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Analysis of the Thermostable Direct Hemolysin (tdh) Gene and the tdh-Related Hemolysin (trh) Genes in Urease-Positive Strains of Vibrio parahaemolyticus Isolated on the West Coast of the United States

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Received 2 December 1996/Returned for modification 4 April 1997/Accepted 7 May 1997

Urease-positive (Ure+) and urease-negative (Ure−) strains of Vibrio parahaemolyticus isolated from patients on the West Coast of the United States between 1979 and 1995 were analyzed for the thermostable direct hemolysin (tdh) gene and the tdh-related hemolysin (trh) genes (trh1 and trh2). The DNA colony hybridization method with the polynucleotide probes was used to determine the distribution of the genes. Of 60 Ure+ strains, 59 strains (98%) had the trh (either trh1 or trh2) gene and 54 strains (90%) carried the tdh gene. The absence of the trh gene or a related sequence in an exceptional Ure− strain was confirmed by Southern blot analyses. The stronger correlation with the trh gene than with the tdh gene was mostly attributable to strains possessing only the trh2 gene. Of 25 Ure− strains, 20 strains (80%) had the tdh gene but none had the trh gene. These results indicate a very strong correlation between the Ure+ phenotype and the trh gene and are consistent with those reported for strains isolated in Asia. The Ure+ strains carrying the trh genes were not restricted to a unique group of the strains. The O4:K12 strains carrying the trh1 gene have predominantly been isolated since 1979. However, strains of various non-O4:K12 serovars carrying either the trh1 or the trh2 gene became predominant after 1992. In addition, analysis by the arbitrarily primed PCR method revealed two subgroups within the selected Ure+ O4:K12 strains. Hybridization tests with oligonucleotide probes demonstrated that the trh1 sequences of the West Coast strains differ to some extent from those of Asian strains. Nevertheless, a PCR method previously established to detect both the trh1 and the trh2 genes in Asian strains could detect 98% of those genes in the West Coast strains.

Vibrio parahaemolyticus can cause gastroenteritis in humans through seafood consumption. The early epidemiological investigations revealed a very strong association between the Kanagawa phenomenon (KP) and gastroenteritis (14, 28). KP is a beta-type hemolysis on a special blood agar medium, Wagatsuma agar, induced by thermostable direct hemolysin (TDH) that is produced almost exclusively by clinical strains (34). Therefore, TDH has been considered a major virulence factor of V. parahaemolyticus. Investigations of patients with traveler’s diarrhea originating from an outbreak of gastroenteritis in the Maldives in 1985 revealed that KP-negative strains of V. parahaemolyticus produce a TDH-related hemolysin (TRH) but not TDH (4, 5). TRH and TDH share a common epitope(s) (5). The similarity of the two hemolysins was also confirmed at the molecular genetic level; the tdh and trh genes encoding TDH and TRH, respectively, shared ca. 70% nucleotide sequence identity (11, 18, 22). Molecular epidemiological evidence supporting the association of the strains possessing the tdh or the trh gene with gastroenteritis was obtained (29).

Among the biochemical characteristics of V. parahaemolyticus, urease activity has usually been considered negative; less than 10% of the strains were positive for urease activity in most investigations reported between 1963 and 1974 (3, 27, 36, 37). Isolation of urease-positive (Ure+) strains from patients with gastroenteritis in various parts of the world attracted attention and recently stimulated many publications (1, 2, 6, 10, 13, 23, 25). The KP-negative phenotype of the Ure+ strains was pointed out in some of the reports (2, 10, 13). Very recent reports on examinations of clinical strains isolated in Asian countries and Brazil indicated that the ratio of Ure+ strains among the clinical strains is gradually increasing and that a strong correlation exists between urease production and the presence of the trh gene rather than that of the tdh gene (12, 24, 26, 30–32). The correlation between urease production and possession of the trh gene suggests that urease production can be used as one of the virulence markers. The correlation was perfect in some of the studies (12, 24, 30, 32) but was not in other studies (26, 31). Unlike in the tdh genes, significant nucleotide sequence variation exists in the trh genes in different strains isolated in Asia, and the trh genes could be clustered into two subgroups, trh1 and trh2, which share 84% sequence identity (11, 18). However, this has not been fully considered in examination of the trh gene in Ure+ strains so far. Ignorance of the sequence variation might affect the results of oligonucleotide-based gene detection, such as hybridization with an oligonucleotide probe and PCR.

Many of the V. parahaemolyticus strains isolated recently on the West Coast of the United States demonstrated urease activity (7). There are reports that suggested a possible association of the Ure+ phenotype and the presence of the tdh gene in the strains isolated in this region (1, 8, 9). However, the strains isolated in the United States have not been examined...
for the presence of the \(trh\) gene to our knowledge. The present study was undertaken to examine the distribution of the \(trh\) genes and its correlation with the Ure+ phenotype in the strains isolated from the patients on the West Coast since 1979. We used suitable methods to distinguish and characterize the \(tdh\), \(trh1\), and \(trh2\) genes in the test strains.

### MATERIALS AND METHODS

#### Bacterial strains

The following strains of \(V.\) *parahaemolyticus* isolated from patients and submitted to the Microbial Diseases Laboratory, California Department of Health Services, were examined (Tables 1 and 2): 5 strains representing the Ure+ strains isolated between 1979 and 1987 (1) and 55 Ure+ and 25 urease-negative (Ure−) strains isolated between 1988 and 1995. All isolates were identified as \(V.\) *parahaemolyticus* by the presence of standard cultural and biochemical characteristics (7). Also included in this study were control strains of \(V.\) *parahaemolyticus* isolated in Asian countries (Tables 1 and 2): WP1 (20); AQ4037 (22); AT4 (11); and 225, 250, and 275 (30). Unless otherwise specified in Tables 1 and 2, the strains listed in the tables were isolated from fecal samples. The urease activities of the test strains were examined by the urea agar method (Christensen’s method). The NaCl concentration of the medium was increased to 1% (7).

#### \(O:K\) serovar

The \(V.\) *parahaemolyticus* strains were grown and the \(O:K\) serovars were determined as described previously (30).

#### Polynucleotide and oligonucleotide probes

The polynucleotide probe specific to the \(tdh\) gene was prepared with a 415-bp DNA fragment internal to the \(tdh1\) gene of strain WP1 (17, 20). The polynucleotide probe specific to the \(trh1\) gene was prepared with a 334-bp DNA fragment internal to the \(trh1\) gene of AQ4037 (29). The polynucleotide probe specific to the \(trh2\) gene was prepared with a 419-bp DNA fragment internal to the \(trh2\) gene of strain AT4 (11). The DNA fragments were labeled by the random priming method with \(^{32}\)P-labeled dCTP (11). The oligonucleotide probes specific to the \(trh1\) and \(trh2\) genes are the A1 and A2 probes described previously (11). The sequences of the A1 and A2 probes are identical to those of the \(trh1\) gene of AQ4037 and the \(trh2\) gene of AT4, respectively, and both are 20-mer synthetic oligonucleotides. The oligonucleotides were labeled at their 5′ ends with \(^{32}\)P-labeled ATP (11).

#### DNA colony hybridization test

DNA colony blots were prepared as described previously (17). The DNA colony hybridization tests with polynucleotide probes were carried out under high-stringency conditions as described previously (17). The DNA colony hybridization tests with oligonucleotide probes were performed under high-stringency conditions specifically determined for each probe (11).

#### Southern blot analysis

Cellular DNA was extracted by the method described previously (21). Digestion of DNA with restriction enzymes and gel electrophoresis were carried out as described previously (19). The Southern blots were prepared as described previously (20). Hybridization with the polynucleotide probe was performed under high-stringency conditions (solution containing 50% formamide) or under reduced-stringency conditions (solution containing 35% formamide) as described previously (22).

### TABLE 1. \(O:K\) serovar and presence or absence of the \(tdh\), \(trh1\), and \(trh2\) genes in \(Ure+\) strains of \(V.\) *parahaemolyticus* isolated from patients on the West Coast of the United States

<table>
<thead>
<tr>
<th>Yr of isolation</th>
<th>No. of strains</th>
<th>(O:K) serovar(^a)</th>
<th>Presence of the following gene(^b):</th>
<th>Strain selected or isolated from an exceptional source(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1979</td>
<td>4:12</td>
<td>+ + −</td>
<td></td>
<td>6190</td>
</tr>
<tr>
<td>1981</td>
<td>4:12</td>
<td>+ + −</td>
<td></td>
<td>7912</td>
</tr>
<tr>
<td>1982</td>
<td>4:12</td>
<td>+ + −</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1983</td>
<td>4:12</td>
<td>+ + −</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1984</td>
<td>4:12</td>
<td>+ + −</td>
<td></td>
<td>1516</td>
</tr>
<tr>
<td>1987</td>
<td>4:12</td>
<td>+ + −</td>
<td></td>
<td>3215</td>
</tr>
<tr>
<td>1988</td>
<td>4:12</td>
<td>+ + −</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1989</td>
<td>4:12</td>
<td>+ + −</td>
<td></td>
<td>5791</td>
</tr>
<tr>
<td>1990</td>
<td>4:12</td>
<td>+ + −</td>
<td></td>
<td>4500, 4735, 5435</td>
</tr>
<tr>
<td>1991</td>
<td>4:12</td>
<td>+ + −</td>
<td></td>
<td>2448</td>
</tr>
<tr>
<td>1992</td>
<td>4:12</td>
<td>+ + −</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1993</td>
<td>4:12</td>
<td>+ + −</td>
<td></td>
<td></td>
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<tr>
<td>1994</td>
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<td></td>
</tr>
<tr>
<td>1995</td>
<td>4:12</td>
<td>+ + −</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) UT, untypeable.

\(^b\) +, present; −, absent.

\(^c\) Source of isolation, if other than feces, is indicated in parentheses.

### TABLE 2. \(O:K\) serovar and presence or absence of the \(tdh\), \(trh1\), and \(trh2\) genes in \(Ure−\) strains of \(V.\) *parahaemolyticus* isolated from the patients on the West Coast of the United States

<table>
<thead>
<tr>
<th>Yr of isolation</th>
<th>No. of strains</th>
<th>(O:K) serovar(^a)</th>
<th>Presence of the following gene(^b):</th>
<th>Strain selected or isolated from an exceptional source(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1988</td>
<td>4:12</td>
<td>+ + −</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1989</td>
<td>4:12</td>
<td>+ + −</td>
<td></td>
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<td>1990</td>
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<td>1994</td>
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<td>+ + −</td>
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<tr>
<td>1995</td>
<td>4:12</td>
<td>+ + −</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) UT, untypeable.

\(^b\) +, present; −, absent.

\(^c\) Source of isolation, if other than feces, is indicated in parentheses.
Hemolysin Genes (tdh, trh) of V. ParaHaemolyticus

Persistence of Ure+ O4:K12 strains. Eight representative O4:K12 strains, isolated between 1979 and 1995, that carried both tdh and trh1 genes and four tdh- and trh1-bearing strains of non-O4:K12 serovars were compared genetically to investigate whether a single clone of the O4:K12 serovar persisted on the West Coast. The polymorphisms of the length of the restriction fragments carrying the tdh and trh genes in the selected strains were first compared (Fig. 1). Previous studies demonstrated variations in the lengths of the HindIII fragments carrying the tdh and trh genes (11, 20). Therefore, HindIII-digested DNAs of the selected strains were compared by the Southern blot hybridization method. All selected strains with the exception of an O4:K9 strain (Fig. 1A, lane 11) had a 2.8-kb tdh-bearing HindIII fragment (Fig. 1A) and a 6.6-kb trh1-bearing HindIII fragment (Fig. 1B). Only the trh1-bearing fragments gave weak hybridization signals with the trh2-specific probe, confirming the absence of the trh2 gene (Fig. 1C). The results indicate that the nucleotide sequences around the tdh and trh1 genes in the selected strains are well conserved.

FIG. 1. Results of Southern blot hybridization analysis of the HindIII-digested cellular DNAs for selected strains of V. paraHaemolyticus carrying both tdh and trh1 genes. The blots were examined by Southern blot hybridization analysis with the polynucleotide probes for the tdh (A), trh1 (B), and trh2 (C) genes under high-stringency conditions. The test strains (listed in Table 1) were treated with an hybridization buffer, 2.8 kb (lanes 1 to 10 and 12) and 6.6 kb (lane 11) in panels A, 6.6 kb (all lanes) in panels B and C.

RESULTS

Gene distribution and serovar. The distribution of the tdh, trh1, and trh2 genes was determined by the DNA colony hybridization method with specific polynucleotide probes under high-stringency conditions. The O:K serovars of the strains were also determined. The results for Ure+ and Ure− strains are summarized in Tables 1 and 2, respectively. Five representative Ure+ strains of the O4:K12 serovar isolated between 1979 and 1987 (1) were shown to have not only the tdh gene but also the trh1 gene (Table 1). Of the Ure+ strains isolated between 1988 and 1992, the O4:K12 strains carrying the tdh and trh1 genes were detected most frequently (Table 1). This type of strain also persisted after 1992, but the tdh- and trh1-bearing Ure+ strains of non-O4:K12 serovars became predominant during this period (Table 1). In addition, five Ure+ strains carrying only the trh2 gene have been isolated since 1991 (Table 1). To sum up, of the 60 Ure+ strains isolated on the West Coast of the United States since 1979, 59 strains had either the trh1 or the trh2 gene. This correlation of the Ure+ phenotype with the trh gene (98%) was stronger than that with the tdh gene (90%). The stronger correlation was mostly attributable to the strains possessing only the trh2 gene.

Compared with the Ure− strains, Ure− strains were isolated less frequently after 1990 (Table 2). Of 25 Ure− strains isolated between 1988 and 1995, 20 strains (80%) had the tdh gene, but none had the trh gene. This and the results presented above indicate that the Ure+ phenotype is much more closely correlated with the presence of the trh gene than with that of the tdh gene.

PCR. PCR for the detection of both trh1 and trh2 genes in the test organism was performed with the R2 and R6 primers described previously (33), except that Taq polymerase (Perkin-Elmer Cetus Corp., Norwalk, Conn.) was replaced with TaKaRa Taq (Takara Shuzo Co., Ltd., Otsu, Shiga, Japan) and that a Hybrid thermal reactor (model HB-TR1L; Hybaid Ltd., Middlesex, England) was used.

AP-PCR. Cellular DNA extracted as described above was used as the template for arbitrarily primed PCR (AP-PCR). PCR amplification was performed in a 30-μl mixture composed of 0.2 mM (each) the four deoxynucleotide triphosphates, 2.5 μg of template DNA, 2.5 U of polymerase (Takara Ex Taq; Takara Shuzo Co., Ltd.), and added as primer ingredients supplied by the manufacturer of the polymerase were used for the reaction. The oligonucleotide primers included in RAPD Analysis Primer Set (Pharmacia Biotech, Inc., Uppala, Sweden) were evaluated in the preliminary experiment, and primer 1 (5′-d[GGTGCGGGAA]-3′) and primer 2 (5′-d[GTTTCGCTCC]-3′), which gave many sharp amplicon bands, were selected as the amplification primers. The reaction mixture was heated at 95°C for 4 min and was subjected to 45 cycles of PCR amplification in the Hybrid thermal reactor; 1 cycle consisted of denaturation at 95°C for 1 min, primer annealing at 36°C for 1 min, and extension at 72°C for 2 min. A 10-μl portion of the PCR products was resolved on a 1.5% agarose gel. TDH detection. The TDH produced by V. ParaHaemolyticus was detected by the immunological method based on the reverse-phase latex agglutination reaction with rabbit anti-TDH immunoglobulin G. The test strain was grown in a broth medium composed of 2% Bacto Peptone (Difco Laboratories,Detroit, Mich.), 0.5% NaCl, and 5% NaCl (pH 7.8), with or without shaking (180 rpm), at 37°C for 18 h. Appropriate dilutions of the culture supernatant that gave a positive reaction.
had nearly identical AP-PCR patterns (Fig. 2B, lanes 3 and 9). The two subgroups within the O4:K12 groups distinguished by AP-PCR, however, shared several common amplicons. The results suggest that the O4:K12 strains carrying the \textit{tdh} and \textit{trh1} genes are fairly uniform but that there are at least two subgroups that have persisted over 13 or 14 years.

**Rare Ure+ strain lacking the \textit{trh} gene.** Unlike other Ure+ strains, one Ure+ strain, strain 2448, lacked the \textit{trh} gene and had only the \textit{tdh} gene (Table 1). This was confirmed by Southern blot hybridization analysis under high-stringency conditions (Fig. 3A). The hybridization analysis was also performed under reduced-stringency conditions (Fig. 3B). Only the \textit{tdh} sequences exhibited weak hybridization signals with the \textit{trh1} and \textit{trh2} probes, indicating that the \textit{trh1}- or \textit{trh2}-related sequence that is distinct from the \textit{tdh} gene is also absent from strain 2448.

We were interested in comparing the level of TDH produced by strain 2448 with those produced by other Ure+ strains possessing the \textit{trh} genes and by KP-positive strains (discussed below). The TDH level of strain 2448 was much higher than those of the other Ure+ strains carrying the \textit{trh} gene but was not as high as those of KP-positive strains (strains WP1, 1171, and 7018; Table 3).

**Comparison of the gene detection methods.** We used the DNA colony hybridization method with specific polynucleotide probes under high-stringency conditions as the “gold standard” (Table 1). This is because it allows for as much as a 20% mismatch with the specific probes (15), and therefore, the target genes with significant sequence variation can still be detected. The hybridization signals of representative strains obtained with the \textit{tdh}, \textit{trh1}-, and \textit{trh2}-specific gene probes are presented in Fig. 4A to C. Significant strain-to-strain variation in the \textit{trh1} and \textit{trh2} gene sequences was documented previously (11). The two subgroups of the \textit{trh} gene, however, could be distinguished by comparing the intensities of the hybridization signals obtained with the \textit{trh1}- and \textit{trh2}-specific gene probes since the two gene probes shared only 72% sequence identity (Fig. 4B and C).

The significant strain-to-strain sequence variation was reported previously in the variable regions of the \textit{trh} gene, in which \textit{trh1} and \textit{trh2} sequences differed greatly (11). When a set of oligonucleotide probes was used to detect one such region of the \textit{trh} genes (A1 and A2 probes specific to \textit{trh1} and \textit{trh2} sequences, respectively) in the DNA colony hybridization test under high-stringency conditions, 44% of the \textit{trh1}-bearing strains and 95% of the \textit{trh2}-bearing strains isolated in Asia gave positive results with the specific probes (11). The test strains isolated on the West Coast of the United States were examined by the same method in this study. The results obtained with the representative strains are shown in Fig. 4D and E. None of 53 \textit{trh1}-bearing strains gave positive results with the A1 probe (compare Fig. 4B and D). Four (80%) of five \textit{trh2}-bearing strains gave positive results with the A2 probe (compare Fig. 4C and E). The sequences of the variable region used for probe preparation were obtained from the Asian strains (11). The results therefore indicate that the \textit{trh1} sequences of the West Coast strains differ to some extent from those of the Asian strains but that the \textit{trh2} sequences are fairly conserved in both the West Coast and Asian strains.

We established previously a PCR method with oligonucleotide primers targeted to the conserved regions of the \textit{trh} sequences so that all \textit{trh1} and \textit{trh2} genes present in Asian strains could be detected (33). We examined whether this PCR method can be used to detect the \textit{trh1} and \textit{trh2} genes carried by the West Coast strains. All 54 \textit{trh1}-bearing strains and 4 of 5 \textit{trh2}-bearing strains gave positive results. The 98% specificity

FIG. 2. Results of the AP-PCR assay for selected strains of \textit{V. parahaemolyticus} carrying both \textit{tdh} and \textit{trh1} genes. The results obtained with primer 2 and primer 1 are presented in panels A and B, respectively. Lanes 1 and 2, molecular size markers: bacteriophage \textit{\lambda} DNA digested with HindIII (lane 1) and \textit{E. coli} 887 (lane 2). Test strains (listed in Table 1) are 6190 (lane 3), 7912 (lane 4), 1516 (lane 5), 3215 (lane 6), 5791 (lane 7), 4693 (lane 8), 6978 (lane 9), 9796 (lane 10), 7093 (lane 11), 4950 (lane 12), 8738 (lane 13), and 9638 (lane 14).

regardless of the serovars and that a technique with higher resolution is needed to compare the selected strains.

Therefore, an AP-PCR method was used next. Two primers were used, and there was no essential difference in the results obtained with the two primers (primer 2 in Fig. 2A; primer 1 in Fig. 2B). On the whole, the AP-PCR patterns of the O4:K12 strains (lanes 3 to 10) differed significantly from those of the non-O4:K12 strains (lanes 11 to 14). Of eight selected O4:K12 strains, six strains isolated between 1982 and 1995 exhibited identical AP-PCR patterns (Fig. 2A and B, lanes 4 to 8 and 10). Two remaining O4:K12 strains isolated in 1979 and 1994 shared an identical AP-PCR pattern (Fig. 2A, lanes 3 and 9) or
suggests that this PCR method is also applicable to the detection of the trh sequences in the strains isolated on the West Coast.

**DISCUSSION**

It was demonstrated in this study that the trh genes are distributed in Ure+ strains isolated from the clinical specimens on the West Coast of the United States. The Ure+ phenotype was almost perfectly correlated with the possession of the trh (trh1 or trh2) gene. This is consistent with the observation made with Asian strains. None of the five trh2-positive strains had either the tdh or the trh1 gene (Table 1). Ninety-five percent of the trh2-bearing strains isolated in Asia had neither the tdh nor the trh1 gene (11). Therefore, it seems to be a worldwide trend that the trh2 gene usually does not coexist with the tdh and trh1 genes.

The Ure+ O4:K12 strains have frequently been isolated from both environmental and clinical sources on the West Coast (1, 8, 9, 23). The present study revealed that the Ure+ strains isolated after 1992. These represent the strains isolated on the West Coast. The Ure strains isolated from both environmental and clinical sources on the West Coast (1, 8, 9, 23). The present study revealed that the Ure strains from both environmental and clinical sources on the West Coast.

![Fig. 3. Southern blot analysis of the DNA extracted from V. parahaemolyticus 2448. Lanes 1, 4, and 7, HincII-digested DNA; lanes 2, 5, and 8, SalI-digested DNA; lanes 3, 6, and 9, EcoRI-digested DNA. The blots onto which the DNAs in lanes 1 to 3, 4 to 6, and 7 to 9 were transferred were hybridized with the polynucleotide probes for the tdh, trh1, and trh2 genes, respectively, under high-stringency (A) and low-stringency (B) conditions.](http://jcm.asm.org/)

TABLE 3. Levels of TDH produced by selected strains of *V. parahaemolyticus*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Urease</th>
<th>Presence of the following gene:</th>
<th>TDH titre$^a$ of:</th>
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<tbody>
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<td></td>
<td></td>
<td>tdh</td>
<td>trh1</td>
</tr>
<tr>
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<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
<td>9796</td>
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<tr>
<td>890</td>
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<td>-</td>
</tr>
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</table>

$^a$ Reciprocal of the highest dilution of the culture supernatant that gave a positive reaction. NT, not tested.
specific effects of urease and the trh gene on the transcriptional level of the tdh gene.

ACKNOWLEDGMENTS

We thank Takeshi Honda for providing the strains of V. parahaemolyticus isolated in Thailand.

This research was supported in part by the Ohyama Health Foundation.

REFERENCES


