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Relationship between the Effects of Stress Induced by Human Bile Juice and Acid Treatment in *Vibrio cholerae*

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ABSTRACT

The effects of low pH and human bile juice on *Vibrio cholerae* were investigated. A mild stress condition (exposure to acid shock at pH 5.5 or exposure to 3 mg of bile per ml for 20 min) slightly decreased (by ≤1 log unit) *V. cholerae* cell viability. However, these treatments induced tolerance to subsequent exposures to more severe stress. In the O1 strain, four proteins were induced in response to acid shock (ca. 101, 94, 90, and 75 kDa), whereas only one protein (ca. 101 kDa) was induced in response to acid shock in the O139 strain. Eleven proteins were induced in response to bile shock in the O1 strain (ca. 106, 103, 101, 96, 88, 86, 84, 80, 66, 56, and 46 kDa), whereas only one protein was induced in response to bile shock in the O139 strain (ca. 88 kDa). *V. cholerae* O1 and O139 cells that had been preexposed to mild acid shock were twofold more resistant to pH 4.5 (with times required to inactivate 90% of the cell population [*D*-values] of 59 to 73 min) than were control cells (with *D*-values of 24 to 27 min). Likewise, cells that were preexposed to mild bile shock (3 mg/ml) were almost twofold more tolerant of severe bile shock (30 mg/ml; *D*-values, 68 to 87 min) than were control cells (with *D*-values of 37 to 43 min). These protective effects persisted for at least 1 h after the initial shock but were abolished when chloramphenicol was added to the culture during the shock. Cells preexposed to acid shock exhibited cross-protection against subsequent bile shock. However, cells preexposed to bile shock exhibited no changes in acid tolerance. Bile shock induced a modest reduction (0 to 20%) in enterotoxin production in *V. cholerae,* whereas acid shock had no effect on enterotoxin levels. Adaptation to acid and bile juice and protection against bile shock in response to preexposure to acid shock would be predicted to enhance the survival of *V. cholerae* in hosts and in foods. Thus, these adaptations may play an important role in the development of cholera disease.

Cholera is an acute intestinal infection caused by *Vibrio cholerae,* usually of serogroup O1. To successfully infect a human host, *V. cholerae* bacteria must colonize the intestine and produce copious amounts of cholera toxin, a potent enterotoxin. In its severe form, cholera causes periodic voluminous ''rice water'' diarrhea that rapidly leads to dehydration *(13, 19, 28).*

As *V. cholerae* enters the human body through the digestive tract, it encounters a variety of stressful stimuli, such as extremely acidic pH, elevated temperature, and bile salts *(6, 18).* The bacterium must endure these stressors until it can reach the intestinal epithelium, where it can proliferate. The mechanisms by which bacteria survive in stressful environments represent an intriguing biological issue that applies to understanding the pathogenicity of various microorganisms. These mechanisms constitute the last line of defense of bacteria in their battle for life in hostile conditions *(10).* Stress proteins are synthesized when an invading pathogen finds itself in a hostile medium inside the host. Various forms of stressful stimuli can lead to the increased synthesis of stress proteins *(15).* It is generally believed that these proteins serve a protective function, although under nonstressful conditions, many of these proteins also play fundamental roles in normal cellular physiology *(32).*

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The ability to respond and adapt to low pHs and bile juice is an important aspect of the life cycles of many pathogenic organisms that colonize the gastrointestinal tract. The bacterial response to low-pH environments has been studied for several microorganisms *(12, 20, 31). Salmonella* Typhimurium can survive for extended periods at a pH as low as 4 by altering the expression of at least 52 proteins *(9).* This tolerance for stress can be even higher if the organism is first adapted to a moderately acidic pH before being exposed to an extremely low pH. This adaptation is called the acid tolerance response *(9, 10). V. cholerae* is a neutralophilic bacterium that is sensitive to mildly acidic conditions. Its sensitivity to acid in vivo explains the high infection doses that are required in vaccine studies involving human volunteers *(3).* Cells that are acid adapted prior to intragastric inoculation into animals survive much better than unadapted cells, implicating the acid tolerance response in the epidemic spread of *V. cholerae (24).*

Intestinal pathogenic bacteria are also likely to encounter bile, which is secreted into the lumen of the duodenum from the gallbladder through the bile duct. Bile is composed primarily of bile salts, anionic detergents with bactericidal activity. Thus, resistance to bile is essential for the survival of enteric pathogens. The exposure of *Enterococcus fecalis* to a sublethal challenge with bile salts was found to induce the synthesis of 18 proteins; the mean bacterial survival time was also increased in the presence of

higher concentrations of bile salts *(8). V. cholerae* exhibits enhanced resistance to bile, and this resistance has been suggested to result from the modulation of outer membrane proteins by ToxR, a transmembrane DNA-binding protein *(27).* However, little is known about how exposure to various stresses affects the survival of this microorganism in response to subsequent, more severe stresses.

Bacterial cells that have undergone acid adaptation or acid shock may exhibit cross-protection against other stresses, such as heat *(7, 22),* osmotic stress *(4, 22),* and sodium lactate *(11).* During the processing of certain foods or during passage thorough the digestive tract, *V. cholera* would be exposed to acidic conditions that could impart tolerance to further exposure to acid or to human bile juice. Furthermore, such exposure could alter the enterotoxin production of *V. cholera.*

In the present study, we have demonstrated that acid shock induces protection against human bile in *V. cholerae.* We also characterize the effects of acid and bile shock on protein synthesis and enterotoxin production. Our findings suggest that preexposure to mild acid- or bile-induced shock may play an important role in bacterial adaptation to subsequent, more severe stresses.

MATERIALS AND METHODS

Culture conditions. The toxigenic *V. cholerae* strains 7677 Ogawa O1 (clinical isolate from Thailand) and 1837 Ogawa O139 (clinical isolate from Bangladesh) were maintained as a stock culture in Luria-Bertani (LB) agar (1% NaCl, 1% pancreatic digest of casein, 0.5% yeast extract, and 1.5% agar) at room temperature. These strains were originally provided by Elisa Elliot (U.S. Food and Drug Administration, Washington, D.C.). Active cultures were obtained by transferring aliquots of the culture into test tubes containing 10 ml of LB broth (the same composition as described above but without agar), and incubated overnight (16 to 18 h) at 37°C. All experiments were performed at least in triplicate.

Bile samples. The bile of deceased individuals was kindly provided by the Legal Medicine Department of the University Hospital of the Autonomous University of Nuevo León, Monterrey, Nuevo León, Mexico. Samples were collected from deceased people ≤8 h after death, and these samples were stored at 4°C until they were tested for sterility by inoculation onto blood agar, onto nutrient agar, and into brain heart infusion broth incubated under aerobic conditions, as well as into fluid thioglycollate medium (Difco Laboratories, Detroit, Mich.), onto blood agar, and onto nutrient agar under anaerobic conditions. Only samples that were free of bacterial contamination were used for further study. The samples were pooled and freeze-dried *(16).* Vials were stored frozen at −20°C until they were needed.

Acid or bile juice tolerance assay. Aliquots (40 μl each) of activated cultures of *V. cholerae* were inoculated into tubes containing 4 ml of LB broth and grown at 37°C until an optical density at 600 nm of 0.10 to 0.15 (ca. 1×10^8 CFU/ml) was reached (mid-log phase). Cultures were acid shocked from pH 3 to pH 5.5 (in pH increments of 0.5) with the addition of 1 M HCl (Sigma-Aldrich Química) to LB broth (pH was measured at the beginning of the treatment with a pH meter) or bile shocked at 1.0, 2.0, 2.5, 3.0, and 5.0 mg/ml for 20 min and incubated at 37°C. During the treatments, cell viability decreased at pH levels of <5.5 and at bile juice concentrations of >3.0 mg/ml (data not shown).

For the acid or bile tolerance assay, cells that reached mid-log phase were shocked either at pH 5.5 or with 3.0 mg of bile per ml for 20 min. Bacterial cells were then harvested by centrifugation and resuspended in fresh LB broth. Cells were then subjected to tolerance assays with lethal treatments involving a pH of 4.5 or 30 mg of bile per ml. Cell viability was determined by plate counts in LB agar. For this purpose, aliquots of cultures were obtained 30, 60, and 90 min after the addition of acid or bile *(14).* The plates were incubated at 37°C for 24 to 36 h. The duration of the acquired tolerance was determined as follows. After the cultures were acid (pH 5.5) or bile (3 mg/ml) shocked, cells were harvested by centrifugation and resuspended in fresh LB broth, and after 30 and 60 min they were challenged at pH 4.5 or with 30 mg of bile per ml as described above. Tolerance was determined by the time required to inactivate 90% of the population (*D*-value) *(17),* which was determined from the death curves. The *t* test was used to analyze the differences between the slopes of the various curves *(33).*

Cross-protection. To determine whether bile tolerance was induced as a result of a previous acid shock, several culture tubes were challenged at pH 5.5 for 20 min. The cultures were immediately centrifuged, and cells were resuspended in fresh LB broth plus bile (final concentration, 30 mg/ml). Control tubes were not acid shocked. Numbers of viable cells were determined at 30-min intervals, and *D*-values were determined as described above. The experiment was also performed in reverse to determine whether a prior bile shock (3 mg/ml for 20 min) was able to confer acid tolerance at pH 4.5.

Effect of chloramphenicol on adaptation. To determine the MIC of chloramphenicol for *V. cholerae,* strains were inoculated into 10-ml volumes of LB medium containing chloramphenicol concentrations ranging from 30 to 200 μg/ml at pH 6.5. The cultures were incubated at 37°C for 16 h, and then viable counts were determined as described above.

The MIC of chloramphenicol for *V. cholerae* growth was found to be 60 μg/ml. The acid, bile juice, and cross-protection assays were then repeated with the addition of either chloramphenicol at a final concentration of 100 μg/ml or an identical volume of sterile distilled water during the sublethal treatment. Unadapted cells were also treated with and without chloramphenicol.

Radiolabeling of proteins. Cells were grown in culture tubes (13 by 100 mm) containing 2 ml of LB broth at pH 7.0 and incubated at 37°C. When the cell cultures reached an A_{600} of 0.10 to 0.15 (mid-exponential phase), they were shocked with either acid or bile juice under the indicated conditions. After 5 min of the sublethal shock, 100 μCi of ^{35}S-labeled methionine and cysteine (^{35}S Cell-Labeling mix, Amersham Pharmacia Biotech, Piscataway, N.J.) was added to each sample, and the incubation was continued under the same stress conditions for an additional 15 min. Unlabeled amino acids (final concentration, 40 μg of methionine plus 10 μg of cysteine [Sigma-Aldrich Química, Mexico, D.F., Mexico] per ml) were added to the tubes and the samples were rapidly cooled on ice to quench the uptake of radioactivity. Cells were pelleted by centrifugation for 10 min at 10,000 × *g* at 4°C. The pellet was then washed twice with 30 mM Tris-HCl buffer (pH 7.6).

Cells were solubilized as described by Dascher et al. *(5),* with some modifications. Briefly, the pellet was resuspended in 2 ml of TBS buffer (0.41 M Tris, 0.40 M boric acid, 1% [wt/vol] sodium dodecyl sulfate [SDS] [pH 8.6]) containing 5% sucrose and 5 μg of DNase per ml (Sigma-Aldrich Química). The mixture was

TABLE 1. D-*values for acid-shocked* V. cholerae *cells*[a]

V. cholerae strain (serogroup)	Time after acid shock (min)	*D* (min)[b] Control cells	*D* (min)[b] Acid-shocked cells
C7677 (O1)	0	24 ± 1	73 ± 11
	30	30 ± 3	69 ± 12
	60	27 ± 6	73 ± 9
	90	22 ± 2	23 ± 3
	0 + chloramphenicol	25 ± 6	24 ± 1
1837 (O139)	0	27 ± 5	59 ± 10
	30	28 ± 10	53 ± 9
	60	32 ± 5	46 ± 7
	90	24 ± 5	25 ± 6
	0 + chloramphenicol	21 ± 4	20 ± 1

[a] Cells were acid shocked at pH 5.5 for 20 min and were then either challenged at pH 4.5 (0 h) or returned to pH 7.0 for 30 and 60 min prior to challenge.
[b] Mean ± standard deviation.

TABLE 2. D-*values for bile-shocked* V. cholerae *cells*[a]

V. cholerae strain (serogroup)	Time after bile shock (min)	*D* (min)[b] Control cells	*D* (min)[b] Bile-shocked cells
C7677 (O1)	0	37 ± 6	68 ± 8
	30	33 ± 8	65 ± 10
	60	38 ± 7	66 ± 5
	90	38 ± 3	39 ± 5
	0 + chloramphenicol	36 ± 5	38 ± 7
1837 (O139)	0	43 ± 6	87 ± 7
	30	40 ± 1	85 ± 10
	60	40 ± 7	75 ± 11
	90	42 ± 7	49 ± 9
	0 + chloramphenicol	41 ± 6	40 ± 8

[a] Cells were shocked with bile juice (3.0 mg/ml) for 20 min and were then either challenged with bile juice (30 mg/ml) (0 h) or returned to fresh LB broth for 30 and 60 min prior to challenge.
[b] Mean ± standard deviation.

incubated at 37°C for 30 min and was then frozen at −20°C for 12 to 14 h to disrupt the cells. To determine the amount of radioactively labeled methionine and cysteine incorporated into protein, 5 μl of the sample was placed in the center of a Whatman GF/A paper filter (Whatman International Ltd., Maidstone, UK). The sample was allowed to dry, and the filter was then placed in 10% trichloroacetic acid for 5 min to precipitate the protein. The filter was then washed 10 times with saline, dehydrated with absolute ethanol, and allowed to dry. Radioactivity was measured with a scintillation counter (Model Delta 300, TM Analytic, Elk Grove Village, Ill.).

Gel electrophoresis. Radioactive samples were mixed with 2× sample buffer (3% Tris, 20% β-mercaptoethanol, 10% SDS, 0.02% bromophenol blue, 40% glycerol [pH 6.8]), heated at 95°C for 3 min, and then centrifuged to remove debris. SDS–polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli *(21)* with a 4% (wt/vol) stacking gel and a 10% (wt/vol) separating gel. Protein samples containing 100,000 cpm were applied to each lane. Myosin (molecular mass, 205 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97.4 kDa), bovine albumin (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa) were obtained from Sigma-Aldrich Química and used as molecular weight standards. Gels were stained with Coomassie brilliant blue R-250 and then dried at 60°C under vacuum prior to exposure to Kodak X-OMAT AR film for 3 days at −70°C.

Effect of acid or bile juice on enterotoxin formation. Tubes (13 by 100 mm) of LB broth were inoculated with the activated cultures (1% inoculum). Tubes were then incubated in a water bath at 37°C, and when bacterial cultures reached an A_{600} of 0.1 to 0.15 (mid-exponential phase), they were subjected to either acid (pH 5.5) or bile (3.0 mg/ml). After 20 min, cells were centrifuged (8,000 × *g* at 37°C for 10 min), resuspended in 2 ml of fresh medium, and incubated overnight at 37°C. Cells were then pelleted by centrifugation (10,000 × *g* at 4°C for 20 min), and the supernatant was removed, freeze-dried, and then resuspended in a small volume of distilled water for enterotoxin determination as previously described *(29)*.

RESULTS

The results obtained indicate that sublethal acid (pH 5.5) or bile (3.0 mg/ml) shock increased the subsequent tolerance of *V. cholerae* to a homologous lethal stress (pH 4.5 or 30 mg of bile per ml; $P \leq 0.05$). For example, after a sublethal acid shock, the *D*-value (at pH 4.5) for strain 7677 increased from 24 to 73 min (Table 1). After a sublethal bile shock, cells were approximately twofold more resistant to 30 mg of bile per ml (Table 2). In both cases, the acquired tolerance was maintained for 60 min after the acid or bile shock treatment. After 90 min, no significant difference from the tolerance of control samples was observed.

We next investigated whether adapted cells acquired cross-tolerance to the other stress. Acid-adapted cells showed significant cross-protection against bile. With bile at 30 mg/ml, the *D*-value for cells that had previously been acid shocked was twofold higher than that for cells that had

TABLE 3. D-*values for previously acid-shocked (pH 5.5)* V. cholerae *cells at a bile concentration of 30 mg/ml*

V. cholerae strain (serogroup)	Time after acid shock (min)	*D* (min)[a] Control cells	*D* (min)[a] Acid-shocked cells
C7677 (O1)	0	24 ± 7	55 ± 3
	30	30 ± 3	56 ± 8
	60	27 ± 6	47 ± 2
	90	22 ± 7	21 ± 9
	0 + chloramphenicol	25 ± 6	24 ± 1
1837 (O139)	0	27 ± 5	65 ± 10
	30	28 ± 10	57 ± 9
	60	32 ± 5	56 ± 7
	90	24 ± 5	25 ± 4
	0 + chloramphenicol	21 ± 4	20 ± 1

[a] Mean ± standard deviation.

TABLE 4. *Effect of acid or bile shock on enterotoxin formation in* V. cholerae

V. cholerae strain (serogroup)	Sublethal stress	Enterotoxin concn (mg of enterotoxin/mg of protein)[a]	
		Control cells	Shocked cells
C7677 (O1)	pH 5.5	31 ± 5	30 ± 6
	Bile juice (3.0 mg/ml)	27 ± 3	20 ± 3
1837 (O139)	pH 5.5	28 ± 7	31 ± 1
	Bile juice (3.0 mg/ml)	29 ± 5	32 ± 1

[a] Mean ± standard deviation.

not been acid shocked (Table 3). However, pretreatment with bile did not induce cross-protection against acid challenge. Moreover, bile adaptation led to increased sensitivity to the effects of acid shock. The *D*-value at pH 4.5 for bile-preshocked cells of strain 7677 was 17 min, compared with 20 min for control cells. For strain 1837, *D*-values were 6 and 8 min for preshocked and control cells, respectively.

Chloramphenicol treatment eliminated tolerance to acid and bile, along with the cross-protection observed (Table 3). These results, similar to those of several previous studies on other bacteria *(8, 12, 15),* suggest that proteins synthesized during adaptation play a role in survival during subsequent, more drastic conditions.

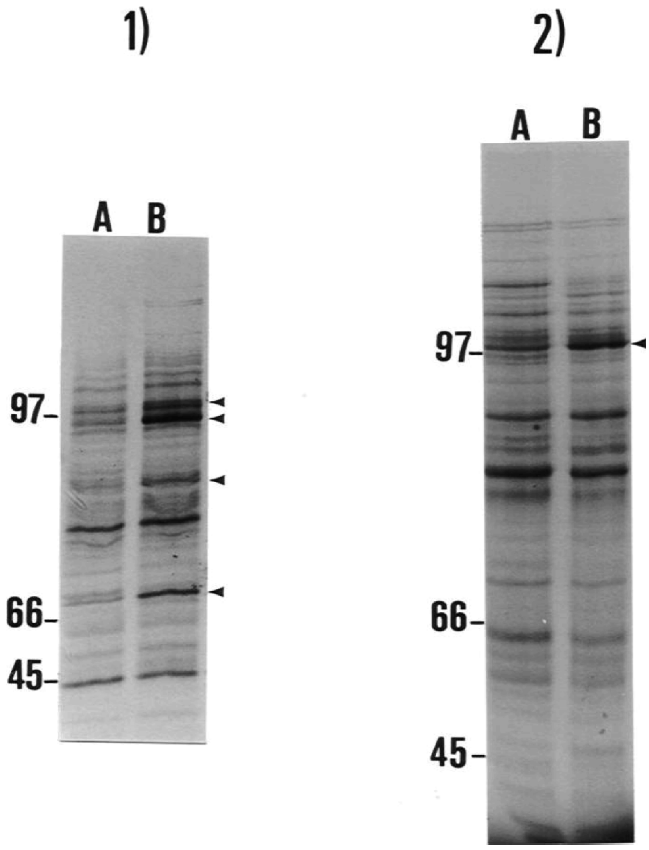

FIGURE 1. *Autoradiographic protein profiles of* V. cholerae *strains 7677 (O1) [1] and 1837 (O139) [2]. Cells were radiolabeled for 25 min through the addition of ^{35}S-labeled methionine and cysteine after exposure to mild acid shock as described in "Materials and Methods." Lane A, control cells; lane B, acid-shocked cells. The migration positions and sizes of protein standards (in kilodaltons) are indicated on the left; arrows at the right indicate the positions of proteins induced in response to acid shock.*

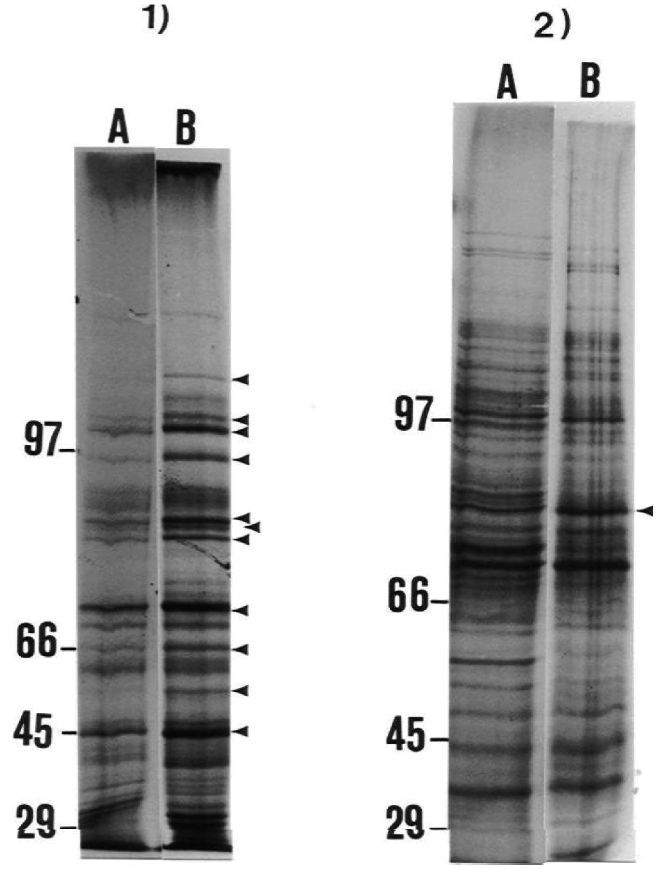

FIGURE 2. *Autoradiographic protein profiles of* V. cholerae *strains 7677 (O1) [1] and 1837 (O139) [2]. Cells were radiolabeled for 25 min through the addition of ^{35}S-labeled methionine and cysteine after sublethal bile shock as described in "Materials and Methods." Lane A, control cells; lane B, bile-shocked cells. The migration positions and sizes of protein standards (in kilodaltons) are indicated on the left; arrows at the right indicate the positions of proteins induced in response to bile shock.*

The enterotoxin levels measured after sublethal acid shock were similar to those observed for control samples (without the sublethal shock) for both strains (Table 4). However, when bacteria were subjected to sublethal bile shock, a modest but significant ($P \leq 0.05$) decrease in enterotoxin production was observed for the O1 strain. No difference in enterotoxin production was observed for the O139 strain.

Analysis of cellular protein synthesis with SDS-PAGE and autoradiography revealed sets of proteins induced by acid shock (Fig. 1) and by bile shock (Fig. 2). However, the two strains of *V. cholerae* differed with respect to their numbers of stress-induced proteins. Four proteins (ca. 101, 94, 90, and 75 kDa) were strongly induced by acid shock in the O1 strain, whereas only one protein (ca. 101 kDa) was induced by acid shock in the O139 strain. Eleven proteins (ca. 106, 103, 101, 96, 88, 86, 84, 80, 66, 56, and 46 kDa) were induced by bile shock in the O1 strain, whereas

only one protein (ca. 88 kDa) was detected in response to bile shock for the O139 strain.

DISCUSSION

The results obtained in the present study show some agreement with previously reported results indicating that bile can induce the expression of specific proteins in response to stresses such as membrane perturbation, oxidative stress, and DNA damage *(2).* Grupta and Chowdhury *(13)* reported that the O1 strain of *V. cholerae* exhibits decreased expression of the enterotoxin gene in response to crude ox bile juice shock. In this study, we observed only a slight reduction in the production of toxin in the O1 strain after exposure to sublethal conditions involving human bile juice, and we observed no reduction for the O139 strain. Most experiments on the effect of bile on *V. cholera* have employed ox bile. However, the composition and properties of animal bile differ from those of human bile *(1).* It is important to note that this study employed bile from deceased people who had no apparent biliary disease.

V. cholerae O139 appears to have been derived from the pandemic El Tor biotype but has lost the characteristic O1 somatic antigen; it has gained the ability to produce a polysaccharide capsule, and it seems to have retained the epidemic potential of O1 strains. *V. cholerae* O139 also lacks several kilobases of the genetic material necessary for the production of the O1 antigen *(28).* Events like these could be the reason for the differences found between the O1 and O139 strains. It will be interesting to identify the proteins induced in response to stress for each serotype to determine specific roles in physiology or pathogenesis.

Although little is known about the relationship between the acid and bile responses, it has been established that OmpU plays an important role in bacterial survival in response to these stresses *(23, 25).* Recent data indicate that the treatment of *V. cholerae* with bile results in a ToxR-dependent increase in levels of *ompU* transcription *(26).* Although it was demonstrated that *ompU* transcription apparently remains unaffected by organic acid stress, its presence is necessary for *V. cholerae* to survive this condition *(23).*

Since *V. cholerae* is sensitive to mild acidic conditions and its sensitivity to acid in vivo leads to the requirement for high doses for infection *(3),* any conditions that alter its acid tolerance may be very important for its pathogenicity. Here, the cells were treated at pHs of 5.5 and 5.0. Apparently, these conditions are drastic for this bacterium, since it has been reported that *V. cholerae* is sensitive to low pH; however, studies on the effect of pH on the viability of vibrios have indicated that vibrios can survive in media at pHs as low as 4.8. Also, in some foods this bacterium can survive at pHs as low as 4.5 *(30).* Our results show that exposure to acid or human bile stresses can allow *V. cholerae* cells to rapidly develop tolerance to these stresses and to homologous stresses. This tolerance to both severe acid and severe bile treatments required protein synthesis, since treatment with chloramphenicol during preshock prevented the development of this resistance. The cross-protective effects observed show that *V. cholerae* is remarkably adaptable.

Stress response is a temporal phenomenon that depends on each organism. For example, in *Clostridium perfringens* the adaptive response is maintained 2 h after the sublethal shock occurs *(14).* In the present study, *V. cholerae* was found to maintain tolerance to a low pH and to bile for 60 min after the sublethal shock occurred.

V. cholerae could contaminate some foods that are acid processed and consumed in a short time. This situation would be important, since shocked bacteria could become tolerant to acidic conditions during food preparation or passage through the stomach. Moreover, *V. cholerae* exposed to mild acidity becomes acid habituated, and if it is subsequently ingested by humans, it could resist stomach acidity and subsequent exposure to bile juice. This report describes the initial experiments of a long-term study designed to eventually delineate the cellular and physiological responses of *V. cholerae* to severe environmental conditions. In conclusion, the results obtained in the present study show that *V. cholerae* can survive in these vastly different environments and, furthermore, that it can both distinguish and appropriately adjust to alternative surroundings.

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