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Molecular Epidemiologic Evidence for Association of Thermostable Direct Hemolysin (TDH) and TDH-Related Hemolysin of *Vibrio parahaemolyticus* with Gastroenteritis

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The Kanagawa phenomenon induced by the thermostable direct hemolysin (TDH) of *Vibrio parahaemolyticus* is almost exclusively associated with clinical strains, and TDH has been considered an important virulence factor. However, Kanagawa phenomenon-negative strains isolated from patients with diarrhea have recently been shown to produce TDH-related hemolysin (TRH). We studied the distribution of the *tdh* gene encoding TDH and the *trh* gene encoding TRH in vibrios by hybridization analyses. The presence or absence of the *tdh* gene and the *trh* gene in 285 strains of *V. parahaemolyticus* was examined by the DNA colony hybridization test with a *tdh* gene-specific probe and a newly constructed *trh* gene-specific probe. For assessment of the importance of TRH, many Kanagawa phenomenon-negative clinical strains (35.4% of all strains) were included. Of 214 clinical strains of *V. parahaemolyticus*, 112 strains (52.3%) had the *tdh* gene only, 52 strains (24.3%) had the *trh* gene only, and 24 strains (11.2%) carried both the *tdh* and the *trh* gene. The coexistence of the *tdh* and *trh* genes in these 24 strains was confirmed by Southern blot hybridization analysis. Of 71 environmental strains, 5 strains (7.0%) hybridized very weakly with the *trh* gene probe and none hybridized with the *tdh* gene probe. These results suggest that TRH as well as TDH is an important virulence factor of *V. parahaemolyticus*. Among 118 strains of other *Vibrio* species examined for the *trh* gene, only 1 strain of *Vibrio furnissii* gave a very weak hybridization signal. Among 48 representative *trh* gene-positive strains of *V. parahaemolyticus*, only 18 strains (37.5%) were found to produce TRH in culture medium when examined by a sensitive enzyme-linked immunosorbent assay method.

The thermostable direct hemolysin (TDH) of *Vibrio parahaemolyticus* induces beta-type hemolysis around colonies on a special blood agar, Wagatsuma agar. This phenomenon, the Kanagawa phenomenon (KP), was found to be manifested by almost all clinical isolates but by only 1 to 2% of the environmental isolates tested (15, 23). These epidemiological findings and lack of success in identifying any other potent virulence factor were, until recently, the main lines of evidence for the hypothesis that TDH is the major virulence factor of *V. parahaemolyticus*. Since results in the hemolysis assay on Wagatsuma agar could be influenced by other hemolysins, immunological methods were devised for specific detection of TDH produced in vitro (3, 10). We made a DNA probe specific for the gene (*tdh*) encoding TDH to detect the genetic potential of *V. parahaemolyticus* to produce TDH regardless of the actual level of TDH production (17). This probe was also used to detect genes encoding hemolysins similar to TDH in *Vibrio hollisae*, *Vibrio cholerae* non-O1, and *Vibrio mimicus* isolated from patients with diarrhea (8, 17, 20).

Recently, several *V. parahaemolyticus* isolates from patients with diarrhea were found not to produce a detectable level of TDH or to possess the *tdh* gene as determined by a hybridization test with the *tdh* gene probe under high-stringency conditions (4). The isolation of these strains from patients could disprove the hypothesis that TDH is the major virulence factor of *V. parahaemolyticus*. One such strain (5) and some other KP-negative strains (6), however, were shown to produce a TDH-related hemolysin (TRH). TRH produced by strain 4037 was purified and characterized (5), and the gene (*trh*) encoding TRH was cloned from this strain and sequenced (21). Comparative analysis revealed that the *tdh* and *trh* genes have 68.6% nucleotide sequence homology, suggesting that they were probably derived from a common ancestor and evolved by random drift and clonal separation (21).

Therefore, a survey of the presence of the *trh* gene in clinical and environmental strains of *V. parahaemolyticus* should provide information on the significance of TRH as a virulence factor. In this study, we constructed a gene probe specific for the *trh* gene and examined the distributions of the *trh* gene and the *tdh* gene in various strains of *V. parahaemolyticus*, including many KP-negative clinical strains, and in related vibrios. The presence of the *tdh* and/or *trh* gene was very closely associated with clinical strains of *V. parahaemolyticus*, indicating that TRH as well as TDH is an important virulence factor of *V. parahaemolyticus*.

MATERIALS AND METHODS

Bacterial strains. Clinical strains (from patients with diarrhea) and environmental strains (from seafood or environmental waters) of *V. parahaemolyticus*, *V. cholerae* non-O1, and *V. mimicus* were originally isolated at Osaka Airport

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Quarantine Station, Kobe Quarantine Station, and Osaka Prefectural Institute of Public Health and were obtained through the Department of Bacteriology and Serology, Research Institute for Microbial Diseases, Osaka University. The KP reactions and O:K serovars of the *V. parahaemolyticus* strains had been determined at the time of their isolation. Other *Vibrio* species used were laboratory stock strains. The origins of *Escherichia coli* HB101 and DH5 used to propagate plasmids were reported previously (18, 19).

General genetic techniques. Total cellular DNA was extracted by standard procedures (1), and plasmid DNA was extracted by an alkaline lysis method (12) and purified by CsCl-ethidium bromide equilibrium density gradient centrifugation (12). Digestion of DNA with restriction enzymes, gel electrophoresis, isolation of DNA fragments from the gel, ligation, and transformation were performed as previously described (18).

DNA probes. pBIK9 contained the *trh* gene on a cloned 2.3-kb *Ava*I fragment (21). Analysis of the *trh* gene sequence revealed *Hpa*I and *Nde*I sites within the coding region. pBIK9 was digested with *Hpa*I and *Nde*I, and a 324-bp *Hpa*I-*Nde*I DNA fragment internal to the *trh* gene was isolated. This fragment had 71.0% nucleotide sequence homology with the corresponding region of the *tdh* gene. After repair of its *Nde*I end with the Klenow fragment of DNA polymerase I, the *Hpa*I-*Nde*I fragment was inserted into the *Sma*I site within the multiple cloning site of pUC9 (25), resulting in pKTN101. pKTN101 was digested with *Bam*HI and *Eco*RI, which cleaved the nucleotide sequences immediately flanking the inserted *Hpa*I-*Nde*I fragment, and a 334-bp fragment was isolated. This fragment was used as a probe for the *trh* gene. The DNA fragment used as a probe for the *tdh* gene was the 415-bp sequence derived from the internal fragment covering 71% of the *tdh* coding sequence and prepared from pCVD518 as described previously (17). Probe DNAs were labeled by the random priming method with ^{32}P-labeled dCTP (2).

DNA colony hybridization test. DNA colony blots were prepared and the hybridization test was performed under high-stringency conditions as described previously (17).

Southern blot hybridization analysis. Total DNA extracted from the test organism was digested to completion with *Hin*dIII, and the digest was separated by electrophoresis in 1.0% agarose gel and transferred to a nitrocellulose filter as described by Southern (24). The blot was first hybridized with the *trh* gene probe under high-stringency conditions as described by Moseley et al. (16) and autoradiographed. The same blot was then hybridized with the *tdh* gene probe under high-stringency conditions and autoradiographed. The *trh* gene probe- and *tdh* gene probe-positive bands were identified by comparison of the two autoradiographs.

Purification of TRH produced in *E. coli*. TRH was expressed from the cloned *trh* gene (21) under the control of the *tac* promoter in *E. coli*. A 0.9-kb *Ava*I-*Hin*dIII fragment carrying the *trh* gene was obtained from pBIK8 (21), and the ends were repaired with the Klenow fragment of DNA polymerase I and cloned into the *Nco*I site of ptac-85 (13) so that the *trh* gene was placed under the control of the inducible *tac* promoter, resulting in pKTN102. *E. coli* DH5 (F^- *endA1 hsdR17* [r_K^- m_K^+] *supE thi-1* λ^- *recA1 gyrA96 relA1*; originally purchased from Bethesda Research Laboratories) harboring pKTN102 was grown in LB broth (14) supplemented with ampicillin (200 μg/ml) with shaking (200 rpm) for 14 h at 37°C, and isopropyl-β-D-thiogalactoside was added at a final concentration of 1 mM to derepress the *tac* promoter. The cells were incubated with shaking for 2 h at 37°C, then harvested by centrifugation (12,000 × *g*, 20 min), and sonicated, and the supernatant was obtained by ultracentrifugation (145,000 × *g*, 2 h).

TRH was purified from the supernatant by a series of chromatographies, and chromatographic fractions were assayed for TRH by the Ouchterlony gel diffusion method with anti-TDH polyclonal rabbit serum. The purity of the TRH preparation was assessed by sodium dodecyl sulfate-polyacrylamide (1.5%) slab gel electrophoresis (SDS-PAGE) by the method of Laemmli (11), followed by staining with Coomassie brilliant blue. The supernatant was applied to a column of DEAE-cellulose (James River Corp.) equilibrated with 10 mM phosphate buffer (pH 7.0), and material was eluted with a linear gradient of 0 to 1.0 M NaCl. TRH in the eluate was precipitated with ammonium sulfate (80% saturation) and dissolved in and dialyzed against 20 mM Tris hydrochloride (Tris-HCl) buffer (pH 7.0). Then it was applied to a column of Sephadex G-200 (Pharmacia) equilibrated with 20 mM Tris-HCl buffer (pH 7.0). TRH in the eluate was precipitated with ammonium sulfate and dissolved in and dialyzed against 25 mM piperazine hydrochloride buffer (pH 5.6).

Chromatofocusing column chromatography was carried out next. For this, the TRH preparation was applied to a column of polybuffer exchange PBE 94 (Pharmacia) equilibrated with 25 mM piperazine hydrochloride buffer (pH 5.6), and material was eluted with a linear gradient of pH 5.0 to 4.0 achieved with Polybuffer (pH 4.0; Pharmacia). The fraction containing TRH was concentrated by ultrafiltration on a YM-10 membrane (Amicon) and subjected to Mono Q high-pressure liquid chromatography. The preparation was loaded onto a Mono Q anion-exchange column (Pharmacia) equilibrated with 20 mM Tris-HCl buffer (pH 7.0), and the column was developed with a linear gradient of 0 to 0.25 M NaCl. The TRH recovered was finally applied to a DEAE 3SW (TOSOH) column equilibrated with 25 mM phosphate buffer (pH 6.5). The column was developed with a linear gradient of 0 to 0.3 M NaCl, and the TRH recovered was concentrated by ultrafiltration and used as purified TRH.

Antiserum to TRH. Japanese white rabbits were immunized by a first injection of the purified TRH in Freund complete adjuvant, a booster injection of the purified TRH in a 1:1 mixture of Freund complete and incomplete adjuvants 4 weeks later, and another injection of TRH in Freund incomplete adjuvant 1 week later. One week after the final booster injection, the animals were bled and their serum was obtained.

Bead ELISA to detect TRH and TDH. The polyclonal anti-TRH rabbit serum was used for a bead enzyme-linked immumosorbent assay (bead ELISA) to detect TRH. The bead ELISA was performed as described previously (22). Briefly, in this ELISA method, anti-TRH immunoglobulin G (IgG) was immobilized on polystyrene beads to bind TRH in the test solution, and the bound TRH was then treated with the Fab' fragments of anti-TRH IgG conjugated with horseradish peroxidase. Then tetramethylbenzidine was added as a substrate for horseradish peroxidase. After incubation, the enzyme reaction was stopped with sulfuric acid, and the resulting yellow color was measured at 450 nm. Plasmid pKTN209 used in the experiment to assess the specificity of the bead ELISA for TRH is a derivative of pUC118 (26) containing the *tdh*2 gene (19) on a 0.9-kb *Sph*I-*Eco*RI DNA fragment (Baba et al., unpublished data). The bead ELISA for detecting TDH was performed as reported previously (22). TDH was purified by the method described previously (9) and used in the bead ELISA.

TABLE 1. Detection of the *tdh* and *trh* genes in 285 strains of *V. parahaemolyticus* by the DNA colony hybridization test

Gene present[a]		No. of *V. parahaemolyticus* strains		
		Clinical		Environmental (KP negative)
tdh	*trh*	KP positive	KP negative	
+	+	12[b]	12	0
+	−	96	16	0
−	+	4	48[c]	5[d]
−	−	1	25	66

[a] Results of the DNA colony hybridization test.
[b] Two strains gave a weakly positive *trh* reaction.
[c] Thirteen strains gave a weakly positive *trh* reaction.
[d] All five strains gave a very weakly positive *trh* reaction.

V. parahaemolyticus strains were grown overnight in 5 ml of SPP medium (6) for TRH production or in peptone-NaCl broth (10) for TDH production with shaking (200 rpm) at 37°C, and the culture supernatants were obtained by centrifugation (13,800 × *g*) and stored at −20°C until used for the bead ELISA. Test samples were appropriately diluted (to utilize the standard curve obtained with purified TRH) and assayed by the bead ELISA method for TRH or for TDH. Samples that gave an OD_{450} more than three times higher than that of a negative control (10^{-3}-diluted spent culture medium of a *trh* gene- and *tdh* gene-negative *V. parahaemolyticus* strain) were judged as positive.

RESULTS

DNA colony hybridization test. A *trh* gene probe derived from 65.5% of the coding sequence of the *trh* gene was constructed as described in Materials and Methods. A *tdh* gene probe was derived from an internal DNA fragment (71% of the coding sequence) of the *tdh* gene (17). These two specific gene probes were used to detect *tdh* and *trh* gene sequences in test organisms by the DNA colony hybridization test under high-stringency conditions. In all, 214 clinical and 71 environmental strains of *V. parahaemolyticus* were examined, and the results are summarized in Table 1. Of the 214 clinical strains, including both KP-positive and KP-negative strains, 112 strains (52.3%) had the *tdh* gene only, 52 strains (24.3%) had the *trh* gene only, 24 strains (11.2%) had both genes, and 26 strains (12.5%) had neither gene. Of 101 KP-negative strains from clinical sources, 60 strains (59.4%) carried the *trh* gene, while 28 strains (27.7%) had the *tdh* gene. Of 71 KP-negative strains from environmental sources, 66 strains (93.0%) contained neither gene and 5 strains (7.0%) reacted with the *trh* gene probe, although very weakly.

There were a total of 81 *trh* gene-positive strains, of which 76 strains (93.8%) were from patients who had diarrhea upon their return from various parts of the world. The O:K serovars of 74 of the *trh* gene-positive clinical strains are known and are widely diverse (Table 2).

Besides these *V. parahaemolyticus* strains, 118 strains belonging to six *Vibrio* species were tested with the *trh* gene probe (Table 3). One strain of *V. furnissii* gave a weakly positive reaction, but the other strains were negative.

Southern blot hybridization analysis. Twenty-four clinical strains of *V. parahaemolyticus* gave positive reactions with both the *tdh* and the *trh* gene probe (Table 1). For confirmation that the positive reactions were specific reactions, i.e., that the two probes hybridized to discrete sequences, not to sequences homologous to both the *tdh* gene and *trh* gene, these 24 *tdh* and *trh* gene-positive strains were analyzed by Southern blot hybridization. *Hin*dIII sites are present outside the coding sequences of the *tdh* and *trh* genes of *V. parahaemolyticus* (18, 19, 21). Therefore, samples of total DNA extracted from the test organisms were digested with *Hin*dIII and analyzed by Southern blot hybridization with the *tdh* and *trh* gene probes as described in Materials and Methods. In all strains, discrete *tdh* and *trh* gene-positive bands were detected. The *trh* gene-positive bands ranged between 4.2 and 6.7 kb in size, and only one band was detected in each strain; the *tdh* probe-positive bands ranged between 0.7 and 9.0 kb in size, and one or two bands were detected in each strain. An autoradiograph of representative strains is shown in Fig. 1.

TABLE 2. O:K serovars of *trh* gene-positive *V. parahaemolyticus* strains

O:K serovar	No. of strains	O:K serovar	No. of strains
1:1	3	5:61	1
1:5	1	5:UT	1
1:9	1		
1:23	1	6:18	4
1:41	1	6:46	2
1:56	4		
1:58	5	8:9	1
1:69	2	8:21	1
1:UT[a]	5		
		10:71	1
2:3	1		
		11:5	1
3:6	13	11:22	1
3:29	1	11:UT	2
3:UT	5		
		12:52	1
4:12	1		
4:49	1	13:59	6
4:53	1	13:64	1
4:63	3		
		UT:UT	1
5:15	1		

[a] UT, Untypeable with available antisera.

Bead ELISA method for TRH. TRH produced in *E. coli* containing the cloned *trh* gene was purified by a series of chromatographic procedures. SDS-PAGE analysis of the TRH-containing fractions of the final chromatography (DEAE 3SW column) is shown in Fig. 2. The two major fractions (lanes 3 and 4 in Fig. 2) were pooled, concentrated, and used as purified TRH preparation. Antiserum raised to this purified TRH was used in the bead ELISA for TRH.

The sensitivity and specificity of this method were first

TABLE 3. Detection of the *trh* gene in *Vibrio* species by the DNA colony hybridization test

Organism	No. of strains[a]	
	Gene present	Gene absent
V. cholerae non-O1	0	92
V. mimicus	0	14
V. fluvialis	0	5
V. hollisae	0	3
V. furnissii	1[b]	2
V. damsela	0	1

[a] Results of DNA colony hybridization: reaction (gene present) or no reaction (gene absent).
[b] Very weak positive reaction.

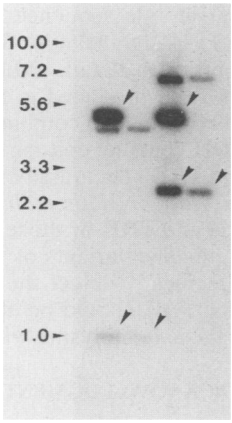

FIG. 1. Southern blot analysis of *V. parahaemolyticus* strains possessing both the *tdh* and *trh* genes. *Hin*dIII digests of sample DNAs were separated by gel electrophoresis, blotted onto a nitrocellulose sheet, and hybridized and autoradiographed sequentially with the *trh* and *tdh* gene probes as described in Materials and Methods. The probe-positive bands indicated by the arrows are the *tdh* probe-positive bands. The other bands are the *trh* probe-positive bands. Positions and sizes (in kilobases) of the molecular size markers are indicated on the left.

assessed by using purified TRH and TDH (Fig. 3). The lower limit of detection was 5 pg of purified TRH in the volume of sample used (500 μl). A good standard curve was obtained in the range of 5 to 500 pg of TRH per 500 μl (0.115 to 1.25 A_{450} units). Although TDH and TRH have a common epitope(s) (5, 7), sample solution containing as much as 5 ng of TDH gave no reaction, and thus this assay system appeared to be highly specific for TRH.

The supernatants of the cell lysates of three *E. coli* strains, DH5(pKTN102), producing TRH; DH5(pKTN209), producing TDH; and DH5, were diluted 1:10,000 and examined by bead ELISA for TRH. The diluted DH5(pKTN102) sample contained TRH at 1 ng/ml (upper limit of the reliable standard curve), whereas TRH was not detected in the two other samples; the A_{450} values were 0.03 and 0.02 unit for the diluted DH5(pKTN209) and DH5 samples, respectively. These results confirmed the specificity of this assay system.

Production of TRH and TDH by *trh* gene-positive strains.

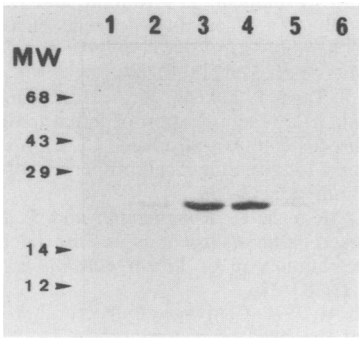

FIG. 2. SDS-PAGE of purified TRH. Six consecutive fractions, four fractions (lanes 2 to 5, respectively), containing TRH and their neighboring fractions (lanes 1 and 6), of the DEAE 3SW column chromatography were examined. The gel was stained with Coomassie brilliant blue. Molecular weights of the markers are shown (MW, in thousands). Two fractions (lanes 3 and 4) were pooled and used as purified TRH.

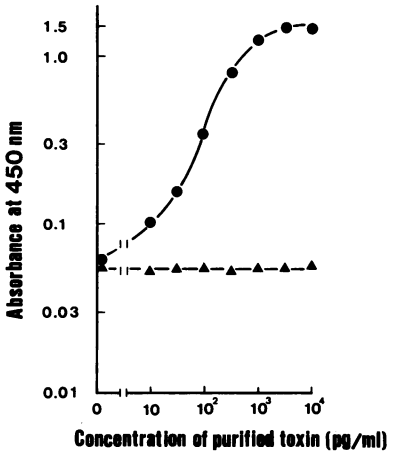

FIG. 3. Sensitivity and specificity of bead ELISA for TRH. Purified TRH (●) and purified TDH (▲) at various concentrations were examined by bead ELISA for TRH as described in the text.

Production of TRH in the culture supernatant by representative *trh* gene probe-positive strains of *V. parahaemolyticus* were examined by the sensitive bead ELISA for TRH. Of 48 strains examined, 18 strains (37.5%) produced TRH detectable by the bead ELISA method (Table 4). The amount of TRH detected ranged between 600 pg/ml and 21 ng/ml of culture supernatant.

The presence or absence of the *tdh* gene in the *trh*-bearing strains did not appear to influence the production of TRH because the ratio of TRH-producing strains to non-TRH-producing strains did not differ significantly between the *tdh*-positive and -negative groups (Table 4). The amount of TDH produced in the culture supernatants by 24 *tdh* gene- and *trh* gene-positive strains ranged between 350 pg/ml and 5 ng/ml.

DISCUSSION

TDH is produced almost exclusively by clinical strains of *V. parahaemolyticus* (3, 15, 23) and is a putative major virulence factor. TRH, a TDH-related hemolysin, was found to be produced by clinical strains that did not produce TDH (5, 6), and so was presumed to be another important virulence factor of *V. parahaemolyticus* (6). One problem in assessing the significance of putative virulence factors by examining the association of their production by clinical and nonclinical strains is that in vitro culture conditions may affect the production of these virulence factors. To circumvent this problem, we used specific gene probes to determine the genetic potential of the test organisms to produce the virulence factors.

TABLE 4. Production of TRH from *trh* gene-positive *V. parahaemolyticus* strains detected by the bead ELISA

trh gene present	*tdh* gene present	TRH production[a]	No. of strains
+	+	+	10[b]
+	+	−	15
+	−	+	8
+	−	−	15[c]

[a] Detected by the bead ELISA method with polyclonal anti-TRH serum.
[b] One strain gave a weakly positive *trh* reaction.
[c] Three strains gave a weakly positive *trh* reaction.

In previous studies, only 4 to 12% of clinical strains of *V. parahaemolyticus* were found to be KP negative (15, 23). In this study, we purposely included many KP-negative clinical strains (35.4% of the total strains examined) to assess the importance of TRH, because TRH has been reported to be produced by KP-negative clinical strains (5, 6). Of 214 clinical strains, 190 strains (88.8%) had the *tdh* and/or the *trh* gene; 78 strains did not contain the *tdh* gene, but 52 (66.7%) of these 78 strains carried the *trh* gene. On the other hand, 66 (93.0%) of 71 environmental strains did not have either gene (Table 1). Accordingly, the presence of the *tdh* gene and/or the *trh* gene is very closely associated with strains of clinical origin, suggesting that TRH as well as TDH is an important virulence factor of *V. parahaemolyticus*. Thus, a KP-positive phenotype associated only with the *tdh* gene is not a hallmark of virulent strains.

TRH was first found to be produced by a *V. parahaemolyticus* O3:K6 strain implicated in an outbreak of gastroenteritis (4, 5). *V. parahaemolyticus* strains belonging to three other O:K serotypes were recently shown to produce TRH (6). In the present study, hybridization tests with a *trh* gene-specific probe disclosed that the *trh* gene is widely distributed in various *V. parahaemolyticus* strains, as evidenced by the diverse O:K serotypes of the strains possessing the *trh* gene (Table 2), and some strains were found to contain both the *tdh* and the *trh* genes (Table 1, Fig. 1). These findings suggest that the *tdh* and the *trh* genes were derived from a common ancestor at an early stage in evolution and that one or both of the genes have been inherited by various strains. Fifteen clinical and five environmental strains of *V. parahaemolyticus* (Table 1) and one strain of *V. furnissii* (Table 3) gave very weak hybridization signals with the *trh* gene probe under high-stringency conditions, and the intensities of the hybridization signals varied slightly from strain to strain. These strains probably possess atypical *trh* genes with varied nucleotide sequences. A family of *tdh*-related hemolysin genes not detectable by hybridization with the *tdh* or *trh* gene probe under high-stringency conditions may be present in some of the 26 *tdh* gene-negative and *trh* gene-negative clinical strains (Table 1). We are currently investigating this possibility, because demonstration of such genes would provide further support for the hypothesis that TDH and related hemolysins are important virulence factors of *V. parahaemolyticus*.

We examined the production of TRH by representative *trh* gene-positive strains. Although we used a sensitive, specific detection method, we found that only 40.6% of the test strains produced detectable TRH. Most KP-positive strains possessing two *tdh* gene copies (designated *tdh*1 and *tdh*2) produce TDH in culture supernatants at microgram per milliliter concentrations (unpublished observation), but the amounts of TRH produced by the *trh* gene-positive strains were much less (600 pg/ml to 21 ng/ml). One possible explanation for this is that the culture conditions that we used (6) were not suitable for maximum production of TRH. TRH was discovered only recently, and the optimal in vitro culture conditions for its production by various strains have not been studied extensively, so expression of the *trh* gene under the culture conditions we used may not have been efficient.

Interestingly, 25 of 48 test strains also had the *tdh* gene. All 25 strains produced TDH, but in much smaller amounts (350 pg/ml to 5 ng/ml) than those produced by typical KP-positive strains. The production of TRH and TDH in *trh* gene-positive strains may be regulated by some as yet unknown mechanism. Alternatively, the *trh* genes in many strains may have nucleotide sequence variations that alter the antigenicity of TRH significantly. We are now investigating these possibilities in detail. Other workers (7) have attempted to detect TRH produced in the culture supernatant by an ELISA method with a combination of monoclonal antibodies to a TDH-specific epitope and to an epitope common to TDH and TRH. Their assay system would not be suitable for strains with both the *tdh* gene and *trh* gene producing both TDH and TRH or those with variants of the *trh* gene encoding antigenic variants of TRH. However, our gene probes that specifically detect the genetic potential to produce TDH and/or TRH should be effective for studying the associations of these hemolysins with disease.

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