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MINIREVIEW

Thermostable Direct Hemolysin Gene of *Vibrio parahaemolyticus*: a Virulence Gene Acquired by a Marine Bacterium

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INTRODUCTION

Bacteria in the genus *Vibrio* are widely distributed in the aquatic environment and are considered to be autochthonous bacteria in marine and estuarine waters. Some *Vibrio* species can also cause infections in humans and have been isolated from a variety of intestinal and extraintestinal sites (4, 21). Pathogenic mechanisms are poorly understood for the majority of *Vibrio* species except for *Vibrio cholerae* O1 and O139 (1, 4, 8, 21, 24). Bacteria belonging to the *V. cholerae* O1 and O139 serogroups are almost invariably pathogenic, because of the presence of the potent enterotoxin cholera toxin. For other *Vibrio* species, individual strains may or may not possess virulence factors or the virulence factors that are critical for disease may not be known. Such *Vibrio* species are thus considered to be potentially pathogenic from the public health perspective. *Vibrio parahaemolyticus* has been recognized as an agent of gastroenteritis associated with consumption of seafood, but not all strains of this species are considered to be truly pathogenic. One putative virulence factor that has long been epidemiologically associated with disease is one of the hemolysins produced by *V. parahaemolyticus* that produces beta-type hemolysis on a special blood agar medium, Wagatsuma agar (34, 50). Almost all *V. parahaemolyticus* strains isolated from clinical specimens demonstrate this hemolytic activity, which has been called the Kanagawa phenomenon (KP) (34, 50), whereas only 1 to 2% of strains from nonclinical sources are KP positive (34, 50). Accordingly, this hemolysin has been considered an important virulence factor, and the KP reaction has been used as a marker for virulent strains. For this reason, this particular hemolysin has been the subject of extensive studies by many workers (reviewed in references 14, 30, and 56). The hemolysin was named thermostable direct hemolysin (TDH) on the basis of its characteristics: TDH was not inactivated by heating at 100°C for 10 min, and the hemolytic activity was not enhanced by the addition of lecithin, indicating a direct action on erythrocytes (52). The biological activities of TDH include hemolysis of various species of erythrocytes, cytotoxicity, lethal toxicity for small experimental animals, and increased vascular permeability in rabbit skin (14, 30, 56). Although the capacity of TDH to induce fluid accumulation (FA) in the rabbit ileal loop was suggested in some studies (see below), the association of TDH and/or the KP with enterotoxigenicity is quite unclear from the older literature. Definitive evidence for the importance of TDH in the enteropathogenicity of *V. parahaemolyticus* has only recently been obtained by using genetically altered strains and more-sensitive assays. The molecular studies of the gene encoding TDH (*tdh*) have also revealed other interesting features regarding sequence divergence, mobility, and regulation.

In this review, we summarize the results of these genetic studies of the *tdh* gene which indicate that TDH is a major virulence determinant of KP-positive *V. parahaemolyticus* strains and that the KP-positive phenotype results from high-level expression of a particular *tdh* gene. These attributes make the KP phenotype a good marker for a virulent strain. We also speculate about the origin and variation of the *tdh* gene and about why the virulence genes, the *tdh* and *tdh*-related hemolysin (*trh*) genes, are present in only a small population of *V. parahaemolyticus*. The extensive data available for the *tdh* gene help us to better understand potentially pathogenic bacteria such as *V. parahaemolyticus* in the natural environment.

tdh GENES IN *V. PARAHAEMOLYTICUS* STRAINS

The *tdh* gene (*tdh*1 in Table 1) was first cloned from a KP-positive strain, WP1 (23), and probes derived from this cloned gene facilitated detection and analysis of other *tdh* genes in various strains (Table 1). The *tdh* genes are usually, but not exclusively, located in the chromosome. All of the cloned *tdh* genes encode predicted protein products composed of 189 amino acid residues (including signal peptides) which have hemolytic and other biological activities. The nucleotide sequences of the various *tdh* genes are well conserved (>97% identity), and the protein products are immunologically indistinguishable.

KP-positive strains. In addition to the *tdh*1 gene, strain WP1 contained a second, nonidentical *tdh* gene named *tdh*2 (43). In strain TY4750, these genes have been named *tdhS* and *tdhA* for *tdh*1 and *tdh*2, respectively (20). A survey of KP-positive and KP-negative *V. parahaemolyticus* strains revealed that all KP-positive strains contained two *tdh* gene copies (43). The duplication of the *tdh* genes initially suggested the possibility that both *tdh*1 and *tdh*2 products are needed for production of functional TDH because the dimeric, but not a monomeric, form of TDH showed hemolytic activity (57). However, the production of TDH with typical biological activities from each of the two *tdh* genes expressed individually in *Escherichia coli* argued against this possibility (42, 43). Although the products of the *tdh*1 and *tdh*2 genes are immunologically indistinguishable, the predicted amino acid sequences of the gene products (mature proteins) differ at seven residues (43). The amino acid sequence of purified TDH determined by Edman degradation

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TABLE 1. *tdh* and *trh* genes for which the nucleotide sequences were determined

Designation	Origin				Location[b]	% Similarity to the *tdh*2 gene[a]	Reference(s)
	Organism						
	Sp.	Strain	Source[c]	KP[d]			
*tdh*1	*V. parahaemolyticus*	WP1	CL	+	C	97.0	42, 43, 58
*tdh*2	*V. parahaemolyticus*	WP1	CL	+	C	100	43
tdhS	*V. parahaemolyticus*	T4750	CL	+	C	97.2	20
tdhA	*V. parahaemolyticus*	T4750	CL	+	C	99.6	20
tdhX	*V. parahaemolyticus*	TH3766	CL	+[e]	C	98.4	64
*tdh*3	*V. parahaemolyticus*	AQ3776	CL	−	C	98.6	43
*tdh*4	*V. parahaemolyticus*	AQ3776	CL	−	P	98.6	43
*tdh*5	*V. parahaemolyticus*	AQ3860	CL	−	C	98.9	3
tdh/*I*	*V. parahaemolyticus*	TH012	CL	−	C	98.4	12
NAG-*tdh*	*V. cholerae* non-O1	91	CL	NT	P	98.6	2
Vm-*tdh*	*V. mimicus*	6	CL	NT	C	97.0	60
Vh-*tdh*	*V. hollisae*	9041	CL	NT	C	93.3	65
*trh*1 (= *trh*)	*V. parahaemolyticus*	AQ4037	CL	−	C	68.6	46
trhX	*V. parahaemolyticus*	TH3766	CL	+[e]	C	68.1	64
*trh*2	*V. parahaemolyticus*	AT4	EN	−	C	68.8	25

[a] Percent similarity in the 567-bp coding region.
[b] C, presumed to be chromosomal (11, 36); P, plasmid borne.
[c] CL, clinical; EN, environmental.
[d] KP, beta-hemolysis on Wagatsuma agar unless otherwise specified. +, positive; −, negative; NT, not tested.
[e] Determined by the modified Elek test (11, 13).

(61) was much more similar to the sequence predicted from the *tdh*2 gene (different at two residues) than that from the *tdh*1 gene (different at nine residues) (43). This result suggested preferential expression of the *tdh*2 gene. Isogenic mutants of a KP-positive strain, defective in either *tdh*1 or *tdh*2, were constructed by allelic exchange methods and were used to assess the contribution of each gene to production of TDH. The KP phenotype and >90% of the total TDH protein production were attributable to expression of the *tdh*2 gene (43, 45). The *tdh*1 gene was not completely silent, however, and accounted for 0.5 to 9.4% of total TDH under various culture conditions (45).

One exceptional KP-positive strain carrying a *tdh* gene (*tdhX*) and a *trh* gene (*trhX* [see below]) has recently been reported (64). The *tdhX* gene was closely related to the *tdh*/*I* gene of a KP-negative strain (see below) but was shown to be expressed (64).

KP-intermediate and -negative strains. Some strains of *V. parahaemolyticus* show weak hemolysis on Wagatsuma agar. Eighty-six percent of the strains showing this phenotype possessed the *tdh* gene (40). However, in contrast to the gene duplication found with KP-positive strains, these KP-intermediate strains had only one copy of the *tdh* gene (43).

When strains showing no hemolysis on Wagatsuma agar were examined, only 16% of such KP-negative strains were positive for the *tdh* gene in a hybridization test, indicating that most KP-negative strains do not possess the genetic potential to produce TDH (40). The KP-negative, *tdh*-positive strains usually had one chromosomal *tdh* gene copy. One exceptional strain had a second *tdh* gene copy on a 35-kb plasmid. The chromosomal (*tdh*3) and plasmid-borne (*tdh*4) genes of this strain and the *tdh* genes (*tdh*5 and *tdh*/*I*) of other KP-negative strains were cloned and characterized. Despite nucleotide sequence variations, the genes of KP-negative strains encoded TDH proteins very similar to TDH proteins encoded by the *tdh*1 and *tdh*2 genes of KP-positive strains, i.e., they had hemolytic and other biological activities and were immunologically indistinguishable (3, 12, 66). These results suggested that low-level expression of the *tdh* genes may be the reason for the KP-negative phenotype of these *tdh*-bearing strains.

Regulation of the *tdh* gene expression. The negligible expression of the *tdh*1 gene in a KP-positive strain and the presumed low-level expression of the *tdh* genes of KP-negative strains, e.g., *tdh*3 and *tdh*4, were hypothesized to be due at least partly to differences in promoter strength because the nucleotide sequences of the presumed promoter regions of the *tdh*1, *tdh*3, and *tdh*4 genes differed slightly from that of the *tdh*2 gene (43). Northern (RNA) blot analysis confirmed that there were differences in steady-state levels of *tdh* mRNA among these genes (43). In fact, the basal-level expression from the presumed native promoter of the cloned *tdh*2 gene was 21- to 26-fold higher than that of those of the *tdh*1, *tdh*3, and *tdh*4 genes in an *E. coli* background (29). Furthermore, expression of the *tdh* genes was influenced by the products of the *V. parahaemolyticus toxRS* (Vp-*toxRS*) operon cloned from a KP-positive strain; overexpression of the Vp-*toxRS* operon increased expression of the *tdh*1 and *tdh*2 genes 1.25- and 5.07-fold, respectively, in an *E. coli* background (29). Vp-ToxR was shown to be involved in transcriptional regulation of *tdh* expression in a KP-positive *V. parahaemolyticus* strain in a culture medium-dependent manner (29). Therefore, the very high level expression of the *tdh*2 gene relative to the *tdh*1 gene is due to differences in the basal-level production of mRNA and in the degree of transcriptional activation by Vp-ToxRS. The nucleotide sequence around 144 bp upstream of the coding region is important for the transcriptional activation of the *tdh*2 gene by Vp-ToxRS (29). The *tdh*3 and *tdh*4 genes, expression of which is increased 3.9- and 11-fold, respectively, by Vp-ToxRS (29), differ from the *tdh*2 gene in this upstream region, as does *tdh*1 (59).

The Vp-*toxRS* operon was so named because of structural and functional similarities to the *V. cholerae toxRS* operon, which encodes a transcriptional activator of the cholera enterotoxin gene (32, 33). Vp-*toxR* and -*toxS* genes share 52 and 62% nucleotide sequence homology, respectively, to the *toxR* and *toxS* genes of *V. cholerae* (29). The amino acid sequences of the Vp-ToxRS contain regions similar to the proposed transmembrane and activity domains of *V. cholerae* ToxRS. Like *V. cholerae* ToxRS, Vp-ToxRS modulates expression of not only an enterotoxin gene (*tdh*) but also other genes; comparison of profiles of total cellular and extracellular proteins of

a wild-type strain and the isogenic Vp-*toxR* mutant strain suggested that production of proteins other than TDH is also under Vp-ToxRS control (36). The Vp-*toxRS* sequence is present in all strains of *V. parahaemolyticus* regardless of the KP phenotype (29), and thus, Vp-ToxRS is considered a global regulator of *V. parahaemolyticus* genes.

CONTRIBUTION OF THE *tdh* GENE TO ENTEROTOXICITY OF *V. PARAHAEMOLYTICUS*

To assess the role of TDH in enterotoxicity of *V. parahaemolyticus*, many workers employed the ligated rabbit ileal loop model and tested for induction of FA in the loop. Whole cultures, culture filtrates, and purified TDH were examined. While the results of the studies with whole cultures were generally in favor of the enterotoxic role of TDH, studies with the other preparations gave confusing results. When whole cultures were tested, KP-positive strains gave positive FA results more frequently than did KP-negative strains (51, 62). Culture filtrates required concentration to induce a positive FA reaction (51), but false-positive results could be obtained by concomitant concentration of NaCl in the culture medium (5, 22). Very large amounts of purified TDH (125 μg or more) were required to demonstrate significant FA, and severe destructive histopathological changes were observed in the inoculated ileal loop at the high dosages (35, 68). Furthermore, some studies suggested that FA was caused by a virulence mechanism in addition to TDH (63) or by a toxin(s) other than TDH (18).

In order to obtain conclusive evidence concerning the role of TDH in the enteropathogenicity of *V. parahaemolyticus*, we compared a KP-positive strain and its isogenic TDH-negative mutant in which both the *tdh*1 and *tdh*2 genes were specifically inactivated by successive allelic exchange procedures (38). Whole-culture preparations of the parent strain gave positive FA results in the rabbit ileal loop assay, while the TDH-negative mutant induced no FA in this model. Additional evidence for enterotoxic activity of TDH was obtained with rabbit ileal tissue mounted in Ussing chambers, a sensitive technique for studying intestinal ion transport. Culture filtrates of the TDH-positive strain induced an increase in short circuit current, whereas no such increase was observed with the isogenic TDH-negative strain (38). Returning the cloned *tdh* gene to the isogenic mutant complemented the mutation and restored the ability to increase short circuit current. The Ussing chamber activity could also be neutralized by the addition of anti-TDH serum. In contrast to the severe destructive histological changes observed when very large amounts of TDH were added to rabbit ileal loops (35, 68), the very small amounts of TDH present in these Ussing chamber experiments (estimated to be on the order of nanograms [38]) produced no histological change (38).

These experiments are consistent with a mechanism whereby TDH alters ion flux in intestinal cells, thereby leading to a secretory response and diarrhea. Additional Ussing chamber experiments using purified toxin indicate that TDH induces intestinal chloride secretion and that calcium may be the intracellular mediator of this effect (48). The ability of TDH to stimulate ion flux was previously suggested by studies using nonintestinal tissue including myocardial tissue (53), rat erythrocytes (55), and human erythrocytes (19). There is evidence that, in erythrocytes, TDH acts as a pore-forming toxin (16, 19), but the relevance of these observations for intestinal cells is not known.

When the Vp-*toxR* gene of a KP-positive strain was specifically inactivated by allelic exchange, the mutant strain produced lower levels of TDH in vitro and induced very little FA in the rabbit ileal loop compared with the parent strain (29). The results suggest that Vp-ToxR is an essential regulator for TDH-mediated enterotoxic activity of *V. parahaemolyticus.*

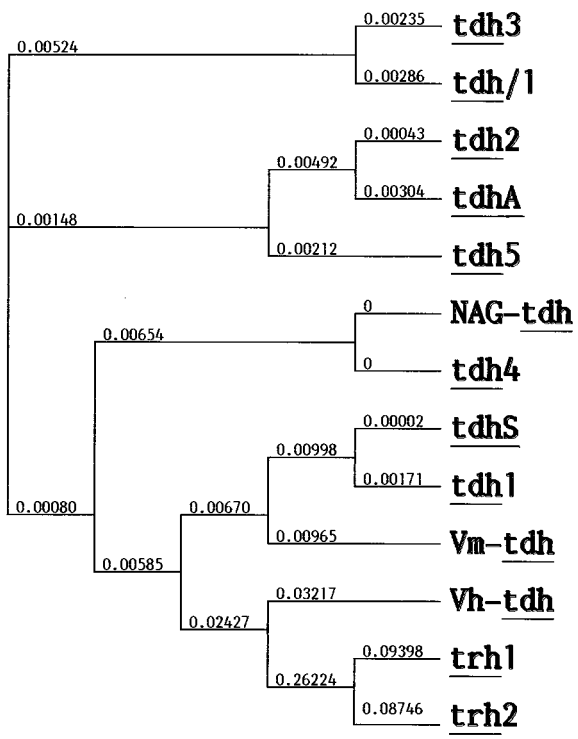

FIG. 1. Phylogenetic relationship among the *tdh* and *trh* genes. See Table 1 for gene designations. Evolutionary distances (indicated by the numerals on the branches of the phylogenetic tree) were calculated by the neighbor-joining method (41, 49). The branch length of the tree is not proportional to the evolutionary distance.

MOBILITY AND DIVERSIFICATION OF THE *tdh* GENE

Studies using a *tdh*1 gene probe led to the discovery of homologous genes in three *Vibrio* species other than *V. parahaemolyticus* (17, 40, 44) and a *tdh*-related hemolysin (*trh*) in *V. parahaemolyticus* (46). Subsequent comparative analyses of these genes and *V. parahaemolyticus tdh* genes revealed dynamic aspects of the *tdh* gene.

***tdh* genes in other vibrios.** Like *V. parahaemolyticus*, *Vibrio hollisae*, *Vibrio mimicus*, and *V. cholerae* non-O1 are commonly isolated from the marine environment and occasionally from diarrheal stool specimens. While all strains of *V. hollisae* so far examined, including an isolate from a coastal fish, were positive for the *tdh* gene (37, 40, 41), only some strains of *V. mimicus* and *V. cholerae* non-O1 isolated from patients with diarrhea contained sequences homologous to the *tdh* probe (17, 44). The *tdh* genes of the three species, like *V. parahaemolyticus tdh* genes, had open reading frames of 567 bp, and the coding sequences were highly homologous to *V. parahaemolyticus tdh* genes (Table 1).

Among the *tdh* gene sequences cloned from *V. parahaemolyticus* and the other *Vibrio* species, the *V. hollisae tdh* gene (Vh-*tdh*) was most distantly related to the other *tdh* genes while the other *tdh* genes were phylogenetically very close (41) (Fig. 1). This phylogenetic relationship of the *tdh* gene does not correlate with phylogenetic relationships of the *Vibrio* species determined on the basis of 16S rRNA sequences (26),

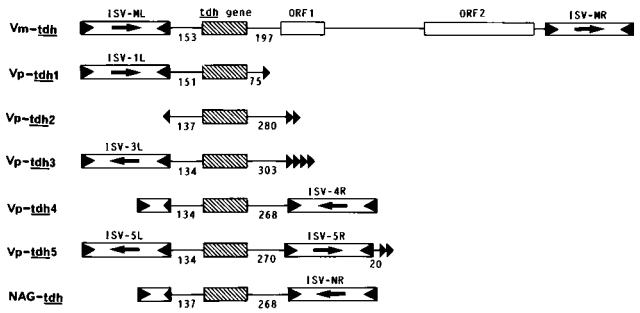

FIG. 2. Schematic representation of the ISVs and related sequences flanking various *tdh* genes (reproduced from Fig. 4 of reference 59). The arrows in the ISVs depict the orientations determined from homology with IS*90*3. Truncated forms of ISVs present upstream of the Vp-*tdh*4 and NAG-*tdh* genes are homologous to a region of ISV-SL. Large arrowheads indicate consensus sequences for the 18-bp terminal inverted repeats of ISVs. Small arrowheads indicate shorter versions (9 bp) of the terminal inverted repeats. ORF, open reading frame. Numerals indicate distances in base pairs between *tdh* coding sequences and terminal inverted repeats or ORF1.

where *V. parahaemolyticus*, *V. cholerae*, *V. mimicus*, and *V. hollisae* form distinct species. This result suggests that the *V. hollisae tdh* may have diverged in concert with the divergence of the species whereas the *tdh* genes of *V. parahaemolyticus*, *V. cholerae*, and *V. mimicus* were more recently shared. Such a scenario would also be consistent with the finding that only some strains of *V. cholerae* and *V. mimicus* contain *tdh* genes whereas all *V. hollisae* strains so far examined contain *tdh* sequences. Interestingly, the homology between the *V. mimicus tdh* gene (Vm-*tdh*) and the *V. parahaemolyticus tdh*1 gene was higher than that between the *tdh*2 and *tdh*1 genes present in the same strain of *V. parahaemolyticus* (60).

The *V. cholerae* non-O1 *tdh* gene (NAG-*tdh*) was present on a plasmid (17) and was 100% identical to the unusual plasmid-borne *tdh* gene (*tdh*4) of *V. parahaemolyticus* AQ3776 (2). In addition, the two *tdh*-bearing plasmids were very similar when compared by restriction endonuclease and Southern blot analysis (2). These results suggest plasmid-mediated *tdh* gene transfer between *V. cholerae* non-O1 and *V. parahaemolyticus*. However, plasmid-borne *tdh* genes are exceptional, and the great majority of *tdh* genes are found on the chromosome (Table 1).

The *tdh* genes of *V. parahaemolyticus* were found to be flanked by nucleotide sequences nearly identical to the terminal inverted repeat sequences of IS*10*2 (3, 39). These observations suggested that the *tdh* gene may be associated with insertion sequences and that transposition of the *tdh* genes may explain the presence of these sequences on chromosomes and plasmids of different *Vibrio* species. Further sequence analysis revealed insertion sequence-like elements (named ISVs), which are similar to IS*90*3 and thus related to IS*10*2 (59) (Fig. 2). The ISVs possessed 18-bp terminal inverted repeats highly homologous to those of IS*90*3 and were 881 to 1,058 bp long. ISVs or portions of the ISVs were found to flank the *tdh*1, *tdh*2, *tdh*3, *tdh*4, *tdh*5, and NAG-*tdh* genes at distances of 75 to 303 bp away from the *tdh* structural gene. In *V. mimicus*, the ISVs are located 153 bp upstream and 3,594 bp downstream of the Vm-*tdh* gene (59). The ISV sequences diverged considerably (up to 33.6% divergence) but shared ca. 50% overall identity with IS*90*3 (59). The ISV homologs not only were associated with the *tdh* gene but also were found in strains of *V. parahaemolyticus*, *V. cholerae* O1, and *V. cholerae* non-O1 which lacked *tdh* sequences. Although IS*90*3 is known to encode an active transposase (7), transposition of the ISVs has not yet been demonstrated. The lack of demonstrable transposition activity may be due to various base changes and deletions in the open reading frame of the ISVs that shares homology with the IS*90*3 transposase gene (59). However, if the ISVs possessed transcriptional activity at some time in the past, this could explain the transfer of the *tdh* gene between chromosome and plasmids. One potential mechanism of transfer of *tdh* genes would be transduction, but there is no available evidence for phage involvement in transfer of *tdh* genes. In another *Vibrio* species, *V. cholerae*, a 2.7-kb repeated sequence named RS1 flanks the cholera enterotoxin gene (*ctx*) (31, 47). This element encodes a site-specific recombination system that allows integration of the *ctx* genes into a specific site in the *V. cholerae* chromosome. RS1 has also been implicated in tandem duplication of the *ctx* gene by an unequal crossover event (6). The exact location of the *tdh* gene in the *V. parahaemolyticus* chromosome is unknown, but the *tdh*1 and *tdh*2 genes are not located in close proximity within the chromosome of KP-positive strains (36). There is also no evidence for ISV-mediated *tdh* duplication.

***trh* genes in *V. parahaemolyticus*.** In an outbreak of gastroenteritis in the Maldives, KP-negative isolates of *V. parahaemolyticus* were found to produce a TDH-related hemolysin (TRH) but not TDH (10, 15). Biological, immunological, and physicochemical characteristics of TRH are similar but not identical to those of TDH (15). Like the *tdh* genes, the gene (*trh*) encoding TRH contains a 567-bp open reading frame (46). The *trh* gene from strain AQ4037 shared 69% identity with the *tdh*2 gene, and the sequence differences were distributed throughout the gene. The hydropathy plots and secondary structures predicted from the deduced amino acid sequences of the two hemolysins were similar, and two cysteine residues necessary for the interchain bond were conserved in both hemolysins (46). These suggested that the *trh* and *tdh* genes probably evolved from a common ancestor by single-base change so that the fundamental structure of the protein was maintained. A hemolysin gene sharing 84 and 69% identity with the *trh* and *tdh*2 genes, respectively, was cloned from a KP-negative strain, and thus the original *trh* gene and the new hemolysin gene were named *trh*1 and *trh*2, respectively (25). Hybridization studies with oligonucleotide probes showed considerable variation in the *trh*1 and *trh*2 sequences from different strains of *V. parahaemolyticus* (25). Although a variant of the *trh*1 gene (*trhX*) showing only 0.9% divergence has recently been reported (64), it appears that there is greater divergence among *trh* genes than among *tdh* genes. Levels of in vitro expression of the *trh*1 genes of various strains were much lower than those of the *tdh* genes of KP-positive strains (54), and expression of the *trh*1 gene was not affected by the presence of Vp-ToxRS (29). The effect of Vp-ToxRS on *trh*2 expression has not been examined.

A survey of 285 strains of *V. parahaemolyticus* revealed that not only were *tdh*-positive strains strongly associated with gastroenteritis but *trh*-positive strains (including the *trh*2 subgroup) were also associated with gastroenteritis (25, 54). Some clinical isolates contained both *tdh* and *trh* genes, while most environmental isolates possessed neither *tdh* nor *trh* sequences (3, 25, 54, 64). If the *tdh* gene of a strain containing both *tdh* and *trh* is significantly closer than other *tdh* genes to *trh*, then it may be possible to infer that the *tdh* and *trh* genes arose by gene duplication followed by accumulation of base changes. However, when sequences of *tdh* genes (*tdhX* and *tdh*5) isolated from strains containing both *trh* and *tdh* were determined, the identity between the *tdh* and the *trh* genes was ca. 68% (3, 64), about as distantly related to the *trh* genes as were the other *tdh* genes (see *tdh*5 in Fig. 1).

CONCLUSIONS AND SPECULATION

The application of molecular genetic techniques to the study of *V. parahaemolyticus* has yielded several insights into the role of TDH in disease. The enterotoxic activity of TDH in KP-positive *V. parahaemolyticus* has been clearly established by comparison of isogenic strains in in vivo and in vitro studies. Furthermore, the use of the KP reaction as an epidemiological marker for virulence has been provided with a reasonable justification. High-level expression of the *tdh*2 gene, one of the two *tdh* gene copies of KP-positive strains, results from the high basal-level production of mRNA which is further enhanced by Vp-ToxRS. The resulting high-level TDH production leads to a KP-positive phenotype. Some *tdh*-positive strains exhibit intermediate or negative KP phenotypes which can be explained by low-level expression of the *tdh* genes. The *tdh* genes in these strains are different from the *tdh*2 gene in that the basal-level expression is very low and the degree of Vp-ToxRS-promoted gene expression varies. Although molecular epidemiological data support the clinical significance of KP-intermediate and -negative strains carrying the *tdh* genes, conclusive evidence of enterotoxigenicity of the *tdh* gene in these strains awaits further investigation. Detailed conditions for *tdh* expression in association with Vp-ToxRS and other regulators, if any, need to be clarified.

The *tdh* gene is absent in most environmental strains of *V. parahaemolyticus* and present in some strains of *V. mimicus* and *V. cholerae* non-O1 and in all strains of *V. hollisae*. Evidence has been obtained indicating that transfer of the *tdh* gene has occurred via plasmids and/or an ISV. The potential mobility of *tdh* suggests that *V. parahaemolyticus* may have acquired the *tdh* gene from another organism(s) in the past, a suggestion consistent with the much lower G+C contents of the *tdh* and *trh* genes (ca. 30%) compared with the average G+C contents of *Vibrio* chromosomes (46 to 49%). We speculate that, subsequent to the acquisition of the *tdh* gene, the *tdh*-bearing genetic units including the ISVs accumulated base changes and underwent ISV-associated genetic rearrangement in some cases. During these changes, the transposase gene of the ISV was mutated and the *tdh* gene was stabilized in the *V. parahaemolyticus* chromosome. A similar hypothesis has been proposed for the evolution of the alpha-hemolysin determinant (*hly*) of *E. coli*, which is present on plasmids as well as in the chromosome (9). Acquisition of this gene from an external source was suggested by the low G+C content of *hly* (27) and the presence of hemolysin genes similar to *hly* in non-*E. coli* species with low G+C content (28). The IS*91*-like elements flanking the *hly* gene were hypothesized to be involved in the spread of *hly* in the past and to have undergone considerable sequence variation during evolution (67). The presence of a family of related virulence genes in various bacterial species may be explained by this kind of gene transfer mechanism.

ISVs or portions of ISVs exist at points 134 to 153 bp upstream of the *tdh* coding sequences except for the *tdh*2 gene. The *tdh*2 gene currently retains only a part (9 bp) of the ISV terminal inverted repeat sequence at the corresponding location (59), suggesting that further upstream sequence was perhaps derived from the chromosome when an ISV-associated rearrangement occurred. The upstream sequence, which is important for high-level expression of the *tdh*2 gene, may be derived from an upstream region of a gene under control of Vp-ToxRS, a global regulator in *V. parahaemolyticus*. Identification of the exact locations of the *tdh* promoter and the sequence needed for Vp-ToxRS stimulation will clarify this point.

Although minor sequence differences are found, the coding sequences of the *V. parahaemolyticus tdh* genes are well conserved (<2.8% divergence). In contrast, the *trh* coding sequences represented by the *trh*1 and *trh*2 subgroups are much more divergent (16% divergence between the two subgroups). It is not known whether most *trh*-positive strains detected by the DNA probes are enterotoxic, but the results of the molecular epidemiological investigation indicate a strong association of the *trh*-positive strains with gastroenteritis. In addition, there are structural and functional similarities between TDH and TRH (including the *trh*1 and *trh*2 gene products) and conservation of key residues needed for at least hemolytic activity. Therefore, the *trh* sequences may have undergone random drift under some selective pressure. Currently, it is not clear whether the *trh* genes are flanked by ISVs or related sequences. If sequence divergence of up to 31% between the *tdh* and *trh* genes simply represents the phylogenetic distance between the two genes, the flanking insertion sequences and regulatory gene sequences of the *trh* genes, if any, may have diverged considerably from those associated with the *tdh* genes. Therefore, it is not clear at present whether the *trh* gene evolved from the *tdh* gene or vice versa in a *V. parahaemolyticus* background or whether the *trh* gene was acquired by *V. parahaemolyticus* independently of the *tdh* gene.

In this review, we have focused on TDH since it is the only virulence factor of *V. parahaemolyticus* whose importance is documented by both experimental and epidemiological data. Although we have gained substantial insights into this virulence factor, much more information on the pathogenicity and phylogeny of this species is needed to achieve a full picture of *V. parahaemolyticus* as a pathogen. In addition to the TDH enterotoxin, *V. parahaemolyticus* no doubt requires intestinal colonization factors to cause disease. A variety of pili and other potential colonization factors have been described for this species, but substantial genetic and epidemiological data to strongly implicate any of the candidate adhesins are lacking. The recent observation that *V. parahaemolyticus* contains a homolog of the *V. cholerae toxRS* regulatory system may lead to discovery of additional virulence factors of *V. parahaemolyticus* that are regulated by this system. From a phylogenetic viewpoint, there is little information about *V. parahaemolyticus* apart from the variation in the *tdh-trh* genes. There are no studies examining the clonal relationships among *V. parahaemolyticus* strains by techniques such as multilocus enzyme electrophoresis. Such studies could yield insights into whether the only difference between pathogenic and nonpathogenic strains of *V. parahaemolyticus* is the presence of *tdh* and/or *trh* genes or whether there are other genes necessary for virulence that may have been acquired concurrently or separately with *tdh* and/or *trh*. The body of knowledge on the *tdh* gene that has been acquired can serve as a paradigm to study the dynamics of bacterial virulence in *V. parahaemolyticus* and in other potentially pathogenic bacterial species in the natural environment.

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