BACTERIAL GENETICS

Evidence for genetic linkage between the *ure* and *trh* genes in *Vibrio parahaemolyticus*

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Although *V. parahaemolyticus* does not generally produce urease, several studies have reported urease-positive *V. parahaemolyticus* isolates from clinical sources. Recently, studies have shown a complete coincidence between the urease-producing phenotype of *V. parahaemolyticus* strains and the possession of the thermostable direct haemolysin (TDH)-related haemolysin (TRH) gene (*trh*). TRH, like TDH, is considered to be an important virulence factor in the pathogenesis of *V. parahaemolyticus* gastroenteritis. The present study attempted to identify the gene *ure* encoding urease in *V. parahaemolyticus* to clarify the relationship between urease production and possession of *trh*. The polymerase chain reaction with mixed oligonucleotide primers targeted for conserved sequences of reported *ure* genes from other species was used to prepare a DNA probe to detect the *V. parahaemolyticus ure* gene. Colony hybridisation with this *ure* probe demonstrated that all the *ure*-positive strains produced urease. Considering the coincidence between production of urease and possession of *trh* in *V. parahaemolyticus*, it was concluded that the presence or absence of the *ure* gene is completely coincident with that of the *trh* gene in *V. parahaemolyticus* strains. Furthermore, the relative location of *ure* and *trh* on *V. parahaemolyticus* chromosomal DNA was analysed by pulsed-field gel electrophoresis. The results showed that, in all the strains examined, *ure* and *trh* were detected on the same *NotI* fragment, showing that the two genes localise within a relatively small portion of the chromosome DNA. These results suggest that the *ure* and *trh* genes are genetically linked in *V. parahaemolyticus* strains.

Introduction

Vibrio parahaemolyticus is an important bacterial pathogen in food-poisoning and traveller's diarrhoea, especially in association with the eating of raw or partially cooked seafood [1]. Almost all the strains isolated from clinical sources were haemolytic on Wagatsuma's agar (a special blood agar), while only a small number of environmental isolates showed haemolysis on this medium [2]. Thus, this haemolysis, known as the Kanagawa phenomenon (KP), is considered a useful marker of virulent *V. parahaemolyticus* strains. The haemolysin responsible for the phenomenon was identified and referred to as the thermostable direct haemolysin (TDH) [1]. Recently, some clinical isolates of KP-negative *V. parahaemolyticus* were found

to produce a haemolysin referred to as the TDH-related haemolysin (TRH), which is physicochemically, immunologically and biologically similar to TDH [3, 4]. TRH-producing *V. parahaemolyticus* has been reported to cause gastroenteritis like TDH-producing isolates [5, 6]. TDH and TRH, encoded by the *tdh* and *trh* genes, respectively, are now considered to be important virulence factors in the pathogenesis of *V. parahaemolyticus* [1].

Like other vibrios, *V. parahaemolyticus* does not generally produce urease. However, several studies have reported urease-positive *V. parahaemolyticus* isolates from clinical sources, and implicated these strains in human gastroenteritis [7–18].

A recent study examined 489 clinical strains of *V. parahaemolyticus* isolated in Thailand, 8% of which were urease positive [17]. Interestingly, the urease-producing phenotype completely coincided with the possession of the *trh* gene in these strains. The finding suggested that the production of urease could be

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considered an indication of virulent (*trh*-possessing) *V. parahaemolyticus* strains in clinical diagnosis [17]. As urease production for strains was not examined by genotype but by phenotype in the previous study [17], the genetic relationship between the production of urease and the possession of the *trh* gene among *V. parahaemolyticus* strains was unclear.

Thus far, neither isolation of urease nor cloning of the gene encoding urease (*ure*) have been reported from *V. parahaemolyticus*. The present study attempted to detect the *ure* gene in *V. parahaemolyticus* strains, to clarify the relationship between the urease-positive phenotype and possession of the *trh* gene in *V. parahaemolyticus* strains. A probe for the detection of the gene was prepared, and the distribution of the *ure* gene among the clinical *V. parahaemolyticus* isolates was examined; its relationship to the production of urease by the strains was then analysed. The relative location of the *ure* and the *trh* genes on the chromosomal DNA of *V. parahaemolyticus* was also determined.

Materials and methods

V. parahaemolyticus strains

One hundred and forty-seven strains of *V. parahaemolyticus* isolated from diarrhoeal patients in Thailand and 10 environmental isolates from the same country were used. The presence or the absence of *tdh* and *trh* had been determined previously by the polymerase chain reaction (PCR) and the urease phenotype had been examined [17, unpublished data]. Of the 147 clinical strains, 100 strains were *tdh*⁺ *trh*⁻ and urease negative, 10 strains were *tdh*⁻ *trh*⁻ and urease negative, 27 strains were *tdh*⁺ *trh*⁺ and urease positive, and 10 were *tdh*⁻ *trh*⁺ and urease positive. The 100 *tdh*⁺ *trh*⁻ strains and 10 *tdh*⁻ *trh*⁻ strains were randomly selected from a total of 396 *tdh*⁺ *trh*⁻ and 56 *tdh*⁻ *trh*⁻ strains in the original population [17]. The 10 environmental strains were isolated from seafood samples from local markets in Bangkok in 1992. All the environmental strains were *tdh*⁻ *trh*⁻ and urease negative. *V. parahaemolyticus* AQ4023 [19], T4750 [20] and *Escherichia coli* HB101 [21] were described previously.

Synthetic oligonucleotide primers for the detection of the *V. parahaemolyticus ure* gene

Two mixed oligonucleotide primers [URE2: 5'-CGTCGAATTC(A/T)(A/G)AT(A/T)(G/C)TIACIGC(T/C)G(G/C)IGGIATIGAT-3' and URE4: 5'-GCTCGAATTC(A/G/A)(G/A)T(G/T)(G/A)TG(G/A)CAIACCATIAICAT(G/A)TC-3'] were designed for PCR to detect the *V. parahaemolyticus ure* gene based on the conserved regions of the reported structural gene sequences of ureases from various bacterial and plant sources stocked in Genbank. Oligonucleotide primers were

synthesised with a DNA synthesizer, Model 381A (Applied Biosystems).

Preparation of template DNA for PCR

V. parahaemolyticus strains were cultured in 1.5 ml of Luria-Bertani (LB: tryptone 1%, yeast extract 0.5%) broth with NaCl 3% and incubated at 37°C overnight with shaking. The cell pellet was harvested by centrifugation at 8000 *g* for 2 min. The supernate was aspirated and the cell pellet was resuspended in 500 µl of distilled water. After boiling at 100°C for 5 min, the suspension was centrifuged at 8000 *g* for 5 min. The supernate, containing the bacterial DNA for use as template in PCR, was kept at -20°C before being assayed.

PCR conditions

The PCR was conducted in a reaction mixture containing 10 mM Tris-HCl (pH 8.9), 80 mM KCl, 1.5 mM MgCl₂, bovine serum albumin 0.5 mg/ml, 0.2 mM dNTP and 0.2 µM of each primer. The total volume of the PCR mixture was 50 µl, including 10 µl of template DNA solution prepared as described above and 1 unit of *Tth* DNA polymerase (Toyobo). A temperature cycle of 94°C for 2 min, 45°C for 3 min and 55°C for 3 min was repeated 35 times. After the reactions, the presence or the absence of amplified DNA fragments was examined by electrophoresis on agarose 0.8% gel followed by ethidium bromide staining.

General molecular genetic methods

General molecular genetic techniques such as DNA digestion with restriction endonucleases, ligation, electrophoresis and transformation were performed as described previously [21].

Nucleotide sequence determination

Nucleotide sequences were determined on a DNA sequencer Model 373A (Applied Biosystems) after sequencing reaction with PRISM Dye Primer Cycle Sequencing Ready Reaction Kit (Perkin-Elmer).

Preparation of digoxigenin-labelled probes

The 334-bp *Eco*RI fragment from pKY298 [19] for the *trh* probe and the 601-bp *Eco*RI fragment from pTI301 (see below) for the *ure* probe were labelled with digoxigenin-11-dUTP by a random primer extension method provided by a DNA labelling and detection kit (Boehringer Mannheim). Labelling procedures were 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.

Colony hybridisation for the *ure* gene

V. parahaemolyticus strains were cultured on marine agar (Difco) with NaCl 3%. After incubation overnight at 37°C, bacterial cultures from single colonies were spotted with sterilised toothpicks on to a nylon membrane (GeneScreen Plus, Dupont). *V. parahaemolyticus* strain AQ4023 was used as a positive control strain and *V. parahaemolyticus* T4750 was used as a negative control strain for urease production. Bacterial immobilisation and denaturation on the membrane were as described previously [21]. The membrane-bound material was hybridised with the digoxigenin-labelled *ure* probe in hybridisation solution containing 5× SSPE (1× SSPE is 0.25 M NaCl, 0.01 M NaH₂PO₄, and 1 mM EDTA, pH 7.4), formamide 50%, sodium-laurylsarcosine (SLS) 0.1%, sodium dodecyl sulphate (SDS) 0.02%, denatured single-stranded salmon sperm DNA 0.1 mg/ml, and blocking reagent from a DIG DNA labelling and detection kit (Boehringer Mannheim) 1%. The hybridisation temperature for the *ure* probe was 37°C. Immunological detection of DNA fragments hybridised with the probes was performed with anti-digoxigenin antibody conjugated with alkaline phosphatase (Boehringer Mannheim). The DNA targets were then detected with a chemiluminescent substrate for alkaline phosphatase, CSPD (Tropix), as recommended by the kit manufacturer.

Extraction of bacterial DNA for conventional Southern assay

The genomic DNA of *V. parahaemolyticus* strains was extracted from overnight cultures in LB broth with NaCl 3% following the method of Wilson [22]. Nucleic acids were precipitated with ethanol, then dissolved in TE buffer. Total genomic DNA was then digested with *Hind*III (Toyobo) overnight at 37°C for Southern analysis. The *Hind*III digests of bacterial genomic DNA were separated by electrophoresis on an agarose 1% gel.

Pulsed-field gel electrophoresis (PFGE)

Samples for PFGE were prepared as described previously [23] with modification. Briefly, bacterial cells were grown overnight with shaking at 37°C in LB broth containing NaCl 3%. Cells were then centrifuged, 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 (lysozyme 1 mg/ml in 10 mM Tris-HCl, (pH 8.0), 1 M NaCl, 100 mM EDTA—sodium deoxycholate 0.2%, SLS 0.5%, RNAase 2 µg/ml) and then deproteinated in proteinase K 1 mg/ml in 0.5 M EDTA (pH 8.0), SLS 1% at 50°C overnight. Cell debris and proteinase K were then removed by four washes in 20 mM Tris-HCl (pH 8.0), 50 mM EDTA for 2 h each at room temperature, of which the second wash was with 1 mM phenylmethylsulphonyl fluoride

(Sigma). The *V. parahaemolyticus* DNA in agarose blocks was digested with the restriction enzyme *Not*I (Toyobo). The restriction DNA fragments were resolved by PFGE in agarose 1% gel (Molecular Biology Certified Agarose, BioRad) on a CHEF DR III System (BioRad). Before Southern transfer, the DNA fragments in the agarose were nicked by exposing the gel to ultraviolet light (UV) in a UV chamber (GS Gene Linker, BioRad).

Southern hybridisation

After electrophoresis, the DNA fragments were transferred on to a nylon membrane (GeneScreen, Dupont) with 6× SSPE as described previously [21], then hybridised with the digoxigenin-labelled *trh* or *ure* probe as described above. The hybridisation temperature for the *trh* probe was 30°C [24] and the temperature for the *ure* probe was 37°C.

Results

Amplification of the *V. parahaemolyticus ure* gene by PCR

To detect the *V. parahaemolyticus ure* gene, a pair of mixed oligonucleotide primers for PCR (URE2 and URE4) were designed and synthesised, based on the nucleotide sequences of the conserved regions in the *ure* genes from the various bacterial and plant sources that have so far been reported. When the PCR was performed with the above two primers, a single DNA fragment of the expected size (615 bp) was amplified in the case of a urease-positive *V. parahaemolyticus* strain AQ4023, while no band was apparent in the case of a urease-negative strain, T4750 (Fig. 1). As both primers were designed to have an *Eco*RI site near the 5' termini, the amplified DNA fragment was digested with *Eco*RI, then ligated to the *Eco*RI site of a plasmid vector pUC119, resulting in the recombinant plasmid pTI301. DNA sequencing of the insert and homology searching in the DNA database, GenBank, revealed that the nucleotide sequence in the fragment had a 64% homology with the corresponding region of the *Klebsiella aerogenes ureC* gene, one of the urease structural genes of the organism [25]. (The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases with the accession number D86348.) This indicates that the expected part of the *ure* gene was cloned from *V. parahaemolyticus*.

Distribution of the *ure* gene among *V. parahaemolyticus* strains

The *Eco*RI-insert of pTI301 (601 bp in size) was labelled with digoxigenin to prepare a probe to detect the *V. parahaemolyticus ure* gene. The distribution of the *ure* gene in clinical and environmental *V. parahaemolyticus* isolates was investigated with this

Fig. 1. PCR for the *V. parahaemolyticus ure* gene. Lane 1, molecular size markers ϕ X174/*Hae*III; 2, *E. coli* HB101; 3, *V. parahaemolyticus* AQ4023 (urease-positive); 4, *V. parahaemolyticus* T4750 (urease-negative).

probe by colony hybridisation (Table 1). All the urease-producing strains hybridised with the *ure* probe, while the urease-negative strains did not. These results indicate that all the strains possessing the *ure* gene produce urease.

Location of the *ure* and *trh* genes on chromosomal DNA of *V. parahaemolyticus* strains

The *trh* gene in *V. parahaemolyticus* has been shown to exist exclusively on chromosomal but not on plasmid DNA [26, unpublished data]. The *ure* gene was also suggested, by Southern hybridisation, to exist exclusively on *V. parahaemolyticus* chromosome but not on

plasmid DNA (data not shown). Consequently, the relative location of the *ure* and *trh* genes on *V. parahaemolyticus* chromosome was investigated. Representative strains from the *trh* type R1–R4 [24] were subjected to PFGE after treatment with a rare-cutting restriction endonuclease, *Not*I, to observe the macro-restriction patterns of chromosomal DNA. The patterns varied among the strains (Fig. 2a). However, the *ure* and *trh* probes both hybridised with the same *Not*I-fragment in each strain by Southern hybridisation (Fig. 2b and c). These results suggest that, on the chromosomal DNA of the *V. parahaemolyticus* strains, the *ure* and *trh* genes localise within a relatively small portion.

Discussion

Detection of urease production is a simple biochemical assay, commonly used in the identification of bacteria. In the family Enterobacteriaceae, the genera *Proteus*, *Klebsiella*, *Citrobacter*, *Yersinia* and some species of *Serratia* and *Enterobacter* produce urease. The production of urease has served as a tool to differentiate many members of Enterobacteriaceae, vibrios and aeromonads. Like other vibrios, most *V. parahaemolyticus* strains do not produce urease, but some are known to do so. Since the first report of Huq *et al.* [10], urease-positive clinical isolates of *V. parahaemolyticus* have been reported from various countries in the world, such as Singapore [14], the USA and Mexico [7, 12, 13, 16], Brazil [15], Malaysia [11], Thailand [17], Nigeria [8] and Japan [9, 18]. In many cases, the urease-positive *V. parahaemolyticus* strains form a minority in the population of clinical isolates, and TDH production is considered to be the most prevalent and important virulence marker of *V. parahaemolyticus*. However, in isolates from some areas, the urease-positive phenotype has been reported to be more closely associated with the pathogenicity of *V. parahaemolyticus* than TDH production [7, 8, 12, 13].

A recent study examined 489 clinical strains of *V. parahaemolyticus* isolated in Thailand, of which 8% of the strains were urease positive [17]. An interesting finding in the study was a perfect coincidence between urease-producing phenotype and the possession of the *trh* gene among *V. parahaemolyticus* strains [17]. This suggests that virulent (*trh*-possessing) *V. parahaemo-*

Table 1. Urease production and distribution of the *ure* gene in clinical and environmental *V. parahaemolyticus* strains

Strain genotype	Number of strains	Urease production	Presence of <i>ure</i> *
Clinical			
<i>tdh</i> ⁺ <i>trh</i> ⁻	100	-	-
<i>tdh</i> ⁺ <i>trh</i> ⁺	27	+	+
<i>tdh</i> ⁻ <i>trh</i> ⁺	10	+	+
<i>tdh</i> ⁻ <i>trh</i> ⁻	10	-	-
Environmental			
<i>tdh</i> ⁻ <i>trh</i> ⁻	10	-	-

* The presence or the absence of the *ure* gene in the *V. parahaemolyticus* strains was determined by colony hybridisation.

Fig. 2. PFGE of *NotI*-digested genomic DNA of *V. parahaemolyticus* strains and detection of *ure* and *trh*. **a**, ethidium bromide staining; **b**, hybridisation with the *trh* probe; **c**, hybridisation with the *ure* probe. PFGE was performed in $1 \times$ TBE buffer (89 mM Tris-89 mM boric acid-2 mM EDTA, pH 8.3) with agarose 1% gel at 6 V/cm for 24 h at 14°C with a ramp time of 6–60 s with clamped homogeneous field electrophoresis. Lane 1, strain VP81 with R1 *trh*; 2, VP389 (R1); 3, VP178 (R2); 4, VP453 (R2); 5, VP207 (R2); 6, VP343 (R3); 7, VP63 (R4); 8, VP466 (R4). Molecular size markers, lambda ladder (concatemers of λ c1857sam7) are noted at the left of panel **a**.

lyticus strains can be indicated by the urease-positive phenotype in clinical diagnosis [17]. At present, it is unknown if the finding is applicable to the clinical *V. parahaemolyticus* isolates from other areas. However, similar results were reported very recently from areas other than Thailand [27, 28].

In this study, a DNA probe was prepared to detect the *V. parahaemolyticus ure* gene. Colony hybridisation with the *ure* probe demonstrated that all the *ure*-positive strains were urease-producers (Table 1). Considering the coincidence between the production of urease and the possession of *trh* in *V. parahaemolyticus* [17], it is concluded that the presence or the

absence of the *ure* gene is completely coincident with that of the *trh* gene in *V. parahaemolyticus* strains. Furthermore, the relative location of *ure* and *trh* on *V. parahaemolyticus* chromosomal DNA was analysed by Southern hybridisation after PFGE. The results showed that, in all the strains examined, the *ure* and *trh* were detected on the same fragments in each strain after *NotI*-digestion of chromosomal DNA (Figs. 2b and c). This indicates that the *ure* and *trh* genes localise within a relatively close range on the chromosomal DNA of *V. parahaemolyticus*. These results, along with the results regarding distribution of the two genes, suggest that the *ure* and *trh* genes are genetically linked in *V. parahaemolyticus* strains.

The present study provided a reason, at the molecular genetic level, why the urease-positive phenotype always associates with the possession of the *trh* gene. This information is important for clinical diagnosis. Instead of using other more specific, but more expensive, laborious assays such as the ELISA, DNA hybridisation or PCR, the possession of the *trh* gene will be known by urease activity, which is easy to assay.

The present findings also provide interesting information from an evolutionary point of view. It has been proposed that at some time in the past, *tdh*, and possibly *trh*, were transferred from another organism into a small population of *V. parahaemolyticus* by plasmid-mediated and insertion sequence-mediated mechanisms [29–31]. As *ure* is genetically linked with *trh* in *V. parahaemolyticus*, as demonstrated in this study, the *ure* gene might also be somehow related to the ancient gene transfer event in *V. parahaemolyticus*. Although a close localisation of *ure* and *trh* on chromosomal DNA was found in this study, exact distances between the two genes have not been determined. More detailed positioning of the genes are now being investigated in this laboratory, together with the analysis of the flanking regions of *ure* and *trh* on chromosomal DNA.

Recently, a number of studies reported the relationship between bacterial pathogenicity and urease production [32–34]. Urease produced by *Helicobacter pylori* has been implicated in pathogenesis by virtue of its role in alkalising the bacterium's micro-environment in the stomach and its toxicity to stomach epithelium [35]. In the case of the enteric pathogen, *Yersinia enterocolitica*, urease was shown to be responsible for acid resistance and to contribute to the virulence of the organism by enhancing the likelihood of bacterial survival during passage through the stomach [33]. In the case of *V. parahaemolyticus*, production of urease does not seem to be essential for the pathogenicity of *V. parahaemolyticus*, as most of the clinical isolates are urease-negative [17]. As the enterotoxicity of purified TRH has been demonstrated [5, 36], it is reasonable to consider that the pathogenicity of urease-positive strains, which, at the same time, have *trh*, is primarily attributable to TRH. At present, we consider that the urease production by *V. parahaemolyticus* strains is merely a marker of *trh* possession. However, we cannot completely exclude the possibility that urease contributes to the pathogenicity of the organism by increasing its chances of survival during passage through the stomach, as has been previously demonstrated for *Y. enterocolitica* infection [33].

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