BACTERIAL PATHOGENICITY

Characterisation of a haemolysin related to Vp-TDH produced by a Kanagawa phenomenon-negative clinical isolate of *Vibrio parahaemolyticus*

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Summary. The production of a family of haemolysins—thermostable direct haemolysin (Vp-TDH), Vp-TDH-related haemolysin (Vp-TRH) and Vp-TDH/I—has been reported in clinical isolates of *Vibrio parahaemolyticus*. This paper describes a fourth type of haemolysin—Vp-TDH/II—produced by a Kanagawa phenomenon-negative clinical isolate of *V. parahaemolyticus* (O13:K, untypable). Vp-TDH/II was purified by ammonium sulphate precipitation and successive filtrations on DEAE-cellulose, hydroxyapatite, Sepharose 4B and Mono Q columns. Vp-TDH/II was biophysicochemically and immunologically similar to, but not identical to Vp-TDH, Vp-TRH and Vp-TDH/I. Vp-TDH/II stimulated vascular permeability in rabbit skin and was lethal to mice. Purified Vp-TDH/II and viable cells of the Vp-TDH/II-producing strain both induced fluid accumulation in ligated rabbit intestine. The plasmid-determined structural gene for Vp-TDH/II was cloned and the nucleotide sequence determined. The deduced amino acid sequence of Vp-TDH/II differed from those of Vp-TDH, Vp-TRH and Vp-TDH/I.

Introduction

Vibrio parahaemolyticus was first isolated in 1950¹ and is considered to be a major cause of gastro-enteritis.^{2–4} The two forms of this organism can be distinguished by the Kanagawa phenomenon, which involves detection of haemolysis on Wagatsuma blood agar medium.^{2,3,5} The Kanagawa phenomenon is thought to be caused by the action of thermostable direct haemolysin (Vp-TDH).^{2,6,7} Most clinical isolates are Kanagawa-positive, whereas most environmental isolates are negative.⁸ Thus, Vp-TDH is thought to be associated with the enteropathogenicity of this organism.^{4,7,8} However, Kanagawa phenomenon-negative strains of *V. parahaemolyticus* can also occasionally cause gastro-enteritis, and these organisms produce a Vp-TDH-related haemolysin (Vp-TRH). Vp-TRH is immunologically and biophysicochemically similar to, but not identical to the Vp-TDH produced by Kanagawa phenomenon-positive strains.^{9–13} A third type of haemolysin—Vp-TDH/I produced by a Kanagawa phenomenon-negative clinical isolate of *V. parahaemolyticus*—is also similar to, but not identical to either Vp-TDH or Vp-TRH.¹⁴

This study describes a new member of the Vp-TDH/TRH family of haemolysins. Named Vp-TDH/II, this enzyme was produced by a Kanagawa phenomenon-negative strain of *V. parahaemolyticus* isolated from a patient with traveller's diarrhoea. This strain (TH103) produced neither Vp-TDH, as determined by the modified Elek method,¹⁵ nor Vp-TRH as determined with a specific ELISA.¹⁶ However, under certain culture conditions, the supernate of the strain had haemolytic activity against human erythrocytes, and the production of a haemolysin belonging to the Vp-TDH/TRH family was suggested by an ELISA incorporating a monoclonal antibody directed against Vp-TDH.¹²

Materials and methods

Bacterial strains

V. parahaemolyticus strain TH103 (serovar O13:K, untypable), shown to be Kanagawa-negative by the modified Elek test,¹⁵ was isolated from a patient with traveller's diarrhoea at Osaka International Airport Quarantine Station. *V. parahaemolyticus* strain N1 was isolated from sea-water and did not produce Vp-TDH, Vp-TRH, Vp-TDH/I or Vp-TDH/II. *Escherichia coli* strain JM109¹⁷ was used for molecular cloning.

Bacterial culture and preparation of haemolysin

Strain TH103 was cultured in SPP broth (NaCl 0.5% w/v, peptone 1% w/v, glucose 0.2% w/v, $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ 0.5% w/v, pH 7.6)¹⁰ at 37°C for c. 16 h with continuous shaking. Crude haemolysin was obtained essentially as described previously.^{10,11} Briefly, the culture was centrifuged at 15000 *g* for 20 min. The supernate was concentrated with a Pellicon PTGC filter with a mol. wt cut-off of 10000 (Millipore). The precipitates obtained by ammonium sulphate fractionation (55% saturation) were dialysed extensively against 0.01 M sodium phosphate buffer, pH 7.0, and used as crude Vp-TDH/II. Further purification was by a series of filtrations on DEAE-cellulose, hydroxyapatite, Sepharose 4B and Mono Q (Pharmacia) columns as described previously.¹¹

Antisera, immunodiffusion tests and SDS-PAGE

Antisera against purified Vp-TDH/II were raised in rabbits by the method described previously for Vp-TRH^{10,11} and Vp-TDH.¹⁵ The double gel diffusion test was performed in Noble Agar (Difco) 1% w/v in 0.01 M sodium phosphate buffer, pH 7.0. Polyclonal antibodies against Vp-TDH, Vp-TRH and Vp-TDH/II were developed as described previously.^{10,11,14,15} SDS-PAGE and non-denaturing PAGE were carried out on slab gels as described previously.¹⁰

Haemolysis assay

Haemolytic activity was assayed by a microtitration plate method with erythrocytes from various animal sources as described previously.¹⁰ Briefly, a mixture comprising 100 μl of toxin preparation diluted with 0.01 M Tris-HCl, pH 7.0, NaCl 0.85% w/v and an equal volume of erythrocytes 2% v/v was incubated at 37°C for 30 min and then centrifuged at 1800 *g* for 2 min. The supernate was transferred to a flat-bottomed 96-well microtitration plate and the absorbance at 540 nm was measured.¹⁰

Rabbit skin vascular permeability factor (PF) test and mouse lethality test

The PF test was carried out essentially as described previously.¹⁸ Two rabbits were challenged by intradermal injection with 0.1 ml of 10-fold dilutions of toxin samples at the indicated times. The diameters of the blue lesions in the skin ("blueing") were measured 1 h after injection of Evans blue 5% w/v solution (1 ml/kg body weight). This experiment was performed in triplicate. Lethal toxicity was assayed in mouse lethality tests¹⁹ by injecting purified toxins intravenously into 5-week-old mice (ddY strain) and observing the mice for 24 h.

Rabbit ileal loop test

The rabbit ileal loop test with purified Vp-TDH/II or a culture of Vp-TDH/II-producing cells (strain TH103) was performed essentially as described previously.²⁰ Toxin was prepared in phosphate-buffered saline (PBS; pH 7.0) at 50, 100 and 200 $\mu\text{g}/\text{ml}$, and 1-ml portions were inoculated into loops prepared in the small intestine of a 1.5-kg rabbit.

Enzymes and chemicals

Restriction endonucleases, T4 DNA ligase, bacterial alkaline phosphatase and DNA polymerase Klenow fragment used in this study were purchased from Toyobo Co. (Osaka, Japan); IPTG and X-Gal were purchased from Sigma and [³²P]dCTP was from Amersham.

DNA manipulation, DNA cloning and DNA sequencing

Total DNA and plasmid DNA from *V. parahaemolyticus* strain TH103 were isolated according to the methods of Birnboim and Doly²¹ and Silhavy *et al.*,²² respectively. Digestion of DNA with restriction endonucleases, DNA ligation and gel electrophoresis, transformation and colony hybridisations were performed as described previously.²³ A 359-bp DNA probe for *tdh* was prepared from pKY199¹⁴ and labelled with ³²P by the random-primed labelling method.²⁴ Total DNA and plasmid DNA were digested with restriction endonucleases and subjected to electrophoresis in agarose 0.8% w/v gels. The digests were transferred to a nitrocellulose membrane by the method of Southern.²⁵ The membrane was pre-hybridised with hybridisation buffer (5 × SSPE,²² formamide 40% v/v, SDS 1% w/v, 5 × Denhardt's solution,²² alkali-denatured sonicated salmon sperm DNA²² 100 $\mu\text{g}/\text{ml}$) for 2 h, and was then hybridised at 42°C for 24 h in the same buffer containing (5 ng/ml) the DNA probe labelled with ³²P. The membrane was then washed three times with 2 × SSPE (containing SDS 1% w/v) for 5 min at room temperature, dried at room temperature and then exposed to X-ray film at −70°C. The procedure for colony hybridisations was the same as that used for Southern blot hybridisations. The oligonucleotide primer for DNA sequencing was synthesised with an automatic DNA synthesiser (Applied Biosystems, Foster City, CA, USA) and purified on a Mono-Q column (Pharmacia). The selected base sequence comprised nucleotides 101–125 of the *tdh* gene, measured from the initiation codon ATG (5'-CCCCGTTCTGAXGAGATATTGTT-3', where X denotes a deoxyuridine where a side-arm was linked). DNA sequencing was performed by the dideoxy-chain termination method of Sanger *et al.*²⁶

Fig. 1. Typical elution profile of Vp-TDH/II from a Mono Q FPLC column. The dotted line indicates the NaCl concentration. The bar indicates fractions containing haemolytic activity.

Results

Purification of Vp-TDH/II

Vp-TDH/II was purified from a 5-L culture, with a final recovery of *c.* 33%, by sequential precipitations with ammonium sulphate (55% saturation), and filtration through DEAE-cellulose, hydroxyapatite, Sepharose 4B and Mono Q FPLC columns. Elution patterns of Vp-TDH/II were similar to those of Vp-TRH.¹¹ A typical elution profile of Vp-TDH/II from a Mono Q FPLC column is shown in fig. 1.

PAGE

SDS-PAGE revealed a single protein band in almost the same position (fig. 2) as Vp-TDH, Vp-TRH and

Vp-TDH/I.^{10,11,14} The mol. wt of the Vp-TDH/II subunit was estimated as *c.* 23 000 from its mobility on SDS-PAGE slab gels. The mol. wt of Vp-TDH/II holotoxin was determined to be *c.* 47 000 by Sepharose 4B gel filtration. Thus, Vp-TDH/II appeared to be composed of two identical subunits, each with a mol. wt of *c.* 23 000. Non-denaturing slab-gel PAGE demonstrated that purified Vp-TDH/II migrated as a single band with a mobility different from that of Vp-TDH, Vp-TRH or Vp-TDH/I (fig. 3).

Immunodiffusion test

The Ouchterlony double gel diffusion test demonstrated that Vp-TDH/II was antigenically indistinguishable from Vp-TDH and Vp-TDH/I. Vp-TDH/II showed only partial identity to Vp-TRH (fig. 4).

Fig. 2. SDS-PAGE of purified toxins. Lanes: 1, Vp-TDH; 2, Vp-TRH; 3, Vp-TDH/I; 4, Vp-TDH/II; 5, marker proteins.

Fig. 3. Non-denaturing slab-gel PAGE of purified toxins. Lanes: 1, Vp-TRH; 2, Vp-TDH/II; 3, Vp-TDH; 4, Vp-TDH/I; 5, Vp-TRH.

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

Table I. Comparison of the lytic activities of Vp-TDH, Vp-TRH, Vp-TDH/I and Vp-TDH/II with erythrocytes from various sources

Source of erythrocytes	Specific activity (HU* of haemolysin/ μ g)			
	Vp-TDH/II	Vp-TDH	Vp-TRH	Vp-TDH/I†
Mouse	33.1	19.7	11.4	0.7
Rabbit	12.8	10.1	1.3	...
Human	24.2	7.3	2.0	21.6
Calf	4.1	2.3	32.2	6.9
Chicken	3.3	1.9	12.3	16.7
Sheep	0.5	0.2	31.1	1.7
Horse	< 0.01	< 0.01	< 0.01	< 0.01

*One HU was defined as the amount of haemolysin causing 50% haemolysis in the haemolysis assay used (see *Materials and methods*).

†Data for Vp-TDH/I taken from Honda *et al.*¹⁴

Haemolytic activity

The specific haemolytic activity of Vp-TDH/II against erythrocytes from various animals was compared with that of Vp-TDH, Vp-TRH and Vp-TDH/I, respectively. As with Vp-TDH, Vp-TRH and Vp-TDH/I, Vp-TDH/II failed to lyse horse erythrocytes. There were, however, differences in the sensitivity of various erythrocytes to Vp-TDH/II and other haemolysins of the Vp-TDH/TRH family. In particular, mouse, human and rabbit erythrocytes showed most sensitivity to Vp-TDH/II (table I). The haemolytic activity of Vp-TDH/II, like that of Vp-TDH⁷ and Vp-TDH/I,¹⁴ but unlike that of Vp-TRH,^{10,11} was stable on heating at 60°, 70°, 80°, 90° or 100°C for 10 min.

Vascular permeability activity

Vp-TDH/II increased skin vascular permeability in rabbits, as did Vp-TDH and Vp-TRH,²⁷ with "blueing" reaching a maximum after 1–4 h. "Blueing" was no longer observed 6–8 h after injection of 0.1–5 μ g of purified Vp-TDH/II into rabbit skin (fig. 5).

Lethal activity for mice

The lethal activity of Vp-TDH/II for mice was examined in comparison with Vp-TDH. Mice died within 1 min of injection of 5.0 μ g of either Vp-TDH/II or Vp-TDH (table II).

Fig. 5. Time course of the "blueing" reaction in the rabbit vascular permeability factor test with purified Vp-TDH/II and Vp-TDH. ○—○, 5.0 μ g of Vp-TDH; ●—●, □—□, 1.0 μ g of Vp-TDH/II and Vp-TDH, respectively; ■—■, △—△, 0.2 μ g of Vp-TDH/II and Vp-TDH, respectively; ▲—▲, 0.04 μ g of Vp-TDH/II. ---+---, buffer only. "Blueing" was measured 1 h after challenge with Evans blue at the times indicated. Values represent the mean of three separate experiments.

Table II. Lethal activities of Vp-TDH/II and Vp-TDH following intravenous challenge of individual mice

Amount of toxin injected ($\mu\text{g}/\text{mouse}$)	Survival time after injection of	
	Vp-TDH/II	Vp-TDH
5.0	< 1 min	< 1 min
	< 1 min	< 1 min
2.5	25 min	34 min
	35 min	36 min
	39 min	51 min
0.5	No death	No death

Table III. Fluid accumulation in the rabbit, ileal loop test after challenge with Vp-TDH/II or viable cells of *V. parahaemolyticus* strains TH103 and N-1

Sample	Mean (SD) fluid accumulation (ml/cm)*
Vp-TDH/II ($\mu\text{g}/\text{loop}$)	
200	0.83 (0.11)
100	0.77 (0.16)
50	0.08 (0.05)
0	0
Live cells (cfu/loop)	
Strain TH103	
10^8	1.15 (0.29)
10^6	0.69 (0.18)
10^3	0.14 (0.07)
0	0
Strain N-1†	
10^8	0

*Values shown are the mean of three experiments.

†Strain N-1 is a marine isolate of *V. parahaemolyticus* which produces neither Vp-TDH, Vp-TRH, Vp-TDH/I nor Vp-TDH/II.

Rabbit ileal loop test

Purified Vp-TDH/II caused marked fluid accumulation (FA) in ligated rabbit intestines. The minimum dose of purified Vp-TDH/II that resulted in a FA ratio of > 0.5 ml/cm was *c.* 100 $\mu\text{g}/\text{loop}$ of intestine. Cultures of *V. parahaemolyticus* strain TH103 also caused FA, whereas *V. parahaemolyticus* strain N-1, which did not produce Vp-TDH, Vp-TRH, Vp-TDH/I or Vp-TDH/II, failed to induce FA (table III).

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

V. parahaemolyticus strain TH103 was observed to carry a 37-kb plasmid (data not shown). Total DNA and plasmid DNA of this strain were digested with three restriction endonucleases, *Eco*RI, *Hind*III and *Pst*I. The digested DNA was transferred to a hybridisation membrane in a Southern blot procedure and was then hybridised with a ^{32}P -labelled 359-bp DNA probe corresponding to the *tdh* gene. The *Eco*RI, *Hind*III and *Pst*I digests contained DNA fragments of

Fig. 6. Southern blot hybridisation analysis of total and plasmid DNA from *V. parahaemolyticus* strain TH103. **a**, agarose 1% w/v gel of total DNA (lanes 1, 2 and 3) and plasmid DNA (lanes 4, 5 and 6) digested with *Eco*RI (lanes 1 and 4), *Hind*III (lanes 2 and 5) or *Pst*I (lanes 3 and 6). **b**, autoradiograph of the corresponding Southern blot following hybridisation with the ^{32}P -labelled DNA probe for the *tdh* gene. Size marker positions of 19.3 and 4.3 kb were plotted from lambda DNA digested with *Sty*I.

17 kb, 5 kb and 16 kb, respectively, that hybridised with the *tdh* gene probe (fig. 6).

Plasmid DNA digested with *Hind*III was ligated into the *Hind*III site of plasmid vector pBR322²⁸ and *E. coli* strain JM109 was transformed with the recombinant DNA. Clones harbouring recombinant plasmids containing the structural gene *tdh/II* were identified by colony hybridisation with the *tdh* gene probe. The recombinant plasmid pKN1, which contained a 5-kb fragment of *V. parahaemolyticus* DNA, hybridised with the *tdh* gene probe.

The 5-kb *Hind*III insert of pKN1 was subcloned into the *Hind*III site of pUC19 and the resultant recombinant plasmid pKN2, with the insert in opposite orientations in relation to the *lac* promoter of the vector, was isolated and the DNA sequence of *tdh/II* was determined from double-stranded DNA by the dideoxy-chain termination sequencing method. The results showed that there was a 567-bp open reading frame in the 5-kb region that encoded a protein of 189 amino acids (designated Tdh/II) (fig. 7). The Shine-Dalgarno sequence²⁹ was observed 7 bp upstream of the initiation codon. The promoter region could not be specified because there were several regions with similarity to consensus *E. coli* promoter sequences.³⁰ An inverted repeat sequence, which may serve as a transcriptional termination sequence for the gene, was found 13 bp downstream of the *tdh/II* termination site. The whole structural gene showed a high degree of homology with the previously reported sequence of the *tdhA* gene of Kanagawa phenomenon-positive *V. parahaemolyticus* strain T4750 (98.5%),³¹ and the *tdh/I* gene of the Kanagawa phenomenon-negative *V. parahaemolyticus* strain TH012 (98.7%).¹⁴

The *tdh/II* gene was plasmid-borne, whereas most genes of this family, including *tdh*, *trh* and *tdh/I*, exist on the chromosome. The *tdh/II* gene appeared to be identical to *tdh4*, which is a plasmid-located gene

Fig. 7. Nucleotide sequence of *tdh/II* in comparison with the *tdhA*, *trh* and *tdh/I* genes. Solid and broken boxes indicate the initiation and terminal codons, respectively. The Shine-Dalgarno sequence is underlined. Identical (–) and deleted (*) nucleotides are indicated. Two horizontal arrows in opposite directions show an inverted repeat sequence. ○ designates where *tdh/II* differed from the sequence of *tdh4* reported by Nishibuchi and Kaper.³⁰

<i>Tdh/II</i>	1	MKYRYFAKKS	FLFISMLAAF	KTFAFELPSV
<i>Tdh/I</i>				
<i>Tdh A</i>				
<i>Trh</i>		..LKLYFAF.	.A.....	..ID...I..
	31	PFPAPGSDEI	LFVVRDTTFN	TNAPVNVKVS
			A..	
				E..
		...S.....L	N..IK	..ES...AI..
	61	DFWTNRNVKR	KPYKDVYQGS	VFTTSGTKWL
				
				
		.Y.....I..	S.H...	I.....S...
	91	TSYMTVNIND	KDYTMAAVSG	YKNGHSAVFV
			R.....	
				H.....
		SA.I.....G	NN.....L..	..D.L.T..T
	121	KSDQVQLQHS	YNSVANFVGE	DEDSIPSKMY
				
			D...S...	
		..EKTS.NQN	.S..SD....	N.E.L..VT.
	151	LDETPEYFVN	VEAYESGSGN	ILVMCISNKE
				
				
			N.H	MF.....S
	181	SFFECEYQN		
		H.K		
		K..Q		
		..D..MS.N		

Fig. 8. Comparison of the amino acid sequence of *Tdh/II* (Vp-TDH/II), deduced from the nucleotide sequence of *tdh/II*, and the amino acid sequences deduced from the *tdhA*, *trh* and *tdh/I* genes, respectively. An arrow indicates the putative cleavage site.

derived from *V. parahaemolyticus*.³² However, *tdh/II* differed from *tdh4* in the open reading frame 63 bp and 86 bp upstream of the initiation codon (fig. 7). The deduced sequence of the Vp-TDH/II protein differed by 6, 53 and 4 amino acid residues, respectively, from the amino acid sequences of Vp-TDH,³¹ Vp-TRH¹³ and Vp-TDH/I¹⁴ (fig. 8).

Discussion

Vp-TDH produced by Kanagawa phenomenon-positive strains of *V. parahaemolyticus* is thought to be an important factor in the pathogenicity of this organism.^{5,7} Production of two new haemolysins, Vp-TRH^{10,11} and Vp-TDH/I,¹⁴ by Kanagawa phenomenon-negative clinical isolates of *V. parahaemolyticus* has been reported, and it has been demonstrated that these haemolysins are immunologically and biophysicochemically related to, but not identical to the Vp-TDH produced by Kanagawa phenomenon-positive strains of *V. parahaemolyticus*. The present report describes the production of a fourth type of Vp-TDH-related haemolysin, named

Vp-TDH/II, by a Kanagawa phenomenon-negative clinical isolate of *V. parahaemolyticus*. Purified Vp-TDH/II was shown to be similar to Vp-TDH and Vp-TDH/I when analysed by SDS-PAGE, the Ouchterlony immunodiffusion test and the heat stability test. However, Vp-TDH/II differed from Vp-TDH and Vp-TDH/I when analysed by non-denaturing PAGE, in its sensitivity to various erythrocytes, and in its amino-acid sequence as deduced from nucleotide analysis of the *tdh/II* gene. Vp-TDH/II showed only partial antigenic similarity to Vp-TRH and had a different sensitivity to heat treatment. The sequence of the Vp-TDH/II protein suggested that Vp-TDH/II was more closely related to Vp-TDH and Vp-TDH/I than to Vp-TRH.

No immunological difference was observed between Vp-TDH, Vp-TDH/I and Vp-TDH/II, which suggests that the amino-acid residues of Vp-TDH/II which are different from Vp-TDH and Vp-TDH/I may not be involved in forming antigenic epitopes. However, differences between these haemolysins in terms of their electrophoretic mobility and haemolytic activity with various erythrocytes may result from these different amino-acid residues. These amino-acid substitutions may affect molecular charge and receptor recognition by the haemolysin or the lytic process of the haemolysin.

Four *tdh* genes related to Vp-TDH of *V. parahaemolyticus* have been reported by Nishibuchi and Kaper.³² The nucleotide sequence of one of those genes, *tdh4*, was identical with that of *tdh/II* reported here. Moreover, both *tdh/II* and *tdh4* were plasmid-borne. The presence of *tdh/II* on a mobile element such as a plasmid may explain the presence of similar, related genes in other vibrios, including *V. cholerae* non-O1³³ and *V. mimicus*.³⁴ However, Nishibuchi and Kaper³² reported that *V. parahaemolyticus* strain AQ3766 carrying *tdh4* failed to produce a detectable amount of Vp-TDH because of the lack of a *tdh*-specific RNA transcript. In contrast, *V. parahaemolyticus* strain TH103 used in this study produced Vp-TDH/II from a gene, *tdh/II*, which was identical to *tdh4*. Therefore, it appears that Vp-TDH/II is a fourth type of haemolysin belonging to the Vp-TDH/TRH family that can be produced by *V. parahaemolyticus*. The fact that Vp-TDH/II exhibits biological activities similar to those found with other haemolysins of the Vp-TDH/TRH family, such as lethality, fluid accumulation in rabbit ileal loops and the production of increased vascular permeability, suggests that Vp-TDH/II plays a role in the pathogenicity of *V. parahaemolyticus*.

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