Close proximity of the *tdh*, *trh* and *ure* genes on the chromosome of *Vibrio parahaemolyticus*

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The distribution and location of the virulence-factor genes of *Vibrio parahaemolyticus*, *tdh* and *trh*, and the structural gene of urease, *ureC*, were examined on the genomic DNAs of 115 clinical isolates of *V. parahaemolyticus*. The majority of strains (81%) had two copies of *tdh* on the chromosome, and no copies of *trh* or *ure*. Southern hybridization with a *tdh* probe, after pulsed-field gel electrophoresis of *NotI*-digested genomic DNA of each strain revealed only single bands, suggesting that the two copies of *tdh* exist on single *NotI* fragments in each strain. Of the 115 strains, 7% had the *tdh*, *trh* and *ure* genes on chromosomal DNA. The three genes were also detected on single *NotI* fragments in these strains. More detailed analysis revealed that the three genes were localized within 40 kb. By long and accurate polymerase chain reactions (LA-PCR), the distance between *trh* and *ure* was shown to be less than 8.5 kb. These results reveal a close proximity of the *tdh*, *trh* and *ure* genes on the chromosome of pathogenic *V. parahaemolyticus* strains.

Keywords: *Vibrio parahaemolyticus*, *tdh*, *trh*, *ure*, virulence-factor genes

INTRODUCTION

Vibrio parahaemolyticus is an important cause of seafood-related gastroenteritis and traveller's diarrhoea (Honda & Iida, 1993). *V. parahaemolyticus* strains isolated from diarrhoeal patients produce either the thermostable direct haemolysin (TDH) or the TDH-related haemolysin (TRH) or both, while almost all isolates from the environment do not produce these haemolysins (Honda & Iida, 1993). TDH and TRH, encoded by *tdh* and *trh*, respectively, are each composed of 165 amino acids and share an approximately 67% identity in their amino acid sequences. Biochemical, epidemiological and genetic studies have shown that the two haemolysins contribute to the pathogenicity of the organisms (Honda & Iida, 1993; Honda *et al.*, 1990b; Nishibuchi *et al.*, 1992; Sakazaki *et al.*, 1968; Shirai *et al.*, 1990; Xu *et al.*, 1994). Several lines of evidence suggest that at some time in the past, the *tdh* gene (and possibly *trh* also) was acquired by ancestral *V. parahaemolyticus* strains from another organism through genetic transfer events mediated by insertion-

sequence-like elements (Terai *et al.*, 1991; Nishibuchi & Kaper, 1995).

Although *V. parahaemolyticus* does not generally produce urease, there have been some reports of clinical isolates that produce this enzyme. We recently demonstrated that the urease-positive phenotype of *V. parahaemolyticus* strains is always associated with the possession of the *trh* gene (Suthienkul *et al.*, 1995), and that the association is due to a genetic linkage between the structural gene of urease (*ure*) and *trh* on the chromosome of *V. parahaemolyticus* strains (Iida *et al.*, 1997).

In this study, to provide insight into the putative ancient transfer of virulence-factor genes, we analysed the distribution of the *tdh*, *trh* and *ure* genes among clinically isolated *V. parahaemolyticus* strains, and determined the location of the genes on the genomic DNA.

METHODS

Bacterial strains. The 115 *V. parahaemolyticus* strains used in this study were isolated from patients with traveller's diarrhoea at the Osaka International Airport quarantine station from October 1991 to October 1992. The countries and areas

Abbreviations: LA-PCR, long and accurate polymerase chain reaction; TDH, thermostable direct haemolysin; TRH, TDH-related haemolysin.

from which the patients are thought to have become infected by *V. parahaemolyticus* are diverse, including Hong Kong, South Korea, Thailand, the Philippines, Indonesia, Singapore, Taiwan, Vietnam, Malaysia, India, China, and the Maldives. Urease production by these strains was examined as previously described (Suthienkul *et al.*, 1995).

Nucleic acid techniques. Southern hybridization was performed with probes specific for *tdh* or *trh* as previously described (Suthienkul *et al.*, 1996). The probes were labelled with digoxigenin-11-dUTP using a random primer extension method provided by a DNA labelling and detection kit (Boehringer Mannheim). Labelling was done according to the manufacturer's protocol. The labelled DNA fragments were separated from unincorporated nucleotides by ethanol precipitation and redissolved in TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0), then stored at -20°C until use. For the *tdh* genes, the hybridization temperature was 37°C , and for the *trh* genes, either less or more stringent conditions were used as previously described (Suthienkul *et al.*, 1996). Restriction enzymes were purchased from Toyobo. Extraction of genomic (Wilson, 1987) and plasmid (Kado & Liu, 1981) DNA from *V. parahaemolyticus* was performed as previously described.

Possession of *tdh* and *trh* genes. The presence or absence of *tdh* and *trh* genes in *V. parahaemolyticus* strains was examined by Southern hybridization as described above. For the *trh* gene, hybridization was done in less stringent conditions.

Pulsed-field gel electrophoresis (PFGE). Samples for PFGE were prepared as described by Smith & Cantor (1987) with minor modification. Briefly, bacterial cells were grown overnight with shaking at 37°C in LB broth containing 3% NaCl. Cells were then harvested by centrifugation, resuspended with 10 mM Tris/HCl (pH 8.0), 1 M NaCl, 50 mM EDTA, and mixed with an equal volume of molten low-melting-point 1.6% agarose (LMP Agarose; Life Technologies). Agarose blocks were incubated for 2 h at 37°C in lysozyme solution [1 mg lysozyme ml^{-1} in 10 mM Tris/HCl (pH 8.0), 1 M NaCl, 100 mM EDTA, 0.2% sodium deoxycholate, 0.5% sodium lauroylsarcosine (SLS), 2 μg RNase ml^{-1}] and then deproteinized in 1 mg proteinase K ml^{-1} in 0.5 M EDTA (pH 8.0), 1% SLS at 50°C overnight. Cell debris and proteinase K were then removed in a sequence of four 2 h washes: washes 1, 3, and 4 were in 20 mM Tris/HCl (pH 8.0), 50 mM EDTA at room temperature; wash 2 was in the same solution containing 1 mM phenylmethylsulphonyl fluoride (Sigma). The *V. parahaemolyticus* DNA in agarose blocks was digested with restriction enzymes described in the text. PFGE was performed as previously described (Iida *et al.*, 1997) using a contour-clamped homogeneous electric field method on a CHEF DR III System (Bio-Rad). Southern hybridization after PFGE was carried out by a previously described method (Iida *et al.*, 1997). The probe for the *V. parahaemolyticus ure* gene has been previously described (Iida *et al.*, 1997).

PCR. Following the manufacturer's instructions, long and accurate polymerase chain reaction (LA-PCR) was performed using the LA PCR kit version 2 (Takara Shuzo). Oligonucleotide primers were synthesized using an Applied Biosystems DNA synthesizer, model 381A. The oligonucleotide primers for PCR between *trh* and *ureC* were p83 (*trh*-sense: 5'-GGCTCAAAATGGTTAAGCG-3'), p84 (*trh*-antisense: 5'-CATTTCCGCTCTCATATGC-3'), URE5 (*ureC*-sense: 5'-ACTAATGCTACAACAGTCAC-3') and URE6 (*ureC*-antisense: 5'-ATGCTGGAATGATGTTAGGT-3'). The design of URE5 and URE6 was based on the partial nucleotide

sequence of the *V. parahaemolyticus ureC* gene that we determined in previous work (Iida *et al.*, 1997). Extracted genomic DNA (100 ng) from *V. parahaemolyticus* AQ4673 was used as a template for PCR in a scale of 50 μl reaction solution. PCR conditions were as follows. After heating at 94°C for 1 min, a cycle of 98°C for 10 s, 55°C for 10 s, and 68°C for 15 min was repeated 30 times. This was followed by an extension step at 72°C for 10 min. The PCR was done on a Perkin Elmer thermal cycler type 9600. Amplified DNA fragments were detected by ethidium bromide staining after agarose gel electrophoresis on 0.8% gels.

RESULTS

Distribution of *tdh* and *trh*

First, to determine the distribution of *tdh* and *trh* in clinical *V. parahaemolyticus* strains, we examined the presence or the absence of the two genes in a total of 115 *V. parahaemolyticus* strains isolated from diarrhoeal patients at the Osaka International Airport quarantine station. The results showed that 106 strains (92.2%) possessed *tdh* and that 19 strains (16.5%) had *trh*. Eleven strains (9.6%) were positive for both *tdh* and *trh*, and only one strain (0.9%) had neither *tdh* nor *trh*.

Clonal relationships among strains

To analyse the clonal relationships among the *V. parahaemolyticus* strains, restriction fragment length polymorphism of *tdh* and *trh* was examined. A previously described Southern hybridization method (Suthienkul *et al.*, 1996) was employed to examine the genomic DNA after it was digested with *Hind*III and the DNA fragments were hybridized with *tdh* and *trh* probes. Strains possessing *tdh* were divided into six types, D1, D2, D3, D6, D7 and D8, depending on differences in the restriction fragment patterns (Fig. 1, Table 1). Some *tdh* genes were found on plasmid DNA (Fig. 1b). *trh*-possessing strains were divided into four types, R1, R2, R3 and R4, as previously reported for Thai isolates (Suthienkul *et al.*, 1996) (Table 1), but some *trh* bands in type R1 strains hybridized with the *trh* probe only in less stringent conditions (Table 1). All the *trh* bands existed exclusively on chromosomal but not on plasmid DNA (data not shown).

The chromosomal and episomal distribution for *tdh* and *trh* among the tested strains is summarized as follows: 93 strains had two copies of *tdh* on the chromosome and no *trh* (this group contains the type D1/None strains, i.e. those having the type D1 *tdh* and no *trh*), one strain had one copy of *tdh* on the chromosome and no *trh* (D7/None), one strain had one *tdh* on a plasmid and no *trh* (D8/None), eight strains had one *trh* on the chromosome (None/R1, None/R3 and None/R4), eight strains had one *tdh* and one *trh* both on the chromosome (D2/R2 and D6/R1), three strains had one *trh* on the chromosome and one *tdh* on plasmid (D3/R4), and one strain had neither *tdh* nor *trh* (None/None). These results suggest that in the majority of the strains, *tdh* and *trh* are present on the chromosomal DNA.

Fig. 1. Eight types of *Hind*III restriction fragment pattern of *tdh* in representative strains. (a) Genomic DNA; (b) plasmid DNA. The lanes contain the following types of restriction fragment patterns: 1, D1; 2, D2; 3, D3; 4, D4; 5, D5; 6, D6; 7, D7; 8, D8. Types D4 and D5, which were previously observed among Thai isolates (Suthienkul *et al.*, 1996), were not found in this study. The *tdh* bands in types D3, D5 and D8, and one of two bands in type D4, were on plasmid DNA, since the extracted plasmid fractions showed the same positive reaction with the *tdh* probe as in (b). Hybridization with the *tdh* probe was performed at 37 °C as described previously (Suthienkul *et al.*, 1996).

Table 1. Relationships among the types of *Hind*III restriction fragment pattern (RFP) for *tdh* and *trh* in clinical *V. parahaemolyticus* isolates at Osaka Airport

The numbers in parentheses represent the number of strains that hybridized with the *trh* probe only in less stringent conditions (Suthienkul *et al.*, 1996).

RFP of <i>trh</i> type:	No. of strains whose RFP of <i>tdh</i> was type:							
	None*	D1	D2	D3	D6	D7	D8	Total
None†	1	93	0	0	0	1	1	96
R1	5 (3)	0	0	0	1	0	0	6
R2	0	0	7	0	0	0	0	7
R3	2 (2)	0	0	0	0	0	0	2
R4	1 (1)	0	0	3 (3)	0	0	0	4
Total	9	93	7	3	1	1	1	115

* None, a type that produced no *tdh* bands.

† None, a type that produced no *trh* bands.

Fig. 2. PFGE of *Not*I-digested genomic DNA of *V. parahaemolyticus* strains possessing only *tdh*. Electrophoresis was performed with an electric field of 6.0 V cm⁻¹ on 1% agarose gels in 1 × TBE buffer at 14 °C. The pulse times increased linearly from 5 to 50 s within 24 h. (a) Ethidium bromide staining; (b) Southern hybridization with a *tdh* probe. Lane 1, Molecular size markers, lambda ladder (concatemers of λ c1857sam7; band sizes are 48.5, 97, 145.5 kb, etc., from bottom to top); lanes 2–10, *V. parahaemolyticus* strains AQ4680, AQ4690, AQ4700, AQ4710, AQ4730, AQ4740, AQ4750, AQ4760 and AQ4780, respectively. All the strains possess only *tdh*, and belong to the D1/None type.

Two copies of *tdh* in type D1/None strains are on single *Not*I fragments

The predominant type of strain, D1/None, had two copies of *tdh* on the chromosome (Fig. 1). Some other types of strain had both *tdh* and *trh* on chromosomal

DNA: for example, D2/R2 and D6/R1. Consequently, we analysed the relative location of the genes on chromosomal DNA using PFGE followed by Southern hybridization.

Fig. 2 shows data on the genomic DNA from type

Fig. 3. PFGE of *NotI*-digested genomic DNA of *V. parahaemolyticus* strains possessing both *tdh* and *trh*. Electrophoresis was performed as in Fig. 2, except that the pulse times increased linearly from 5 to 50 s within 22 h. (a) Ethidium bromide staining; (b) Southern hybridization with a *tdh* probe. The results of hybridization with a *trh* probe or a *ureC* probe were basically identical to those with the *tdh* probe (data not shown). Lanes 1–8, AQ4673, AQ4686, AQ4696, AQ4713, AQ4758, AQ4761, AQ4781 and AQ4739, respectively. AQ4739 belongs to the D6/R1 type, and the other strains to the D2/R2 type.

Fig. 4. PFGE of the restriction-enzyme-digested genomic DNA of *V. parahaemolyticus* strain AQ4700, which possesses only *tdh*. Electrophoresis was performed as in Fig. 2, except that the pulse times increased linearly from 1 to 40 s within 20 h. (a) Ethidium bromide staining; (b) Southern hybridization with a *tdh* probe. Lane 1, Molecular size markers, lambda ladder (concatemers of λ c1857sam7: band sizes are 48.5, 97, 145.5 kb, etc., from bottom to top). Genomic DNA of *V. parahaemolyticus* strain AQ4700 was digested with *NotI* (lane 2), *SfiI* (lane 3), *SmaI* (lane 4), *NotI* and *SfiI* (lane 5), and *NotI* and *SmaI* (lane 6).

D1/None strains and Fig. 3 shows data for strains possessing both *tdh* and *trh* on the chromosome. The genomic DNA was digested with a rare-cutting restriction enzyme, *NotI*. The ethidium-bromide-stained DNA patterns (Figs 2a and 3a) had no obvious correlation with the *HindIII* fragment patterns of *tdh* and/or *trh* (Fig. 1, Table 1).

After Southern hybridization with the *tdh* probe, the *NotI*-digested genomic DNA of each of the type D1/None strains showed only one band (Fig. 2b). This result was unexpected since these strains have two copies of *tdh* (Fig. 1a). This indicates that, in each of the type D1/None strains, two copies of *tdh* exist on single *NotI* fragments. The size of the *NotI* fragments that possessed the *tdh* varied, but was within the range 300–700 kb. This means that for type D1/None strains, the distance between the two copies of *tdh* on chro-

mosomal DNA is less than this range. When the genomic DNA of AQ4700, a D1/None strain, was digested with other restriction enzymes such as *SfiI* or *SmaI*, or with combinations of enzymes with *NotI*, in all cases the two copies of *tdh* were detected on distinct bands, and each band was 40–100 kb in size (Fig. 4). Thus, although the two copies of *tdh* are close to each other on the chromosome (Fig. 2), the distance between the two does not seem to be near as that between *tdh* and *trh* of the type D2/R2 strain (see below).

***tdh* and *trh* in types D2/R2 and D6/R1 strains are also on single *NotI* fragments**

Strains possessing both *tdh* and *trh* on the chromosome were also examined (Fig. 3). After PFGE, the *NotI* digests of the genomic DNA of those strains were

Fig. 5. Relative location of the *tdh*, *ureC* and *trh* genes on the chromosome of *V. parahaemolyticus* AQ4673 as determined by Southern hybridization. Genomic DNA was digested with the indicated restriction enzymes, then the *ure*, *trh* or *tdh* gene was detected by Southern hybridization after PFGE. Electrophoresis was performed with an electric field of 6.0 V cm^{-1} on 1% agarose gels in $1 \times \text{TBE}$ buffer at 14°C . The pulse times increased linearly from 2 to 20 s within 12 h.

Fig. 6. Schematic representation of the relative location and direction of the *trh* and *ureC* genes on the chromosome of *V. parahaemolyticus* AQ4673. For comparison, the rough organization of the *ure* gene cluster of *Proteus mirabilis* (Nicholson *et al.*, 1993) is indicated at the bottom.

hybridized with the *tdh* or *trh* probe. In each of the strains, the probes for *tdh* and *trh* hybridized with common single bands, ranging from 150 to 350 kb in size. These results suggest that the two genes exist within this range on the chromosomal DNA of the strains tested.

Close proximity of *tdh*, *trh* and *ureC* in types D2/R2 and D6/R1 strains

Previously, we have shown that the urease-producing phenotype of *V. parahaemolyticus* isolated in Thailand is completely coincident with the possession of *trh*, and that this is due to a genetic linkage between *trh* and *ure* (Suthienkul *et al.*, 1995; Iida *et al.*, 1997). We investigated whether this was also the case with the strains tested in this study. All the *trh*-possessing strains were shown to produce urease, while none of the *trh*-negative strains produced the enzyme (data not shown). Thus, the coincidence of possession of *trh* and urease-producing phenotype was confirmed with the strains used in this study. Since we had discovered that *ure* was present only in the chromosome but not on a plasmid (Iida *et al.*, 1997), we analysed the relative locations of *trh* and *ure* on the chromosome of the strains in this study. PFGE followed by Southern hybridization with a probe (Iida *et al.*, 1997) for a structural gene of urease, *ureC* (Mobley *et al.*, 1995) demonstrated that, in strains possessing both *tdh* and *trh*, *ureC* was on the same *NotI* fragments

as *tdh* and *trh* (Fig. 3). More detailed analysis, using more frequently cutting restriction enzymes than *NotI*, of the distance and location of *tdh*, *trh* and *ureC* was done on strain AQ4673, which possesses all the three genes on the chromosome (type D2/R2) (data not shown). The results showed that the three genes were on a common *BamHI* fragment of approximately 40 kb. When *EcoRI* was used for digestion, *trh* and *ureC* appeared to be on a similar fragment of approximately 15 kb, but *tdh* was on an obviously distinct *EcoRI* fragment. When DNA was digested with *HindIII*, each gene was found on distinct fragments. We have schematically summarized these data in Fig. 5.

Distance between *trh* and *ureC*

Since the distance between *trh* and *ureC* in strain AQ4673 was apparently less than 15 kb (Fig. 5), we attempted to determine the exact distance between the two genes by means of LA-PCR. To cover all the four possibilities of relative directions between *trh* and *ureC*, we tried four combinations of primers, p83/URE5, p83/URE6, p84/URE5 and p84/URE6 (p83 and p84 are primers for *trh*, and URE5 and URE6 for *ureC*). Only one combination of primers (p84/URE6) amplified a single DNA fragment after PCR, when tested with the genomic DNA of AQ4673 (data not shown). The size of the amplified fragment was approximately 8.5 kb. Taking into account the positions of the primers and the hitherto unknown open reading frames between *trh* and *ureC*, since the genes that are required for the bio-synthesis of urease normally make up clusters (Mobley *et al.*, 1995), the distance between the *trh* and urease gene cluster should be only a few kilobases. Fig. 6 presents a schematic representation of the relative location of *trh* and *ure*.

DISCUSSION

V. parahaemolyticus is a causative agent of gastroenteritis, especially associated with consumption of seafood (Honda & Iida, 1993). *V. parahaemolyticus* is a normal marine inhabitant, and most environmental isolates are non-pathogenic to humans. It has generally been considered that only a part of the population of *V. parahaemolyticus* has ability to cause human gastroenteritis. To discriminate the pathogenic and non-pathogenic strains, a blood agar medium designated Wagatsuma agar was devised. On plates of this medium, almost all the strains isolated from the diarrhoeal faeces of patients give clear haemolysis, while environmental isolates are non-haemolytic (Sakazaki *et al.*, 1968; Miyamoto *et al.*, 1969). The haemolysis on Wagatsuma agar was named as the Kanagawa phenomenon, and it has been used to discriminate pathogenic *V. parahaemolyticus* strains. TDH is a protein toxin which has been identified as the agent responsible for the Kanagawa phenomenon and thus has been recognized as an important virulence factor of *V. parahaemolyticus*. Honda *et al.* (1988) found that clinical isolates of *V. parahaemolyticus* with a Kanagawa-phenomenon-nega-

tive phenotype, a rare phenotype among clinical isolates of the organism, produce TDH-related haemolysin (TRH). The amino acid sequence of TRH is approximately 67% identical to TDH, and TRH has similar biological activities such as haemolytic activity, cytotoxicity, cardiotoxicity and enterotoxicity (Honda & Iida, 1993). Thus, TRH is also believed to be an important virulence factor in Kanagawa-phenomenon-negative *V. parahaemolyticus*. Several groups of researchers have examined the distribution of the genes that encode TDH (*tdh*) and TRH (*trh*) in clinical and environmental isolates (Shirai *et al.*, 1990; Suthienkul *et al.*, 1995; Osawa *et al.*, 1996). In general, most clinical *V. parahaemolyticus* strains possess *tdh*, while few environmental strains do. Although strains possessing *trh* are occasionally isolated from the environment (seawater and seafood, etc.), they are also almost exclusively found in clinical samples (Shirai *et al.*, 1990; Honda *et al.*, 1990a, 1992). By homologous recombination techniques, the contribution of TDH and TRH to the diarrhoeagenicity of *V. parahaemolyticus* has been demonstrated (Nishibuchi *et al.*, 1992; Xu *et al.*, 1994).

In this study, we have demonstrated that *tdh* and *trh* are closely linked on the chromosome in *V. parahaemolyticus* strains that possess both genes. This result was unexpected, because the distributions of the two genes in clinical *V. parahaemolyticus* strains seem to be almost independent of each other (this study; Suthienkul *et al.*, 1995). The strong association observed between the restriction fragment patterns of *tdh* and *trh* (Table 1; Suthienkul *et al.*, 1996) might be partly related to the linkage of the two genes on chromosome.

In addition to the relation between *tdh* and *trh*, the relation between *trh* and *ure* genes also provided interesting results. We have previously reported on the complete association of a urease-producing phenotype with the possession of *trh* (Suthienkul *et al.*, 1995), and several other groups have reported similar findings for clinical *V. parahaemolyticus* strains (Magalhaes *et al.*, 1992; Obata *et al.*, 1996; Okuda *et al.*, 1997; Osawa *et al.*, 1996; Suzuki *et al.*, 1995). More recently, we demonstrated that the association is due to a genetic linkage between the urease structural gene (*ure*) and *trh* in *V. parahaemolyticus* strains (Iida *et al.*, 1997). In this study, we further analysed the relative location of the *ure* and *trh* genes on the chromosome, and determined the distance between the two genes. The results of LA-PCR have revealed that the distance is within a range of a few kilobases. These results confirm the genetic linkage between *trh* and *ure*. The observed genetic linkage suggests the possibility that *trh* and *ure* were involved in a gene-transfer event in the past (see below).

The clustering of virulence genes on chromosomal DNA was recently reported for several pathogenic bacteria, such as uropathogenic and enteropathogenic *Escherichia coli*, *Salmonella* species, *Yersinia pestis* and *Helicobacter pylori* (Groisman & Ochman, 1996). Usually, the clustering regions, or pathogenicity islands,

have a considerably lower GC content than that of the entire genomic DNA of the bacteria in which they are found. This suggests that they were somehow transferred into those pathogenic organisms from another organism. The mechanisms are unknown, but one possibility involves a bacteriophage-mediated process. The close proximity of *tdh*, *trh* and *ure* (in D2/R2 and D6/R1 strains) and two copies of *tdh* (in D1/None strains) on chromosomal DNA of clinical *V. parahaemolyticus* strains as demonstrated in this study seems to suggest that pathogenic *V. parahaemolyticus* strains also have a clustering of virulence genes on the chromosome. The fact that the GC content of *tdh* and *trh* is considerably lower than the mean GC content of the genomic DNA of *V. parahaemolyticus* (Iida & Yamamoto, 1990; Nishibuchi & Kaper, 1995) tempts us to consider the possibility that pathogenic *V. parahaemolyticus* might have a pathogenicity island on the chromosome. However, further evidence is needed to establish this. Nishibuchi and his colleagues, on the other hand, reported that *tdh* is flanked by sequences homologous to insertion sequences (Terai *et al.*, 1991), and suggested that pathogenic *V. parahaemolyticus* acquired the *tdh* gene through a mechanism involving the insertion sequences (Nishibuchi & Kaper, 1995). More precise localization of *tdh*, *trh* and *ure* and determination of the nucleotide sequences around the genes, which we are currently involved in, could reveal the way in which the gene transfer events happened in the past.

Although TDH and TRH are believed to be the primary virulence factors of *V. parahaemolyticus* in gastroenteritis infection, the enterotoxicity of purified TDH and TRH is not strong enough in itself to cause the severe clinical symptoms of pathogenic *V. parahaemolyticus*. For example, the quantity of TDH (or TRH) needed to cause a level of fluid accumulation similar to cholera toxin, a strong diarrhoeagenic protein toxin, in a rabbit ligated ileal loop model, was several hundred times more than that of cholera toxin (Honda *et al.*, 1990b). Thus, although we cannot exclude the contribution of TDH and TRH to the pathogenicity of *V. parahaemolyticus*, we must also look for extra factor(s) that contribute to the pathogenicity of the organism. If pathogenic *V. parahaemolyticus* strains have clusters of virulence genes on their chromosomal DNA, more precise and extensive analyses of the regions around *tdh* and *trh* may reveal virulence factor genes of *V. parahaemolyticus* that are as yet unknown.

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