BACTERIAL GENETICS

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

TETSUYA IIDA*, ORASA SUTHIENKUL*, KWON-SAM PARK†, GUANG-QING TANG*, RYOHEI K. YAMAMOTO*, MASANORI ISHIBASHI†, KOICHIRO YAMAMOTO* and TAKESHI HONDA*

*Department of Bacterial Infections, Research Institute for Microbial Diseases, Osaka University, Osaka 565 and †Department of Food Microbiology, Osaka Prefectural Institute of Public Health, Osaka 537, Japan

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

Received 24 Oct. 1996; accepted 4 Dec. 1996.
Corresponding author: Dr T. Iida.
‡ Present address: Department of Microbiology, Faculty of Public Health, Mahidol University, Rajvithi Road, Bangkok 10400, Thailand.
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 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- strains in clinical diagnosis [17], as described previously [21].

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 EcoRI fragment from pKY298 [19] for the trh probe and the 601-bp EcoRI fragment from pT1301 (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 onto 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 (Boehringer Mannheim). The recommended 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 HindIII (Toyobo) overnight at 37°C for Southern analysis. The HindIII 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 agarose 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 deproteinised in proteinase K 1 mg/ml in 0.5 mM 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 Not1 (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 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 EcoRI site near the 5' termini, the amplified DNA fragment was digested with EcoRI, then ligated to the EcoRI 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 EcoRI-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 φX174/HaeIII; 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 (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, NotI, 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 NotI-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. paraheamolyticus strains possess the trh gene exclusively.
**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 × 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, trh, 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 alkalinising 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].

We thank Yuji Isegawa for helpful suggestions. This study was supported by a grant-in-aid for Scientific Research from the Ministry of Education, Science and Culture of Japan and the Japan Society for Promotion of Science.

References