

## Effects of Acid Stress on *Vibrio parahaemolyticus* Survival and Cytotoxicity

P. S. MARIE YEUNG AND KATHRYN J. BOOR\*

Department of Food Science, Cornell University, Ithaca, New York 14853, USA

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### ABSTRACT

For several foodborne bacterial pathogens, an acid tolerance response appears to be an important strategy for counteracting acid stress imposed either during food processing or by the human host. The acid tolerance response enhances bacterial survival of lethal acid challenge following prior exposure to sublethal acidic conditions. Previous studies have revealed relationships between a foodborne pathogen's ability to survive acid challenge and its infectious dose. *Vibrio parahaemolyticus* is capable of causing gastroenteritis when sufficient cells of pathogenic strains are consumed. This study was designed to characterize acid sensitivities and to compare the effects of sublethal acid exposure (adaptation) on survival capabilities and cytotoxicities of different *V. parahaemolyticus* strains. Survival of acid challenge by stationary-phase cells differed by up to 3 log CFU/ml among the 25 isolates tested. No differences in acid resistance were found between strains when they were grouped by source (clinical isolates versus those obtained from food). Survival at pH 3.6 for log-phase cells that had been previously exposed to sublethal acidic conditions (pH 5.5) was enhanced compared with that for cells not previously exposed to pH 5.5. However, for stationary-phase cells, exposure to pH 5.5 impaired both subsequent survival at pH 3.6 and cytotoxicity to human epithelial cells. Relative cytotoxicities of nonadapted stationary-phase cells were 1.2- to 4.8-fold higher than those of adapted cells. Sublethal acid exposure appears to impose measurable growth phase-dependent effects on subsequent lethal acid challenge survival and cytotoxicity of *V. parahaemolyticus*.

Though genetically and phenotypically diverse, foodborne pathogens encounter similar stresses, such as exposure to acidic conditions, that are imposed by food processing procedures and by their human hosts. The acid tolerance response (ATR) has been recognized as an important strategy employed by foodborne pathogens to counteract acid stress. Various pathogens, including *Salmonella* Typhimurium, *Listeria monocytogenes*, and *Escherichia coli* O157:H7, have enhanced survival capabilities during exposure to a lethal acid challenge when these pathogens have had prior exposure to sublethal acidic conditions (9, 15, 36). Previous studies with *Vibrio cholerae* and *L. monocytogenes* have revealed that ATR also contributes to enhanced bacterial virulence in mouse models (31, 36). Thus, in addition to enhancing bacterial survival, ATR also may influence bacterial pathogenesis.

*Vibrio parahaemolyticus*, a gram-negative microorganism found in the marine environment, is capable of causing gastroenteritis; typical clinical signs include diarrhea, abdominal pain, nausea, vomiting, headache, fever, and chills. Individual factors that have been associated with virulence in *V. parahaemolyticus* include a thermostable direct hemolysin, a thermostable direct hemolysin-related hemolysin, and urea hydrolysis (19, 20, 23, 33, 34). Although an ATR has been reported previously for at least two *V. parahaemolyticus* strains (24, 43), specific mechanisms that may contribute to the ability of pathogenic *V. parahaemolyticus* strains to endure the acidic environment of the human stomach and to subsequently mount an infection are unknown.

To provide an overview of the acid resistance characteristics of *V. parahaemolyticus*, the objectives of this study were (i) to determine the innate acid sensitivities of multiple, diverse *V. parahaemolyticus* isolates, (ii) to determine for multiple strains the effect of prior exposure to sublethal acidic conditions (i.e., acid adaptation) on subsequent strain survival at a lower pH, and (iii) to determine the effect of acid adaptation on subsequent *V. parahaemolyticus* strain cytotoxicity in HeLa cells. We also evaluated the relative acid sensitivities among *V. parahaemolyticus* isolates classified according to isolation source.

### MATERIALS AND METHODS

**Bacterial isolates.** Isolates used in this study were provided by the U.S. Food and Drug Administration (Table 1). Serotypes were determined previously by the Centers for Disease Control and Prevention. Upon receipt, all isolates were streaked for isolation of single colonies on tryptic soy agar (Difco Laboratories, Becton Dickinson, Sparks, Md.) supplemented with 2% NaCl (TSAS). Isolates were stored in tryptic soy broth supplemented with 2% NaCl (TSBS) and 20% glycerol at  $-80^{\circ}\text{C}$ . Isolates were subcultured on TSAS at least once before each experiment.

**Acid sensitivity assays.** To evaluate strain sensitivity to acid, growth kinetics and survival of each strain were measured under various conditions. Overnight cultures ( $37^{\circ}\text{C}$ , 12 to 16 h, 250 rpm) were used to inoculate TSBS (1% inoculum, vol/vol) that had been adjusted with 6 N HCl to pH 7.2, 6.5, 5.5, or 4.2. Growth kinetics were determined by monitoring the optical density at 600 nm ( $\text{OD}_{600}$ ) for each strain grown in microtiter plate wells. The

\* Author for correspondence. Tel: 607-255-3111; Fax: 607-254-4868; E-mail: kjb4@cornell.edu.

TABLE 1. *Vibrio parahaemolyticus* isolates used in this study and their innate acid sensitivities

Laboratory no.	FDA no.	Serotype	Source	Place of isolation	Year of isolation	Acid sensitivity <sup>a</sup>	
						Mean $\pm$ SE	n
FSL-Y1-003	T-3980	O4:K13	Clinical	Japan	Unknown	2.3 $\pm$ 0.8	4
FSL-Y1-010	T-3979	O5:K15	Clinical	Japan	Unknown	5.4 $\pm$ 0.5 <sup>b</sup>	5
FSL-Y1-012	48432	O4:K12	Clinical	Washington	1991	3.3 $\pm$ 0.5	4
FSL-Y1-013	47978	O6:K18	Clinical	Washington	1991	3.1 $\pm$ 1.0 <sup>c</sup>	4
FSL-Y1-014	AN-5034	O4:K68 <sup>d</sup>	Clinical	Bangladesh	1996	1.9 $\pm$ 0.7	4
FSL-Y1-015	AN-16000	O1:KUT <sup>de</sup>	Clinical	Bangladesh	1998	3.5 $\pm$ 0.7 <sup>c</sup>	4
FSL-Y1-016	TX-2103	O3:K6	Clinical	Texas	1998	2.5 $\pm$ 0.6	4
FSL-Y1-017	NY-3064	O3:K6	Clinical	New York	1998	2.8 $\pm$ 0.5	3
FSL-Y1-021	U-5474	Old O3:K6 <sup>f</sup>	Clinical	Bangladesh	1980	3.6 $\pm$ 0.8	3
FSL-Y1-023	VP86	O3:K6	Clinical	Calcutta	1996	3.8 $\pm$ 0.3	4
FSL-Y1-024	VP199	O3:K6	Clinical	Calcutta	1997	4.7 $\pm$ 1.1 <sup>b</sup>	3
FSL-Y1-025	VP208	O3:K6	Clinical	Calcutta	1997	2.4 $\pm$ 0.3	4
FSL-Y1-026	VP155	O3:K6	Clinical	Calcutta	1996	4.0 $\pm$ 1.0 <sup>c</sup>	5
FSL-Y1-046	BAC-98-03255	O3:K6	Clinical	New York	Unknown	2.2 $\pm$ 0.8	5
FSL-Y1-004	CRAB	Unknown	Food	Washington	1972	2.9 $\pm$ 1.2	5
FSL-Y1-001	92000713 (1)	O8	Food (clam)	Unknown	1992	3.2 $\pm$ 0.5	4
FSL-Y1-002	NY477	O4:K8	Food (oyster)	New York	1977	3.4 $\pm$ 1.1	4
FSL-Y1-005	5C-1C	O1	Food (oyster)	Washington	1988	4.3 $\pm$ 1.0 <sup>c</sup>	3
FSL-Y1-006	M350A	O5	Food (oyster)	Washington	1994	3.4 $\pm$ 0.6	5
FSL-Y1-011	8332924	O1:K56	Food (oyster)	Gulf	1983	4.0 $\pm$ 0.5	3
FSL-Y1-059	98-792-807 (27)	O8:K74	Food (oyster)	Galveston Bay	1998	4.0 $\pm$ 1.0 <sup>c</sup>	7
FSL-Y1-068	DI-A6-031699	O4:K9	Food (oyster)	Alabama	Unknown	5.2 $\pm$ 1.0 <sup>b</sup>	3
FSL-Y1-069	DI-B11-031699	O4:K22	Food (oyster)	Alabama	Unknown	3.1 $\pm$ 0.9	3
FSL-Y1-073	DI-F8-031699	O11:KUT	Food (oyster)	Alabama	Unknown	3.0 $\pm$ 0.5	3
FSL-Y1-081	DI-A6-020800	O11:K61	Food (oyster)	Alabama	Unknown	3.8 $\pm$ 0.9	3

<sup>a</sup> Log CFU/ml reduction (mean  $\pm$  SE) of stationary-phase cells following exposure to TSBS pH 3.6 adjusted with 6 N HCl at 37°C for 30 min. n = the number of independent experiments conducted for each isolate.

<sup>b</sup> CFU/ml fell below the detection limit of the assay (300 CFU/ml) in two independent experiments for these strains.

<sup>c</sup> CFU/ml fell below the detection limit of the assay (300 CFU/ml) in one experiment for these strains.

<sup>d</sup> Strains deemed genetically similar to O3:K6 strains by arbitrarily primed PCR, ribotypes, and pulsed-field gel electrophoresis pattern analyses (10, 30).

<sup>e</sup> UT, untypeable.

<sup>f</sup> O3:K6 strain isolated before 1996 that is genetically different from the O3:K6 strains isolated after 1996.

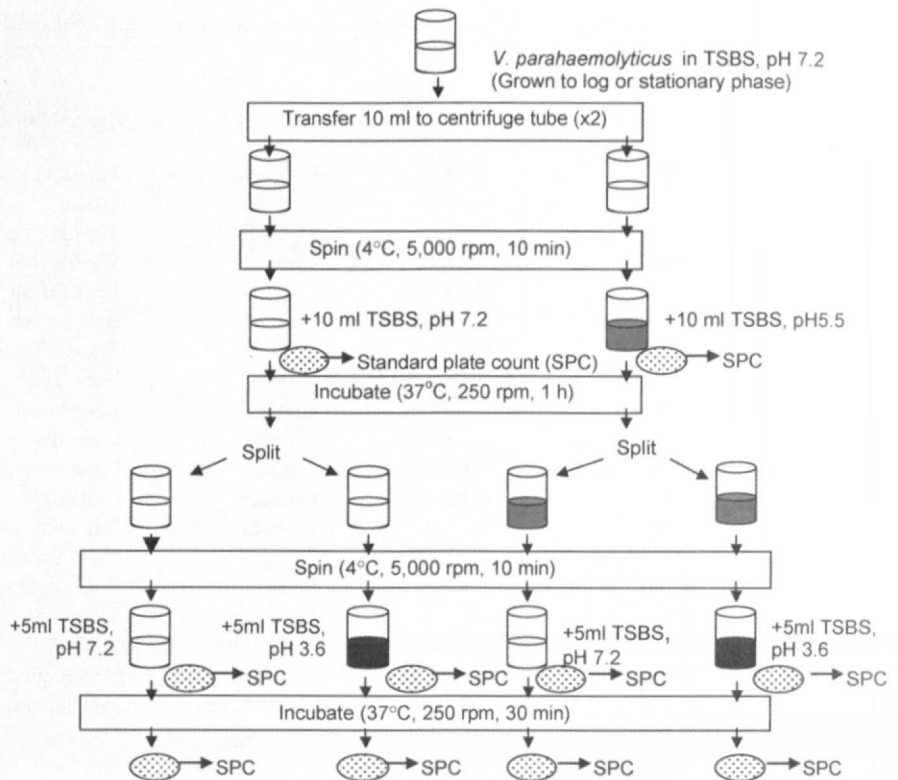
microtiter plates were incubated statically at 37°C in a microplate analyzer (Fusion Universal, Packard Instrument Co., Meriden, Conn.). Readings were taken every 10 min for ~5 h.

To determine bacterial survival following acid exposure, bacterial colonies were enumerated by standard procedures following exposure to acidic conditions. Stationary-phase cells were obtained by growing *V. parahaemolyticus* cells to a mean ( $\pm$  standard error [SE]) OD<sub>600</sub> of 1.1  $\pm$  0.1. Culture samples were centrifuged, and the cell pellets were resuspended in TSBS with pH levels ranging from 3.0 to 6.5 (adjusted with 6 N HCl) and incubated at 37°C for 30 min or for 1 h with shaking at 250 rpm. Based on results from these preliminary trials, pH 3.6 was selected for subsequent lethal acid challenge conditions for all 25 strains. Stationary-phase cultures were serially diluted in phosphate-buffered saline, and 0.1 ml of the appropriate dilution was spread plated onto TSAS plates. Plates were incubated at 37°C for 16 to 18 h, and then colonies were counted. Only data from plates bearing 30 to 300 colonies were used in the calculations, hence the effective lower limit for detection of assay survivors was 300 CFU/ml. To enable calculations and statistical analyses, when the number of colonies present on a plate from the lowest dilution was <30, we used 300 CFU/ml as the number of survivors present after acid challenge. Experiments were conducted at least three times for each isolate.

**ATR assays.** To determine the effect of ATR on subsequent survival, log- and stationary-phase *V. parahaemolyticus* cells were subjected to an adaptation treatment (TSBS, pH 5.5  $\pm$  0.1 for 1 h at 37°C) prior to exposure to an acid challenge (TSBS, pH 3.6  $\pm$  0.1 for 30 min at 37°C). Log-phase cells were obtained by growing *V. parahaemolyticus* cells to an OD<sub>600</sub> of 0.4  $\pm$  0.05. Stationary-phase cells also were obtained. To control for possible effects of the addition of fresh medium during the acid adaptation procedure, nonadapted cells were prepared in parallel with the adapted cells by incubating log- or stationary-phase cells in TSBS at pH 7.2 rather than at pH 5.5 for 1 h prior to acid challenge. To monitor viability of nonstressed cells throughout the experiment, additional controls were prepared by incubating both adapted and nonadapted cells in TSBS at pH 7.2 in parallel with those incubated at pH 3.6 during the acid challenge procedure. A schematic representation of the experimental procedure is shown in Figure 1. Samples were taken (Fig. 1) to count the CFU present. At least two independent experiments were conducted on each isolate. For each sample, survival percentage = 100  $\times$  (colony counts at 30 min after pH 3.6 acid challenge/colony counts obtained immediately following exposure to pH 3.6).

**Cytotoxicity assays.** To assess the relationship between ATR and virulence-associated characteristics, we used a tissue culture

FIGURE 1. Experimental procedure to measure survival of *V. parahaemolyticus* cells following acid adaptation.



model to measure HeLa cell cytotoxicity induced by acid-adapted or nonadapted *V. parahaemolyticus* cells harvested in stationary phase. Two tubes of 500- $\mu$ l aliquots of *V. parahaemolyticus* cultures (typically  $8 \times 10^8$  to  $2 \times 10^9$  CFU/ml) were centrifuged at 13,000 rpm ( $17,900 \times g$ ) for 5 min and resuspended in 500  $\mu$ l of TSBS pH 7.2 and TSBS pH 5.5, respectively. After incubation for 1 h at 37°C, a 15- $\mu$ l aliquot from a 10-fold dilution (1.5  $\mu$ l of the undiluted suspension) was used to infect monolayers of HeLa cells grown in Dulbecco's modified Eagle's medium (Gibco BRL, Life Technologies, Rockville, Md.) in microtiter plates. At 2 h postinfection, cytotoxicity was assessed by measurement of released host cell lactate dehydrogenase (LDH) using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, Wis.). LDH concentration was measured by reading absorbance at 490 nm using the microplate analyzer. Relative cytotoxicity calculations were based on the following formula: cytotoxicity percentage =  $100 \times (A_{\text{sample}} - A_{\text{spontaneous}}) / (A_{\text{total}} - A_{\text{spontaneous}})$ , where  $A_{\text{sample}}$  is the absorbance of infected host cells and

$A_{\text{spontaneous}}$  is the absorbance of uninfected host cells. Cytotoxicity for the internal control (nonadapted *V. parahaemolyticus* FSL-Y1-014) was set as 100% ( $A_{\text{total}}$ ) and cytotoxicity for other samples was expressed relative to this control. The cytotoxicity differences between adapted and nonadapted cells were analyzed using the two-sample *t* test and the Mann-Whitney U test for normally and nonnormally distributed data, respectively (Minitab version 12.0, Minitab, Inc., State College, Pa.).

To ensure that bacterial numbers were similar for all cultures at key points during infection, bacterial cells were counted immediately following infection (0 h) and at 2 h postinfection. Non-adapted and adapted cells were present at similar numbers under these conditions for a given isolate (mean  $\pm$  standard deviation [SD] =  $0.27 \pm 0.33$  log CFU/ml).

## RESULTS

**Acid sensitivity of *V. parahaemolyticus* strains.** Initially, growth characteristics at various pH levels were determined for seven strains (FSL-Y1-003, FSL-Y1-005, FSL-Y1-011, FSL-Y1-013, FSL-Y1-014, FSL-Y1-015, and FSL-Y1-023), which were selected from our collection to represent different serotypes and isolation sources. Overall, growth kinetics were similar for these seven strains. A representative growth curve is illustrated in Figure 2. None of the *V. parahaemolyticus* strains were able to reproduce at pH 4.2. In these seven strains, 1 h of exposure at pH 4.2 resulted in mean ( $\pm$ SD) reduction of  $0.6 \pm 0.5$  log CFU/ml, with a maximum 1.5 log CFU/ml reduction (log reduction) in one strain. No colonies were found after 1 h of exposure at pH 3.0. Based on these results, we selected exposure to pH 3.6 for 30 min as the parameters for lethal acid challenge in assays with all 25 *V. parahaemolyticus* isolates shown in Table 1. Overall, exposure to acid challenge resulted in reductions ranging from 1.9 to 5.4 log

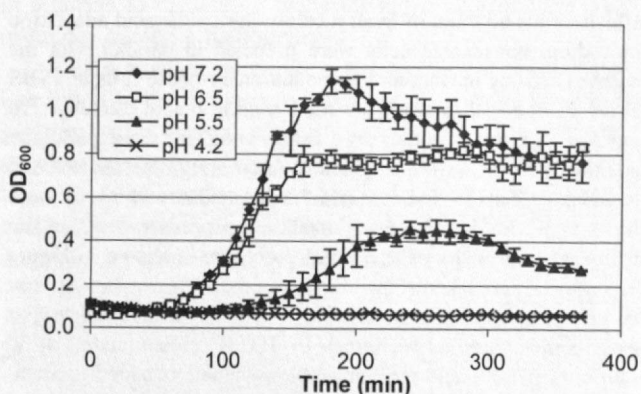


FIGURE 2. Growth of *V. parahaemolyticus* FSL-Y1-003 in TSBS at 37°C at various pH levels. Error bars represent the SDs from the mean. Experiments were conducted at least in triplicate.



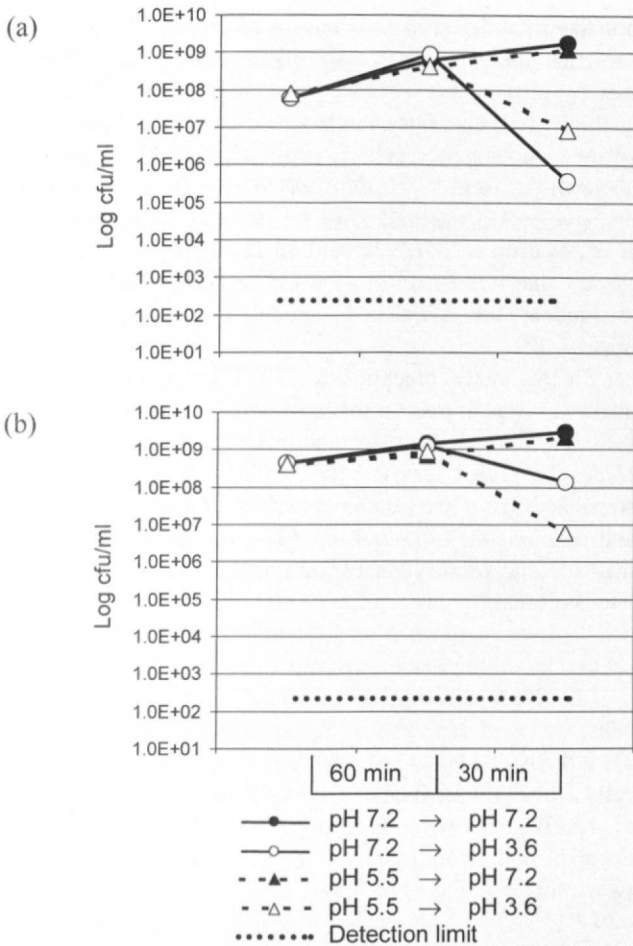


FIGURE 3. Survival of *V. parahaemolyticus* FSL-Y1-011 following exposure to different pH levels in one representative experiment. Prior to the experiment, cultures were grown in TSBS until the  $OD_{600}$  reached 0.4 to obtain log-phase cells (a) and grown in TSBS until the  $OD_{600}$  was  $>1.0$  to obtain stationary-phase cells (b).

CFU/ml among these stationary-phase cultures. When isolates were categorized by source, 30 min of pH 3.6 exposure resulted in  $3.3 \pm 1.0$  and  $3.6 \pm 0.7$  log reductions for the clinical and food isolates, respectively. Relative survival of clinical and food isolates did not differ ( $P = 0.34$ ), indicating that the clinical isolates in our collection are not inherently more acid resistant than the food isolates, which had not been associated with foodborne illness.

**ATR is induced in log-phase cells.** To determine whether the effect of acid adaptation on subsequent survival differed between log-phase and stationary-phase *V. parahaemolyticus* cells, four food isolates (FSL-Y1-001, FSL-Y1-005, FSL-Y1-006, and FSL-Y1-011) and three clinical isolates (FSL-Y1-013, FSL-Y1-015, and FSL-Y1-017) representing different serotypes were tested. All adapted cells harvested in log phase survived acid challenge more effectively than did nonadapted cells. Conversely, with the exception of FSL-Y1-013, all adapted stationary-phase cells did not survive as well as did their nonadapted counterparts. Representative data are provided in Figure 3, and a data summary from all tested strains is provided in Figure 4. For the seven isolates, mean log reductions for log-phase adapt-

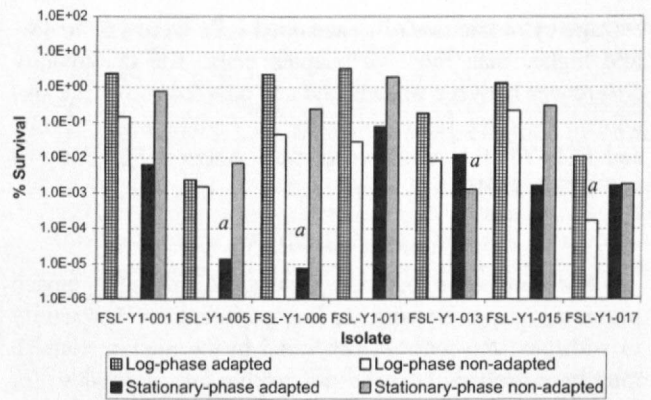


FIGURE 4. Mean relative survival of *V. parahaemolyticus* following acid challenge (30-min exposure at pH 3.6) in duplicate experiments. Prior to the experiment, cultures were grown until  $OD_{600}$  reached 0.4 and  $>1.0$  to obtain log-phase and stationary-phase cells, respectively. Nonadapted and adapted cells (columns 2 and 4 in Fig. 1) are those that were preexposed to pH 7.2 and pH 5.5, respectively, for 1 h. For bars labeled a, colony numbers in a single experiment fell below the detection limit of the assay (300 CFU/ml), hence the true survival percentage for these cultures may be lower than that shown.

ed and nonadapted cells were 2.6 and 3.9, respectively. With the exception of FSL-Y1-013, mean log reductions for stationary-phase adapted and nonadapted cells were 5.0 and 3.3, respectively. Survival of stationary-phase *V. parahaemolyticus* FSL-Y1-013 cells differed from that of all other strains. Adapted stationary-phase FSL-Y1-013 cells survived better than did nonadapted cells, with log reductions of 3.9 and 4.9, respectively.

**Virulence-associated characteristics of acid-adapted and nonadapted cells.** Host cell LDH release was measured to reflect cytotoxicity resulting from interactions with bacterial pathogens (38). Relative percentage cytotoxicity in HeLa cells ranged from 1.4 to 103.2% and 6.7 to 133.8% for adapted and nonadapted cells, respectively (Fig. 5). With the exception of FSL-Y1-013, the mean relative per-

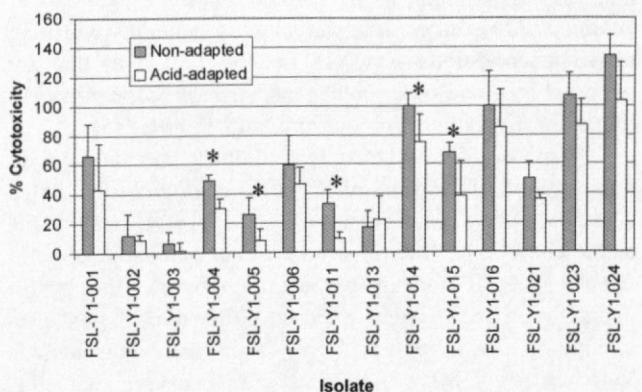


FIGURE 5. Relative cytotoxicity percentage in HeLa cells at 2 h postinfection for acid-adapted and nonadapted *V. parahaemolyticus* cultures. Error bars represent the SDs from the mean for at least three independent experiments. An asterisk indicates that the cytotoxicity differences between acid-adapted and nonadapted cells for a given isolate are significantly different ( $P < 0.05$ ).

centage cytotoxicities of nonadapted cells were 1.2- to 4.8-fold higher than those of adapted cells. The cytotoxicity differences between nonadapted and adapted cells were significant for three food isolates (FSL-Y1-004, FSL-Y1-005, and FSL-Y1-011) and two clinical isolates (FSL-Y1-014 and FSL-Y1-015).

### DISCUSSION

For foodborne pathogens, survival through stomach passage is a prerequisite for human infection. The ability to withstand the reduced pH found in the human stomach may be correlated with an organism's infectious dose (3, 16, 41). Therefore, growth or survival capabilities of foodborne pathogens, such as *Aeromonas* (35), *L. monocytogenes* (14), *Salmonella* (4), *E. coli* (37), *Shigella flexneri* (40), and *Vibrio vulnificus* (26), under different acidic conditions represent important areas of study. In a few previous studies, survival of log-phase *V. parahaemolyticus* under acidic conditions has been examined. For example, Wong et al. (43) reported a 1-log reduction for a clinical *V. parahaemolyticus* strain following 60 min of exposure to pH 4.4. Koga et al. (24) reported a 2-log reduction for another clinical isolate following 60 min of exposure to pH 2.0. Both groups tested *V. parahaemolyticus* cells that had been harvested in log phase.

Our data indicate wide variation in acid sensitivities among different *V. parahaemolyticus* strains. Stationary-phase cultures of the *V. parahaemolyticus* isolates in our collection were highly sensitive to pH 3.0. Among the isolates tested, the most and least acid sensitive strains were FSL-Y1-010 and FSL-Y1-014, respectively. These two isolates showed an average of a 5.4- and 1.9-log reduction, respectively, following 30 min of exposure to pH 3.6. The presence or absence of urease activity could affect the relative acid sensitivities of the *V. parahaemolyticus* strains examined in this study. Urease production can contribute to enhanced acid tolerance in *Yersinia enterocolitica* and *Helicobacter pylori* (39, 44). Therefore, it would be useful to examine the association between urease production and acid tolerance of specific *V. parahaemolyticus* strains. Our findings suggest that, as with *Vibrio cholerae* (42) and *V. vulnificus* (25), *V. parahaemolyticus* is generally sensitive to acidic conditions (i.e., pH 3.6 for  $\geq 30$  min) that are tolerated by some other pathogens, such as *Staphylococcus aureus*, *Salmonella* Typhimurium, and *E. coli* (35).

Response to acid stress is a complex phenomenon. In this study, the sensitivity of *V. parahaemolyticus* to a lethal acid challenge was affected by prior exposure to sublethal acidic conditions. Among several foodborne bacterial pathogens, an ATR appears to be an important strategy for counteracting acid stress imposed either during food processing or inside the human host. Many pathogens are able to mount an ATR that enhances bacterial survival of a lethal acid challenge following prior exposure to sublethal acidic conditions (3, 17, 32). Exposure to sublethal acidic conditions can confer cross protection to other stresses, e.g., heat, osmotic gradients, and bile acid (24, 43); therefore, the ability to mount an ATR may provide an organism with relative survival or growth advantages over other microflora that do

not have similar acid tolerance mechanisms. Exposure to sublethal acid stress may also influence bacterial pathogenesis by altering the virulence-associated characteristics of acid-adapted cells. For example, acid-adapted cells were superior to nonadapted cells in colonizing suckling mice (*V. cholerae* (31) and *V. parahaemolyticus* (43)) and were more invasive in Caco-2 and J774.A1 cells (*L. monocytogenes* (11)). Marron et al. (29) tested an *L. monocytogenes* LO28 mutant that was unable to induce an ATR and found that this mutant had a reduced capacity to cause infection in mice.

In this study, preexposure to pH 5.5 improved subsequent survival at pH 3.6 for log-phase *V. parahaemolyticus* cells but not in general for stationary-phase cells. Growth-phase-dependent survival capabilities following mild acid stress also have been documented for *Bacillus cereus* (22) and *Salmonella* Typhimurium (27). For example, in contrast to relative survival of similarly treated lag-, log-, or late stationary-phase cultures, stationary-phase *B. cereus* cells grown at pH 6.0 or 7.0 showed decreased survival following subsequent exposure to pH 4.0 (22).

Acid-adapted stationary-phase *V. parahaemolyticus* cells, which generally had impaired survival capabilities at pH 3.6, also had decreased cytotoxicity in human epithelial cells. The cytotoxicities of all but one isolate decreased 1.2- to 4.8-fold following preexposure to pH 5.5. Under our experimental conditions, the cytotoxicity differences between adapted and nonadapted cells were significant for 5 of 14 (36%) isolates, suggesting that acid exposure has a substantial negative impact on virulence in some *V. parahaemolyticus* strains. However, as with strain ST550 (43), sublethal acid exposure increased the relative survival and cytotoxicity characteristics of FSL-Y1-013, supporting the hypothesis that mild acid treatment may enhance virulence capabilities for some *V. parahaemolyticus* strains. However, these data indicate that prior exposure to sublethal acidic conditions does not universally enhance survival and virulence characteristics for *V. parahaemolyticus* under all conditions.

The reduced cytotoxicity of acid-adapted cells could be a consequence of physical injury. *V. cholerae* cells grown at pH 6.0 had reduced expression of various enzymes necessary for lipopolysaccharide biosynthesis, resulting in alterations in the outer membrane and increased susceptibility to hydrophobic drugs (e.g., erythromycin) (18). Acid exposure also represses motility in *V. cholerae* and *Salmonella* Typhimurium (1, 18). Although changes in membrane structure and motility may not necessarily lead to decreased virulence, these findings suggest that significant physiological changes could occur in *V. parahaemolyticus* following acid exposure.

Apart from the possible intrinsic physiological differences in response to acid stress among strains, the use of different experimental conditions and virulence models may have contributed to variations in observed acid-mediated effects (e.g., survival capabilities and virulence) in previous studies. Experimental variations that influence acid sensitivity include growth phase (22, 27), growth medium (21, 22), growth conditions (12), and type of acid stress (31).

In addition, bacterial virulence responses may also differ in different hosts. Merrill and Camilli (31) identified a set of genes that are involved in *V. cholerae* acid tolerance in an adult rabbit ligated-loop model. However, fewer than half of these identified genes were induced when *V. cholerae* was inoculated into infant mice. Our results further illustrate the necessity for application of identical experimental conditions to enable direct comparisons of acid tolerances among *V. parahaemolyticus* strains. For example, the protocol used in our ATR experiments (i.e., resuspension of cells in fresh medium with each incubation period) enhanced survival of stationary-phase cells relative to that seen in the acid sensitivity experiments for five of seven *V. parahaemolyticus* strains. The stationary-phase *V. parahaemolyticus* cells used in this study appeared to be more susceptible to acid stress than did the acid-treated log-phase *V. parahaemolyticus* cells used in studies by Koga et al. (24) and Wong et al. (43). Culture growth phase clearly contributes to the experimental outcome of *V. parahaemolyticus* acid tolerance studies.

Estuarine and brackish waters are natural habitats for *V. parahaemolyticus* (5, 6). These cells usually experience little variation in environment conditions, i.e., temperatures of 10 to 30°C, pH of 7 to 8.5, and salinity of 0.8 to 3‰ (8, 13). This pathogen has been reported as highly sensitive to heat (55°C), hydrostatic pressure, and low-temperature pasteurization (2, 7). Because *V. parahaemolyticus* is not typically required to survive under extreme ranges of environmental conditions in nature, many strains may lack effective mechanisms for surviving conditions such as acid stress, which can be tolerated by some other foodborne pathogens.

In this study, the majority of *V. parahaemolyticus* strains tested were highly sensitive to a pH (3.6) that can be tolerated by other major foodborne pathogens (e.g., *E. coli* (35)). Log-phase but not stationary-phase *V. parahaemolyticus* displayed an ATR following exposure to sublethal acidic conditions. Exposure to these conditions impaired subsequent survival and cytotoxicity of stationary-phase cells for all strains except FSL-Y1-013. The recent publication of the *V. parahaemolyticus* genomic sequence (28) will provide the opportunity to use microarrays and proteomics to dissect the complex phenomenon of bacterial behavior following acid exposure and to identify genetic responses important for *V. parahaemolyticus* virulence.

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