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Production of Thermostable Direct Hemolysin by *Vibrio parahaemolyticus* Enhanced by Conjugated Bile Acids

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The effects of conjugated bile acids, glycocholic acid, and taurocholic acid (TC) on production of thermostable direct hemolysin (TDH) by *Vibrio parahaemolyticus* were determined by a reversed passive latex agglutination assay against TDH. The amount of TDH excreted in growth medium containing either glycocholic acid or taurocholic acid (5 mM/liter) was, on a per-cell basis, 4- to 16-fold greater than that excreted in medium without the bile acids. The amounts of TDH released from lysed cells grown with the bile acids (5 mM/liter) were 4- to 32-fold greater than those from lysed cells grown without, suggesting that the bile acids enhanced synthesis of TDH within bacterial cells. These data imply that the conjugated bile acids play a key role in the pathogenicity of *V. parahaemolyticus*.

Vibrio parahaemolyticus is a halophilic bacterium widely distributed in estuarine and marine environments (8) and often causes acute gastroenteritis in humans who consume raw or improperly cooked seafood (1, 2). Past epidemiological studies (11, 16) revealed a strong association of thermostable direct hemolysin (TDH) produced by members of this species with its etiology. TDH has been purified and characterized as a thermostable cytotoxic protein that exhibits cardiotrophism in rats and mice (6, 7) and fluid-accumulating activity in the rabbit ileal loop (12). Several in vitro studies demonstrated that production of TDH was influenced by concentrations of NaCl (11), sugars (4), amino acids (3, 9), and phosphate (10) in growth media. Little information on its ability to produce TDH within the human intestine, however, is available.

In the intestine, the bacteria may encounter concentrations of bile that has a strong detergent-like property. Noh and Gilliland (13) have reported that the presence of 0.3% oxgall in reaction buffer increased the cellular permeability of *Lactobacillus acidophilus*, thereby enhancing its enzymatic activity. Glycocholic acid (GC) and taurocholic acid (TC) are glycine- and taurine-conjugated cholic acids, respectively, and the major, if not only, constituents of human bile. A recent study (5) has demonstrated that GC and TC at concentrations ranging from 0.03 to 0.3% enhanced excretion of bacterial β-glucuronidase by *Escherichia coli* and *Clostridium perfringens*. We therefore postulated that the bile acids may have similar effects on production of bacterial toxins, for example, TDH of *V. parahaemolyticus*. In this study, we aimed to determine whether the presence of the bile acids enhances TDH production by *V. parahaemolyticus*.

Three strains of *V. parahaemolyticus*, K-10100, K-10297, and K-10300, isolated from feces of patients from past food poisoning outbreaks in Kanagawa Prefecture, Japan, were used. As described in our previous report (15), K-10297 and K-10300 carry the TDH gene whereas K-10100 does not. Cultures of the strains were maintained on heart infusion (HI) agar (Difco Laboratories, Detroit, Mich.) supplemented with 2% NaCl throughout the study. A reversed passive latex agglutination assay (KAP-RPLA kit; Denka Seiken Co., Tokyo, Japan) which employs polyvalent capture antibody against TDH (14) was used to detect and quantify TDH produced in a growth medium by the bacterium, according to the manufacturer's instructions. In this assay, a minimum of ~1 to 2 ng/ml of TDH can be detected at a titer of 1:2.

In the first experiment, each bacterial strain was grown in HI broth (Difco) supplemented with 2% NaCl, pH 7.8, and incubated at 37°C for 6 h with agitation to obtain good mid-exponential growth. After incubation, the cells were harvested by centrifugation (4,500 × *g*, 20 min, 4°C) and washed three times with sterile HI broth. The suspension was then adjusted to an optical density (OD) at 660 nm of 0.4 by using sterile HI broth. Exactly 0.1 ml of the suspension was added to six different sterile broth media (100 ml each) as follows: (i) basal medium (BM; pH 7.8) consisting of 2 g of Polypeptone (Difco), 0.5 g of D-mannitol, and 5 g of NaCl; (ii) BM supplemented with 5 mM GC (Sigma Chemicals, St. Louis, Mo.), referred to as BM+GC; (iii) BM supplemented with 5 mM TC (Sigma), referred to as BM+TC; (iv) BM + 5 mM cholic acid (Sigma), referred to as BM+CH; (v) BM supplemented with 5 mM glycine (Sigma); and (vi) BM supplemented with 5 mM taurine (Sigma). The initial concentration of bacteria in the media was adjusted to ca. 2.0×10^5 CFU/ml. The bacterial cells were then incubated at 37°C for 18 h. It should be noted that the use of BM was specifically recommended by the manufacturer of the KAP-RPLA kit for the maximum production of TDH by the bacterium (14).

After incubation, the growth in each medium was measured as the OD at 660 nm, and the OD was subsequently adjusted to 0.1 by using sterile BM. After this OD adjustment, all preparations contained the same approximate number of CFU (ca. 1.0×10^8 CFU/ml), regardless of the presence of the bile acids in the growth medium. Cells were then centrifuged (15,800 × *g*, 20 min, 4°C), and the supernatant was collected for a subsequent assay by KAP-RPLA. Since the KAP-RPLA kit was provided with purified TDH (100 ng), the TDH was aseptically dissolved in the above-described six media at a final concentration of 50 ng/ml (equivalent to an RPLA titer of 1:32), and the mixtures were incubated at 37°C for 18 h. After incubation, the mixtures were assayed by KAP-RPLA for a possible effect of the bile acids on TDH itself. All assays were performed in triplicate.

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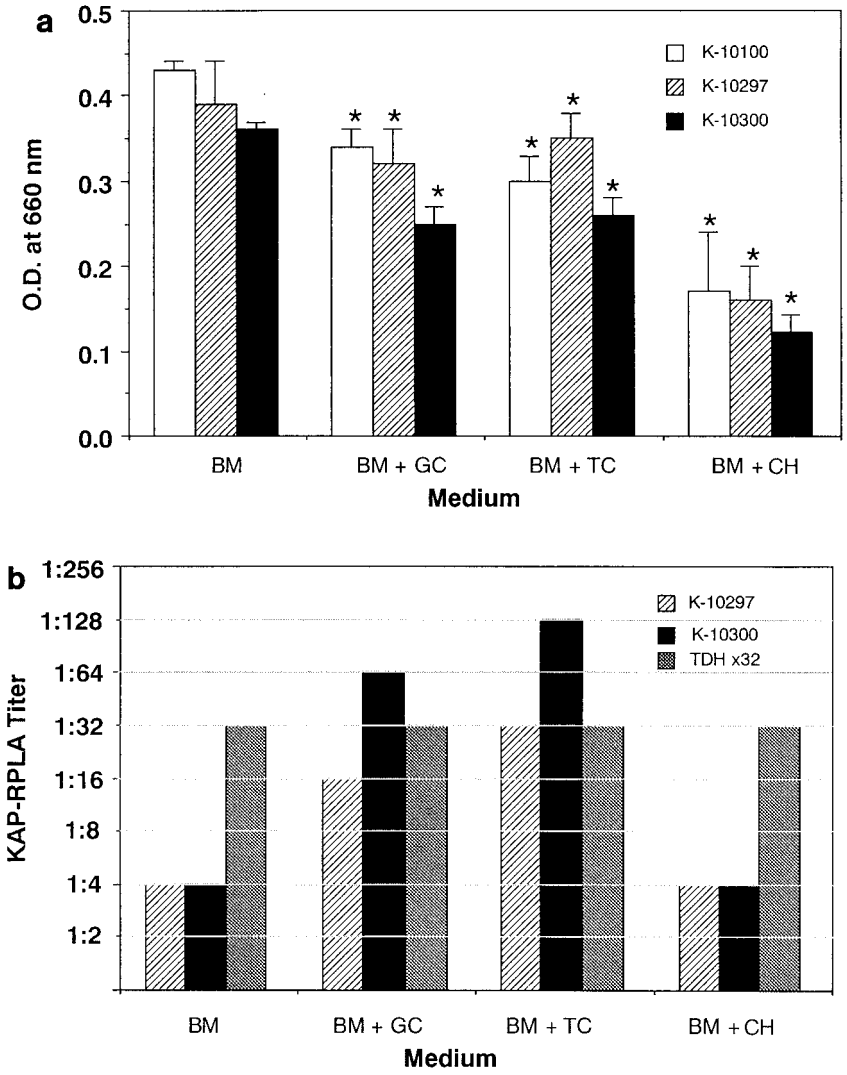

FIG. 1. Effects of the bile acids on growth of three strains of *V. parahaemolyticus* (a) and corresponding RPLA titers of TDH excreted in the medium per equivalent numbers of cells (OD at 660 nm, 0.1 or ca. 1.0×10^8 CFU/ml) over 18 h (b). Error bars indicate standard errors of the means. *, $P < 0.05$. RPLA titers are the results of triplicate tests. THD ×32, purified TDH whose KAP-RPLA titer is known to be 1:32. CH, cholic acid.

Growth was significantly ($P < 0.01$, Student t test) inhibited by the presence of the bile acids for all three strains tested, with cholic acid being the most inhibitory (Fig. 1a). In spite of the growth suppression, the amounts of TDH excreted per equivalent numbers of cells of K-10297 and K-10300 in the media containing TC and GC increased 4- and 32-fold over those excreted in the media without the bile acids (Fig. 1b). No such increase, however, was observed for the strains grown in the medium containing cholic acid (Fig. 1b). The presence of the bile acids did not affect the purified TDH, in which the RPLA titer remained constant (1:32) both before and after incubation in any medium tested (Fig. 1b). Growth of K-10297 and K-10300 and the RPLA titers measured in the spent BM supplemented with either glycine or taurine were comparable to those in the unsupplemented spent BM (data not shown).

In the second experiment, the two TDH-producing strains, K-10297 and K-10300, in HI broth supplemented with 2% NaCl was inoculated into BM, BM+GC, and BM+TC (200 ml each) at an initial CFU count of 2.0×10^5/ml and were incubated at 37°C for 6 h with agitation. After incubation, the cells were harvested by centrifugation (4,500 × g, 10 min, 4°C) and washed three times in phosphate buffer (0.1 M KH_2PO_4 per liter, pH 7.8) containing 5% NaCl. The bacterial pellet was aseptically resuspended in 5 ml of the phosphate buffer to obtain a dense bacterial suspension with an OD of 0.8 (CFU ranging from 1.7×10^9 to 2.0×10^9 [Table 1]). The bacterial cells thus prepared were lysed by sonication with cooling for 10 min, using a Bioruptor (Tosho Electric Co. Ltd., Tokyo, Japan). After sonication, the ODs of all cell suspensions decreased to less than 0.06, with a corresponding decrease in CFU of less than 2.5×10^6 (Table 1), indicating that more than 99% of the cells were lysed by the sonication. The sonicated aliquots were clarified by centrifugation (15,800 × g, 20 min, 4°C), and the supernatants were collected for a subsequent assay by KAP-RPLA. The triplicate assays confirmed that the amounts of TDH released from the lysed cells grown in BM+GC and BM+TC increased 4- to 32-fold over those grown in BM not containing the bile acids (Table 1).

The results of the present study demonstrate that the bile acids enhance the production of TDH by *V. parahaemolyticus*.

TABLE 1. Effects of GC and TC on amount of TDH produced by two *V. parahaemolyticus* strains[a]

Strain	Growth medium	Value before sonication		Value after sonication		
		OD	CFU	OD	CFU	TDH titer
K-10297	BM	0.80	$(1.8 \pm 0.2) \times 10^9$	0.05 ± 0.01	$(1.5 \pm 0.3) \times 10^6$	1:16
	BM+GC	0.80	$(2.0 \pm 0.1) \times 10^9$	0.05 ± 0.01	$(1.4 \pm 0.2) \times 10^6$	1:64
	BM+TC	0.80	$(2.1 \pm 0.1) \times 10^9$	0.05 ± 0.02	$(1.8 \pm 0.3) \times 10^6$	1:128
K-10300	BM	0.80	$(1.9 \pm 0.9) \times 10^9$	0.05 ± 0.01	$(2.1 \pm 0.3) \times 10^6$	1:8
	BM+GC	0.80	$(2.1 \pm 0.3) \times 10^9$	0.06 ± 0.01	$(2.5 \pm 0.4) \times 10^6$	1:256
	BM+TC	0.80	$(1.7 \pm 0.9) \times 10^9$	0.06 ± 0.02	$(2.1 \pm 0.3) \times 10^6$	1:256

[a] Where applicable, GC and TC were used at a concentration of 5 mM/liter. TDH titers were determined by KAP-RPLA. All values are the results of triplicate tests.

TDH is known to be a dimer of polypeptides which can be fragmented by treatment with sodium dodecyl sulfate (SDS) to yield two subunits, each of 21 kDa (17). Since the salts of GC and TC have a strong detergent or surfactant property (18), we initially considered the possibility that, like SDS, the salts might split TDH into fragments, thereby increasing its total immunoreactivity. This was, however, not the case since the purified TDH sustained its original immunoreactivity even after a prolonged incubation with the bile salts.

It has been demonstrated for *E. coli* and *C. perfringens* that GC and TC enhance the extracellular secretion of bacterial β-glucuronidase possibly because of their biochemical property which increases cellular permeability (5). We thus presumed that the bile acids acted similarly on *V. parahaemolyticus* cells, with a resultant increase of TDH in the spent medium. Alternatively, we considered the possibility that the observed TDH increase may simply be caused by active cell lysis of the strains due to a strong detergent effect of the bile acids. However, the observed increase of TDH is more likely to reflect enhanced synthesis of TDH within the bacterial cell rather than increased cellular permeability or active cell lysis since the lysate of intact *V. parahaemolyticus* cells grown with the bile acids had a TDH level at least severalfold greater than that of cells grown without. It has been reported for *L. acidophilus* that a fresh bile solution made of oxgall increased the cellular permeability of the bacterium, allowing more substrate to enter the cells (13). A similar course of events may apply to TDH-producing *V. parahaemolyticus*, and we postulated that glycine and taurine that can be derived from the conjugated bile acids might be utilized by the bacterium as essential substrates for TDH synthesis. This possibility was, however, unlikely since the supplementation of the growth media with amino acids failed to enhance TDH production. The evidence provided in the present study suggests that the bile acids enhance synthesis of TDH within the cell through a mechanism yet to be defined, with a tendency of TC having greater effect than GC.

This article is the first report that conjugated bile acids enhance TDH production by *V. parahaemolyticus*. This in turn suggests that the bile acids play a key role in the pathogenicity of *V. parahaemolyticus* during its natural infection of the human intestine. A similar effect may apply to other enterotoxin-producing bacteria, such as *Vibrio cholerae*, enterotoxigenic *E. coli*, and *C. perfringens*. Further studies are currently in progress to test this possibility.

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