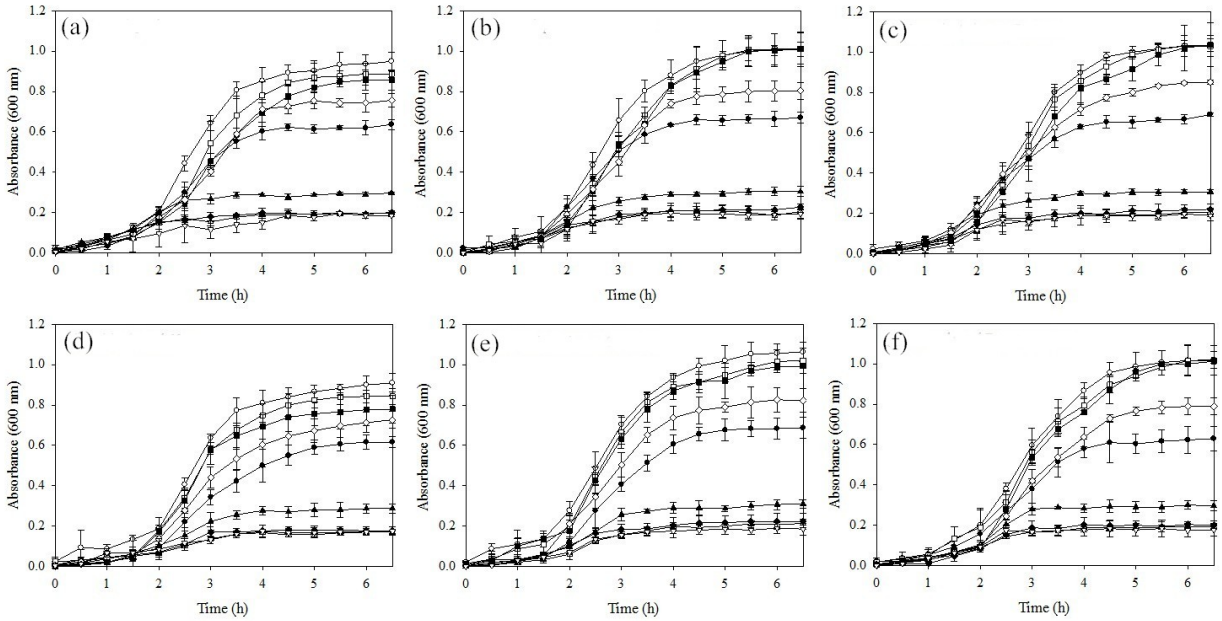

Fig. 2 Growth of *V. parahaemolyticus* strains in the media supplemented with various carbon sources. (a) Non-adapted 690; (b) non-adapted BCRC 13023; (c) non-adapted BCRC 13025; (d) acid-adapted 690; (e) acid-adapted BCRC 13023; (f) acid-adapted BCRC 13025. ○, Glucose; ●, fructose; □, galactose; ■, maltose; ∆, sucrose; ▲, lactose; ◇, mannitol; ◆, sorbitol; ▽, basal medium contains 2% tryptone, 3% NaCl and 0.25% K_2HPO_4 (pH 7)

C. Fatty Acid Profiles of Acid-Adapted and Non-Adapted V. parahaemolyticus

Table II shows the fatty acid profiles of acid-adapted and non-adapted *V. parahaemolyticus*. The major fatty acids in three strains of *V. parahaemolyticus* are palmitic acid (16:0), palmitoleic acid (16:1) and octadecenoic acid (18:1). It was found that acid-adapted *V. parahaemolyticus* 690, BCRC 13023 and BCRC 13025 had significantly higher ($P < 0.05$) amounts of palmitic acid than the respective non-adapted strains. However, the amounts of octadecenoic acid in strain 13025 and the amounts of linoleic acid (18:2) in strains 690 and BCRC 13025 decreased after acid adaptation. Slightly different changes of fatty acid proportion were observed among acid-adapted strains. Generally, Acid-adapted *V. parahaemolyticus* had significantly higher ($P < 0.05$) saturated fatty acids (SFA) content and lower ($P < 0.05$) unsaturated fatty acids (USFA) content than non-adapted *V. parahaemolyticus*. Additionally, acid adaptation increased the ratio of SFA/USFA in cells of the three strains of *V. parahaemolyticus*. An increased level of SFA and a decreased level of USFA in acid-adapted bacteria have also been reported in several studies [28], [30], [31]. These changes in fatty acid composition can decrease the membrane fluidity, which may have resulted in increased acid tolerance [20] and decreased TDH secretion in acid-adapted *V. parahaemolyticus* (Table I).

TABLE I
GROWTH OF *V. PARAHAEMOLYTICUS* STRAINS AND CORRESPONDING KAP-RPLA TITERS OF TDH EXCRETED IN TSB-3% NACL

Strain	Treatment	Time (h)	Titer	OD at 600 nm
690	Non-adapted	6	1:2	1.030 ± 0.046 f[2]
		12	1:8	1.063 ± 0.031 de
		18	1:16	1.220 ± 0.026 bc
	Acid-adapted	6	ND[1]	1.043 ± 0.040 f
		12	1:2	1.177 ± 0.025 cde
		18	1:8	1.227 ± 0.015 bc
BCRC 13023	Non-adapted	6	ND	1.133 ± 0.015 e
		12	1:2	1.217 ± 0.015 bcd
		18	1:8	1.228 ± 0.016 bc
	Acid-adapted	6	ND	1.147 ± 0.025 e
		12	1:2	1.213 ± 0.032 bcd
		18	1:4	1.223 ± 0.038 bc
BCRC 13025	Non-adapted	6	ND	1.130 ± 0.044 e
		12	1:2	1.240 ± 0.026 ab
		18	1:8	1.290 ± 0.044 a
	Acid-adapted	6	ND	1.133 ± 0.015 e
		12	ND	1.247 ± 0.015 ab
		18	1:4	1.260 ± 0.036 ab

[1]Values in the same column with different letters (a-f) are significantly different ($P < 0.05$)
[2]ND: Non-detected

TABLE II
FATTY ACID COMPOSITION OF *V. PARAHAEMOLYTICUS* STRAINS

Fatty acid[1]	Fatty acid proportion (%)[2]					
	690		BCRC 13023		BCRC 13025	
	Non-adapted	Acid-adapted	Non-adapted	Acid-adapted	Non-adapted	Acid-adapted
12:0	2.47±0.04 a[3]	2.27±0.17 a	2.6±0.29 a	2.83±0.20 a	2.35±0.04 a	2.60±0.06 a
14:0	5.71±0.25 a	6.04±0.19 a	6.71±0.19 a	6.40±0.45 a	5.52±0.07 a	6.34±0.44 a
15:0	0.05±0.01 a	0.10±0.05 a	0.41±0.03 a	0.44±0.05 a	0.05±0.01 a	0.09±0.01 a
16:0	25.63±1.13 b	28.13±0.54 a	26.98±0.58 b	29.78±0.55 a	25.75±0.72 b	29.06±0.36 a
16:1	32.56±0.43 a	29.57±1.52 a	33.98±0.71 a	31.16±0.33 a	33.26±0.80 a	29.09±0.56 a
17:0	0.28±0.03 a	0.34±0.02 a	0.29±0.04 a	0.78±0.30 a	0.37±0.03 a	0.39±0.03 a
18:0	2.66±0.08 a	4.74±0.50 a	2.63±0.35 a	3.64±0.91 a	2.76±0.20 a	6.21±0.33 a
18:1	29.69±1.40 a	27.94±0.95 a	25.77±0.51 a	24.19±0.26 a	29.00±0.12 a	25.68±0.15 b
18:2	0.95±0.10 a	0.42±0.04 b	0.62±0.13 a	0.78±0.06 a	0.93±0.03 a	0.54±0.07 b
SFA	36.80±1.18 b	42.06±0.88 a	39.63±0.48 b	43.88±0.22 a	36.81±0.94 b	44.70±0.64 a
USFA	63.20±1.18 a	57.94±0.88 b	60.37±0.48 a	56.12±0.22 b	63.19±0.94 a	55.30±0.64 b
SFA/USFA	0.58±0.03 b	0.73±0.03 a	0.66±0.01 b	0.78±0.01 a	0.58±0.02 b	0.81±0.02 a

[1]12:0, Lauric acid; 14:0, myristic acid; 15:0, pentadecanoic acid; 16:0, palmitic acid; 16:1, palmitoleic acid Δ9; 17:0, heptadecanoic acid; 18:0, stearic acid; 18:1, *cis*- and *trans*-octadecenoic acid Δ9 and Δ11; 18:2, *cis*- and *trans*-linoleic acid Δ9, 12; SFA, saturated fatty acid; USFA, unsaturated fatty acid
[2]Values are proportions of fatty acid (%)
[3]Values in the same row with different letters (a, b) are significantly different ($P < 0.05$)

IV. CONCLUSION

In conclusion, this study demonstrated the acid adaption responses among three strains of *V. parahaemolyticus*. Acid-adapted and non-adapted *V. parahaemolyticus* showed similar growth behaviors, while lower TDH secretion and higher ratio of SFA/USFA were observed in acid-adapted *V. parahaemolyticus* than in non-adapted *V. parahaemolyticus*. These findings provided information in understanding the growth and some physiological characteristics of *V. parahaemolyticus* exposed to mild acid conditions.

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