JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 2004, p. 4284–4292 Vol. 42, No. 9
0095-1137/04/$08.00+0 DOI: 10.1128/JCM.42.9.4284–4292.2004

Rapid and Specific Detection of *tdh*, *trh*1, and *trh*2 mRNA of *Vibrio parahaemolyticus* by Transcription-Reverse Transcription Concerted Reaction with an Automated System

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Received 29 December 2003/Returned for modification 28 March 2004/Accepted 29 May 2004

Vibrio parahaemolyticus* strains carrying the thermostable direct hemolysin (TDH) *tdh* gene, the TDH-related hemolysin (*trh*) gene, or both genes are considered virulent strains. We previously demonstrated that the transcription-reverse transcription concerted (TRC) method could be used to quantify the amount of mRNA transcribed from the *tdh* gene by using an automated detection system. In this study, we devised two TRC-based assays to quantify the mRNAs transcribed from the *trh*1 and *trh*2 genes, the two representative *trh* genes. The TRC-based detection assays for the *tdh*, *trh*1, and *trh*2 transcripts could specifically and quantitatively detect 10^3 to 10^7 copies of the corresponding calibrator RNAs. We examined by the three TRC assays the total RNA preparations extracted from 103 strains of *Vibrio parahaemolyticus* carrying the *tdh*, *trh*1, or *trh*2 gene in various combinations. The *tdh*, *trh*1, and *trh*2 mRNAs in the total RNA preparations were specifically quantified, and the time needed for detection ranged from 9 to 19 min, from 14 to 18 min, and from 9 to 12 min, respectively. The results showed that this automated TRC assays could detect the *tdh*, *trh*1, and *trh*2 mRNAs specifically, quantitatively, and rapidly. The relative levels of TDH determined by the immunological method and that of *tdh* mRNA determined by the TRC assays for most *tdh*-positive strains correlated. Interestingly, the levels of TDH produced from the strains carrying both *tdh* and *trh* genes were lower than those carrying only the *tdh* gene, whereas the levels of mRNA did not significantly differ between the two groups.

Vibrio parahaemolyticus can cause seafood-borne gastroenteritis in humans. However, not all strains are considered virulent strains. The Kanagawa phenomenon, β-type hemolysis in Wagatsuma agar was formerly considered the marker of virulent strains because early studies demonstrated that most clinical strains, but few environmental strains, exhibit this phenomenon (16, 29). Thermostable direct hemolysin (TDH) is responsible for the Kanagawa phenomenon. Subsequently, clinical strains lacking the ability to produce TDH but producing a TDH-related hemolysin (TRH) were discovered (5, 6). The strains carrying the *tdh* gene encoding TDH, the *trh* gene encoding TRH, or both genes are now considered to be virulent strains (19); the strains carrying these hemolysin genes are strongly associated with clinical cases (11, 30). Therefore, in investigations of the infection by *V. parahaemolyticus*, the *V. parahaemolyticus* strains isolated from clinical specimens are usually examined for the *tdh* and *trh* genes in most laboratories. The strains carrying the *tdh* gene, the *trh* gene, or both genes are then examined for the O:K serotype and, if possible, the DNA fingerprint for epidemiological investigation.

Our study group reported five variants of the *tdh* genes, *tdh*1 and *tdh*2 from a Kanagawa phenomenon-positive strain and *tdh*3, *tdh*4, and *tdh*5 genes from Kanagawa phenomenon-negative strains, and these five variants of the *tdh* gene share >97% sequence identity (19). The *trh* gene shares ~68% sequence identity with the *tdh* gene (11). Strain-to-strain nucleotide sequence variation in the *trh* gene is known, and the *trh* sequence variants can be separated into two groups representing two *trh* genes: the *trh*1 and *trh*2 that share an 84% sequence identity (11). Expression levels of the *tdh* and *trh* genes vary. Of various *tdh* and *trh* genes, the *tdh*2 gene is expressed at a very high level and is directly responsible for the Kanagawa phenomenon (19, 26). The high-level *tdh*2 expression was shown to be due to a strong promoter activity (26). The biological activities of TDH include hemolysis of various species of erythrocytes, cytotoxicity, lethal toxicity for small experimental animals, increased vascular permeability in rabbit skin, and enterotoxicity to rabbits (19). TRH has biological activities similar to TDH (6). The strains carrying the *tdh* gene are isolated more often than *trh*-bearing strains from clinical specimens (11, 30). The expression levels of the virulence genes, the *tdh* or *trh*, are likely to influence the virulence of the strains carrying these genes. Therefore, it is important to examine whether the *tdh* and *trh* genes are expressed in all strains carrying these genes and, if so, to quantitatively determine how much each gene is expressed.

The PCR method is a powerful tool that allows easy and quick detection of the *tdh* and *trh* genes with a high sensitivity and specificity (35). However, this detection method can reveal

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only the presence or absence of these genes. We demonstrated that the transcription-reverse transcription concerted (TRC) method could be applied to quantify the amount of *tdh*-specific mRNA produced in a Kanagawa phenomenon-positive *V. parahaemolyticus* strain and its derivatives (9). The TRC method is a method of real-time monitoring of isothermal RNA sequence amplification with a fluorescent probe in which the amplification reaction and its monitoring are carried out by using a simple automated system. The steps in the TRC method include the following: the amplification reaction starts with the binding of the oligonucleotide, named the scissors probe, to the target mRNA at the specific site and is followed by cleavage of the mRNA at the binding site by using the RNase H activity of avian myeloblastosis virus (AMV) reverse transcriptase. The trimmed mRNA is used as the template and participates in the synthesis of the first-strand cDNA with the antisense primer and then the promoter-bearing double-stranded DNA with the promoter primer, with AMV reverse transcriptase. The RNA fragments are synthesized by a T7 RNA polymerase by using the synthesized double-stranded DNA as the template. The synthesized RNAs are subsequently recycled as RNA templates to synthesize double-stranded DNA. The amplified RNA fragments are detected by using the intercalation activating fluorescent (INAF) DNA probe to emit enhanced fluorescence by binding to a complementary sequence. The entire reaction and fluorescence detection are carried out in a specialized and automated instrument called a TRC monitor, which is composed of a round isothermal incubation block and a rotating fluorescence scanning unit (9).

In the present study, we developed assays for the rapid and quantitative detection of the *trh*1 and *trh*2 mRNAs by using the TRC method. We then examined 103 *V. parahaemolyticus* strains carrying the *tdh*, *trh*1, or *trh*2 gene in various combinations that represent the strains isolated from various locations in the world over a 24-year period by using the TRC-based assays for detection of the *tdh*-, *trh*1-, and *trh*2-specific mRNAs to evaluate the features of this method: specificity, rapidity, and quantification.

MATERIALS AND METHODS

Bacterial strains. *V. parahaemolyticus* strains used in the present study are from our laboratory stock cultures and are listed in Table 1. Their O:K serovars were determined by the slide agglutination method by using specific rabbit anti-O and anti-K sera as described previously (31).

Detection of the *tdh*, *trh*1, and *trh*2 genes. The presence or absence of the *tdh*, *trh*1, and *trh*2 genes in the test strain was determined by the DNA colony hybridization method with the specific DNA probes as described previously (11, 25). Briefly, the DNA probes specific to the *tdh*, *trh*1, and *trh*2 genes were, respectively, 415-, 334-, and 419-bp DNA fragments isolated from recombinant plasmids and were labeled by the random priming method with ^{32}P-labeled dCTP. DNA colony blots were prepared on nitrocellulose membrane, and hybridization was performed under high-stringency conditions (in solution containing 50% formamide). Hybridization signals on the X-ray film were judged visually. The colony blot that gave strong and weak signals with the *trh*1 and *trh*2 probes, respectively, were judged to be positive for the *trh*1 gene. The colony blot that gave weak and strong signals with the *trh*1 and *trh*2 probes, respectively, were judged to be positive for the *trh*2 gene.

Isolation of total RNA from *V. parahaemolyticus*. The test strain of *V. parahaemolyticus* was grown in Luria-Bertani broth containing 1% NaCl (14) with shaking (160 rpm) at 37°C until early log phase (optical density at 600 nm of 1.0). Bacterial cells were collected by centrifugation (15,000 rpm) on a tabletop centrifuge (Centrifuge 5415C; Eppendorf, Hamburg, Germany) from a 1-ml culture, and the total RNA was extracted by using an RNeasy minikit (Qiagen, Hilden, Germany) according to the manufacturer's specifications. The concentration of the extracted total RNA in RNase-free water was adjusted to 500 ng/μl and stored at −20°C. The stored RNA solution was thawed and diluted 10-fold with RNase-free water immediately before use in the TRC reaction.

Detection of TDH production. Relative levels of TDH produced in the culture broth by *V. parahaemolyticus* strains were compared by using a commercially available TDH detection kit that was based on the reversed-phase latex agglutination reaction with rabbit anti-TDH immunoglobulin G. The test strain was grown in Luria-Bertani broth containing 1% NaCl with shaking (160 rpm) at 37°C for 18 h. A 50-μl portion of the culture was inoculated into 5-ml of the fresh broth medium and incubated with shaking (160 rpm) at 37°C for 18 h. Twofold dilutions of the culture supernatant were prepared and an agglutination test by using a commercially available TDH detection kit (KAP-RPLA; Denka Seiken Co., Ltd., Tokyo, Japan) was performed according to the manufacturer's instructions. This kit consisted of the latex particles coated with rabbit anti-TDH immunoglobulin G, those coated with immunoglobulin G from a nonimmunized rabbit (a control), purified TDH (a control), and dilution buffer. The agglutination test was carried out in a 96-well V-bottom microtiter plate for 20 h at room temperature. The reciprocal of the highest dilution that gave a positive reaction was defined as the TDH titer.

Preparation of standard RNAs as calibrators. Standard RNA containing the target region for TRC amplification was prepared by in vitro transcription of the T7 promoter-bearing double-stranded DNA as the template with T7 RNA polymerase. The DNA templates were synthesized from the total DNA extracted from the control strain of *V. parahaemolyticus* by using PCR (95°C for 30 s, 55°C for 30 s, and 72°C for 3 min; 30 cycles) with a pair of synthetic oligonucleotide primers. The control strains producing the target mRNA were as follows: AQ3815 for the *tdh*-specific mRNA (18); AQ4037 for the *trh*1-specific mRNA (20); AT4 for the *trh*2-specific mRNA (11). The respective genes in the control strains were well characterized (11, 17, 18, 20, 26), and we have been using these strains as the control strains for the DNA colony hybridization tests and PCR assays to detect these genes. The primer pairs for each target mRNA were as follows: 5′-AATTCTAATACGACTCACTATAGGGAGAATTCTGGCAAAG TTATTAATC-3′ and 5′-TTTTATTGTTGATGTTTACATT-3′ for the *tdh*-specific mRNA; 5′-AATTCTAATACGACTCACTATAGGGAGATCGAGCAAT CTTGCTCAAAACCA-3′ and 5′-GTTTAATTTTGTGACATACATTC-3′ for the *trh*1-specific mRNA; and 5′-AATTCTAATACGACTCACTATAGGGAGA TCGAGCAATTTTGCTTAAAATCAT-3′ and 5′-ATTTAAATTTGTGATTT ACATTC-3′ for the *trh*2-specific mRNA. The long primer in each promoter primer pair has the T7 RNA polymerase-binding sequence at its 5′ end (indicated by the underline) to provide the preferred transcription initiation site. The resultant RNA fragments were purified by gel filtration with CHROMA-SPIN 30 columns (Clontech, Palo Alto, Calif.). The concentration of the RNA obtained was estimated by using a high-pressure liquid chromatography system (GPC, G4000SW column; Tosoh Corp., Tokyo, Japan). The calibrator set was prepared by diluting the RNA obtained to the appropriate concentrations with TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]).

TRC reactions. Synthetic oligonucleotides used for the TRC reaction include a pair of amplification primers (designated as the promoter primer and the antisense primer), a scissors probe to initiate the TRC reaction, and an INAF probe to detect the RNA amplicons. The nucleotide sequence of these oligonucleotides for detection of the *tdh*-specific mRNA were described previously (9), and those for the *trh*1- and *trh*2-specific mRNAs are summarized in Table 2. The sizes of the *trh*1- and *trh*2-specific mRNA amplicons were expected to be 154 and 243 bases, respectively. In the INAF probe, oxazole yellow was linked at the nucleotide indicated by the asterisk (Table 2). The sequences indicated by the underlines in the promoter primers are the T7 RNA polymerase-binding sequences (Table 2). The nucleotide sequences of these oligonucleotides were selected by considering the secondary structure of the target transcript as described in Results. The secondary structures of the transcripts from the *trh*1 and *trh*2 genes were predicted by using Zucker's computer program obtained through the "mFold" server on the web (http://bioinfo.math.rpi.edu/zuckerm/). The 3′-OH of scissors probes and INAF probes were capped with an amino group and glycolic acid, respectively, to avoid undesired enzymatic elongation by AMV reverse transcriptase.

The protocol of the TRC assay for detection of the *tdh*-specific mRNA was described previously (9). The same protocol was used for the detection of *trh*1- and *trh*2-specific mRNAs. Briefly, 20 μl of the TRC buffer for each target mRNA was added to 5 μl of RNA extract in a thin-wall PCR tube (Applied Biosystems, Foster City, Calif.), followed by the addition of 5 μl of the enzyme mix. TE buffer in place of the RNA extract was used to serve as the negative control in TRC assays. The TRC buffer contained 90 mM Tris-HCl (pH 8.6), 195 mM KCl, 28 mM $MgCl_2$, 1.5 mM dithiothreitol, 0.38 mM deoxynucleoside triphosphate, 4.5 mM nucleoside triphosphate, 5.4 mM isonine 5′-triphosphate, 0.3 U of RNase

TABLE 1. Detection of *tdh*, *trh*1, and *trh*2 genes and *tdh*1-, and *trh*2-specific mRNAs in various *V. parahaemolyticus* strains

Strain	Isolation				Detection[a] of:			Detection[b] of mRNA specific to:			Strain designation in Fig. 4
	Source[c]	Location[d]	Date (yr)	O:K serovar	*tdh*	*trh*1	*trh*2	*tdh*[e]	*trh*1	*trh*2	
AQ3815	C	IT (Philippines)	1983	4:12	+	−	−	+ (14.1)	−	−	57
WP1	C	Japan	Before 1983	4:12	+	−	−	+ (15.3)	−	−	53
VP108	C	India	1996	3:6	+	−	−	+ (16.4)	−	−	43
W-3995	C	Bangladesh	1981	11:46	+	−	−	+ (15.7)	−	−	48
AQ3810	C	Singapore	1983	3:6	+	−	−	+ (16.0)	−	−	44
KX-V138	C	IT (Indonesia)	1995	3:6	+	−	−	+ (17.4)	−	−	26
Y-24634	C	Bangladesh	1983	8:22	+	−	−	+ (17.4)	−	−	27
AL-37223	C	Bangladesh	1996	3:6	+	−	−	+ (16.4)	−	−	36
Q-9604	C	Bangladesh	1977	4:8	+	−	−	+ (15.7)	−	−	47
T-26233	C	Bangladesh	1979	5:15	+	−	−	+ (16.0)	−	−	45
97LVP2	C	Laos	1997	3:6	+	−	−	+ (16.3)	−	−	37
AN-5034	C	Bangladesh	1998	4:68	+	−	−	+ (16.5)	−	−	40
DOH812-14	C	Taiwan	1997	3:6	+	−	−	+ (16.8)	−	−	30
BE98-2029	C	United States	1998	3:6	+	−	−	+ (14.3)	−	−	25
VP26	C	Thailand	1998	3:6	+	−	−	+ (16.5)	−	−	39
VP232	C	India	1998	4:68	+	−	−	+ (15.7)	−	−	49
VP1	C	Korea	1998	3:6	+	−	−	+ (16.6)	−	−	33
D-1-81	C	Japan	1981	4:10	+	−	−	+ (16.9)	−	−	29
D-21-82	C	Japan	1982	1:38	+	−	−	+ (15.7)	−	−	50
D-53-83	C	Japan	1983	1:38	+	−	−	+ (15.8)	−	−	46
D-7-84	C	Japan	1984	4:8	+	−	−	+ (14.8)	−	−	56
D-14-85	C	Japan	1985	2:3	+	−	−	+ (16.5)	−	−	42
D-73-86	C	Japan	1986	3:33	+	−	−	+ (15.2)	−	−	54
D-19-88	C	Japan	1988	4:53	+	−	−	+ (13.9)	−	−	58
D-26-90	C	Japan	1990	4:63	+	−	−	+ (16.6)	−	−	35
F192-1	E (seafood)	Philippine	1999	5:68	+	−	−	+ (16.8)	−	−	31
D-1-93	C	Japan	1993	1:56	+	−	−	+ (15.5)	−	−	51
D-4-94	C	Japan	1994	4:55	+	−	−	+ (12.4)	−	−	59
D-3-96	C	Japan	1996	1:56	+	−	−	+ (15.0)	−	−	55
TCVP60	C	Thailand	1999	4:68	+	−	−	+ (16.5)	−	−	41
TCVP94	C	Thailand	1999	1:25	+	−	−	+ (17.0)	−	−	28
TCVP197	C	Thailand	1999	1:41	+	−	−	+ (16.8)	−	−	32
00F390-1	E (seafood)	Indonesia	2000	12:UT	+	−	−	+ (18.4)	−	−	24
PS02839	C	Vietnam	1997	3:75	+	−	−	+ (16.6)	−	−	38
PS06011	C	Vietnam	1997	4:12	+	−	−	+ (16.6)	−	−	34
PS14945	C	Vietnam	1999	11:UT	+	−	−	+ (15.4)	−	−	52
58	E	India	2000	7:19	+	−	−	+ (10.5)	−	−	60
91A1437	C	United States	1991	1:UT	+	+	−	+ (16.7)	+ (14.6)	−	10
VP104	C	India	1996	1:UT	+	+	−	+ (17.9)	+ (16.1)	−	2
AQ3855	C	IT (Philippines)	1983	6:18	+	+	−	+ (17.1)	+ (15.0)	−	3
AQ4889	C	IT (Thailand)	1993	4:12	+	+	−	+ (16.6)	+ (16.0)	−	14
KX-V47	C	IT (Thailand)	1995	5:UT	+	+	−	+ (16.9)	+ (14.9)	−	5
TX-98-792-807-27	E (oyster)	United States	1998	8:74	+	+	−	+ (19.3)	+ (16.2)	−	1
AQ3776	C	IT (Thailand)	1983	10:71	+	+	−	+ (12.8)	+ (15.0)	−	22
AQ3860	C	IT (Philippines)	1983	6:46	+	+	−	+ (16.4)	+ (15.1)	−	13
94A-7175	C	United States	1994	4:9	+	+	−	+ (17.0)	+ (15.4)	−	23
225	C	Thailand	1990	5:11	+	+	−	+ (15.9)	+ (14.6)	−	18
91A-3992	C	United States	1991	1:56	+	+	−	+ (16.9)	+ (14.4)	−	6
90A-5598	C	United States	1990	4:63	+	+	−	+ (16.7)	+ (16.0)	−	9
88A-5320	C	United States	1988	4:12	+	+	−	+ (16.6)	+ (17.4)	−	11
AQ4781	C	IT (Hong Kong)	1992	1:UT	+	−	+	+ (16.4)	−	+ (10.5)	15
AQ4969	C	IT (Indonesia)	1994	1:UT	+	−	+	+ (16.9)	−	+ (12.1)	7
275	C	Thailand	1990	1:1	+	−	+	+ (13.9)	−	+ (13.9)	4
AQ3857	C	IT (Hong Kong)	1983	1:1	+	−	+	+ (14.7)	−	+ (12.0)	20
AQ4433	C	IT (Thailand)	1989	1:UT	+	−	+	+ (15.4)	−	+ (11.7)	19
AQ4405	C	IT (Thailand)	1989	3:72	+	−	+	+ (13.5)	−	+ (11.8)	21
AQ4704	C	IT (Singapore)	1992	1:UT	+	−	+	+ (15.8)	−	+ (11.7)	17
AQ4815	C	IT (Thailand)	1993	1:69	+	−	+	+ (16.9)	−	+ (11.0)	8
AQ4966	C	IT (Thailand)	1994	1:UT	+	−	+	+ (16.3)	−	+ (11.9)	16
KX-V132	C	IT (Philippines)	1995	3:UT	+	−	+	+ (16.6)	−	+ (10.9)	12
90A-7408	C	United States	1990	4:12	−	+	−	−	+ (15.8)	−	
AQ3749	C	IT (Singapore)	1983	3:6	−	+	−	−	+ (15.7)	−	
AQ4019	C	IT (Maldives)	1985	3:6	−	+	−	−	+ (16.6)	−	
AQ4037	C	IT (Maldives)	1985	3:6	−	+	−	−	+ (14.2)	−	
AQ4235	C	IT (Thailand)	1987	3:6	−	+	−	−	+ (15.4)	−	
AQ4299	C	IT (Thailand)	1987	3:6	−	+	−	−	+ (15.8)	−	

Continued on following page

TABLE 1—*Continued*

Strain	Isolation				Detection[a] of:			Detection[b] of mRNA specific to:			Strain designation in Fig. 4
	Source[c]	Location[d]	Date (yr)	O:K serovar	*tdh*	*trh*1	*trh*2	*tdh*[e]	*trh1*1	*trh*2	
AQ4644	C	IT (Thailand)	1991	3:6	−	+	−	−	+ (14.8)	−	
AQ4733	C	IT (Singapore)	1992	3:6	−	+	−	−	+ (15.7)	−	
AQ4853	C	IT (Hong Kong)	1993	3:6	−	+	−	−	+ (15.0)	−	
AQ4901	C	IT (Thailand)	1993	3:6	−	+	−	−	+ (15.0)	−	
AQ3960	C	IT (Hong Kong)	1984	4:63	−	+	−	−	+ (15.9)	−	
AQ4093	C	IT (Maldives)	1986	3:6	−	+	−	−	+ (15.6)	−	
AQ4095	C	IT (Maldives)	1986	3:6	−	+	−	−	+ (14.1)	−	
AQ4129	C	IT (Philippines)	1986	3:6	−	+	−	−	+ (15.2)	−	
95A-4675	C	United States	1995	11:15	−	−	+	−	−	+ (12.4)	
AT4	E(seawater)	Japan	Before 1987	4:37	−	−	+	−	−	+ (10.1)	
93A-5463	C	United States	1993	5:UT	−	−	+	−	−	+ (10.8)	
91A-5992	C	United States	1991	3:59	−	−	+	−	−	+ (9.8)	
VP18	C	India	1994	1:UT	−	−	+	−	−	+ (11.2)	
VP41	C	India	1995	1:33	−	−	+	−	−	+ (11.1)	
X-13261	C	Bangladesh	1982	4:UT	−	−	+	−	−	+ (11.1)	
W-7529	C	Bangladesh	1981	13:UT	−	−	+	−	−	+ (10.8)	
W-8274	C	Bangladesh	1981	1:UT	−	−	+	−	−	+ (9.4)	
AB-8950	C	Bangladesh	1986	1:25	−	−	+	−	−	+ (10.3)	
Q-6030	C	Bangladesh	1977	5:UT	−	−	+	−	−	+ (10.2)	
Q-14337	C	Bangladesh	1977	3:UT	−	−	+	−	−	+ (12.0)	
U-5880	C	Bangladesh	1980	1:56	−	−	+	−	−	+ (10.8)	
U-12122	C	Bangladesh	1980	1:UT	−	−	+	−	−	+ (12.4)	
U-11868	C	Bangladesh	1980	4:11	−	−	+	−	−	+ (10.2)	
AQ4135	C	IT (Thailand)	1986	1:UT	−	−	+	−	−	+ (10.4)	
BAC-98-3574	C	United States	1998	4:55	−	−	+	−	−	+ (9.8)	
VP230	C	India	1998	1:UT	−	−	+	−	−	+ (11.5)	
VP276	C	India	1998	2:28	−	−	+	−	−	+ (10.0)	
68/5	E (seafood)	Thailand	1998	11:36	−	−	+	−	−	+ (10.0)	
AN-20865	C	Bangladesh	1998	1:UT	−	−	+	−	−	+ (15.0)	
VP6	C	India	1994	1:UT	−	−	+	−	−	+ (10.7)	
DOH272	C	Taiwan	1996	3:6	−	−	−	−	−	−	
VP56	E	Japan	1983	2:28	−	−	−	−	−	−	
KV9	E	Korea	1998	5:17	−	−	−	−	−	−	
4060	E	Thailand	1998	3:12	−	−	−	−	−	−	
F25-1	E (seafood)	Malaysia	2000	5:UT	−	−	−	−	−	−	
APCC VP00017	E	Japan	2000	3:6	−	−	−	−	−	−	
PS01972	C	Vietnam	1997	8:39	−	−	−	−	−	−	

[a] Examined by DNA colony hybridization methods (11, 25).
[b] Examined by TRC methods.
[c] C, clinical; E, environmental. More detailed information for the environmental strain is indicated in the parenthesis if available.
[d] IT, international travelers. The origin of travel is indicated in parentheses.
[e] The detection time in minutes (the time to reach the cutoff value after initiation of the reaction) is indicated in parentheses.

inhibitor (TaKaRa, Shiga, Japan)/μl, 1.5 μM promoter primer, 1.5 μM antisense primer, 0.24 μM scissors probe, 23 nM INAF probe, and 16% dimethyl sulfoxide. The enzyme mix consisted of 0.72 mg of bovine serum albumin/ml, 12% sorbitol, 1.3 U of AMV reverse transcriptase (TaKaRa)/μl, 34 U of T7 RNA polymerase (Life Technologies, Rockville, Md.)/μl, and 0.006 U of RNase (TaKaRa)/μl. The TRC reaction tube containing the reaction mix was set in the "TRC monitor" instrument. The temperature of the incubation block was maintained at 41°C, and the RNA amplicons were detected by scanning the fluorescence with the

TABLE 2. Oligonucleotide primers and probes used in TRC assays for the *trh*1- and *trh*2-specific mRNAs

Target mRNA, primer, and probe	Position[a]	Sequence (5′–3′)[b]
*trh*1		
Promoter primer	429–453	AATTCTAATACGACTCACTATAGGGAGATATTCTTCTGTTAGTGATTTCGTTG
Antisense primer	562–582	ATGATGATTTATTGGAAATAC
Scissor probe	414–433	GAATAGTTCTGATTTAGGCT
INAF probe	545–561	ACATAACAAA*CATATGCC
*trh*2		
Promoter primer	99–121	AATTCTAATACGACTCACTATAGGGAGAAAATCATTCGCGATTGATCTGCCA
Antisense primer	322–341	GTGACCATTGATGTTGACTG
Scissor probe	84–103	GATTTAGATATTGAAAATAT
INAF probe	259–278	CGATTGA*CCGTATACATCTT

[a] The numbers correspond to the positions in the nucleotide sequence deposited in GenBank (accession numbers for the *trh*1 [AB112353] and *trh*2 [M88112] genes).
[b] The underlined sequence is the T7 RNA polymerase-binding sequence. The base linked to oxazole yellow is indicated by the asterisk.

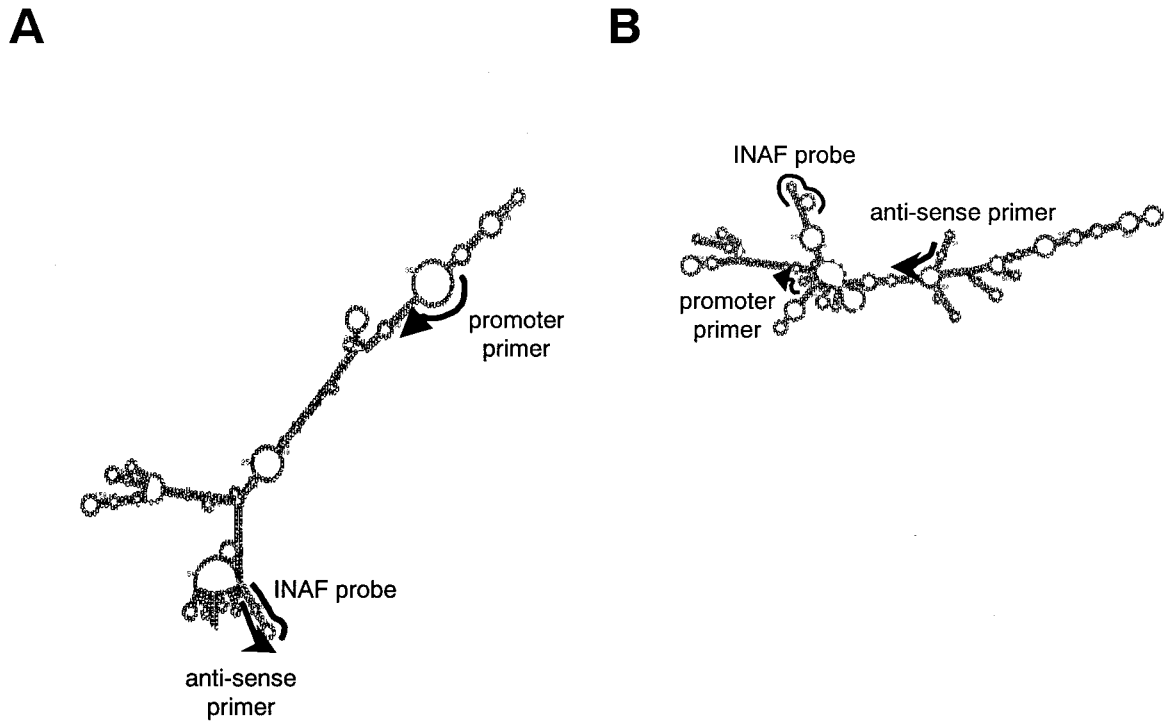

FIG. 1. Secondary structures of the *trh*1- and *trh*2-specific mRNA sequences predicted by the Zucker's computer program. (A) *trh*1-specific mRNA; (B) *trh*2-specific mRNA. The binding sites of the promoter and antisense primers and INAF probe on the mRNA sequence are indicated by bold lines. Arrowheads indicate the 3′ positions of the primers.

light-emitting diode source (470 nm) and the photomultiplier for fluorescence (520 nm) collection in the instrument at 1-min intervals. Time for fluorescence enhancement to reach a cutoff value of 1.2 was defined as the detection time in the TRC assay.

RESULTS

Sensitivity and specificity of the TRC assays. We developed the TRC assays for *trh*1- and *trh*2-specific mRNAs in the present study. The secondary structures of mRNA sequences encoded by the *trh*1 or *trh*2 gene were predicted by Zucker's computer program (Fig. 1). Primers and probes were designed to bind to the sites relatively free from the secondary structure of the transcripts (Table 2 and Fig. 1). We carried out the TRC assays to detect *tdh*, *trh*1, and *trh*2 mRNAs by using the calibrator RNAs synthesized in vitro (Fig. 2A, C, and E). The profiles shown in Fig. 2A, C, and E indicate the fluorescence enhancement relative to the reaction time. Similar profiles were obtained for all of the TRC assays. The detection time, the time to reach the cutoff value 1.2, yielded a very good linear relationship compared to the number of initial copies ranging from 10^3 to 10^7 (Fig. 2B, D, and F). This cutoff value was determined to be $\geq$3 times the standard deviation of the fluorescence enhancement value of the negative control at 15 min of reaction time. The results indicated that the three TRC assays are quantitative and rapid and that their sensitivity limit is $\sim 10^3$ copies of mRNA.

Next, we evaluated the specificity of the three TRC assays with control strains carrying the *tdh*, *trh*1, and *trh*2 genes in various combinations (Fig. 3). The results presented in Fig. 3 show that the three TRC assays could detect the mRNAs transcribed only from the corresponding target genes and suggest that these TRC assays are specific.

Examination of the various *V. parahaemolyticus* strains by using the TRC assays. We further evaluated the three TRC assays for their specificity and rapidity of detection with the total RNA extracted from 103 test strains listed in Table 1. These strains were selected from our stock culture collection so as to represent various toxin gene profile groups (combinations of the *tdh*, *trh*1, and *trh*2 genes). These strains were isolated mostly from clinical sources in various parts of the world between 1977 to 2000 and belonged to various O:K serovars. The results of the TRC assays are shown in Table 1. The results of the detection of the *tdh*-, *trh*1-, and *trh*2-specific mRNAs by the TRC assays agreed absolutely with the presence of the *tdh*, *trh*1, and *trh*2 genes detected by the DNA colony hybridization tests. The detection time by the TRC assays, the time to reach the cutoff value after initiation of the reaction, ranged from 9 to 19 min, from 14 to 18 min, and from 9 to 12 min for the *tdh*-, *trh*1-, and *trh*2-specific mRNAs, respectively. These results indicate that the TRC assays can specifically and rapidly detect the *tdh*-, *trh*1-, and *trh*2-specific mRNAs produced in *V. parahaemolyticus* strains.

Comparison between *tdh* mRNA transcription level and TDH production level. The levels of TDH produced can vary among strains. In the present study we focused on TDH because a commercial kit for detection of TDH, but not TRH, is available. Examination of the twofold dilutions of the culture supernatant by using this detection kit (KAP-RPLA) and determining the end point of the positive reaction allows comparison of the level of TDH produced. We defined the level of

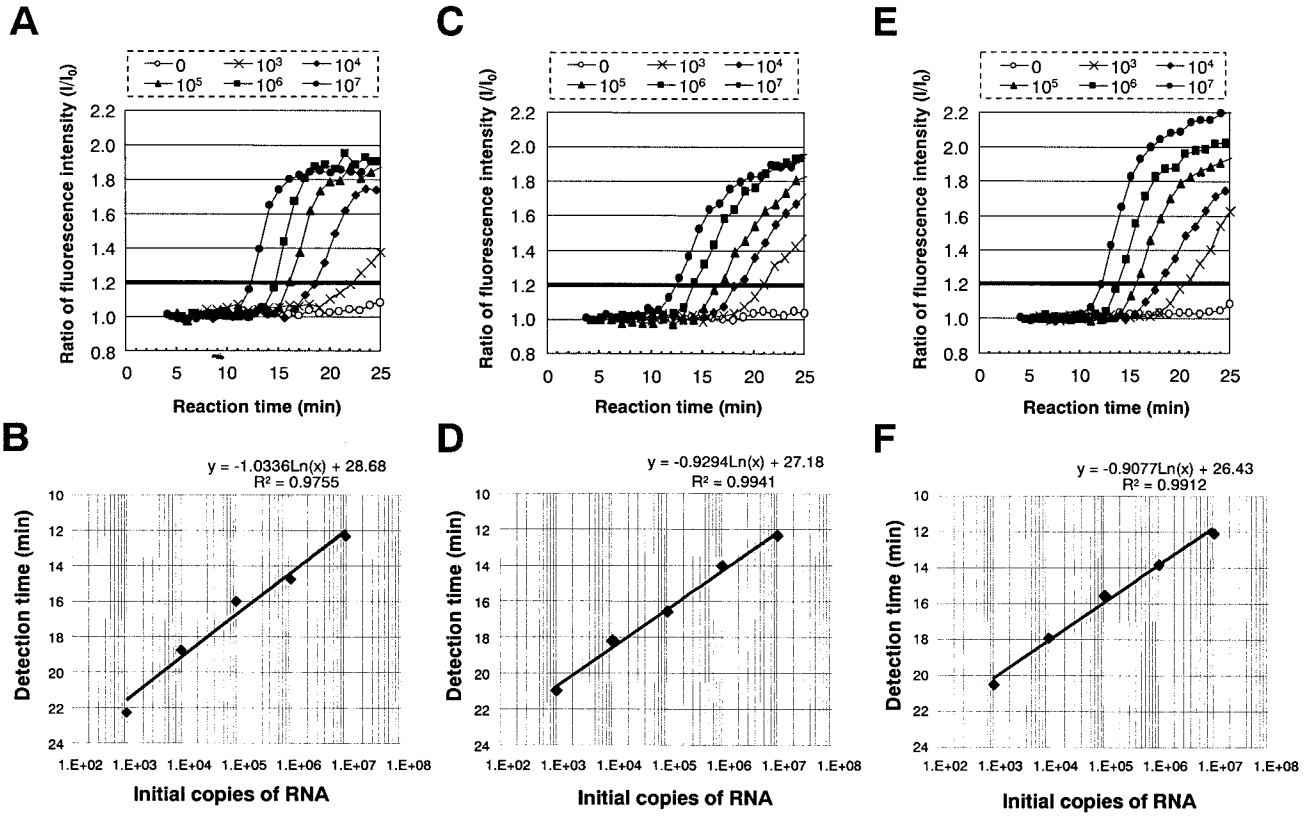

FIG. 2. Real-time monitoring of the TRC reactions for the RNA calibrators. (A, C, and E) Fluorescent profile of the TRC reaction for the RNA calibrators (A, *tdh* RNA; C, *trh*1 RNA; and E, *trh*2 RNA); the initial copies of the calibrator mRNA (0, 10^3, 10^4, 10^5, 10^6, and 10^7) are indicated. (B, D, and F) Correlation between the initial copies of the mRNA calibrators and their detection times (B, *tdh* RNA; D, *trh*1 RNA; F, *trh*2 RNA).

TDH determined by this way as the "TDH titer." Determination of the copy number of the *tdh*-specific mRNA from the calibration curve in the TRC reaction allows comparison of the levels of the *tdh* transcription in the different strains. We were interested in comparing the level of the *tdh*-specific mRNA determined by the TRC assay to the level of TDH produced in test strains to examine whether the level of the *tdh* transcription is reflected in the level of TDH produced. We could detect

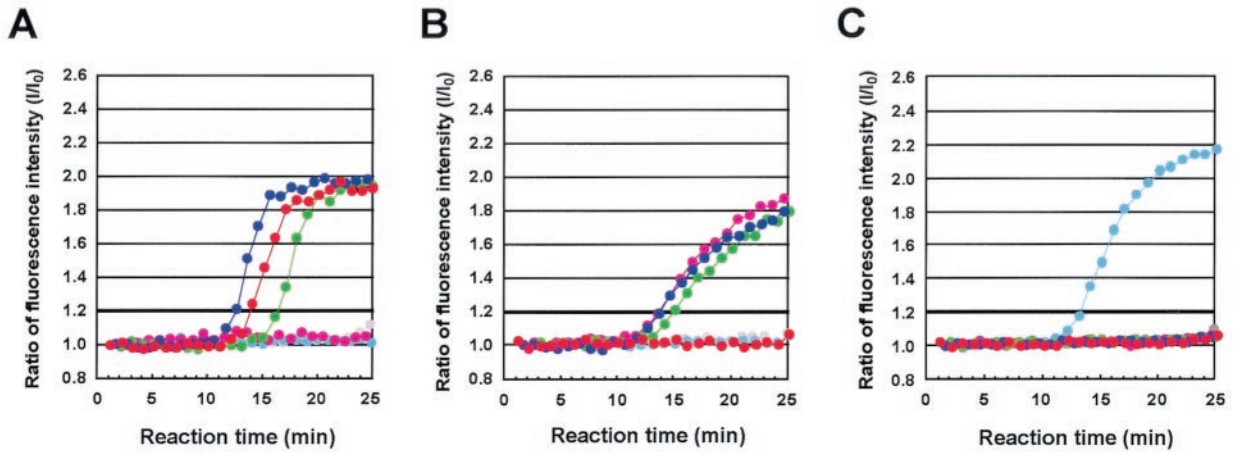

FIG. 3. Real-time monitoring of TRC reactions for RNA preparations of control strains of *V. parahaemolyticus* by using TRC assays to detect *tdh*, *trh*1, and *trh*2 mRNAs. The profiles of the TRC reactions for detection of the *tdh* mRNA (A), the *trh*1 mRNA (B), and the *trh*2 mRNA (C) are shown. The total RNA extracted from AQ3815 (red dots; *tdh*1 positive, *tdh*2 positive, *trh*1 negative, and *trh*2 negative), AQ3776 (blue dots; *tdh*3 positive, *tdh*4 positive, *trh*1 positive, and *trh*2 negative), AQ3860 (green dots; *tdh*5 positive, *trh*1 positive, and *trh*2 negative), AQ4037 (pink dots; *tdh* negative, *trh*1 positive, and *trh*2 negative), AT4 (pale-blue dots; *tdh* negative, *trh*1 negative, and *trh*2 positive), and DOH272 (gray dots; *tdh* negative, *trh*1 negative, and *trh*2 negative) were examined.

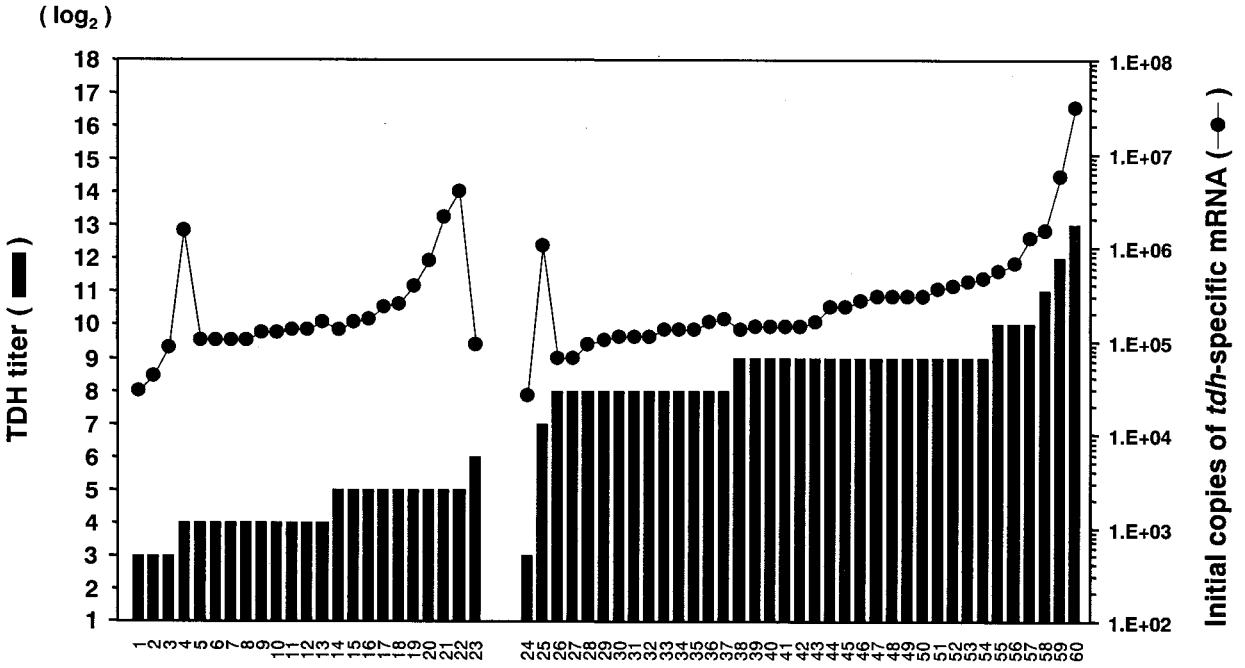

FIG. 4. Comparison between levels of TDH and those of *tdh*-specific mRNA for *tdh* gene-bearing strains of *V. parahaemolyticus*. The level of TDH production in each strain is expressed as the TDH titer (explained in the text). The initial copy of the *tdh*-specific mRNA was determined from the calibration curve in the TRC reaction (Fig. 2B). Designations of the test strains are indicated in Table 1. The strains from 1 to 23 carried the *tdh* and *trh* (*trh*1 or *trh*2) genes. The strains from 24 to 59 carried the *tdh* gene alone.

the transcription of the *tdh* gene in all *tdh*-bearing strains (Fig. 4 and Table 2). The initial copies of mRNA in these strains were greater than 10^4 copies, and every score was over the threshold, 10^3 copies. A good correlation between the TDH titer and the quantity of the *tdh*-specific mRNA was observed among the strains carrying the *tdh* gene alone with one exception (Fig. 4, strain 25). The strains carrying *tdh* and *trh* genes (Fig. 4, strains 1 to 23) produced less TDH than did those carrying the *tdh* gene alone (Fig. 4, strains 24 to 60) with one exception (Fig. 4, strain 24). The mean and standard error of the TDH titers of the *tdh*- and *trh*-positive strains and the *tdh*-positive strains were 23.0 ± 2.7 and 792.4 ± 234.5, respectively, the difference being ~35-fold. However, the levels of the *tdh*-specific mRNA in the *tdh* and *trh* gene-positive strains were not comparable to their low TDH titers. The mean and standard error of the initial copies of the *tdh*-specific mRNA of the *tdh* and *trh* gene-positive strains and that of the *tdh* gene-positive strains were 473,099 ± 188,596 and 1,274,237 ± 833,297, respectively, and the difference was only 2.7-fold.

DISCUSSION

The TRC assays for the detection of the *tdh*, *trh*1, and *trh*2 mRNAs are specific, sensitive (detecting 10^3 mRNA copies), and quantitative (range, 10^3 to 10^7 mRNA copies). The TRC reaction is carried out, measured, and recorded by using a simple and automated system, and the results can be obtained rapidly (25 min after onset of the reaction). The easiness and rapidity give these TRC assays advantages over conventional PCR assays in examination of clinical strains for their ability to produce TDH or TRH. In addition, the PCR assays thus far reported (2, 35) could not distinguish the *trh*1 and *trh*2 genes that share 84% sequence identity. We have previously distinguished these two *trh* genes in the test strains by the relative intensities of the hybridization signals with the *trh*1- and *trh*2-specific DNA probes in the DNA colony hybridization tests (11). The TRC assays are much easier than the DNA colony hybridization tests, and they can be one of the tools for epidemiological investigation. Furthermore, the TRC assays combined with appropriate RNA preparation methods will be very useful if there is a need for detection of viable virulent strains in clinical and environmental samples.

The TRC assays are more advanced than the PCR or hybridization assays in that the TRC assays detect the *tdh* and *trh* genes that are actually expressed but not silent genes. In the present study, we examined 103 strains representing the strains of various toxin gene combinations by the TRC assays and revealed that all *tdh* and *trh* genes in the strains were actually transcribed although the levels of transcription among the strains varied. The result suggests that the PCR assays can be used to evaluate the ability of the test strain to produce TDH and TRH in clinical and environmental studies, although the various amounts of the toxins cannot be measured.

Infection by the strain capable of colonizing the host intestine and producing TDH or TRH at a high level is expected to cause damages to the tissues of the susceptible host, and it will result in clinical symptoms. Various clinical symptoms have been reported from *V. parahaemolyticus* infections. These include diarrhea, abdominal pain, headache, vomiting, fever, weakness, chill, tenesmus, and nausea (15, 27). However, contribution of TDH or TRH to the clinical symptoms of the

patients has not been established. The amount of the *tdh*-specific mRNA determined by the TRC assay generally reflected the level of TDH production (Fig. 4, discussed below). A future study on the association of the expression level of the *tdh* and *trh* genes in the clinical strains with the clinical symptoms of the patients would reveal relative importance of TDH and TRH in each of clinical symptoms. Recently, we have investigated 548 cases of *V. parahaemolyticus* infection in Vietnam between 1997 and 1999, and the symptoms of the patients were recorded (36). The characteristics of the strains isolated from individual patients varied considerably. These characteristics included *tdh* and *trh* gene profiles, O:K serotype, and DNA fingerprints (4). These strains and clinical information would be suitable for the study on the association of *tdh* and *trh* expression with clinical symptoms.

By using the TRC assays we found that the relative level of TDH production from the *tdh* gene in test strains can usually be predicted by the level of the *tdh* transcription, but this correlation cannot be observed in some strains, notably those carrying both *tdh* and *trh* genes (Fig. 4). We found in a previous study that the strains carrying both *tdh* and *trh* genes produced TDH at lower levels than did the strains carrying a single *tdh* gene (32). We therefore compared the TDH titer and the level of the *tdh*-specific mRNA produced from the strains belonging to these two groups. The difference in the amount of mRNA, 2.7-fold, was much lower than the difference in TDH titer at 37-fold, between the two groups. This result suggests that low-level TDH production in the former group is due to the mechanisms not only at a transcriptional level but also at a translational level. We consider that the low-level *tdh* transcription is due in part to a weak *tdh* promoter (26). In addition, effect of the *trh* gene on *tdh* transcription needs to be evaluated. A small portion of *V. parahaemolyticus* strains produce urease (1, 3, 7, 10, 12, 21, 23, 24, 37), and almost all urease-positive strains carry the *trh* (either *trh*1 or *trh*2) gene (8, 22, 24, 28, 31, 33, 34). We demonstrated that urease production influenced the transcription of neither the *trh* nor *tdh* gene (17). Furthermore, the urease gene cluster did not influence production of TDH from a Kanagawa phenomenon-positive strain (17). Accordingly, a non-urease factor or factors including the *trh* gene is likely to be responsible for the low-level translation from the *tdh* mRNA.

00F390-1 (Fig. 4, strain 24) was exceptional among the strains carrying the *tdh* gene alone in that it produced TDH and *tdh*-specific mRNA at the lowest levels. This may be due to a mutation in the *tdh* promoter. Analysis of the *tdh* promoter sequence would confirm this hypothesis (26).

The mechanism responsible for the exceptionally high *tdh* transcription in some strains also needs to be explained. The high level of the *tdh*-specific mRNA in strain AQ3776 belonging to the *tdh*- and *trh*-positive group (Fig. 4, strain 22) was much higher than those of the other strains in this group. It was previously reported that this strain is exceptional in that it carries the two *tdh* genes (*tdh*3 and *tdh*4); the *tdh*3 gene is on the chromosome; however, the *tdh*4 gene exists on a plasmid (18). The high-level *tdh* transcription in AQ3776 may be due to the copy number effect of the *tdh*4 gene. TDH production was not very high compared to the high-level *tdh* transcription in this strain. The translation from the *tdh* transcript may be repressed by some mechanism, but this aspect has not been studied thus far. The *tdh* transcriptions were very high in the two strains among the strains carrying the *tdh* gene alone (Fig. 4, strains 59 and 60). The exact mechanism involved in the very high *tdh* transcriptions in these strains is not known. The *tdh* promoters in these strains may have up mutation. Alternatively, *tdh* transcription may be stimulated by some factor more strongly in these strains than in other strains. The transmembrane regulator encoded by the *toxRS* genes stimulates *tdh* gene variants to different degrees (13). A further study is needed to examine these possibilities.

ACKNOWLEDGMENTS

We are grateful to Yohko Takeda for technical assistance.

This research was supported, in part, by a grant-in-aid for scientific research from the Ministry of Education, Science, Sports, and Culture of Japan.

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