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 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
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 *tdb* 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 *tdb* 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 DNA from *V. parahaemolyticus* was performed as previously described.

**Possession of *tdb* and *trh* genes.** The presence or absence of *tdb* 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, 30 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 lysyme 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 laurylsarcosine (SLS), 2 μg RNase ml⁻¹) and then deproteinated 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), 30 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 was prepared as previously described (Iida et al., 1997).

**RESULTS**

**Distribution of *tdb* and *trh***

First, to determine the distribution of *tdb* 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 *tdb* and that 19 strains (16.5 %) had *trh*. Eleven strains (9.6 %) were positive for both *tdb* and *trh*, and only one strain (0.9 %) had neither *tdb* nor *trh*.

**Clonal relationships among strains**

To analyse the clonal relationships among the *V. parahaemolyticus* strains, restriction fragment length polymorphism of *tdb* 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 *tdb* and *trh* probes. Strains possessing *tdb* 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 *tdb* 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 *tdb* and *trh* among the tested strains is summarized as follows: 93 strains had two copies of *tdb* on the chromosome and no *trh* (this group contains the type D1/None strains, i.e. those having the type D1 *tdb* and no *trh*), one strain had one copy of *tdb* on the chromosome and no *trh* (D7/None), one strain had one *tdb* on a plasmid and no *trh* (D8/None), eight strains had one *tdb* on the chromosome (None/R1, None/R3 and None/R4), eight strains had one *tdb* and one *trh* both on the chromosome (D2/R2 and D6/R1), three strains had one *trh* on the chromosome and one *tdb* on plasmid (D3/R4), and one strain had neither *tdb* nor *trh* (None/None). These results suggest that in the majority of the strains, *tdb* and *trh* are present on the chromosomal DNA.
tdh, trh and ure on genome of V. parahaemolyticus

Fig. 1. Eight types of HindIII 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 HindIII 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).

<table>
<thead>
<tr>
<th>RFP of trh type:</th>
<th>No. of strains whose RFP of tdh was type:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None†</td>
</tr>
<tr>
<td>None†</td>
<td>1</td>
</tr>
<tr>
<td>R1</td>
<td>5 (3)</td>
</tr>
<tr>
<td>R2</td>
<td>0</td>
</tr>
<tr>
<td>R3</td>
<td>2 (2)</td>
</tr>
<tr>
<td>R4</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Total</td>
<td>9</td>
</tr>
</tbody>
</table>

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

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

After Southern hybridization with the \textit{tdh} probe, the \textit{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 \textit{tdh} (Fig. 1a). This indicates that, in each of the type D1/None strains, two copies of \textit{tdh} exist on single \textit{NotI} fragments. The size of the \textit{NotI} fragments that possessed the \textit{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 \textit{tdh} on chromosomal DNA is less than this range. When the genomic DNA of AQ4700, a D1/None strain, was digested with other restriction enzymes such as \textit{SfiI} or \textit{SmaI}, or with combinations of enzymes with \textit{NotI}, in all cases the two copies of \textit{tdh} were detected on distinct bands, and each band was 40–100 kb in size (Fig. 4). Thus, although the two copies of \textit{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 \textit{tdh} and \textit{trh} of the type D2/R2 strain (see below).

\textit{tdh} and \textit{trh} in types D2/R2 and D6/R1 strains are also on single \textit{NotI} fragments

Strains possessing both \textit{tdh} and \textit{trh} on the chromosome were also examined (Fig. 3). After PFGE, the \textit{NotI} digests of the genomic DNA of those strains were
gated whether this was also the case with the strains producing phenotype was confirmed with the strains used in this study. All the trh-possessing strains were shown to produce urease, while none of the trh-negative strains tested. The pulse times increased linearly from 2 to 20 s within 12 h.

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 60 V cm⁻¹ on 1% agarose gels in 1 x TBE buffer at 14 °C. 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.

Fig. 6. Schematic representation of the relative location and direction of the trh and ure 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.

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 biosynthesis 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.

ACKNOWLEDGEMENTS

This work was supported by a grant-in-aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

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Received 2 February 1998; revised 29 April 1998; accepted 1 June 1998.