Acid adaptation induces cross-protection against some environmental stresses in *Vibrio parahaemolyticus*

Tetsuro Koga,* Fumie Sakamoto, Akiko Yamoto, and Kenji Takumi

Department of Food Microbiology, Tokushima University School of Medicine, Tokushima 770–8503, Japan

1Department of Food and Nutrition, Okayama Women’s College, Kurashiki 710–8511, Japan

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The relationship of acid adaptation to the resistance of other environmental stresses was examined in *Vibrio parahaemolyticus*. Acid-adapted cells were found to have increased resistance to various stresses, including heat, crystal violet, bile, and deoxy cholic acid. However, heat-adapted cells showed no increased resistance against acid stress. Adaptation required protein synthesis, since treatment with chloramphenicol during adaptation to pH 5.3 prevented the development of acid resistance. Acid-adapted cells showed an increased amount of outer membrane protein with an apparent molecular weight of 27,000. These results show that acid-induced cross-protection involved changes in outer membrane protein composition and the known enhancement of intracellular pH homeostasis.

Key words——acid adaptation; cross-protection; *Vibrio parahaemolyticus*

The ability to survive in the acidic conditions of specific foods and the gastrointestinal tract is important to food-borne pathogens. They must survive extremely acidic environments, as well as moderately acidic environments containing weak acids. The ability to survive in the extreme acidic conditions of the stomach can contribute to virulence by increasing the possibility of intestinal colonization. Acidification by weak acid is used extensively in food processing to control growth and survival of spoilage-causing and pathogenic microorganisms (Brown and Booth, 1991). However, many bacteria have been shown to develop acid tolerance on exposure to acid shock. Therefore the ability of food-borne pathogens, including *Salmonella typhimurium* (Foster and Hall, 1990), *Escherichia coli* (Goodson and Rowbury, 1989), and *Listeria monocytogenes* (Kroll and Patchett, 1992), to adapt to acidic conditions is a concern in food safety (Brown and Booth, 1991). Adaptation enhances tolerance to stresses and may promote survival in adverse environments. Cross-protection induced by acid adaptation against distinct environmental stresses has previously been reported for *S. typhimurium* (Leyer and Johnson, 1993).

*Vibrio parahaemolyticus* is a marine bacterium that causes gastroenteritis associated with the consumption of contaminated seafoods (Miwatani and Takeda, 1976). The bacterium can survive diverse environmental conditions: outside the host in seawater and inside the host. The ability of this bacterium to withstand acidic conditions is also an important factor of survival in acidic foods, food processing treatment, and the gastrointestinal tract. We have previously shown that nutrient-starved cells of this bacterium induces cross-protection against heat, osmotic, or H2O2 challenge (Koga and Takumi, 1995a). However, concerning *V. parahaemolyticus*, acid adaptation and cross-protection against some environmental stresses induced by acid stress have not been examined.

In this study, we describe that acid adaptation of *V. parahaemolyticus* induces cross-protection against some environmental stresses and that the response involves changes in the composition of outer membrane proteins.

Materials and Methods

Bacterial strains and growth conditions. *V. parahaemolyticus* Na2 (Nakasone and Iwanaga, 1991), a clinical isolate with a positive Kanagawa phenomenon that correlates well with the human pathogenicity of the bacterium was used throughout this study. The
bacterium was grown in LB broth (Bertani, 1951) containing 3% NaCl (3% NaCl-LB broth) at 37°C with aeration in a shaking water bath. For acid adaptation, when the culture reached \( A_{660} \) of 0.10, it was acidified to a pH of 5.3 with 4 N HCl. It was allowed to grow until it reached an \( A_{660} \) of 0.4, and the cells were harvested by centrifugation. The nonadapted control was grown to an \( A_{660} \) of 0.4 in nonacidified 3% NaCl-LB broth, and the cells were harvested by centrifugation. The acid-adapted and nonadapted cell pellets were washed once in 0.1M phosphate buffer (PB) containing 0.5 M NaCl (0.5 M NaCl-PB; pH 7.2). These cells were used to determine the resistance to stress. Colony-forming units (CFU) were determined on 3% NaCl-LB agar (Bertani, 1951) plates by using a surface spread plate technique.

**Measurement of acid tolerance.** Cell suspension was diluted to about \( 5 \times 10^7 \) CFU/ml in 3% NaCl-LB medium preadjusted with HCl to pH 2.0, then incubated at room temperature. A portion of each sample was taken periodically during each treat, serially diluted in 0.5 M NaCl-PB, and plated onto 3% NaCl-LB agar plates to determine the CFU. The survival rate (%) was expressed as the fraction of CFU at a given time divided by the CFU immediately before each treatment.

**Measurement of tolerance to other stresses.** For heat challenge, cell suspension was diluted to about \( 5 \times 10^7 \) CFU/ml in 0.5 M NaCl-PB prewarmed in a 47°C water bath. For crystal violet challenge, cell suspension was diluted to about \( 5 \times 10^7 \) CFU/ml in 3% NaCl containing crystal violet (30 \( \mu \)g/ml). For bile challenge, cell suspension was diluted to about \( 5 \times 10^7 \) CFU/ml in 0.5 M NaCl-PB containing bile (5.0%, Ox Gall, Sigma Chemical Co., St. Louis, MO). For deoxy cholic acid (DOC) challenge, cell suspension was diluted to about \( 5 \times 10^7 \) CFU/ml in 0.5 M NaCl-PB containing DOC (0.1%). Cell viability was determined at time intervals as described above, and the survival rate (%) was expressed as described above.

**Preparation of outer membrane.** Cell envelope pellets were prepared from acid-adapted and nonadapted cells as described previously (Koga and Takumi, 1994). The cell envelope preparation was then treated with sodium lauroyl sarcosinate (Sarkosyl), as described previously (Koga and Takumi, 1995b). The Sarkosyl-insoluble material was used as the outer membrane preparation.

**SDS-polyacrylamide gel electrophoresis (SDS-PAGE).** SDS-PAGE was performed according to the method of Laemmli (Laemmli, 1970) by using a separation gel of 12% acrylamide. Samples (about 10 \( \mu \)g of protein) were dissolved in the sample buffer of Laemmli by heating at 100°C for 5 min. Protein bands were visualized with Coomassie brilliant blue R-250.

For molecular weight estimation, a low molecular weight electrophoresis calibration kit (Pharmacia Fine Chemicals, Uppsala, Sweden) was used as molecular weight standards.

**Results**

**Induction of acid tolerance response**

Acid adaptation has been shown to enhance the resistance of *S. typhimurium* to lethal concentration of HCl (Foster and Hall, 1990). We were interested in determining whether acid adaptation would also increase the tolerance of *V. parahaemolyticus* to acid challenge. Aliquots from the acid-adapted cells and the nonadapted cells were challenged by adjusting pH of the medium to 2.0, and their viability was determined. They were more thermally tolerant than their nonadapted counterpart (Fig. 2). After 5 min of exposure, little difference in survival was observed between the two cultures after 30 min of exposure to acid; about 40-fold difference in the number of survivors was observed after 60 min.

**Tolerance of acid-adapted cells toward thermal stress**

Since heat is commonly used to inactivate pathogens in food processing, we initially investigated whether acid adaptation influenced thermal tolerance. Acid-adapted cells were challenged at 47°C, and their viabilities were determined. They were more thermally tolerant than their nonadapted counterpart (Fig. 2). After 5 min of exposure, little difference in survival was observed between the two cultures, but thereafter the nonadapted cells died off more rapidly than the acid-adapted cells. Cell death resulted in a 200-fold difference in the number of survivors after 10 min, and this difference decreased slightly over the remainder of the incubation. But the reproducibility of this difference...
after 20 min exposure was confirmed.

**Tolerance of acid-adapted cells toward hydrophobic dye crystal violet**

We tested whether acid-adapted cells had different tolerance toward the hydrophobic dye crystal violet that interacts with cell surfaces. Acid-adapted and nonadapted cells were exposed to 30 μg/ml of crystal violet. Acid-adapted cells exhibited increased tolerance when compared with nonadapted cells (Fig. 3). Approximately 16% of the original acid-adapted cell population was viable after 20 min of exposure, whereas the nonadapted culture died rapidly, showing 12% survival after 10 min exposure and decreasing about 1,500-fold during the 20 min.

**Tolerance of acid-adapted cells toward bile and DOC**

In the intestine, the bacteria may encounter bile and bile acid that have a strong detergent-like property. We were interested in determining whether acid-adapted cells of *V. parahaemolyticus* would increase the tolerance to these agents.

Acid-adapted cells showed higher resistance against both bile and DOC challenge in comparison with nonadapted cells (Figs. 4 and 5). The acid-adapted cells had about fivefold increased tolerance compared with nonadapted cells during the first 10 min of exposure to bile (5.0%), and though the killing rate decreased after this time, about fourfold difference in the number of survivors was still observed after 20 min (Fig. 4). When challenged with DOC (0.1%), acid-adapted cells had about sixfold increased tolerance compared with nonadapted cells during the first 10 min of exposure, and about 17-fold difference in the number of survivors observed after 30 min (Fig. 5).

**Survival of heat-adapted cells toward acid stress**

The heat-shock response conferred global protection against multiple physical and chemical stresses in many bacteria. Since the results presented above indicated that acid-adapted cells also induce thermal
tolerance, we examined whether heat-adapted cells are able to induce acid tolerance. Heat-adapted cells did not exhibit increased resistance against acid stress: Heat-adapted cells and nonadapted cells showed similar sensitivity to acid (Fig. 6). Although little difference in survival was observed between nonadapted and heat-adapted cells, these cells died off quicker than the acid-adapted cells. The heat-adapted cells exhibited lower acid resistance than the acid-adapted cells, in which a 20-fold difference was observed after 60 min.

Adaptive acid tolerance response requires protein synthesis

Acid tolerance could involve the physiological acti-

vation of a preexisting protective system or the induction of specific proteins that provide acid tolerance response. To determine whether synthesis of new protein is required for acid adaptation, cultures were treated with protein synthesis inhibitor chloramphenicol. Concentration as high as 5 μg/ml of chloramphenicol added at the same time the cells were exposed to the inducing pH of 5.3 did not fully inhibit induction of the acid tolerance response (data not shown); however, an inhibition of induction was achieved when chloramphenicol was added 10 min before exposure to pH 5.3. Chloramphenicol treatment eliminated the adaptive acid tolerance response, as shown in Fig. 7. This result suggests that proteins synthesized during adaptation at pH 5.3 are involved in the survival during subsequent exposure to pH 2.0.

Acid adaptation alters the composition of outer membrane protein

The production of outer membrane proteins is regulated by several environmental factors such as the osmolarity of the medium, carbon source, and temperature (Nikaido and Vaara, 1985). We were interested in whether the profile of outer membrane proteins was altered in cells adapted to acid. V. parahaemolyticus was adapted to acid as described above, and the composition of outer membrane proteins was examined by SDS-PAGE (Fig. 8). The protein with apparent molecular weights of about 40,000 (40K protein), 36,000 (36K protein), and 28,000 (28K protein) were the major outer membrane proteins of nonadapted cells grown in 3% NaCl-LB broth. However, when the outer membrane proteins of acid-adapted cells were analyzed, the production of a protein with apparent molecular weights of 27,000 (27K protein) and the reduction of 28K protein were observed. The amount of other major outer membrane proteins (40K and 36K proteins) were not affected by the acid adaptation.
Influence of NaCl concentration in the growth medium on the outer membrane protein composition

Because we have previously shown that different concentrations of NaCl in the growth medium change the composition of outer membrane protein from V. parahaemolyticus 3283-61 (Koga and Kawata, 1983), we examined the effect of NaCl concentration on the composition of outer membrane protein from this strain. Cells grew most rapidly in 3% NaCl-LB medium, but lower growth rates were observed in 7% NaCl-LB and 0.5% NaCl-LB media. SDS-PAGE profiles of the outer membrane fractions isolated from cells grown exponentially in LB medium containing different concentrations of NaCl are shown in Fig. 9. When the cells were grown in 0.5% NaCl-LB broth and 1% NaCl-LB broth, the amount of 27K protein increased compared with those of cells grown in 3% NaCl-LB broth. However, the amount of other major outer membrane proteins were little affected by the concentrations of NaCl in the medium. The outer membrane protein profile from cells grown in 7% NaCl-LB broth was similar to that from cells grown in 3% NaCl-LB broth. Thus the composition of outer membrane proteins from cells grown in 0.5% NaCl-LB broth and 1% NaCl-LB broth was similar to that of cells from acid-adapted cells grown in 3% NaCl-LB broth.

Acid resistance of the cells grown in LB broth containing different concentrations of NaCl

To determine whether the cells grown in 1, 3, and 7% NaCl broth showed variable resistance to acid stress, the viability of these cells was compared (Fig. 10). The cells grown in 1% NaCl-LB broth showed more acid tolerance than the cells grown in 3% NaCl broth. On the other hand, the cells grown in 7% NaCl-LB broth showed similar acid tolerance to that of the cells grown in 3% NaCl-LB broth. For example, after 60 min challenge, the viability of the cells grown in 1% NaCl-LB broth was 25%, whereas those of cells grown in 7% NaCl-LB broth and 3% NaCl-LB broth were 1.6 and 1.0%, respectively. These results suggest that the cells grown in 1% NaCl-LB broth, which produced an increased amount of 27K protein, showed greater resistance against acid challenge than the cells grown in 3% NaCl- and 7% NaCl-LB broth did.

Discussion

V. parahaemolyticus encounters a wide variety of environments during its life cycle. One component of the environment that fluctuates widely is pH. In nature, V. parahaemolyticus can experience and survive dramatic acid stresses that occur in diverse ecological niches ranging from seawater to the gastrointestinal tract of the host. The physiological state of the test organism could influence survival in foods, resistance to food processing and to chemical preservatives, and survival in the gastrointestinal tract of the host. Non-stressed cells grown in rich media in the laboratory probably do not accurately represent the physiology of cells found in the food and host environments. In this study, we examined whether acid adaptation cross-protected cells against environmental stresses. Our results show that acid adaptation affects the resistance of cells to a variety of stresses.

Acid-adapted V. parahaemolyticus had increased tolerance toward heat. The exposure of V. parahaemolyticus to starvation has been demonstrated to enhance resistance to heat, osmotic stress, and hydrogen peroxide (Koga and Takumi, 1995a). Starvation for carbon in E. coli also enhanced resistance to heat, oxidative stress, and osmotic stress (Jenkins et al., 1988). Heat shock proteins have been found to be induced in S. typhimurium and E. coli during starva-
tion for amino acids (Grossman et al., 1985; VanBogelen et al., 1987) and exposure to hydrogen peroxide (Christman et al., 1985; VanBogelen et al., 1987) or acid (Foster, 1991; Heyde and Portaliere, 1990). Although adaptation to a number of stresses or starvation increases thermal tolerance in bacteria by a mechanism involving heat shock proteins, the synthesis of heat shock proteins has not been shown to enhance acid resistance. Foster and Hall (1990) reported that heat shock did not increase acid resistance. In this study we found that heat shock did not increase acid resistance; however, acid adaptation increased resistance to heat. The results were consistent with those of Leyer and Johnson (1993) in *S. typhimurium*. Apparently, the presence of heat shock proteins is not sufficient to enhance acid resistance, but proteins expressed during acid adaptation do provide heat resistance. Protein synthesis during acid tolerance response induction was investigated by using the protein synthesis inhibitor chloramphenicol. The acid tolerance response in *V. parahaemolyticus* is shown here to depend, at least partially, on the de novo synthesis of proteins. This is also true for the acquisition of acid tolerance in several other organisms including *S. typhimurium* (Foster and Hall, 1990), *E. coli* (Raja et al., 1991), *Aeromonas hydrophila* (Karem et al., 1994), and *Streptococcus faecalis* (Kobayashi et al., 1986). We found that acid-adapted cells were more resistant to surface-active agents, and they were more tolerant than nonadapted cells toward crystal violet. The uptake of crystal violet depends on the permeability of the outer membrane, and mutants that have an altered outer membrane are more susceptible to the dye (Gustafsson et al., 1973). Acid-adapted cells were also more tolerant toward bile and DOC, which have a strong detergent-like property. Once the bacteria reached the intestinal tract, their ability to survive depended on their resistance to bile (Gilliland et al., 1984). Therefore it seems that acid-adapted bacteria is also favored to survive in the intestinal tract.

The involvement of the outer membrane in acid resistance has been reported. Several reports have demonstrated by the construction of lacZ fusion that genes responsible for acid adaptation alter the protein content of outer membranes (Heyde and Portaliere, 1987; Thomas and Booth, 1992). Of 18 proteins expressed in mildly acidic conditions (pH 5.8), 14 were associated with a total membrane fraction (Foster and Hall, 1990). Laub et al. (1989) reported that changes in the outer membrane lead to alteration susceptibility to penicillins when *Salmonella* spp. were grown at acidic pH (pH 5.2).

Leyer and Johnson (1993) reported that OmpC was expressed, OmpF was repressed, and at least one unidentified protein was present in the outer membrane of acid-adapted *S. typhimurium*. In this study, we found the enrichment of 27K protein and the reduction of 28K protein in the outer membranes of acid-adapted *V. parahaemolyticus*. Since we previously reported that the composition of outer membrane proteins of *V. parahaemolyticus* is affected by changing NaCl concentration in the growth medium (Koga and Kawata, 1983), we examined the effect of NaCl in the growth medium on the composition of outer membrane proteins and on the acid resistance of this bacterium. Increasing the NaCl concentration in a range of 3 to 7% resulted in almost no change of outer membrane protein composition, whereas decreasing NaCl to 1% resulted in an increase of 27K protein. The cells grown in 1% NaCl-LB broth were more resistant to acid than the cells grown in 3% NaCl-LB broth, and the cells grown in 7% NaCl-LB broth showed similar acid tolerance to the cells grown in 3% NaCl-LB broth. These results suggest that acid adaptation involves changes in the outer membrane, which show an increase of 27K outer membrane protein. This information supports the theory that acid adaptation of *V. parahaemolyticus* involves changes in the outer membrane structure and an increase in intracellular pH homeostasis by enhanced buffering, as proposed previously (Foster and Hall, 1991).

**References**


