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Effect of Bile on *Vibrio parahaemolyticus*†

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Many enteric pathogens are thought to enter a viable but nonculturable state when deprived of nutrients. Virulent strains of the enteric pathogen *Vibrio parahaemolyticus* are rarely isolated from their low-nutrient aquatic environments, possibly due to their nonculturability. Host factors such as bile may trigger release from dormancy and increase virulence in these strains. In this study, the addition of bile or the bile acid deoxycholic acid to estuarine water-cultured bacteria led to an increase in the direct viable count and colony counts among the virulent strains. This effect was not demonstrated in the nonvirulent strains, and it was reversed by extraction of bile acids with cholestyramine. Bile-treated *V. parahaemolyticus* had lower levels of intracellular calcium than untreated cells, and this effect coincided with an increase in the number of metabolically active cells. Chelation of intracellular calcium with BAPTA/AM (R. Y. Tsien, Biochemistry 19:2396–2402, 1980) produced similar results. Addition of bile to *V. parahaemolyticus* cultures in laboratory medium enhanced factors associated with virulence such as Congo red binding, bacterial capsule size, and adherence to epithelial cells. These results suggest that a bile acid-containing environment such as that found in the human host favors growth of virulent strains of *V. parahaemolyticus* and that bile acids enhance the expression of virulence factors. These effects seem to be mediated by a decrease in intracellular calcium.

Many enteric pathogens are capable of surviving diverse environmental conditions. These bacteria may persist outside of the host in an aqueous, low-nutrient environment by entering a viable nonculturable state (60, 65). This physiological state is characterized by the lack of detectable growth but apparent metabolic activity (47, 60). The dormant bacteria may become active upon ingestion by a host, and dormant cells of several pathogens have induced disease after oral administration to test animals (12, 27, 35, 61). The ingested microorganisms must adhere, replicate, and produce virulence factors before they are flushed from the intestine (44, 45). Thus, the ability to respond rapidly is essential for the success of the pathogen and requires the bacterium to sense its surroundings (44, 45).

The autochthonous estuarine bacterium *Vibrio parahaemolyticus* may consist of two classes of strains (29, 32, 36). One class, Kanagawa phenomenon positive (K+), causes hemolysis when grown on high-[Na^+] blood agar, and strains of this class cause food-related gastroenteritis (37, 46). Kanagawa phenomenon-negative (K−) strains are less frequently associated with human disease. A paradox associated with this organism is that hemolytic strains of the bacterium are commonly isolated from gastroenteritis victims but rarely from seafoods or estuarine water (4, 29, 46, 71). One possible explanation for this phenomenon may be that pathogenic strains are more sensitive to low-nutrient conditions and, while present in the aqueous environment, rapidly become nonculturable (29, 34, 50, 60, 65). Alternatively, pathogenic strains may be at a lower level in the estuarine environment but proliferate more readily in the host and elaborate virulence factors.

In this study, we have examined whether bile or its component bile acids might act on estuarine water-cultured *V. parahaemolyticus* in vitro. The bacteria were cultured until signs of nutrient deprivation were evident, and then the capacity of bile to act as an agent for enumeration of the bacteria by the direct viable count (DVC) and to affect cellular composition and function was evaluated. The findings indicate that bile indeed is active in regulating bacterial physiology and that it may play an important role in the host-pathogen interactions of *V. parahaemolyticus*. Our data suggest that bile might act on the bacteria through a calcium-dependent mechanism and thus may be analogous to the low-calcium response exhibited by other pathogenic bacteria.

MATERIALS AND METHODS

Bacterial cultures and growth conditions. The *V. parahaemolyticus* strains used in this study were P7, 38C1, and V6074 (all K+) and P6, P15, and 38C6 (all K−). Strain P7 was a human clinical isolate, while the remaining bacterial strains were environmental isolates. These bacteria were kindly provided by R. Colwell and R. Twedt or were from this laboratory and have been described previously (50). Culture of bacteria was in proteose peptone beef extract medium (PPBE) (10, 50) or sterile estuarine water (obtained from the Choptank River, a part of the Chesapeake Bay near Cambridge, Md.) at 35°C in a rotary incubator at 200 rpm as described previously (50). Partial composition of the estuarine water was 0.05 mg-atom of nitrogen/liter, 1.2 mg-atom of phosphorus/liter, 15 mg of organic carbon/liter, 0.06 mg-atom of soluble iron as Fe/liter, and 10 ppt salinity (10a, 20a). Aliquots of bacteria were withdrawn for assay after 0, 24, 48, 96, and 192 h of culture in estuarine water or as noted below.

Bacterial enumeration. Bacteria were enumerated by spread plating on PPBE agar with or without added oxgall (bile). The total count (TC) and the DVC were determined as described previously (39, 53). Briefly, bacteria were cultured in estuarine water and then incubated with nalidixic acid (20 μg/ml) and a substrate such as bile (0.015 to 1.5%), sodium deoxycholate (2 mM), sodium glycocholate (15 mM), yeast extract (0.05%), or BAPTA/AM (25 μM) (70) for 6 h at 35°C. Bacteria so treated were fixed with 2.5% formalin, stained with acridine orange (13 μM) (50), and observed by fluorescence microscopy. The TC was the total number of bacteria observed. The DVC was the number of metabolically active bacteria (filaments stained red). The relative proportion of metabolically active bacteria was expressed as (DVC/TC) × 100%. To determine the contribution of bile acids to bacterial activity, cholestyramine-extracted bile (bile treated twice with 60 mg of cholestyramine resin/ml to precipitate bile acids) was also tested (31, 64).

Intracellular calcium levels. Bacterium-free intracellular calcium levels ($[Ca^{2+}]_i$) were assayed as described previously (25) with Fura-2/AM. Cells were cultured and then suspended in assay buffer and loaded with 5 μM Fura-2/AM for 2 h at 35°C (28). Following washing to remove excess dye, calcium-dependent fluorescence was measured with a spectrofluorometer with excitation at 340 nm

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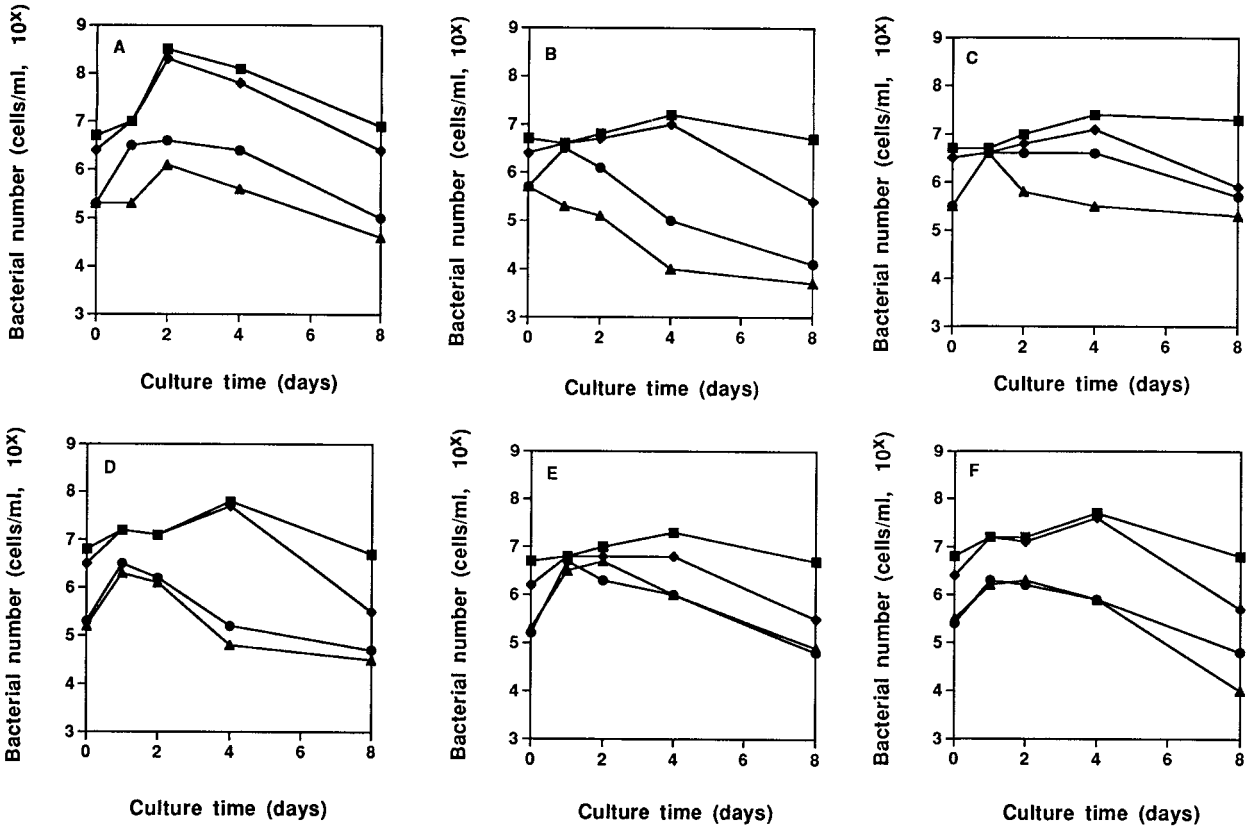

FIG. 1. Enumeration of *V. parahaemolyticus* P7 (A), 38C1 (B), V6074 (C), P6 (D), P15 (E), and 38C6 (F) cultured in estuarine water by TC (■), DVC (◆), and plate count with PPBE (▲) or with PPBE containing 0.15% bile (●).

and emission at 510 nm. $[Ca^{2+}]_i$ was calculated as $K_d \times [(F - F_{min})/(F_{max} - F)]$, with a K_d of 315 nM (24, 25). F is the fluorescence of the sample, while F_{min} is the minimum fluorescence and F_{max} is the maximum fluorescence of the sample in the presence of 100 μM ionomycin–10 mM EGTA or 100 μM ionomycin–10 mM $CaCl_2$, respectively. To determine the effect of bile on estuarine water bacteria (cultured 96 h), the cells were treated for 30 min with bile (0.15%, wt/vol) before assay of $[Ca^{2+}]_i$.

Effect of lanthanum on cellular phosphatidylglycerol levels. Bacteria were cultured in PPBE, estuarine water, or estuarine water containing the calcium antagonist lanthanum chloride (10 μM) (73). After 24 h of growth at 35°C, phospholipids were extracted, separated by thin-layer chromatography, and quantitated (1, 5, 50).

Determination of bacterial capsule size. Bacteria were cultured in PPBE broth or in PPBE broth with bile (1.5%, wt/vol) or 0.1% deoxycholate. Capsule stains were performed on cultures by counterstaining a bacterium-nigrosine smear with crystal violet. Bacteria were observed microscopically, and capsule size was measured with an eyepiece micrometer (3). Capsule size was determined for all cells in each of 10 fields per sample. Capsular materials were also extracted with 1% phenol (15). The extracts were dried and quantitated gravimetrically. To confirm the polysaccharide nature of the extracted capsular materials, the uronic acid content of the extracts was determined with the carbazole reagent of Dische (16).

Congo red dye binding. Bacteria were cultured on brain heart infusion agar containing Congo red (0.025%, wt/vol) with or without bile (1.5%, wt/vol) or 0.1% sodium deoxycholate at 35°C. Colonies were suspended in water, and the dye was extracted with acetone (2). Dye binding was expressed as the A_{488} of the extract divided by the A_{660} of the cell suspension.

Bacterial adhesion to cultured epithelial cells. Bacteria were cultured in PPBE broth overnight or in PPBE broth overnight followed by subculturing in PPBE broth with or without bile (1.5%, wt/vol) or 0.1% deoxycholate for 3 h and then were cultured a further 0.5 h in fresh prewarmed medium. All bacteria (10^6 bacteria in 10 μl added per well) were incubated with Int-407 cell monolayers (10^5 epithelial cells per well) in 24-well tissue culture plates overlaid with antibiotic-free Hanks' balanced salt solution (0.5 ml) for 30 min before washing (five washes in Hanks' balanced salt solution), lysis, and plating on PPBE agar. Results were expressed as a percentage of the inoculum (23).

RESULTS

Bacterial growth in estuarine water. Culture of *V. parahaemolyticus* in estuarine water resulted in an increased plate count by 24 h that subsequently declined following culture for 48 h (Fig. 1). Plate counts increased approximately 1 to 1.5 logs before a decline of from 1 to 2 logs. DVCs were similar to the plate counts at 24 h but continued to increase after the plate count declined. By 96 h of incubation, the DVC was 1.5 to 2 logs greater than the plate count. After 192 h of culture, the DVC declined but was still 0.5 to 1 log greater than the plate count. The TC was similar to the DVC up until 96 h. Subsequently, the TC remained greater than the DVC.

Effect of bile on the enumeration of *V. parahaemolyticus* by plate count. After 96 h of culture, plate counts on agar containing 1.5% bile were reduced, suggesting some inhibitory activity of the medium (data not shown). This effect was particularly pronounced for the K− strains. On plates containing 0.15% bile, little difference in the plate count was observed compared with that from cultures on medium without bile for K− strains (Fig. 1). However, plate counts of the pathogenic strains were approximately 0.5 to 1 log higher on the bile-containing medium.

Effect of bile as an activating agent in the DVC. When bile was utilized to determine the proportion of active bacteria, it was a more effective agent for K+ than K− *V. parahaemolyticus* (Table 1). Yeast extract, a commonly utilized agent for determining the DVC (53), was a more effective substrate than bile at eliciting a higher DVC for K− than K+ strains. Cho-

TABLE 1. Effect of substrate on bacterial activation

Strain	Proportion of bacteria metabolically active on substrate[a]					
	None	Yeast extract	Bile	CEB[b]	DOC[c]	BAPTA/AM
P7	5.0 ± 1.0	58.7 ± 2.6	95.3 ± 4.5	53.9 ± 5.8	99.8 ± 6.2	83.3 ± 11.1
38C1	6.1 ± 1.8	65.7 ± 0.7	95.1 ± 6.8	13.3 ± 5.6	100.0 ± 1.0	83.5 ± 7.6
V6074	2.5 ± 0.6	55.2 ± 0.9	93.1 ± 1.3	15.6 ± 5.9	75.4 ± 5.9	50.6 ± 3.6
P6	3.0 ± 0.5	80.0 ± 4.1	42.4 ± 4.8	37.4 ± 4.6	56.2 ± 4.3	79.7 ± 7.5
P15	0.1 ± 0.1	81.4 ± 2.9	34.6 ± 7.3	27.5 ± 7.3	79.8 ± 1.7	99.0 ± 8.2
38C6	0.5 ± 0.2	83.5 ± 3.0	45.6 ± 2.0	45.6 ± 3.5	13.8 ± 4.8	73.8 ± 7.2

[a] Values were calculated as (DVC/TC) × 100% and are means ± standard deviations.
[b] CEB, cholestyramine-extracted bile.
[c] DOC, deoxycholate.

lestyramine, a bile acid-binding resin, was utilized to remove bile acids from the bile. The DVC was then determined with the cholestyramine-extracted bile. Interestingly, the proportion of viable K+ bacteria enumerated was diminished while the number of K− cells was only slightly affected (Table 1).

Effect of bile acid replenishment on the DVC. Individual bile acids were added to the cholestyramine-extracted bile, and the DVC was determined. When the bile acid deoxycholic acid (in the salt form sodium deoxycholate) was used, the proportion of active K+ cells was increased nearly two- to eightfold relative to that of the bile acid-depleted bile. K− strains were more variable, and the DVC was diminished for one strain and increased only 0.5- to 2.8-fold for the other two isolates (Table 1). Proportions of metabolically active cells enumerated with deoxycholic acid by the DVC assay were 75.4 to 100% as compared to 13.8 to 79.8% for the K+ and K− strains, respectively. Glycochloic acid, another bile acid, was much less effective as an agent in the DVC assay (Table 1; data not shown). However, again the DVC obtained with the bile acid was greater for K+ than for K− bacteria.

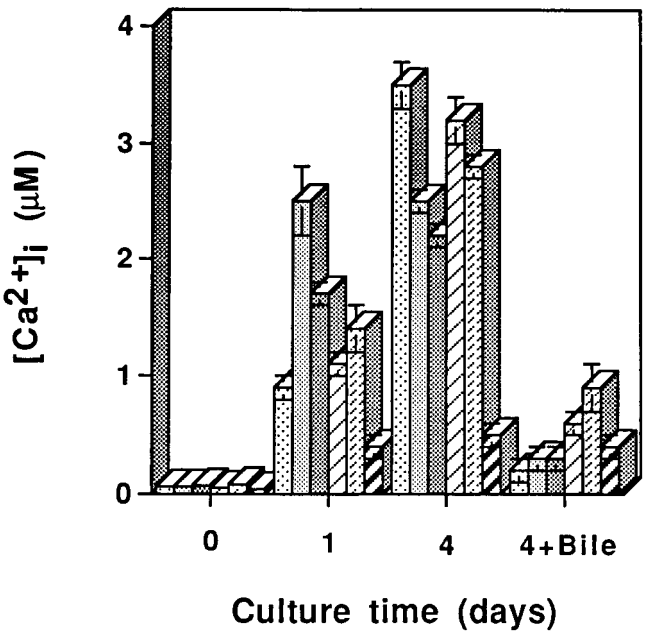

FIG. 2. $[Ca^{2+}]_i$ of estuarine water-cultured bacteria. Bacteria were cultured for 0, 1, or 4 days in estuarine water or for 4 days in estuarine water followed by treatment for 30 min with 0.15% bile. Symbols: ⊡, P7; ▨, 38C1; ▩, V6074; ◪, P6; ◪, P15; and ◪, 38C6.

Effectiveness of a calcium chelator as a substrate in the DVC assay. Bile acids can function as calcium chelators. Therefore, the membrane-permeative calcium chelator BAPTA/AM was assayed as an agent for the determination of the DVC. When it was combined with cholestyramine-extracted bile, enhanced numbers of active bacteria were obtained regardless of the bacterial strain. The proportion of active bacteria ranged from 50.6 to 99.0% with the chelator as compared to 13.3 to 53.9% when the bile acid-depleted bile alone was used (Table 1). A non-membrane-permeative form of the chelator was much less active. BAPTA/AM alone was less effective at increasing the DVC than when it was combined with the cholestyramine-extracted bile. A nutrient source in addition to the chelator appears to be required for the increased DVC.

Calcium level of estuarine water-cultured bacteria. $[Ca^{2+}]_i$ were increased in *V. parahaemolyticus* cultured in estuarine water (Fig. 2). By 24 h, $[Ca^{2+}]_i$ increased nearly 10-fold. A further increase was observed with some bacterial strains after 96 h of incubation.

When bacteria incubated for 96 h in estuarine water were treated briefly with bile, $[Ca^{2+}]_i$ was greatly diminished (Fig. 2). The decrease in cellular calcium was rapid and occurred within the 30-min treatment with bile. A 30-min treatment was selected because no increase in total cell numbers that might result in decreased calcium concentration was observed during this time (data not shown). The concentration of bile (0.15%) used for this treatment was selected because inhibition of plate counts had been observed with the higher concentration (1.5%) for some of the estuarine water-cultured bacteria. No inhibition was observed when 1.5% bile was included in PPBE.

Effect of a calcium antagonist on the phosphatidylglycerol level of estuarine water-cultured bacteria. In many cases, an increase in the phosphatidylglycerol level is observed in bacteria starved for nutrients (50). When *V. parahaemolyticus* was

TABLE 2. Effect of the calcium antagonist lanthanum ($LaCl_3$, 10 μM) on cell membrane phosphatidylglycerol

Strain	Phosphatidylglycerol level (% of total phospholipid)[a] under culture condition		
	PPBE	Estuarine water	Estuarine water + lanthanum
P7	8.2 ± 1.6	26.8 ± 2.7	16.7 ± 6.2
38C1	13.5 ± 2.8	27.1 ± 2.0	13.2 ± 0.3
V6074	14.0 ± 3.0	25.0 ± 1.9	11.2 ± 0.1
P6	11.9 ± 2.1	24.9 ± 1.6	20.8 ± 0.1
P15	12.5 ± 0.2	25.4 ± 1.7	13.8 ± 0.2
38C6	17.6 ± 1.7	27.5 ± 0.4	16.1 ± 0.4

[a] Values are means ± standard deviations.

TABLE 3. Effect of bile or deoxycholate (DOC) on bacterial capsule size

Strain	Bacterial capsule size (μm)[a] under culture condition[b]		
	PPBE	PPBE + bile	PPBE + DOC
P7	2.5 ± 0.1	6.1 ± 0.9	8.2 ± 0.5
38C1	2.5 ± 0.3	6.0 ± 0.6	7.1 ± 0.7
V6074	3.1 ± 0.3	8.5 ± 1.1	9.0 ± 0.4
P6	2.0 ± 0.1	5.8 ± 0.6	6.8 ± 0.9
P15	3.3 ± 0.2	5.5 ± 0.2	7.1 ± 0.8
38C6	2.8 ± 0.5	5.3 ± 0.2	6.9 ± 0.6

[a] Values are means ± standard deviations.
[b] PPBE + bile, broth medium containing 1.5% bile; PPBE + DOC, broth medium containing 0.1% sodium deoxycholate.

cultured in estuarine water, the cellular level of phosphatidylglycerol was increased (Table 2). Addition of the calcium antagonist lanthanum (10 μM) to estuarine water prior to bacterial culture reduced the accumulation of phosphatidylglycerol observed in bacteria cultured in estuarine water alone (Table 2). The low level of lanthanum utilized in this study did not cause precipitation, while higher levels caused the rapid formation of a flocculum in the estuarine water and thus could not be assayed.

Effect of bile on bacterial capsule production. Capsule production was increased for both K+ and K− strains cultured with bile or deoxycholate. By microscopic observation, bile and deoxycholate increased capsule production 1.7- to 2.9-fold and 2.1- to 3.4-fold, respectively (Table 3). The increase in capsule production determined by the gravimetric method after phenol extraction was 1.5- to 3.3-fold for bile-cultured cells (data not shown) (51). The uronic acid content of the extracted materials was 50 to 72%.

Congo red binding was increased by culture with bile or bile acids. Congo red dye binding has been utilized as an indicator of virulence, and the level of dye binding by *V. parahaemolyticus* was measured (2). Some strain variability was evident, but in general, dye binding was increased when bacteria were cultured on medium containing bile or sodium deoxycholate (Table 4). The increase in dye binding following culture on bile-containing medium ranged from 50 to 150%. The elevation of dye binding was even greater when deoxycholate was substituted for bile in the medium. Cells from medium containing sodium deoxycholate bound from 90 to 340% more Congo red dye than cells from BHI medium alone.

Adhesion of bacteria to cultured epithelial cells. Bacteria cultured in PPBE broth or in broth with bile or deoxycholate were assayed for adhesion to Int-407 human intestinal cells. Bacteria cultured in PPBE with 1.5% bile or 0.1% deoxycholate were consistently more adherent than cells cultured without bile (Table 5). Early-log-phase growth was an additional requisite for the increased adherence observed when bacteria were cultured with bile or deoxycholate (data not shown). Additionally, short-term incubation of the bacteria with bile or deoxycholate was inadequate to induce a high level of increased adherence. The three-step culture method with bile or deoxycholate was necessary to observe the increased cellular adherence. Addition of an equivalent amount of bile or deoxycholate to the wells of the tissue culture plate had no observable effect on the epithelial cells and did not result in increased bacterial adhesion (data not shown).

TABLE 4. Culture with bile or deoxycholate (DOC) increases Congo red binding of *V. parahaemolyticus*

Strain	Dye A_{488}/cell A_{660} (mean ± SD)[a] under culture condition[b]		
	BHI	BHI + bile	BHI + DOC
P7	1.0 ± 0.0	1.6 ± 0.1	1.9 ± 0.2
38C1	1.0 ± 0.1	1.9 ± 0.1	3.1 ± 0.2
V6074	1.0 ± 0.1	1.9 ± 0.2	1.9 ± 0.2
P6	1.0 ± 0.1	1.5 ± 0.0	2.2 ± 0.1
P15	1.0 ± 0.0	2.5 ± 0.2	3.3 ± 0.2
38C6	1.0 ± 0.1	1.5 ± 0.1	3.4 ± 0.1

[a] Brain heart infusion broth (BHI) values are normalized to 1.0 for comparison.
[b] BHI + bile, broth medium containing 1.5% bile; BHI + DOC, broth medium containing 0.1% sodium deoxycholate.

TABLE 5. Culture with bile or deoxycholate (DOC) increases adherence of *V. parahaemolyticus* to Int-407 cells

Strain	Adherence[a] under culture condition[b]		
	PPBE	PPBE + bile	PPBE + DOC
P7	5.1 ± 2.9	42.9 ± 11.3	90.3 ± 10.4
38C1	4.9 ± 2.7	45.1 ± 18.2	85.2 ± 9.8
V6074	9.6 ± 4.3	72.5 ± 19.5	94.6 ± 7.2
P6	12.4 ± 5.8	67.7 ± 3.7	88.1 ± 10.2
P15	21.7 ± 2.0	79.0 ± 20.8	88.9 ± 9.3
38C6	23.4 ± 9.7	55.9 ± 13.1	82.5 ± 6.1

[a] Adherence was measured as the percentage of inoculum that adhered to Int-407 cells and is expressed in means ± standard deviations.
[b] PPBE + bile, broth medium containing 1.5% bile; PPBE + DOC, broth medium containing 0.1% sodium deoxycholate.

DISCUSSION

An important aspect of bacterial cell signaling and its central role in regulating responses to intra- and extra-host environments has been suggested by this study. We have shown that bile can enhance the growth of nutrient-deprived *V. parahaemolyticus* and enhance potential virulence characteristics of the microorganism. *V. parahaemolyticus* cultured in estuarine water exhibited characteristics of nutrient deprivation and perhaps dormancy. The dormancy traits exhibited were consistent with those of the viable nonculturable state described by Roszak and Colwell (60) and postulated by others (34, 38, 42, 52, 65). Physiological changes observed in *V. parahaemolyticus* were associated with changes in $[Ca^{2+}]_i$. Intracellular calcium has been shown to regulate motility and protein synthesis in *Escherichia coli*, and nutrient-deprived *E. coli* has increased $[Ca^{2+}]_i$ as does *V. parahaemolyticus* cultured in estuarine water (24, 40, 56, 69). A Ca^{2+}-based regulatory mechanism has been suggested for other bacterial systems (6, 8, 48). The low calcium response of virulent *Yersinia* spp. and the role of calcium accumulation in gram-positive bacteria during sporulation are well known (6, 8, 18, 21, 49, 66, 67). The low calcium response leads to increased survival ability in vivo and cytotoxicity of *Yersinia* spp. (6, 8, 22, 66).

Bile, sodium deoxycholate, and BAPTA/AM were all active substrates in the DVC assay. In the plate count assay, bile was somewhat inhibitory. Virulent strains of the bacterium may be selected for when encountering bile in the host intestine because they appear to be somewhat more resistant to any inhibitory activities. We have observed that K− strains were selectively inhibited by direct plating on bile (1.5%)-containing medium only after culture in estuarine water. The inhibition of growth was not observed when the bacteria were cultured in PPBE medium prior to plating. Bile is a commonly utilized

inhibitor in selective bacterial culture media (17). However, the recommended method for the isolation of *V. parahaemolyticus* from estuarine water or seafoods requires preenrichment in alkaline peptone water (20). Only after this enrichment step are samples plated on bile-containing medium (20). Perhaps if estuarine water samples were plated directly on bile-containing medium, greater levels of K+ *V. parahaemolyticus* would be detected. K+ strains of *V. parahaemolyticus* reportedly are selected for over K− strains by culture on medium containing the salt of the bile acid taurocholic acid (29). We have found that K+ but not K− *V. parahaemolyticus* will grow in medium containing bile (1.5%) and elevated sodium chloride (3.5%) (unpublished results), suggesting that host bile may exert a selective pressure beneficial to pathogenic strains. K+ *V. parahaemolyticus* also multiplies more rapidly in rabbit ligated ileal loops than K− *V. parahaemolyticus* does, perhaps because of its relative resistance to the inhibitory effect of bile (71). The ability to survive the inhibitory effects of host bile may be particularly important for virulence of *V. parahaemolyticus*, since expression of many other components and responses of both K+ and K− isolates are quite similar.

Interestingly, it has been shown that molecules similar in structure to the vertebrate bile acids and found in gut secretions from *Manduca sexta* trigger germination of spores from pathogenic *Bacillus* species ingested by the insect (68, 74). Agents such as chelators and surfactants that displace calcium from the spore may act as germinants (19, 21, 58, 59, 67). A similar phenomenon may occur when the nutrient-deprived vibrio encounters bile in the host intestine. Bile acids are calcium chelators and surfactants and can enter bacterial cells, and we have shown that bile triggers a large and rapid decrease of $[Ca^{2+}]_i$ from *V. parahaemolyticus* (7, 30, 54, 57).

Congo red dye binding has been reported as an indicator of hemin binding and virulence (2, 41, 52). We have shown that culture of *V. parahaemolyticus* with bile or sodium deoxycholate induced enhanced Congo red binding. This finding indicates that the ability to obtain iron in an iron-restricted environment, a characteristic necessary for bacterial survival, is enhanced by a host factor. Wild-type *Shigella* spp. bind Congo red and become more adherent and invasive during culture with sodium deoxycholate, while a *Shigella flexneri* Crb^- (Congo red binding-negative) mutant is unresponsive (2, 14, 43, 55). *Campylobacter jejuni* cultured with bile or deoxycholic acid also binds increased levels of Congo red and adheres to and invades cultured epithelial cells at greater levels (51a). Several virulence-related characteristics may be coregulated by bile, or alternatively, bile may act indirectly by chelating both iron and calcium (57, 63).

Adhesion of *V. parahaemolyticus* to epithelial cell lines has been evaluated in several studies (9, 11, 26, 76). We observed no differences in the adherence of bacterial strains to Int-407 cells. However, culture of bacteria with bile or deoxycholate enhanced adherence to epithelial cells. This response was observed only when bacteria were in the log phase.

Capsule size and composition are major virulence factors for several bacterial pathogens (13, 33, 75). Production of a uronic acid-containing extracellular capsule may enable the bacteria to aggregate and adhere to intestinal cells (62, 72). Other workers have reported that a variant of K+ *V. parahaemolyticus* unable to produce K antigen (capsule) remained highly hemolytic but unable to produce disease in vivo (62). In this study, production of a uronic acid-containing capsule by the bacteria was increased by culture with bile.

Our study suggests a potentially important ecological relationship between a host factor and the pathogen *V. parahaemolyticus*. The signal sensed by the bacterium upon entering the host may mediate both the exit from dormancy and the expression of virulence factors. Further, the apparent resistance of K+ strains to the inhibitory effects of bile may explain their predominance in clinical samples. The rapid changes in $[Ca^{2+}]_i$ that we observed when estuarine water-cultured bacteria were treated with bile may trigger the related responses mediating adherence and colonization of a host. The in vitro observations of the bacterial response to bile are consistent with the expected rapid response of a pathogen encountering a host (45, 51).

Various factors or conditions, including pH, temperature, anaerobiosis, and osmolarity, are known to affect a wide variety of bacterial functions (7, 43–45). All of these conditions may be encountered in a host by the bacterial pathogen. Additionally, factors specific to the host, such as the bile acids, may serve as signals. We have identified a host factor that apparently regulates bacterial responses. Further studies may elucidate whether bile acids are a generic signal for enteric pathogens in vivo and may confirm a calcium-mediated mechanism of action.

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REFERENCES

1. **Ames, B. N., and D. T. Dubin.** 1960. The role of polyamines in the neutralization of bacteriophage deoxyribonucleic acid. J. Biol. Chem. **235:**769–775.
2. **Andrews, G. P., and A. T. Maurelli.** 1992. *mxiA* of *Shigella flexneri* 2a, which facilitates export of invasion plasmid antigens, encodes a homolog of the low-calcium-response protein, LcrD, of *Yersinia pestis*. Infect. Immun. **60:** 3287–3295.
3. **Benson, H. J.** 1979. Microbiological applications: a laboratory manual in general microbiology, 3rd ed. Wm. C. Brown Publishers, Dubuque, Iowa.
4. **Beuchat, L. R.** 1982. *Vibrio parahaemolyticus*: public health significance. Food Technol. **36:**80–92.
5. **Bligh, E. G., and W. J. Dyer.** 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **378:**911–917.
6. **Bolin, I., and H. Wolf-Watz.** 1988. The plasmid-encoded Yop2b protein of *Yersinia pseudotuberculosis* is a virulence determinant regulated by calcium and temperature at the level of transcription. Mol. Microbiol. **2:**237–245.
7. **Borgstrom, B.** 1974. Bile salts—their physiological functions in the gastrointestinal tract. Acta Med. Scand. **196:**1–10.
8. **Brubaker, R. R.** 1983. The Vwa^+ virulence factor of yersiniae: the molecular basis of the attendant nutritional requirement for Ca^{2+}. Rev. Infect. Dis. **5:**S748–S758.
9. **Carruthers, M. M.** 1977. In vitro adherence of Kanagawa positive *Vibrio parahaemolyticus* to epithelial cells. J. Infect. Dis. **136:**588–592.
10. **Chai, T.** 1983. Characteristics of *Escherichia coli* grown in bay water as compared with rich medium. Appl. Environment. Microbiol. **45:**1316–1323.

10a. **Chai, T.** Unpublished results.

11. **Chakrabarti, M. K., A. K. Sinha, and T. Biswas.** 1991. Adherence of *Vibrio parahaemolyticus* to rabbit intestinal epithelial cells in vitro. FEMS Microbiol. Lett. **84:**113–118.
12. **Colwell, R. R., P. R. Brayton, D. J. Grimes, D. B. Roszak, S. A. Huq, and L. M. Palmer.** 1985. Viable but non-cultureable *Vibrio cholerae* and related pathogens in the environment: implications for release of genetically engineered microorganisms. Bio/Technology **3:**817–820.
13. **Costerton, J. W., R. T. Irvin, and K.-J. Cheng.** 1981. The role of bacterial surface structures in pathogenesis. Crit. Rev. Microbiol. **3:**303–338.
14. **Daskaleros, P. A., and S. M. Payne.** 1987. Congo red binding phenotype is associated with hemin binding and increased infectivity of *Shigella flexneri* in the HeLa cell model. Infect. Immun. **55:**1393–1398.
15. **Dazzo, F. B., and W. J. Brill.** 1979. Bacterial polysaccharide which binds *Rhizobium trifolii* to clover root hairs. J. Bacteriol. **137:**1362–1373.
16. **Dische, Z.** 1946. A new specific color reaction of hexuronic acids. Biochem. J. **24:**189–198.
17. **D'mello, A., and W. W. Yotis.** 1987. The action of sodium deoxycholate on *Escherichia coli*. Appl. Environ. Microbiol. **53:**1944–1946.
18. **Eisenstadt, E., and S. Silver.** 1972. Calcium transport during sporulation in *Bacillus subtilis*, p. 180–186. *In* H. O. Halvorson, R. Hanson, and L. L. Campbell (ed.), Spores V. American Society for Microbiology, Washington, D.C.
19. **Ellar, D. J., M. W. Eaton, and J. Postgate.** 1974. Calcium release and germination of bacterial spores. Biochem. Soc. Trans. **2:**947–948.
20. **Elliot, E. L., C. A. Kaysner, and M. L. Tamplin.** 1992. *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, and other *Vibrio spp.*, p. 111–140. *In* Food

and Drug Administration Bacteriological Analytical Manual, 7th ed. AOAC International, Arlington, Va.
20a. **Fisher, T., and R. Harrell.** Personal communication.
21. **Fitz-James, P. C.** 1971. Formation of protoplasts from resting spores. J. Bacteriol. **105:**1119–1136.
22. **Forsberg, A., and R. Rosqvist.** 1994. In vivo expression of virulence genes of *Y. pseudotuberculosis*. Infect. Agents Dis. **2:**275–278.
23. **Galán, J. E., and R. Curtiss III.** 1989. Cloning and molecular characterization of genes whose products allow *Salmonella typhimurium* to penetrate tissue culture cells. Proc. Natl. Acad. Sci. USA **86:**6383–6387.
24. **Gangola, P., and B. P. Rosen.** 1987. Maintenance of intracellular calcium in *Escherichia coli*. J. Biol. Chem. **262:**12570–12574.
25. **Gangola, P., and B. P. Rosen.** 1988. Fura-2 measurements of intracellular $[Ca^{2+}]_i$ in *Escherichia coli*. Prog. Clin. Biol. Res. **252:**215–220.
26. **Gingras, S. P., and L. V. Howard.** 1980. Adherence of *Vibrio parahaemolyticus*. Appl. Environ. Microbiol. **39:**369–371.
27. **Grimes, D. J., R. W. Atwell, P. R. Brayton, L. M. Palmer, D. M. Rollins, D. B. Roszak, F. L. Singelton, M. L. Tamplin, and R. R. Colwell.** 1986. The fate of enteric pathogenic bacteria in estuarine and marine environments. Microbiol. Res. **3:**324–329.
28. **Grynkiewicz, G., M. Poenie, and R. Y. Tsien.** 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem. **260:**3440–3450.
29. **Hackney, C. R., and A. Dicharry.** 1988. Seafood-borne bacterial pathogens of marine origin. Food Technol. **42:**104–109.
30. **Hofmann, A. F., and D. M. Small.** 1967. Detergent properties of bile salts: correlation with physiological function. Annu. Rev. Med. **18:**333–376.
31. **Horan, J., M., N. R. DiLuzio, and J. N. Etteldorf.** 1964. Use of an anion exchange resin in treatment of two siblings with familial hypercholesterolemia. J. Pediatr. **64:**201–209.
32. **Janda, J. M., C. Powers, R. G. Bryant, and S. L. Abbott.** 1988. Current perspectives on the epidemiology and pathogenesis of clinically relevant *Vibrio* spp. Clin. Microbiol. Rev. **1:**245–267.
33. **Jann, K., and B. Jann.** 1992. Capsules of *Escherichia coli*, expression and biological significance. Can. J. Microbiol. **38:**705–710.
34. **Jannasch, H. W.** 1967. Growth of marine bacteria at limiting concentrations of organic carbon in seawater. Limnol. Oceanogr. **12:**264–271.
35. **Jones, D. M., E. M. Sutcliffe, and A. Curry.** 1991. Recovery of viable but non-culturable *Campylobacter jejuni*. J. Gen. Microbiol. **137:**2477–2482.
36. **Joseph, S. W., R. R. Colwell, and J. B. Kaper.** 1980. *Vibrio parahaemolyticus* and related halophilic vibrios. Crit. Rev. Microbiol. **10:**77–124.
37. **Kaper, J. B., and M. Nishibuchi.** 1995. Thermostable direct hemolysin gene of *Vibrio parahaemolyticus*: a virulence gene acquired by a marine bacterium. Infect. Immun. **63:**2093–2099.
38. **Kjelleberg, S., N. Albertson, K. Flardh, L. Holmquist, A. Jouper-Jaan, R. Marouga, J. Ostling, B. Svenblad, and D. Weichart.** 1993. How do non-differentiating bacteria adapt to starvation? Antonie Leeuwenhoek **63:**333–341.
39. **Kogure, K., U. Simidu, and N. Taga.** 1979. A tentative direct microscopic method for counting living marine bacteria. Can. J. Microbiol. **25:**415–420.
40. **Laoudj, D., C. L. Andersen, A. Bras, M. Goldberg, A. Jaq, and I. B. Holland.** 1994. EGTA induces the synthesis in *Escherichia coli* of three proteins that cross-react with calmodulin antibodies. Mol. Microbiol. **13:**445–457.
41. **Lawlor, K. M., P. A. Daskaleros, R. E. Robinson, and S. M. Payne.** 1987. Virulence of iron transport mutants of *Shigella flexneri* and utilization of host iron compounds. Infect. Immun. **55:**594–599.
42. **Matin, A., E. A. Auger, P. H. Blum, and J. E. Schultz.** 1989. Genetic basis of starvation survival in nondifferentiating bacteria. Annu. Rev. Microbiol. **43:**293–316.
43. **Maurelli, A. T., A. E. Hromockyj, and M. L. Bernardini.** 1992. Environmental regulation of *Shigella* virulence. Curr. Top. Microbiol. Immunol. **180:**95–116.
44. **Mekalanos, J. J.** 1992. Environmental signals controlling expression of virulence determinants in bacteria. J. Bacteriol. **174:**1–7.
45. **Miller, J. F., J. J. Mekalanos, and S. Falkow.** 1989. Coordinate regulation and sensory transduction in the control of bacterial virulence. Science **243:**916–922.
46. **Miyamoto, Y., T. Kato, Y. Obara, S. Akiyama, K. Takizawa, and S. Yamai.** 1969. In vitro hemolytic characteristic of *V. parahaemolyticus*: its close correlation with human pathogenicity. J. Bacteriol. **100:**1147–1149.
47. **Morita, R. Y.** 1988. Bioavailability of energy and its relationship to growth and starvation survival in nature. Can. J. Microbiol. **34:**436–441.
48. **Norris, V., M. Chen, M. Goldberg, J. Voskull, G. McGurk, and I. B. Holland.** 1991. Calcium in bacteria: a solution to which problem? Mol. Microbiol. **15:**775–778.
49. **O'Hara, M. B., and J. H. Hageman.** 1990. Energy and calcium ion dependence of proteolysis during sporulation of *Bacillus subtilis* cells. J. Bacteriol. **172:**4161–4170.
50. **Pace, J., and T. Chai.** 1989. Comparison of *Vibrio parahaemolyticus* grown in estuarine water and rich medium. Appl. Environ. Microbiol. **55:**1877–1887.
51. **Pace, J. L.** 1989. Ph.D. thesis. University of Maryland, College Park.
51a. **Pace, J. L., et al.** Unpublished results.
52. **Payne, S. M., and R. A. Finkelstein.** 1977. Detection and differentiation of iron-responsive avirulent mutants on Congo red agar. Infect. Immun. **18:**94–98.
53. **Peele, E., and R. R. Colwell.** 1981. Application of a direct microscopic method for enumeration of substrate-responsive marine bacteria. Can. J. Microbiol. **27:**1071–1075.
54. **Plesiat, P., and H. Nikaido.** 1992. Outer membranes of gram-negative bacteria are permeable to steroid probes. Mol. Microbiol. **6:**1323–1333.
55. **Pope, L. M., K. E. Reed, and S. M. Payne.** 1995. Increased protein secretion and adherence to HeLa cells by *Shigella* spp. following growth in the presence of bile salts. Infect. Immun. **63:**3642–3648.
56. **Postgate, J. R., and J. R. Hunter.** 1962. The survival of starved bacteria. J. Gen. Microbiol. **29:**233–263.
57. **Rajagopalan, N., and S. Lindenbaum.** 1982. The binding of Ca^{2+} to taurine and glycine-conjugated bile salt micelles. Biochim. Biophys. Acta **711:**66–74.
58. **Rode, L. J., and J. W. Foster.** 1960. The action of surfactants on bacterial spores. Arch. Mikrobiol. **36:**67–84.
59. **Rode, L. J., and J. W. Foster.** 1961. Germination of bacterial spores with alkyl primary amines. J. Bacteriol. **81:**768–779.
60. **Roszak, D. B., and R. R. Colwell.** 1987. Survival strategies of bacteria in the natural environment. Microbiol. Rev. **51:**365–379.
61. **Roszak, D. B., D. J. Grimes, and R. R. Colwell.** 1984. Viable but nonrecoverable stage of *Salmonella enteritidis* in aquatic systems. Can. J. Microbiol. **30:**334–338.
62. **Sakazaki, R.** 1971. Present status of studies on *Vibrio parahaemolyticus* in Japan. Symposium of the Division of Microbiology. Food and Drug Administration, Washington, D.C.
63. **Sanyal, A. J., M. L. Shiffman, J. I. Hirsch, and E. W. Moore.** 1991. Premicellar taurocholate enhances ferrous iron uptake from all regions of rat small intestine. Gastroenterology **101:**382–388.
64. **Shi, S. P., C. C. Y. Chang, G. W. Gould, and T. Y. Chang, T. Y.** 1989. Comparison of phosphatidylethanolamine and phosphatidylcholine vesicles produced by treating cholate-phospholipid micelles with cholestyramine. Biochim. Biophys. Acta **982:**187–195.
65. **Stevenson, L. H.** 1978. A case for bacterial dormancy in aquatic systems. Microb. Ecol. **4:**127–133.
66. **Straley, S. C., G. V. Plano, E. Skrzypek, P. L. Haddix, and K. A. Fields.** 1993. Regulation by Ca^{2+} in the *Yersinia* low-Ca^{2+} response. Mol. Microbiol. **8:**1005–1010.
67. **Sudo, S. Z., and M. Dworkin.** 1973. Comparative biology of prokaryotic resting cells, p. 153–203. *In* A. H. Rose and D. W. Tempest (ed.), Advances in microbial physiology, vol. 9. Academic Press, Inc., New York, N.Y.
68. **Swevers, L., J. G. D. Lambert, and A. De Loof.** 1991. Synthesis and metabolism of vertebrate-type steroids by tissues of insects: a critical evaluation. Experientia **47:**687–698.
69. **Tisa, L. S., and J. Adler.** 1995. Cytoplasmic free-Ca^{2+} level rises with repellents and falls with attractants in *Escherichia coli* chemotaxis. Proc. Natl. Acad. Sci. USA **92:**10777–10781.
70. **Tsien, R. Y.** 1980. New calcium indicators and buffers with high selectivity against magnesium and protons: design, synthesis, and properties of prototype structures. Biochemistry **19:**2396–2402.
71. **Twedt, R. M., J. T. Peeler, and P. L. Spaulding.** 1980. Effective ileal dose of Kanagawa-positive *Vibrio parahaemolyticus*. Appl. Environ. Microbiol. **40:**1012–1016.
72. **Waldor, M. K., R. R. Colwell, and J. J. Mekalanos.** 1994. The *Vibrio cholerae* O139 serogroup antigen includes an O-antigen capsule and lipopolysaccharide virulence determinants. Proc. Natl. Acad. Sci. USA **91:**11388–11392.
73. **Weiss, G. B.** 1974. Cellular pharmacology of lanthanum. Annu. Rev. Pharmacol. **14:**343–354.
74. **Wilson, G. R., and T. G. Benoit.** 1990. Activation and germination of *Bacillus thuringiensis* spores in *Manduca sexta* larval gut fluid. J. Invertebr. Pathol. **56:**233–236.
75. **Yamamoto, T., M. J. Albert, and R. B. Sack.** 1994. Adherence to human small intestines of capsulated *Vibrio cholerae* O139. FEMS Microbiol. Lett. **119:**229–235.
76. **Yamamoto, T., and T. Yokota.** 1989. Adherence targets of *Vibrio parahaemolyticus* in human small intestines. Infect. Immun. **57:**2410–2419.