CURRENT MICROBIOLOGY Vol. 44 (2002), pp. 302–305
DOI: 10.1007/s00284-001-0051-3

Current Microbiology
An International Journal

Levels of Thermostable Direct Hemolysin Produced by *Vibrio parahaemolyticus* O3:K6 and Other Serovars Grown Anaerobically with the Presence of a Bile Acid

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Received: 27 August 2001 / Accepted: 15 October 2001

Abstract. Twenty-three *V. parahaemolyticus* strains, including 12 pandemic O3:K6 strains, were examined for their growth and production of thermostable direct hemolysin (TDH) under an anaerobic culture condition with or without presence of a bile acid, taurocholic acid (TCA). Both bacterial growth and TDH production were markedly enhanced by TCA for a majority of the strains, but the scale of the TDH production was disproportionately greater than that of the corresponding growth for 14 strains. Such enhancement was, however, not specific to the pandemic strains.

Vibrio parahaemolyticus is one of the major seafood-borne gastroenteritis-causing bacteria, often associated with consumption of raw or improperly cooked seafood [9]. Almost all clinical isolates induced the Kanagawa phenomenon, a zone of beta type hemolysis around the colony on a special blood agar (Wagatsuma agar) [17]. The phenomenon is caused by thermostable direct hemolysin (TDH) produced by the isolates [17]. Recent reported outbreaks of the food poisoning were, however, frequently associated with a biotype producing TDH-related hemolysin (TRH) [7]. Both hemolysins have been thus considered as major virulence factors of the bacterium [13]. Several *in vitro* studies demonstrated that production of TDH was influenced by concentrations of NaCl [12], sugars [6], amino acids [5], and phosphate [11] in growth media. Furthermore, our previous study [15] has demonstrated that production of TDH by two *V. parahaemolyticus* strains was markedly enhanced when they were grown in a medium containing conjugated bile acids such as glycocholic acid and taurocholic acid.

V. parahaemolyticus can be classified into 13 O serotypes and 71 K serotypes [8]. Although various serovars of the bacterium can cause infections, a serovar O3:K6 has been recognized as a predominant serovar responsible for most outbreaks worldwide since 1996 [4, 10, 14]. Genotypic analyses [1, 3] on the pandemic strains and other recently emerged serovars, such as O4:K68 and O1:K untypeable, revealed that these strains showed an almost identical pulsed-field gel electrophoresis (PFGE) pattern, suggesting that these strains shared the same clonality.

In order to provide an epidemiological ground for their emergence and spread, Wong et al. [21] investigated phenotypic characteristics of the pandemic strains in comparison with those belonging to the same serotype isolated before 1996 and other serovars. No biological trait unique to the pandemic strains has been, however, identified to date. They [21] also indicated that the level of TDH produced in vitro did not link to the high incidence of the pandemic strains. In this connection, we postulated that the pandemic O3:K6 strains could produce TDH at higher level than other serovars upon entrance into the intestinal lumen with a concentration of bile acids, thereby rendering them more virulent than other conventional strains. This possibility was evaluated in the present study by comparing levels of TDH production by the pandemic strains and others in an anaerobic culture condition with presence of a bile acid, taurocholic acid, which might simulate partly an environment within the host's intestine.

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Material and Methods

A total of 23 strains of *V. parahaemolyticus* that had been isolated from clinical specimen, foods, and water samples, and characterized serologically and biochemically at our laboratories were used in the present study. These included 14 strains of O3:K6, consisting of two strains isolated before 1996 and 12 stains isolated since 1996. PFGE analysis was also performed on genomic DNAs of the O3:K6 and O4:K68 strains digested with a restriction enzyme *Sfi*I, following essentially the methods as described elsewhere [2]. Serotypes, sources, localities, and year of isolation of the strains used are listed in Table 1.

The presence of the gene encoding TDH (*tdh*) and that encoding TRH (*trh*) was determined by PCR with the use of primers 5′-GGTACTAAATGGCTGACATC-3′ and 5′-CCACTACCACTCT-CATATGC-3′ for tdh, and 5′-GGTCAAAATGGTTAAGCG-3′ and 5′-CATTTCCGCTCTCATATGC-3′ for trh, following the protocols established by Tada et al. [20]. The conditions for the PCR were as follows: A cycle of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min was repeated 35 times. The length of the resultant amplicons for *tdh* and *trh* were 251 bp and 250 bp, respectively.

Each bacterial strain was grown in heart infusion broth (pH 7.8, Difco) supplemented with 2% NaCl, and incubated at 37°C for 6 h with agitation (120 rpm) 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 artificial seawater (ASW; pH 8.0 [18]). The suspension was then adjusted to an optical density (OD) at 660 nm of 0.4 by using sterile ASW. The suspension was then diluted 10^5 times to make 0.1 ml of the final suspension contain ca. 100∼200 cells. Exactly 0.1 ml of the final suspension was added to two different broth media (10 ml each) as follows: (i) Buffered Peptone Water (pH 7.4; Oxoid) supplemented with 0.5% NaCl (the final concentration of 1% NaCl); (ii) the above medium supplemented with 5 mM taurocholic acid (TCA; Sigma). The bacterial cells were then incubated anaerobically using a commercial kit (AnaeroPack, Mitsubishi Gas Chemical) at 37°C for 24 h. After incubation, the growth in each medium was measured as the OD at 660 nm.

A reversed passive latex agglutination assay kit (KAP-RPLA; Denka Seiken) that employs polyvalent capture antibody against TDH was used to detect and quantify TDH produced by the strains in the above media according to the manufacturer's instructions. Briefly, twofold serial dilutions (25μl) of cell-free supernatant of the bacterial spent media were made in diluent [phosphate buffer (pH 7.0) containing 0.5% bovine serum albumin and 0.1% sodium azide] and dispensed in wells of a 96-well microplate (V-shaped bottom; Greiner). Subsequently, and equal volume of the reactive solution containing the antibody-fixed latex beads was added to each well, mixed thoroughly, and incubated at room temperature (23°C) for 24 h. After incubation, the highest dilution producing dispersed latex particles on the tilted inner side of the well, which were visible to the eye, was read as KPA-RPLA titer to quantify TDH produced. In the absence of TDH, the latex particles were accumulated to the bottom of the well. According to the manufacturer's instruction, the assay could detect a minimum of 1∼2 ng/ml of TDH at the titer value of 1:2. It should be also mentioned that both growth assay and KAP-RPLA were performed in triplicate.

Table 1. Characteristics of *V. parahaemolyticus* strains used

Strain no.	O:K serovar	Year of Isolation	Country of Isolation	Source	PFGE genotype[a]	Presence of: *tdh*	Presence of: *trh*
KE9967	O3:K6	1981	Japan	human	B	+	−
KE9971	O3:K6	1981	Japan	food	B	+	−
KE10457	O3:K6	1998	Japan	human	A	+	−
KE10472	O3:K6	1998	Japan	human	A	+	−
KE10481	O3:K6	1998	Japan	human	A	+	−
KE10484	O3:K6	1988	Japan	human	A	+	−
KE10495	O3:K6	1996	Japan	human	A	+	−
KE10514	O3:K6	1998	Japan	human	UT[b]	+	−
KE10524	O3:K6	1998	Japan	sea water	A	+	−
KE10527	O3:K6	1998	Japan	food	A	+	−
KE10531	O3:K6	1998	Japan	human	A	+	−
NIID965-98	O3:K6	1998	USA/CDC	human	A	+	−
NIID K7	O3:K6	1998	Japan	human	A	+	−
NIID 59-99	O3:K6	1999	Thailand	human	ND[c]	+	−
KE10545	O4:K68	1999	Indonesia	human	ND	+	+
NIID181-99	O4:K68	1999	Thailand	human	A	+	−
NIID 242-200	O4:K68	2000	Korea	human	A	+	−
KE10538	O4:K8	1999	Thailand	human	ND	+	−
KE10540	O3:K46	1999	Thailand	human	ND	+	+
KE10541	O8:K41	1999	Thailand	human	ND	+	−
KE10542	O3:K48	1999	Thailand	human	ND	+	−
KE10471	O4:K6	1997	Japan	human	ND	+	−
KE10579	O1:K1	2000	Japan	sea water	ND	+	+

[a] According PFGE patterns of *Sfi*I digests [1].
[b] UT, Untypeable.
[c] ND, not determined.

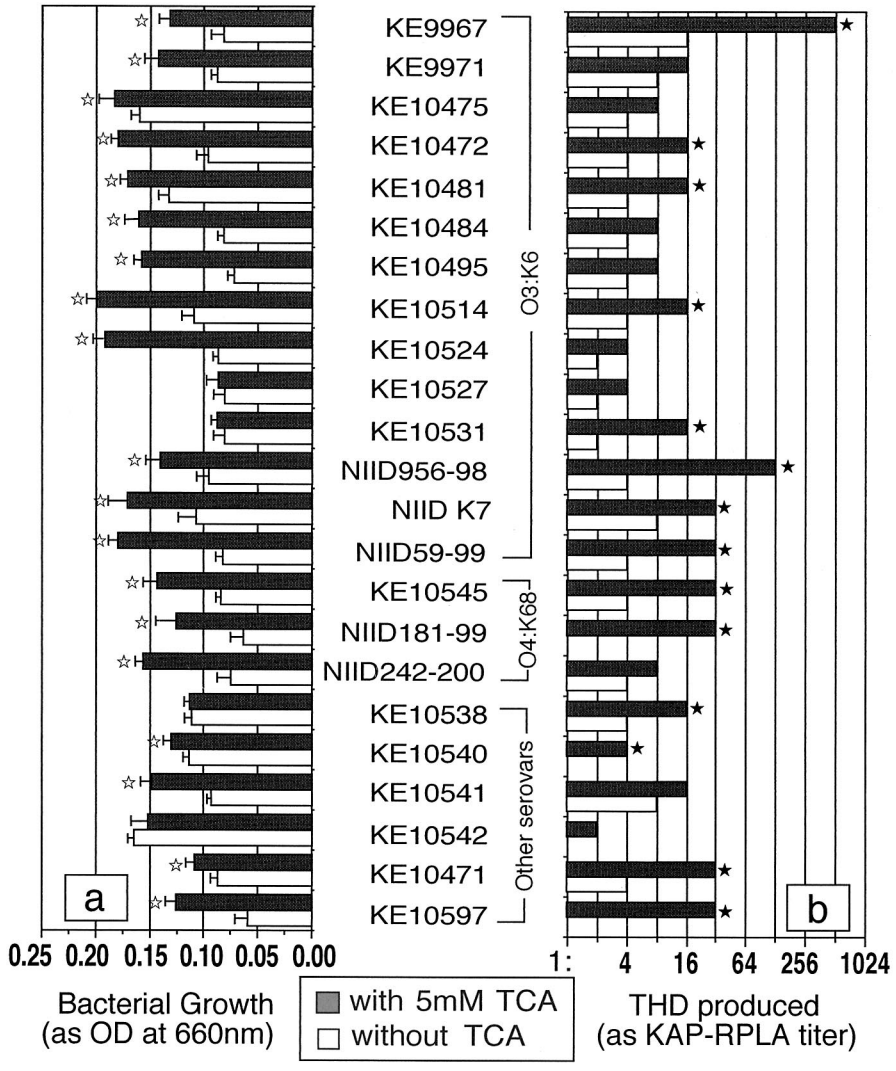

Fig. 1. Effects of TCA on growth and TDH production of *V. parahaemolyticus*. (a) bacterial growth expressed as OD at 660nm; values are means of triplicate tests; bars indicate standard deviations; ☆, significantly greater growth ($p < 0.05$, student-t test). (b) amounts of THD produced expressed as KAP-RPLA titer values with or without presence of 5mM TCA in the growth medium (by triplicate tests); ★, fourfold or more TDH production.

Results and Discussion

All strains possessed *tdh* and three of them also had *trh*. PFGE analysis revealed that the strains belonging to O3:K6 and O4:K68 isolated since 1996 showed very similar restriction patterns, being designated as PFGE type A according to the classification by Arakawa et al. [1] whereas two strains of O3:K6 isolated in 1981 showed patterns different from the above group (Table 1).

Anaerobic growth appeared to be enhanced with presence of TCA for most of the strains irrespective of serovars, in which KE10514 (O3:K6), KE10524 (O3:K6), NIID59–99 (O3:K6), NIID242-200 (O4:K68), and KE10579 (O1:K1) grown with TCA, for example, showed nearly twofold increases in OD as compared with those grown without TCA (Fig. 1a). In a preliminary experiment, we observed that there was an almost linear correlation between OD and cell numbers (i.e. colony forming unit) of the culture when the OD was within a range from 0.05 to 0.4 (data not shown). The observed twofold increase in OD could be thus interpreted as twofold increase in cell numbers.

Bile acids including TCA are found in relatively high concentration in human intestine and levels of the total bile acids in the jejunum range from 5 to 10 mM [19]. Production of TDH was enhanced by presence of 5 mM TCA for both pandemic O3:K6 strains and other serovars (Fig. 1b). Such enhancement could be explained readily by the observed increases in cell numbers for nine strains, whereas fourfold or more increases in KAP-RPLA titer were observed in other 14 strains. This enhanced TDH production was, however, not specific to

the pandemic O3:K6 strains. It should be noted that KE9967 (O3:K6, non-pandemic) and NIID965–98 (O3:K6, pandemic) grown with TCA produced 32 more folds of TDH than did those grown without. It should be also noted that NIID181-99 (O4:K68), KE10540 (O3:K68), KE10542 (O3:K48), and KE10597 (O1:K1) produced sizable amounts of TDH with TCA, while they did not produce any without TCA. The evidence further supports our previous view [15] that the bile acid enhanced synthesis of TDH by *V. parahaemolyticus*, in which the amounts of TDH released from lysed cells grown with the bile acids were four to 32-fold greater than those from lysed cell grown without. Likewise, Pace et al. [16] reported that bile acids enhanced the expression of virulence factors of *V. parahaemolyticus* such as Congo red binding, bacterial capsule size, and adherence to epithelial cells. Bile acids are thus most likely to play a critical role in the pathogenicity of *V. parahaemolyticus* in the human intestine. Admittedly, a limited sample size and diversity of the strains used in the present study made it difficult for us to draw an overall conclusion on the effect of bile acids on TDH production. Further work is in progress to identify any unique traits for the "pandemic" strains.

ACKNOWLEDGMENTS

We are grateful to Centers of Disease Control and Prevention, Atlanta, USA for providing strains. This study was supported by a grant from the Ministry of Health and Welfare of Japan.

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