INFECTION AND IMMUNITY, Sept. 1985, p. 481–486
0019-9567/85/090481-06$02.00/0

Vol. 49, No. 3

Detection of the Thermostable Direct Hemolysin Gene and Related DNA Sequences in *Vibrio parahaemolyticus* and Other *Vibrio* Species by the DNA Colony Hybridization Test

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Received 27 March 1985/Accepted 3 June 1985

A specific gene probe for the *Vibrio parahaemolyticus* thermostable direct hemolysin gene was constructed and used to examine the presence or absence of the thermostable direct hemolysin gene or related DNA sequences in *V. parahaemolyticus* and other vibrios by the DNA colony hybridization method. The gene probe consisted of a 406-base-pair, completely internal fragment covering 71% of the structural gene with *PstI* linkers added to the ends. Six copies of this 415-base-pair *PstI* fragment were cloned into plasmid pBR322, which yielded large amounts of the probe DNA. One hundred forty-one *V. parahaemolyticus* strains were tested with the gene probe, and the results were compared with those of phenotypic assays for the thermostable direct hemolysin. All Kanagawa phenomenon-positive strains were gene positive. However, 86% of the strains that exhibited weak Kanagawa phenomenon and 16% of Kanagawa phenomenon-negative strains also reacted with the gene probe. Immunological methods for the detection of the thermostable direct hemolysin (modified Elek test, enzyme-linked immunosorbent assay) showed better correlation with gene probe results. All gene-positive strains produced hemolysin detectable in the enzyme-linked immunosorbent assay, although occasional strains showed weak reaction. The modified Elek test was slightly less sensitive than the enzyme-linked immunosorbent assay. All gene-negative strains were also negative in these immunological assays. One hundred twenty-one strains of *Vibrio* spp. other than *V. parahaemolyticus* were tested with the gene probe; only *Vibrio hollisae* strains reacted with the probe under stringent conditions.

Vibrio parahaemolyticus is a natural inhabitant of the estuarine or marine environment which often causes seafood-borne gastroenteritis (8). Although the pathogenic mechanism of this organism is still unknown (1), the thermostable direct hemolysin (TDH) or Kanagawa phenomenon-associated hemolysin has been considered an important virulence factor due to the nearly exclusive detection of TDH among strains isolated from cases of gastroenteritis (24, 33). Production of TDH by *V. parahaemolyticus* is routinely tested by β-type hemolysis of erythrocytes incorporated into a special blood agar, Wagatsuma agar (24). This hemolytic reaction, designated Kanagawa phenomenon, is sometimes difficult to ascertain, because the reaction may be weak or induced by other hemolysins. Therefore, an immunoprecipitation test, a modified Elek test, was designed to specifically identify TDH-producing strains (13). However, the level of TDH production varies considerably, and strains producing very low levels of TDH may give false-negative reactions. In addition, culture conditions such as medium composition and incubation period influence TDH production (5, 7). Accordingly, in vitro TDH production does not necessarily reflect in vivo TDH production.

Recently, DNA colony hybridization (DCH) has been used to examine the presence or absence of toxin genes in enteropathogens, and its usefulness has been demonstrated in epidemiological studies (11, 26). By virtue of the reaction between the DNA probe and homologous DNA sequences in test organisms, this method can detect the potential of a bacterium to produce the toxin. Thus, the variability of in vitro assay is avoided.

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Our earlier studies employing a relatively nonspecific probe for the gene encoding TDH (*tdh* gene) revealed that all Kanagawa phenomenon-positive (KP^+) strains possessed DNA sequences homologous to the probe (19). However, Kanagawa phenomenon-negative (KP^-) strains, with one exception, did not possess homologous sequences, demonstrating that not all *V. parahaemolyticus* strains have the genetic potential to produce TDH. The explanation for the single exception was unclear, due to the presence of nonspecific sequences in the probe.

Recently, we characterized the *tdh* gene and determined its nucleotide sequence (29). This information enabled us to construct a specific *tdh* gene probe consisting of a completely internal fragment of the structural gene. The incidence of the *tdh* gene in *V. parahaemolyticus* and its distribution in a variety of human pathogenic and marine vibrios was examined by DCH with the new gene probe. To evaluate the sensitivity and specificity of this genetic technique, DCH was compared with methods that detect phenotypic expression of TDH, including an enzyme-linked immunosorbent assay (ELISA). These studies demonstrate the usefulness of the *tdh* gene probe in examination of the epidemiology and ecology of *V. parahaemolyticus*.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Escherichia coli* HB101 (F^- *hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44* λ^-) (3) was obtained from S. Falkow and used as the host for the recombinant plasmids. *V. parahaemolyticus* strains from various countries such as Bangladesh, India, Japan, the United Kingdom, and the United States were obtained from P. Blake, D. Burstyn, M. I. Huq, S. W.

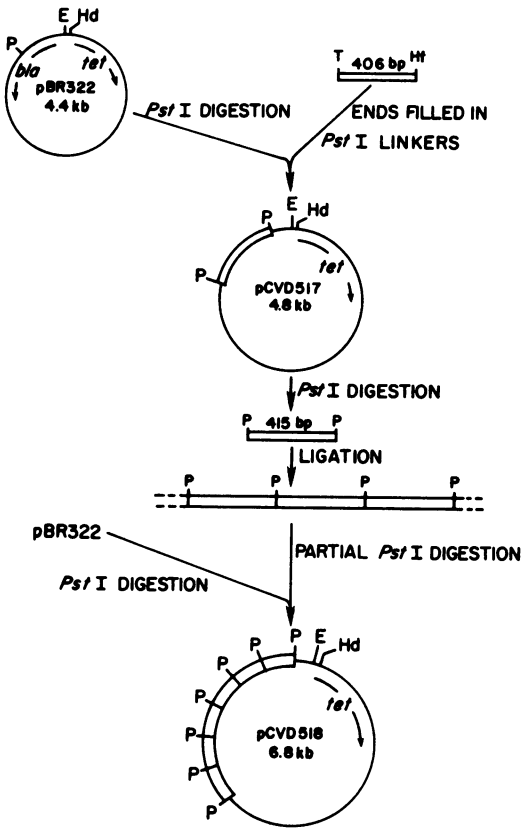

FIG. 1. Cloning of the internal fragment of the *tdh* gene for use as DNA probe in the DCH test. Recognition sites for restriction endonucleases are abbreviated as follows: Hd, *Hin*dIII; Hf, *Hin*fI; T, *Taq*I; E, *Eco*RI; P, *Pst*I. Lines: —, DNA derived from pBR322; ▭ DNA derived from the *tdh* structural gene (see Fig. 2).

Joseph, G.Stelma, R. Twedt, and J. Wells or isolated by the authors. Strains of other *Vibrio* spp. and genera other than *Vibrio* were described previously (30) or obtained from M. M. Levine (*Vibrio cholerae* O1), J. J. Farmer (*Vibrio hollisae*), and J. A. Baross (psychrotrophic *Vibrio* spp.). Plasmid pBR322 (2) was obtained from S. Falkow, and plasmid pCVD512 (*bla*$^+$ *tdh*$^+$) was previously described (29). Plasmids pCVD517 (*tet*$^+$) and pCVD518 (*tet*$^+$) are derivatives of pBR322 containing the cloned probe DNA and were constructed as described below.

Computer analysis. The nucleotide sequence was analyzed for recognition sites of restriction endonucleases by using the Univac 1108 computer. The basic programs were adopted from Gingeras et al. (9).

Molecular cloning. The internal *Taq*I-*Hin*fI fragment of the *tdh* structural gene was cloned into plasmid vector pBR322 (Fig. 1). The 1,275-base-pair (bp) *Hin*dIII fragment of pCVD512 was isolated by agarose gel electrophoresis and digested with *Taq*I and *Hin*fI. A 406-bp *Taq*I-*Hin*fI fragment was isolated, the ends were filled in with the large fragment of DNA polymerase I (Bethesda Research Laboratories), and *Pst*I linkers (Bethesda Research Laboratories) were added as described by Maniatis et al. (23). This DNA fragment was ligated with *Pst*I-digested pBR322 and transformed into *E. coli* HB101.

Tetracycline-resistant, ampicillin-susceptible clones were selected from which a recombinant plasmid, pCVD517 (Fig. 1), was isolated. Next, plasmid pCVD517 was digested with *Pst*I, and the smaller (415-bp) *Pst*I fragment was isolated. Ligation of highly concentrated *Pst*I fragment (4.5 μg of DNA per 20-μl reaction volume) resulted in circularized concatemers of various sizes. Concatemers were size fractionated by agarose gel electrophoresis, and larger molecules were isolated. These cocatemers were partially digested with *Pst*I, ligated with *Pst*I-cut pBR322, and transformed into *E. coli* HB101. Tetracycline-resistant, ampicillin-susceptible transformants were selected and screened for plasmid content. Plasmid pCVD518, the largest plasmid isolated in this manner, contained six copies of the *Pst*I fragment inserted into pBR322 (Fig. 1). The methods used for plasmid screening and purification, isolation of DNA fragments, ligation, transformation, and selection of clones have been previously described (29).

Preparation of DNA probe. Plasmid pCVD518 was digested to completion with *Pst*I, and the 415-bp fragment was isolated by agarose gel electrophoresis and purified by phenol-chloroform extraction. Purified DNA was labeled with ^{32}P by incorporating [α-^{32}P]dATP (New England Nuclear Corp.) to a specific activity of 2×10^8 to 8×10^8 cpm per μg of DNA by nick translation (23). DNase I and DNA polymerase I used were purchased from Sigma Chemical Co. and Bethesda Research Laboratories, respectively. Radiolabeled probe DNA was purified by chromatography on NACS PREPAC as specified by the manufacturer (Bethesda Research Laboratories).

Colony blot and hybridization. Up to 40 test organisms were inoculated onto an autoclaved, gridded nitrocellulose filter (Schleicher & Schuell Co., BA 85/20) overlying an agar plate and incubated. Culture media and incubation conditions employed were as follows: tryptic soy agar (Difco Laboratories) with 0.5% NaCl added, 37°C, overnight for *V. parahaemolyticus* and other human pathogenic vibrios; brain heart infusion agar (Difco) supplemented with 1% NaCl and 0.02% $MgCl_2$, 15°C, 2 days for marine and fish pathogenic vibrios; tryptic soy agar (Difco), 37°C, overnight for organisms other than *Vibrio* spp. After incubation, the filter was removed from the plate and treated with NaOH, neutralized, and baked as described by Moseley et al. (28). Hybridization with the probe DNA was performed under stringent conditions by the method of Moseley et al. (28).

Kanagawa test. The test organism was streaked onto a modified Wagatsuma agar (Eiken, Japan) containing fresh defibrinated human blood (type O). The medium was prepared according to the manufacturer's specification. After incubation at 37°C for 24 h, hemolysis around the bacterial growth was recorded as + (positive; large clear zone of β-type hemolysis), +w (weak reaction; clear but small zone of β-type hemolysis), and − (negative; no hemolysis or non-β-type hemolysis).

Modified Elek test. The immunoprecipitation test to detect TDH in an agar medium was carried out as described by Honda et al. (13).

ELISA. A 50-μl sample of the purified TDH (16) at a concentration of 2 μg/ml in 0.05 M carbonate buffer (pH 9.6) was added to each well of flat-bottomed Dynatech Microelisa-plates (Dynatech, Denkendorf, Germany) to precoat the surface of the microplate. After overnight incubation at 4°C, the plates were washed three times with 0.01 M phosphate-buffered saline–Tween 20 (0.05%). Additional binding sites on the microplate surface were blocked by incubating the wells with 75 μl of 1% bovine serum albumin (Armour Pharmaceutical) in phosphate-buffered saline-Tween 20. Tenfold dilutions of culture supernatant in phosphate-buffered saline-Tween 20 were mixed with equal vol-

umes of anti-TDH of known concentration in test tubes and incubated for 2 h at 37°C. Then 50 μl of each mixture was added into TDH-coated wells in duplicate. The plates were incubated overnight at 4°C and then washed five times with phosphate-buffered saline-Tween 20. A 50-μl sample of diluted (1:1,600) goat anti-rabbit immunoglobulin G conjugated with alkaline phosphatase (Tago) was added, and the plates were incubated overnight at 4°C. The plates were then washed five times with phosphate-buffered saline-Tween 20, and 50 μl of *p*-nitrophenyl phosphate (1 mg/ml; Sigma) in diethanolamine buffer (0.05 M, pH 9.8) was added. The plates were incubated at room temperature for approximately 40 min, and reactions were stopped by adding 50 μl of 3 N NaOH. The intensity of the color reaction was measured at 405 nm in a Dynatech Microelisa-plate reader.

RESULTS

Construction of the *tdh* gene probe. The complete nucleotide sequence of the 1,275-bp *Hin*dIII fragment from plasmid pCVD512, which contains the cloned *tdh* gene (29), was analyzed for the recognition sites of various restriction endonucleases (Fig. 2). There are single recognition sites for *Taq*I and *Hin*fI within the *tdh* structural gene (Fig. 2). This internal, 406-bp *Taq*I-*Hin*fI fagment, representing 71% of the structural gene, was ligated to *Pst*I linkers and cloned into plasmid vector pBR322 as described above (Fig. 1). Two recombinant plasmids constructed, pCVD517 and pCVD518, contained 1 and 6 copies of this fragment, respectively. Although the orientation of the inserts in pCVD518 are not examined, this plasmid was stably maintained under selective pressure. In a preliminary experiment, results obtained with this 415-bp *Pst*I fragment as the probe and those obtained with the probe consisting of the 406-bp *Taq*I-*Hin*fI fragment without added *Pst*I linkers were identical. Thus, there is no nonspecific reaction due to the added *Pst*I linkers. Therefore, this 415-bp *Pst*I fragment, isolated in high yield from pCVD518, was used as the DNA probe for hybridization throughout this study.

Detection of the *tdh* gene in *V. parahaemolyticus* strains. A total of 141 strains of *V. parahaemolyticus* (101 clinical and 40 nonclinical) from various geographical locations were examined by DCH and the Kanagawa test (Table 1). All 66 KP^+ strains possessed sequences homologous to the *tdh* gene probe. Eighty-six percent (12 of 14) of the strains that showed weak Kanagawa phenomenon (KP^{+w}) and 16% (10 out of 61) of KP^- strains were also positive with this specific gene probe. Differences between the Kanagawa test and DCH reactions were found in both clinical and nonclinical isolates.

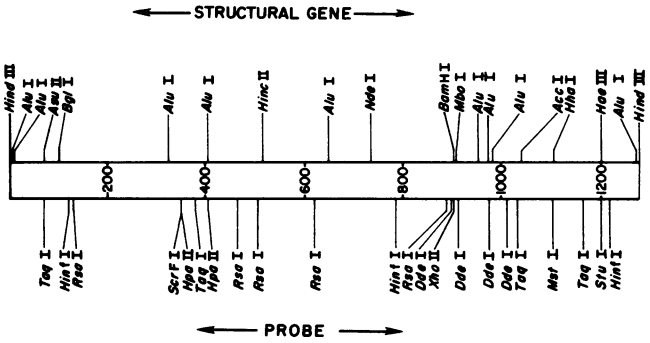

FIG. 2. Predicted fine restriction map of a 1,275-bp *Hin*dIII fragment of pCVD512 containing the *tdh* gene. The scale is in base pairs. The locations of the *tdh* structural gene and the *Hin*fI-*Taq*I fragment used to prepare the gene probe are shown.

TABLE 1. Detection of the *tdh* gene in 141 strains of *V. parahaemolyticus* by the DCH test

Source	No. of strains						Total
	KP^{+a}		KP^{+w}		KP^-		
	$Gene^{+b}$	$Gene^-$	$Gene^+$	$Gene^-$	$Gene^+$	$Gene^-$	
Clinical	63	0	11	2	8	17	101
Nonclinical	3	0	1	0	2	34	40

[a] Kanagawa phenomenon reaction on a modified Wagatsuma agar: +, positive; +w, weak reaction; −, negative.

[b] Results of the DNA colony hybridization. Reaction ($gene^+$) or no reaction ($gene^-$) with the *tdh* gene probe.

Detection of TDH by immunological methods. One hundred representative strains were tested by immunological methods to examine TDH production more specifically, and the results were compared with those of DCH (Table 2). All 100 strains were tested by the modified Elek test; 53 strains were positive, 4 strains showed weak reaction, and 43 strains were completely negative. A total of 50 strains, 3 modified Elek test-positive strains and all 47 strains that exhibited weak or no reaction in the modified Elek test, were further examined with a sensitive ELISA method. In addition to the three modified Elek test-positive strains, four strains that showed weak reaction in the modified Elek test and three modified Elek test-negative strains were clearly ELISA positive. Two modified Elek test-negative strains gave a weak reaction in the ELISA. DCH detected the *tdh* gene in all strains that produce TDH, including the two strains that exhibited weak reaction in the ELISA. None of the 38 non-TDH-producing strains reacted with the gene probe.

Detection of the *tdh* gene in other vibrios. A total of 129 strains were examined by DCH for the presence of DNA sequence homologous to the *tdh* gene probe (Table 3). These strains included 101 *Vibrio* spp. organisms from 19 named species other than *V. parahaemolyticus*, a group of 20 marine psychrotrophic *Vibrio* spp., and 8 strains of unrelated organisms representing seven genera. Of all the test organisms, only *V. hollisae* reacted with the DNA probe. All 11 strains of *V. hollisae* were positive, producing autoradiographic signals that were slightly weaker than those of the *V. parahaemolyticus* strains.

DISCUSSION

Epidemiological surveys reveal that almost all clinical isolates of *V. parahaemolyticus* are KP^+, whereas nonclini-

TABLE 2. Comparison of the DCH test with ELISA and a modified Elek test

No. of strains	Reaction[a]		
	DCH	ELISA	Elek
50	+	NT	+
3	+	+	+
4	+	+	+w
3	+	+	−
2	+	+w	−
38	−	−	−

[a] +, Positive; +w, weak reaction; −, negative; NT, not tested.

TABLE 3. Detection of DNA sequences homologous to the *tdh* gene in *Vibrio* spp. and other organisms by the DCH test[a]

Organism	No. of strains tested	Results[b]
Human pathogenic *Vibrio* spp.		
V. vulnificus	58	−
V. cholerae O1	9	−
V. cholerae non-O1	3	−
V. fluvialis	3	−
V. mimicus	1	−
V. metschnikovii	1	−
V. hollisae	11	+[c]
V. alginolyticus	1	−
V. damsela	2	−
Fish pathogenic or marine *Vibrio* spp.		
V. ordalii	2	−
V. aestuarianus	1	−
V. campbellii	1	−
V. gazogenes	1	−
V. harveyi	1	−
V. natriegens	1	−
V. nereis	1	−
V. nigripulchritudo	1	−
V. pelagius	1	−
V. proteolyticus	1	−
V. splendidus	1	−
Psychrotrophic *Vibrio* spp.[d]	20	−
Other genera		
Escherichia coli	1	−
Klebsiella pneumoniae	1	−
Klebsiella oxytoca	1	−
Serratia liquefaciens	1	−
Streptococcus faecalis	1	−
Staphylococcus aureus	1	−
Pseudomonas sp.	1	−
Acinetobacter sp.	1	−

[a] Hybridization was performed under stringent conditions by the method of Moseley et al. (28).
[b] +, Homologous DNA detected; −, not detected.
[c] All strains were positive.
[d] Marine vibrios capable of inducing the Kanagawa phenomenon (32).

cal isolates are almost always KP^- (23, 32). Despite this strong association, there is no clear evidence that supports a direct relationship between the hemolysin (TDH) responsible for the Kanagawa phenomenon and disease. The presence of TDH could merely be associated with another factor that might be responsible for the disease (15, 31). However, because of the strong association of the Kanagawa phenomenon with virulent isolates, *V. parahaemolyticus* strains are commonly tested for the Kanagawa phenomenon. The Wagatsuma blood agar used for Kanagawa phenomenon detection is not easy to prepare correctly, and reactions may be difficult to interpret with some strains. Some discrepancies have been noted between the Kanagawa phenomenon, as defined by β-hemolysis on Wagatsuma agar, and the actual production of TDH, as determined by immunological methods (13). The cloning and sequencing of the *tdh* gene has made available a sensitive and specific DNA probe to detect strains possessing genes encoding TDH.

In the present study, we surveyed both clinical and nonclinical isolates of *V. parahaemolyticus* to the *tdh* gene. Eighty-one percent (82 of 101) of the clinical isolates and 15% (6 of 40) of nonclinical isolates probably carried the genetic potential to produce TDH (Table 1). Previous studies (24, 33) have demonstrated that KP^+ strains are isolated from only 1 to 2% of water or seafood samples, the presumed reservoir of disease. Since many of the nonclinical strains examined in this survey were isolated from foods suspected to be the source of a disease outbreak, there was an unusually high incidence of gene-positive nonclinical isolates. It has been suggested that environmental KP^- strains possess the genetic potential to produce TDH which is expressed only upon intestinal passage. The DNA probe results show that most nonclinical isolates clearly lack the genetic potential to produce TDH. Thus, it is more likely that the small portion of gene-positive environmental strains are selected upon ingestion.

The results obtained with the specific *tdh* probe developed in this study were compared with those obtained with phenotypic assays for TDH (Table 1). The β-type hemolysis on Wagatsuma agar, i.e., the Kanagawa phenomenon, was the least sensitive technique. Sixteen percent (10 of 61) of KP^- strains were positive by DCH, as were 86% (12 of 14) of KP^{+w} strains. The two strains that were KP^{+w} and gene negative may indicate a nonspecific hemolytic reaction produced by a factor(s) other than TDH. The correlation between DCH and immunological methods was much better; the ELISA was more sensitive than the modified Elek test (Table 2). Of 62 DCH-positive strains, only 2 gave a weak reaction in the ELISA, whereas 5 gave a negative reaction and 4 gave a weak reaction in the modified Elek test. Thus, the DCH technique with the *tdh* gene probe was the most sensitive assay for TDH. Two possible explanations for the failure of the phenotypic assays to definitively identify all gene positive strains are that (i) the level of TDH produced by some strains is too low to be detected, or (ii) the DNA probe is detecting inactive or mutant *tdh* genes. Further studies are ongoing to investigate both possibilities.

The gene probe was also employed to examine *Vibrio* spp. other than *V. parahaemolyticus*. In recent years, a variety of *Vibrio* spp. have been shown to cause disease in humans. A definite pathogenic mechanism has been established only for *V. cholerae* O1. However, all human pathogenic vibrios produce hemolysins (6, 10, 14, 17, 20–22, 34, 35), suggesting a possible role for such factors in disease. One such pathogenic species, *Vibrio vulnificus*, induces the Kanagawa phenomenon on Wagatsuma agar (17) as does a group of psychrotrophic marine vibrios (32). To examine the relationship between TDH and other hemolysins, 19 *Vibrio* spp. including *V. vulnificus* and the psychrotrophic vibrios, were examined by DCH with the *tdh* probe. Only *V. hollisae*, a newly recognized agent of diarrhea (10, 25), possessed sequences homologous to the *tdh* gene probe (Table 3). The KP^+, gene-negative reaction of *V. vulnificus* and the group of psychrotrophic vibrios demonstrates that Kanagawa phenomenon induced by these vibrios is not due to hemolysins completely identical with TDH. Although *V. hollisae* is KP^- on Wagatsuma agar, the concentrated culture supernatant is hemolytic for human (type O) and rabbit erythrocytes, and the supernatant contains antigenic determinants that are similar, but not identical, to TDH in Ouchterlony double diffusion analysis (Nishibuchi and Kaper, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, B90, p. 33). This partial identity seen in Ouchterlony analysis may explain the somewhat weaker reaction of *V. hollisae* with the DNA probe, and further characterization of the hemolysin gene from this species is in progress. If the TDH is a virulence factor of *V.*

parahaemolyticus, the related hemolysin produced by *V. hollisae* may serve a similar function.

There are two possible explanations for the presence of sequences in *V. hollisae* homologous to the *V. parahaemolyticus tdh* gene. First, the *tdh* sequences in these two species could be the result of divergent evolution from a common ancestor. Alternatively, the similarity of these genes could be the result of interspecies transfer. The low overall DNA homology (less than 4%) between these species argues against the first possibility (10). The second hypothesis is supported by the recent isolation of a plasmid-borne *tdh* gene from a clinical strain of *V. parahaemolyticus* (Nishibuchi and Kaper, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, B90, p. 33). Transfer of the *tdh* gene between *V. parahaemolyticus* and *V. hollisae* could be accomplished via such a plasmid and would be one possible explanation for the appearance of the *tdh* gene in both species.

Reports that demonstrate the apparent instability of the *tdh* gene shed light on the origin of KP^- strains from patients with gastroenteritis. Clark and Cherwonogrodzky (7) showed that when KP^+ strains are grown in vitro the number of hemolytic cells decrease with each generation. Burstyn et al. (4) reported that two spontaneous KP^- mutants derived from a KP^+ strain did not revert to KP^+. These KP^- mutants were negative with the *tdh* probe, although the parent strain was positive (unpublished observations). A similar observation was reported by Taniguchi et al. (The 18th Symposium on *V. parahaemolyticus*, October 1984, Hamamatsu, Japan). Thus, KP^- clinical strains may arise from deletion of the *tdh* gene in the bowel or during isolation.

Using the sensitive and specific *tdh* gene probe developed in the present study, we have examined the incidence of *tdh* genes in a variety of isolated strains. The DCH technique has been successfully applied to the direct detection of other enteropathogens in stool and seafood specimens (12, 27) and should be useful in detecting *V. parahaemolyticus* carrying the *tdh* gene in these samples. In addition, DNA hybridization by the Southern blot technique has also been employed to study the epidemiology of bacterial infections (18). We are currently investigating the potential application of this technique to *V. parahaemolyticus*; preliminary results indicate that it will be quite useful. Thus, the use of cloned *tdh* genes should allow significant insight into the epidemiology and pathogenicity of disease due to *V. parahaemolyticus*.

ACKNOWLEDGMENTS

We are grateful to those who supplied valuable bacterial cultures.

This research was supported by Public Health Service grant AI19165 from the National Institute of Allergy and Infectious Diseases. Computer analysis was supported by the Computer Science Center, University of Maryland.

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