Characterization of a new thermostable direct haemolysin produced by a Kanagawa-phenomenon-negative clinical isolate of *Vibrio parahaemolyticus*

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The production of two haemolysins, thermostable direct haemolysin (Vp-TDH) and a Vp-TDH-related haemolysin (Vp-TRH), by clinical isolates of *Vibrio parahaemolyticus* has previously been reported. Here we describe a third type of haemolysin (named Vp-TDH/I), which is produced by a clinical isolate (strain TH012) that is Kanagawa phenomenon negative. Vp-TDH/I was purified by a series of column chromatographies on DEAE-Sephadex A25, hydroxyapatite, Sepharose 4B and Mono Q. By physicochemical, biological and immunological analyses, Vp-TDH/I was demonstrated to be similar, but not identical, to Vp-TDH and Vp-TRH. The gene encoding Vp-TDH/I was cloned and the deduced amino acid sequence of Vp-TDH/I confirmed that Vp-TDH/I has a sequence different from those of previously known Vp-TDH and Vp-TRH. Not only purified Vp-TDH/I but also live cells of the Vp-TDH/I-producing strain induced fluid accumulation in ligated rabbit intestine. We conclude that this clinical isolate produces a new type of Vp-TDH-related haemolysin, which may be involved in the pathogenesis of this organism.

Introduction

Vibrio parahaemolyticus is a major cause of acute gastroenteritis and is found worldwide (Miwatani & Takeda, 1976; Joseph *et al.*, 1982; Janda *et al.*, 1988). This organism is classified into two types by the Kanagawa phenomenon (KP) test, a haemolytic test on Wagatsuma blood agar (Miyamoto *et al.*, 1969; Miwatani & Takeda, 1976; Joseph *et al.*, 1982). Thermostable direct haemolysin (Vp-TDH) has been shown to be responsible for the KP (Honda *et al.*, 1976*b*; Miwatani & Takeda 1976; Miyamoto *et al.*, 1980). It used to be thought that a positive KP test was closely associated with the pathogenicity of *V. parahaemolyticus* in humans (Sakazaki *et al.*, 1968; Miyamoto *et al.*, 1969; Miwatani & Takeda, 1976; Honda *et al.*, 1976*a*; Joseph *et al.*, 1982; Janda *et al.*, 1988). However, more recently some cases of

V. parahaemolyticus gastroenteritis have been found to be due to KP-negative strains (Honda *et al.*, 1987, 1988, 1989*a*; Honda & Miwatani, 1988). Many of these KP-negative strains were found to produce a Vp-TDH-related haemolysin, Vp-TRH (Honda *et al.*, 1988, 1989*a*, *b*; Nishibuchi *et al.*, 1989), which is immunologically and biophysicochemically related, but not identical, to Vp-TDH produced by KP-positive *V. parahaemolyticus*.

A recent survey of 102 KP-negative clinical isolates by ELISA for production of Vp-TDH or Vp-TRH showed that 41 of the strains were Vp-TRH producers and that 22, including strain TH012, were Vp-TDH producers (Honda *et al.*, 1989*b*, 1990*b*).

Here we describe the characterization of a Vp-TDH-like haemolysin, named Vp-TDH/I produced by a KP-negative clinical isolate (TH012) of KP-negative *V. parahaemolyticus*. Vp-TDH/I has both similar and different characteristics to the previously known Vp-TDH (Miwatani & Takeda, 1976; Joseph *et al.*, 1982) and Vp-TRH (Honda *et al.*, 1988, 1989*a*) produced by *V. parahaemolyticus* and has a different amino acid sequence from that of Vp-TDH and Vp-TRH produced by clinical strains of ordinary KP-positive and KP-negative *V. parahaemolyticus*, respectively.

Abbreviations: KP, Kanagawa phenomenon; Vp-TDH and Vp-TRH, *Vibrio parahaemolyticus* thermostable direct haemolysin and TDH-related haemolysin.

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number D90238.

Methods

Bacterial strains. *V. parahaemolyticus* TH012 (serovar O6:K18), a KP-negative strain determined by the modified Elek test (Honda *et al.*, 1980), was isolated from a patient with traveller's diarrhoea at Osaka International Airport Quarantine Station. *Escherichia coli* JM109 (Yanisch-Perron *et al.*, 1985) was used for cloning.

Bacterial cultures and preparation of crude haemolysin. Strain TH012 was cultured in SPP broth (0.5% NaCl, 1% peptone, 0.2% glucose and 0.5% Na₂HPO₄ · 12H₂O, pH 7.6; Honda *et al.*, 1988) at 37 °C for about 18 h with shaking. Procedures essentially as described previously (Honda *et al.*, 1988, 1989a) were used to obtain the crude preparation. In brief, the culture supernatant was obtained by centrifugation (16000 g for 20 min) and concentrated with a Pellicon PTGC filter (*M_r* cut-off 10000; Millipore). The precipitates obtained by ammonium sulphate fractionation (55% saturation) were extensively dialysed against 0.01 M-sodium phosphate buffer (pH 7.0), and used as crude Vp-TDH/I.

Purification of Vp-TDH/I. This was achieved as described previously (Honda *et al.*, 1989a) by a series of column chromatographies on DEAE-Sephadex A25, hydroxyapatite, Sepharose 4B and Mono Q (Pharmacia).

Polyclonal and monoclonal antisera. Antisera against purified Vp-TDH/I were raised in rabbits by the method described previously for Vp-TRH (Honda *et al.*, 1988, 1989a) and Vp-TDH (Honda *et al.*, 1980). The double gel diffusion test was performed in 1% Noble agar in 0.01 M-sodium phosphate buffer (pH 7.0) containing 0.05% Na₂S₂O₃.

Murine monoclonal antibodies (mAbs) against Vp-TDH/I were prepared by the method described previously (Honda *et al.*, 1989b, 1990a). Briefly, after completing the immunization schedule, mouse spleen cells from the immunized mice were fused with X6-Ag8.653 myeloma cells as described previously (Honda *et al.*, 1989b). After selecting hybrid cells by HAT selection, clones producing mAbs were screened by ELISA using Vp-TDH/I-coated plates. The positive hybridomas were finally recloned at least twice by the limiting dilution method, and ascites were induced by intraperitoneal injection of the hybridoma cells into BALB/c mice.

mAbs against Vp-TDH and Vp-TRH were developed as described previously (Honda *et al.*, 1989b, 1990a).

Polyacrylamide gel electrophoresis. SDS slab PAGE and non-denaturing slab PAGE with samples at concentrations of 100 µg ml⁻¹ were carried out as previously described (Honda *et al.*, 1988).

Haemolysin assay. Haemolysin activity was assayed by a microplate method using erythrocytes from various animal sources as described previously (Honda *et al.*, 1988). Briefly, a mixture of 100 µl toxin solution diluted with 10 mM-Tris/HCl (pH 7.0) containing 0.85% NaCl and an equal volume of a 2% (v/v) suspension of erythrocytes was incubated at 37 °C and then centrifuged at 1800 g for 2 min. The supernatant was transferred to a flat-bottomed microtitre plate and the A₅₄₀ measured (Honda *et al.*, 1988).

Rabbit ileal loop test. This test, with purified Vp-TDH/I or live Vp-TDH/I-producing cells, was performed as described by Twedt *et al.* (1980). Toxin was diluted with phosphate-buffered saline (PBS, pH 7.0) to 25, 50, 100, and 200 µg ml⁻¹ and 1 ml of each toxin solution was inoculated into each loop prepared in the intestine of a 1.5 kg rabbit. After 14 h incubation, the ratio (FA ratio) of loop-fluid volume to loop length was determined.

Enzymes and chemicals. Restriction enzymes, T4 DNA ligase, bacterial alkaline phosphatase and DNA polymerase Klenow fragment used in this study were purchased from Toyobo Co. (Osaka, Japan).

DNAase I, IPTG and X-Gal were purchased from Sigma. [α -³²P]dCTP was obtained from Amersham Japan Co. (Tokyo, Japan).

DNA manipulations and DNA sequencing. Total DNA from *V. parahaemolyticus* and plasmid DNA were isolated according to the methods of Birnboim & Doly (1979) and Silhavy *et al.* (1984). Digestions of DNA with restriction enzymes, DNA ligation and gel electrophoresis, transformation and colony hybridizations were performed as described by Maniatis *et al.* (1982). Oligonucleotide primers for DNA sequencing were synthesized by an automatic DNA synthesizer (Applied Biosystems). DNA sequencing was done by the method of Sanger *et al.* (1977) using single-stranded DNA as a template. Single-stranded DNAs were prepared from recombinant plasmids cloned with pUC119 (Vieira & Messing, 1987). DNA sequencing kits were purchased from Takara Shuzo Co. (Kyoto, Japan).

Construction of a recombinant plasmid for use as a *tdh* probe. A 359 bp *TaqI*-*NdeI* fragment of the structural gene (*tdhA*) was isolated from the recombinant plasmid pTI10 (Iida & Yamamoto, 1990). pTI10 contains a 1.2 kb *EcoRI* fragment of *V. parahaemolyticus* T4750 DNA in the *EcoRI* site of pHS398. The *TaqI*-*NdeI* fragment comprises 63% of the coding region of *tdhA*. The fragment was rendered blunt-ended by using the Klenow fragment of DNA polymerase and ligated with *EcoRI* linkers. The linked fragment was digested with *EcoRI*, purified by PAGE and cloned into the *EcoRI* site of pUC18 (Messing, 1983). A recombinant plasmid (pKY199) was isolated and was confirmed to have the *EcoRI*-linked fragment by DNA sequencing. pKY199 was prepared on a large scale and digested with *EcoRI*. The resultant probe fragment was separated by PAGE and extracted. A random primer DNA labelling method (Feinberg & Vogelstein, 1983) was used to label the DNA probe with ³²P.

Results

Purification of Vp-TDH/I

The production of a TDH-related haemolysin (Vp-TDH/I) by strain TH012 was demonstrated by ELISA as described previously (Honda *et al.*, 1989b) and Vp-TDH/I was purified from a large-scale culture (6 litres) by sequential steps of precipitation with ammonium sulphate (55% saturation) and chromatography on DEAE-Sephadex A25, hydroxyapatite, Sepharose 4B and finally Mono Q (fast protein liquid chromatography: FPLC) columns. The elution patterns of Vp-TDH/I from these column chromatographies were very similar to those of Vp-TRH and Vp-TDH as described previously (Honda *et al.*, 1976b, 1988, 1989a). A typical elution profile of Mono Q FPLC is shown in Fig. 1. By this procedure, the final recovery of the toxin was approximately 23% and 1020 µg of purified Vp-TDH/I was obtained from 6 litres of culture.

Characterization of Vp-TDH/I

On SDS slab PAGE (Fig. 2), the purified Vp-TDH/I gave one protein band in almost the same position as those of Vp-TDH and Vp-TRH which were previously

Fig. 1. Typical elution profile of Vp-TDH/I on Mono Q FPLC. —, A_{280} ; ----, NaCl concentration. The bar represents fractions containing haemolytic activity.

Fig. 2. SDS slab PAGE of purified toxins. Lanes: 1, Vp-TDH; 2, Vp-TRH; 3, Vp-TDH/I; 4, M_r markers.

purified (Honda *et al.*, 1976*a*, 1988, 1989*a*). The M_r values of the subunit and holotoxin of Vp-TDH/I were estimated to be about 23 000 and 47 000 from the mobility on SDS slab PAGE and from the elution position on gel filtration (Sephacose 4B) column chromatography, respectively. Thus, like Vp-TDH and Vp-TRH (Honda *et al.*, 1988), Vp-TDH/I is composed of two identical subunits of M_r 23 000.

Despite these similarities of Vp-TDH/I to Vp-TDH and Vp-TRH, conventional slab PAGE showed that purified Vp-TDH/I migrated as a single band and had a different mobility from those of Vp-TDH and Vp-TRH (Fig. 3).

Fig. 3. Non-denaturing slab PAGE of purified toxins. Lanes: 1, Vp-TDH/I; 2, Vp-TDH; 3, Vp-TRH; 4, Vp-TDH/I.

Fig. 4. Ouchterlony double gel immunodiffusion test. Wells: 1, anti-Vp-TDH; 2, anti-Vp-TRH; 3, anti-Vp-TDH/I.

Immunological cross-reactivity of Vp-TDH/I with Vp-TDH and Vp-TRH was analysed using both polyclonal and monoclonal antibodies. An Ouchterlony double gel diffusion test (Fig. 4) demonstrated full antigenic identity between Vp-TDH/I and Vp-TDH, but Vp-TDH/I showed only partial identity to Vp-TRH. This was confirmed by analysis with ELISA using mAbs. Six mAbs were developed against Vp-TDH/I purified in this study. Three reacted similarly with both Vp-TDH and Vp-TRH as well as with Vp-TDH/I, and the other three reacted only with Vp-TDH/I and Vp-TDH (Table 1), suggesting again that Vp-TDH/I is antigenically identical to Vp-TDH, but has only partial identity to Vp-TRH. This result was confirmed by analyses with mAbs

Table 1. Reactivity of mAbs developed against Vp-TDH/I, Vp-TRH and Vp-TDH

Clone*	ELISA activity† in plates coated with:			
	Vp-TDH/I	Vp-TRH	Vp-TDH	BSA
(a) 9E-6H	1970	35	1258	42
1A-24A	1344	31	1744	36
2A-13C	1035	40	911	40
4E-12D	845	935	982	42
9B-11B	826	869	951	61
4E-3D	1047	1098	1273	71
(b) 1-D	1830	76	1666	61
2-D	1211	88	1222	61
3-D	1832	1165	1896	55
5-D	1565	1480	1799	50
8-D	1310	1359	1411	58
(c) 1-R	41	1680	60	39
2-R	56	1281	48	51
4-R	1471	1509	1570	44
5-R	59	1305	86	60
7-R	1168	1756	1108	47

* (a) mAbs against Vp-TDH/I developed in this study; (b) mAbs developed against Vp-TDH (Honda *et al.*, 1989b); (c) mAbs developed against Vp-TRH (Honda *et al.*, 1990).

† Values (A_{405}) were obtained in ELISA performed as described previously (Honda *et al.*, 1989b).

developed against Vp-TDH (Honda *et al.*, 1989b) and Vp-TRH (Honda *et al.*, 1990a) (Table 1).

The haemolytic activities of Vp-TDH/I on erythrocytes from various animals were examined and the results are compared with those of Vp-TDH and Vp-TRH (Honda *et al.*, 1988) in Table 2. Like Vp-TDH and Vp-TRH, Vp-TDH/I did not lyse horse erythrocytes. There were, however, differences in the sensitivity of various erythrocytes to Vp-TDH/I, Vp-TRH and Vp-TDH. In particular, human, monkey and rat erythrocytes were the most sensitive to Vp-TDH/I.

The haemolytic activity (against human erythrocytes) of Vp-TDH/I, like Vp-TDH and unlike Vp-TRH (Honda *et al.*, 1988, 1989a), was stable upon heating at 60 °C or higher temperatures (70, 80, 90 and 100 °C) for 10 min. The purified Vp-TDH/I induced definite fluid accumulation in ligated rabbit intestine. The minimal dose of purified Vp-TDH/I resulting in an FA ratio greater than 0.5 ml cm⁻¹ was about 100 µg per loop of intestine (Table 3a), which is comparable with the dose for Vp-TDH (Miwatani & Takeda, 1976; Miyamoto *et al.*, 1980). Live cells of strain TH012, from which Vp-TDH/I was isolated and purified, could also induce marked fluid accumulation, whereas *V. parahaemolyticus* strain Vp-A10, producing neither Vp-TRH, Vp-TDH/I nor Vp-TDH, did not induce such fluid accumulation (Table 3b).

Table 2. Comparison of lytic activities of Vp-TDH, Vp-TRH and Vp-TDH/I on erythrocytes from various sources

Erythrocyte source	Specific activity [haemolytic units (µg haemolysin) ⁻¹] of		
	Vp-TDH/I	Vp-TDH	Vp-TRH
Human	21.6	1.7	2.0
Calf	6.9	0.8	35.2
Guinea-pig	10.6	1.5	2.3
Sheep	1.7	0.3	34.1
Monkey	38.9	3.3	19.7
Mouse	0.7	6.9	11.8
Chicken	16.7	0.7	19.6
Rat	37.1	9.1	37.1
Horse	<0.01	<0.01	<0.01

Table 3. Fluid accumulation in the rabbit ileal loop test caused by purified Vp-TDH/I (a) and live cells (b) of *V. parahaemolyticus*

Sample	Fluid accumulation (ml cm ⁻¹)
(a) Purified Vp-TDH/I (µg per loop):	
200	1.29 ± 0.25
100	0.79 ± 0.31
50	0.22 ± 0.29
25	0
0	0
(b) Live cells (cells per loop):	
TH012	
10 ⁸	1.36 ± 0.24
10 ⁶	0.97 ± 0.21
10 ⁴	0.44 ± 0.34
0	0
Vp-A10*	
10 ⁸	0.17 ± 0.09
10 ⁶	0

* An isolate of *V. parahaemolyticus* from sea-water, which produced neither Vp-TDH, Vp-TRH nor Vp-TDH/I.

Cloning and nucleotide sequencing of Vp-TDH/I gene (*tdh/I*)

Whole DNA of *V. parahaemolyticus* TH012 was digested with *Hind*III and ligated into the *Hind*III site of vector plasmid pBR322 (Bolivar *et al.*, 1977). *E. coli* JM109 was transformed with the recombinant DNA, and clones harbouring recombinant plasmids containing the structural gene, *tdh/I*, for Vp-TDH/I were screened by colony hybridization with a ³²P-labelled *tdh* gene probe. A recombinant pNY1, which contained a 2.3 kb fragment of *V. parahaemolyticus* DNA, hybridized with the *tdh* gene probe. The 2.3 kb *Hind*III insert was subcloned into the *Hind*III site of pUC119. The resultant recombinants, pKY231 and pKY232, with the insert in opposite

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AACCAACAACCACATGCCAGTTGTTGACAATATCAACAAAAGAGAATCATTATTAAATGTTTTAGCTTATGGTT
--T-----GT-AC-----T-----

TTTATGAATT*~~ccc~~*AAATTA*~~ccc~~*AATGTTTTGCA*TTGTGCTATGTT*GTTT*CAAGCTATTTATT
-A---*AA--ATGA-----AATTC-----TGCAATTGTAATGTTTGTTCAGCTATTTATTGCTTCTGA

TGGTTACACTTTAGAGGCTTTGTTGOGTAATAGTGAATTC

Fig. 5. Nucleotide sequence of *tdh1*, and comparison with those of the *tdhA* and *trh* genes. Boxes of solid and broken lines indicate initiation and terminal codons, respectively. The putative ribosome-binding site is underlined. Identical (–) and deleted (★) nucleotides are indicated. The two horizontal arrows in opposite directions show an inverted repeat sequence. ○, nucleotide of *tdh1* different from that of *tdh3* reported by Nishibuchi & Kaper (1990).

orientations in relation to the *lac* promoter of the vector, were isolated and single-stranded DNA was prepared from them. The DNA sequence of *tdh/l* was determined with the single-stranded DNAs using 20–24-mer synthesized oligonucleotides as primers by the dideoxy chain-termination method.

We found a 567 bp open reading frame in the 2.3 kb region, which can code for a protein of 189 amino acids (designated Tdh/I) (Fig. 5). The deduced M_r of the Tdh/I protein was 21550. A putative ribosome-binding sequence (GAGG) (Shine & Dalgarno, 1974) was observed 7 bp upstream of the initiation codon. Promoter regions could not be specified since there were several pairs of sequences with similarity to the consensus sequences of *E. coli* promoter (−35 and −10) (Rosenberg & Court, 1979). An inverted repeat sequence, which may serve as a transcriptional termination sequence for the gene, was found 13 bp downstream of the termination codon of *tdh/I*. The whole structural gene shows a high degree of homology with the previously reported sequences of the *tdh* of KP-positive *V. parahaemolyticus* strains WP-1 (97.4%) (Nishibuchi *et al.*, 1985) and T4750 (*tdhA* 98.2%, *tdhS* 97.2%) (Iida & Yamamoto 1990).

Since Vp-TDH/I is an extracellular protein, the nascent unprocessed precursor in the cytoplasm should possess a signal peptide for secretion through the cytoplasmic membrane. In the amino-terminal region of the Tdh/I protein deduced from the DNA sequence (Fig. 6), there are a few basic amino acids followed by a highly hydrophobic region that ends with an Ala₂₄ residue, which is the cleavage site for most signal peptides; this suggests that the first 24 amino acids comprise a signal peptide for the Tdh/I protein. This was confirmed by direct sequencing of the Vp-TDH/I protein, which showed that the N-terminal amino acid was Phe₂₅ (data not shown). The deduced sequence of the Tdh/I protein, excluding the putative single peptide, differed in 7 and 55 amino acid residues, respectively, from the amino acid

	Tdh/I	1	MKYRYFAKKS	FLFISMLAAF	KTFAFELPSV	PFPAPGSDEI	LFVVRRDATFN
	TdhA						-----T-----
	Trh		--LKLYFAF-	-L-A-----	---ID---I	---S---L	----NT-IK
	51		TNAPVNVKVS	DFWTNRNVKR	KPYKDVGQGS	VFTTSGTKWL	TSYMTVNIND
			-----E-				-----
			-ES---AI-N	-Y-----I--	---S-H---	I-----S---	SA-I-----G
	101		KDYTHAAVSG	YKRGHSAVFV	KSDQVQLQHS	YNSVANFVGE	DEDSIPSKMY
			-----H-			-D-S-	-----
			NN-----L-	--D-L-T--T	--EKTS-NQN	-S--SD----	N-E-L-VI-
	151		LDETPEYFVN	VEAYESGSGN	ILVMCISNKE	SFFECEHQK	
			-----			-----K-Q	
			-----	-----N-H	MF-----S	--D--MS-N	

Fig. 6. Amino acid sequence of Tdh/I (Vp-TDH/I) deduced from the nucleotide sequence of *tdh/1* and comparison with those of TdhA and Trh deduced from the reported nucleotide sequences of the *tdhA* (reference given in the Results) and *trh* (Nishibuchi *et al.*, 1989) genes, respectively. An arrow indicates the putative cleavage site.

sequences of Vp-TDH (Iida & Yamamoto, 1990) and Vp-TRH (Nishibuchi *et al.*, 1989).

Discussion

Although the production of Vp-TDH-related haemolysin(s) by *V. parahaemolyticus* was suggested by Zen-Yoji *et al.* (1974), detailed purification and characterization of the toxin(s) have not been performed until recently (Honda *et al.*, 1988, 1989a). Two major haemolytic toxins of *V. parahaemolyticus* have been reported to date, namely Vp-TDH (Honda, *et al.*, 1976b; Joseph *et al.*, 1982; Janda *et al.*, 1988) and Vp-TRH (Honda *et al.*, 1988, 1989a). In this paper, we describe the production of a third Vp-TDH-related haemolysin, Vp-TDH/I, by a clinical isolate of KP-negative *V. parahaemolyticus*. Vp-TDH/I was purified and shown to have similar properties to Vp-TDH by SDS-PAGE, immunological tests using polyclonal and monoclonal antibodies and heat-stability tests; but it also has different characteristics from Vp-TDH as demonstrated by non-denaturing PAGE, and by its erythrocyte-sensitivity spectrum and amino acid sequence. Vp-TDH/I shows only partial antigenic identity with Vp-TRH, and some characteristics such as heat stability and erythrocyte sensitivity of Vp-TDH/I are also different from those of Vp-TRH. Moreover, the deduced sequence of Tdh/I protein (Vp-TDH/I) differed in 7 and 55 amino acid residues, respectively, from the amino acid sequence of Vp-TDH (Iida & Yamamoto, 1990) and that of Vp-TRH deduced from DNA analysis (Nishibuchi *et al.*, 1989), suggesting that Vp-TDH/I relates more closely to Vp-TDH than to Vp-TRH. Vp-TDH/I is an additional member of the Vp-TDH family of a haemolysin, which includes the recently described Vp-TDH-related haemolysins produced by *V. cholerae* non-O1 (Yoh *et al.*, 1986a), *V. mimicus* (Honda & Miwatani, 1988) and *V. hollisae* (Yoh *et al.*, 1986b).

Nishibuchi & Kaper (1990) recently described four *tdh* genes (genes related to Vp-TDH) of *V. parahaemolyticus*.

Interestingly, one of these genes (designated *tdh3*) is similar (except for one amino acid and two synonymous [silent] nucleotide substitutions) to the *tdh/I* gene described in this study. Although Nishibuchi & Kaper (1990) reported that the *V. parahaemolyticus* strain carrying the *tdh3* gene did not produce a detectable amount of Vp-TDH due to lack of *tdh*-specific RNA transcript, we found in this study that strain TH012 of *V. parahaemolyticus* does produce Vp-TDH/I, a product of the *tdh/I* (similar to *tdh3*) gene.

The fact that there was no immunological difference between Vp-TDH/I and Vp-TDH suggests that the seven amino acid residues of Vp-TDH/I which are different from Vp-TDH are not involved in forming antigenic epitopes. However, these seven residues affected the electrophoretic mobility and erythrocyte sensitivity, suggesting a molecular charge difference and a difference in receptor recognition by the toxin or lytic process of the toxin, respectively.

One peculiar point is that strain TH012 was judged to be KP-negative, although it was found in this study to produce a Vp-TDH-related haemolysin (Vp-TDH/I). A similar phenomenon was observed in the case of Vp-TRH (Honda *et al.*, 1987, 1988). The main reason for this is that the optimum culture conditions for the production of Vp-TDH and Vp-TDH/I were different, and the conditions established for KP tests [modified Elek test (Honda *et al.*, 1980) and Wagatsuma agar method (Miyamoto *et al.*, 1969; Miwatani & Takeda, 1976)] included a high (3% or more) sodium chloride content, which is not suitable for the production of Vp-TDH/I; strain TH012 produced about 70 times more Vp-TDH/I in a medium containing 0.5% NaCl such as SPP (Honda *et al.*, 1988) than in the medium containing 3% NaCl (data not shown).

The fact that not only live cells of strain TH012 but also purified Vp-TDH/I induced fluid accumulation in rabbit ileal loop experiments suggests the probable importance of Vp-TDH/I and Vp-TDH/I-producing *V. parahaemolyticus* as a cause of gastroenteritis.

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