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Structure, function and regulation of the thermostable direct hemolysin (TDH) in pandemic *Vibrio parahaemolyticus*

Qin Cai[a,b], Yiquan Zhang[b,*]

[a] The Fourth People 's Hospital of Zhenjiang, Zhenjiang, 212001, Jiangsu, PR China
[b] School of Medicine, Jiangsu University, Zhenjiang, 212013, Jiangsu, PR China

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ABSTRACT

Vibrio parahaemolyticus is a leading cause of seafood-associated bacterial gastroenteritis. The pathogen produces the thermostable direct hemolysin (TDH), which is the sole cause of the Kanagawa phenomenon (KP), a special β-type haemolysis in the Wagatsuma agar. TDH also exerts several other biological activities, the major includes lethal toxicity, cytotoxicity, and enterotoxicity. The structure and roles of TDH and the transcriptional regulation of *tdh* genes, are summarized in this review, which will give a better understanding of the pathogenesis of *V. parahaemolyticus*.

1. Introduction

Vibrio parahaemolyticus, a Gram-negative halophilic bacterium, is ubiquitous in coastal habitats and estuaries throughout the world [1]. It is a human pathogen that causes three types of diseases, i.e., gastroenteritis, wound infections, and septicemia [2]. Gastroenteritis is the most common syndrome with symptoms including fever, nausea, watery diarrhea and abdominal cramps, and usually derived from the consumption of raw or undercooked seafood [2,3]. *V. parahaemolyticus* is recognized as a leading cause of seafood-associated bacterial gastroenteritis in many countries including China [1,4–7].

Virulent *V. parahaemolyticus* strains usually produce the thermostable direct hemolysin (TDH) and/or the TDH-related hemolysin (TRH), both of which are also considered molecular markers for the pathogenicity of the species [8–11]. TDH and TRH had been reported to be expressed in very low percentages of nonclinical isolates, but a recent study found that 48% and 8.3% of environmental strains of *V. parahaemolyticus* had the *tdh* and trh gene, respectively [12]. TDH but not TRH can cause the β-type hemolysis in the Wagatsuma agar medium called the Kanagawa phenomenon (KP), and nearly all clinical isolates are KP positive [13,14]. Actually, the KP test had been applied to verify the virulence of *V. parahaemolyticus* isolates from environmental and clinical samples. In this present review, we summarize the structure of TDH and its roles in pathogenesis, and the regulation of its expression in *V. parahaemolyticus*.

2. Distribution of TDH coding genes in *V. parahaemolyticus*

In the 1980s, Kaper et al. first cloned and sequenced the *tdh* gene encoding TDH in *V. parahaemolyticus* [15,16]. After that, several *tdh* homologs were detected by the DNA hybridization test with a *tdh*-specific probe [17–19]. All KP-positive strains carried two chromosomal *tdh* gene copies designated *tdh1* and *tdh2*, respectively [18,20,21], while KP-negative but *tdh*-positive strains possessed the chromosomal *tdh3* and *tdh5* genes, and the plasmid-borne *tdh4* genes [18]. These *tdh* genes had over 96% identity in nucleotide sequences between each other [17,18,20]. However, the expression level of *tdh2* was much higher than that of the other four *tdh* genes [17,18,20], thus the production of *tdh2* gene was predominantly responsible for TDH activities in the KP-positive strains.

The *tdh* homologs are not detected solely in *V. parahaemolyticus* strains. Studies demonstrated that virulence-related genes including *tdh* occurred at high frequencies in non-*V. parahaemolyticus Vibrionaceae* species such as *V. diabolicus* CW-9-11-1, *V. alginolyticus* NS0607, *V. hollisae*, and *V. cholerae* non-01 [22–24]. The high occurrence of *tdh* in other vibrios not only increases the difficulty of tracking of outbreaks of *V. parahaemolyticus* infections but may also indicate other potential roles of TDH in the environmental strains. A strong piece of evidence for this hypothesis is that the densities of *V. parahaemolyticus* remain low, but the proportion of strains carrying *tdh* and *trh* has increased, two years post the 2010 Deepwater Horizon (DH) oil spill [25].

* Corresponding author.
E-mail address: zhangyiquanq@163.com (Y. Zhang).

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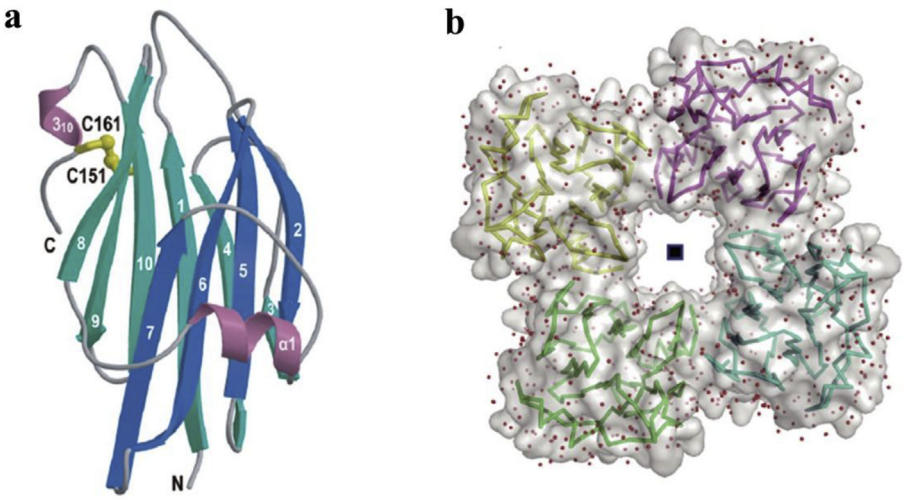

Fig. 1. Structures of TDH in *V. parahaemolyticus* [28]. **a) the ribbon structure of TDH monomer.** The yellow stick represents the intramolecular disulfide bond. **b) Structure of the TDH tetramer.** The different colored wires represent different individual monomer. The red spheres indicate water molecules. The black square represent the crystallographic 4-fold axis. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3. The crystal structure of TDH

TDH is a heat-stable and reversible amyloid toxin that can refold its structure depending on temperature changes [26]. It has been demonstrated that TDH can change its native structure into nontoxic fibrils rich in β-strands after being incubated at 60–70 °C, which can be further dissociated into unfolded states by being heated at 80–100 °C [26]. The unfolded TDH can be reversibly refolded into the native form after being rapidly cooled [26]. The native TDH is a C4-symmetric tetramer in an aqueous environment [27]. The TDH monomer contains 10 β-strands and two helices, α1 and 3_{10} (Fig. 1a) [28]. There is an intramolecular disulfide bond between Cys^{151} in β10 and Cys^{161} in 3_{10} (Fig. 1a) [28]. Four TDH monomers around the center axis form the tetramer with a central pore through the π-cation interaction between adjacent monomers (Fig. 1b) [28]. The C-terminal segment of each TDH monomer acts as a mediator, playing a key role in the oligomerization process [29]. The central pore functions as a channel during hemolysis, allowing both water and ions to flow freely [2,28]. Amino acid mutations of TDH at positions 53, 59, and 63 decreased the protein's hemolytic activity due to the conformational changes [23]. Thus, the maintenance of tetrameric structure with a central pore is indispensable for the TDH activity [28,30].

4. The biological activities of TDH in *V. parahaemolyticus*

TDH has lethal toxicity on mice. An earlier study by Honda et al. showed that the lethal effect of TDH was very rapid, because the injection of 5 μg of purified toxin into mice killed the animals within 1 min [31]. The 50% lethal dose (LD_{50}) of TDH was 1.1 μg per mouse when injected into mice intravenously [32]. In addition, TDH, the sole determinant of KP, is a pore-forming toxin that forms transmembrane pores in phospholipid bilayers of erythrocyte membranes, permeable to water and ions [13,27,33,34]. The imbalance of ions inside and outside of the cells causes membrane permeabilization and erythrocytes to swell, leading to lysis [34,35]. G_{T1} ganglioside was thought to be the receptor sites for TDH on erythrocyte membranes [36,37], however other studies reported contradictory results [38–40].

TDH has been observed to exhibit cytotoxicity in a variety of cell lines [41–44]. TDH induces cytotoxicity by acting both inside and outside of the cultured cells, and kills the cells by promoting apoptosis [41]. The cytotoxic effect of TDH is dependent on lipid rafts, among which sphingomyelin is the most important for the association of TDH with lipid rafts [40]. TDH induces the caspase-1 dependent pyroptosis through activating both the NLRP3 and NLRC4 inflammasomes [45]. Modulation of cytoskeletal organization and Ca^{2+} homeostasis also seems play key roles in TDH-dependent cytotoxicity [42,44,46]. Besides, TDH also can inhibit carcinoma cell proliferation and involves CaSR in its mechanism of action [47]. In addition, a new type of immunotoxin by conjugation of R46E mutation of TDH from *Grimontia hollisae* and an epidermal growth factor receptor-binding peptide showed obvious antitumor activity in a xenograft model of athymic nude mice [48], and thus, it opens a new possibility for the treatment of tumors via enhance of the anticancer drug's effect.

TDH has been also shown to be involved in the enterotoxigenic effect on small intestine and the watery diarrhea induced by *V. parahaemolyticus* infection [49–53]. Interaction of TDH with the colonic epithelial cells elevates the concentration of Ca^{2+} inside the cells, resulting in the secretion of Cl^- into the mucosal side of the epithelial layer from the serosal [50–52]. Although Ca^{2+} is a second messenger of the protein kinase C (PKC) phosphorylation pathway, Ca^{2+}-dependent Cl^- secretion is not associated with this pathway [53].

Thus it can be concluded that TDH is one of the major virulence determinants of *V. parahaemolyticus*, exerting several mainly biological activities including lethal toxicity, hemolytic activity, cytotoxicity, and enterotoxicity [2,14]. However, whether TDH has other unknown biological activities or not, needs to be further investigated.

5. Regulation of TDH expression

All KP-positive *V. parahaemolyticus* strains possess *tdh1* and *tdh2*, and both of them are encoded in the 80 kb pathogenicity island Vp-PAI [18,21]. An eprevious study showed that the transmembrane regulatory protein ToxR is essential for the expression of *tdh2* but not *tdh1* and TDH-mediated enterotoxicity *in vivo* [54]. ToxR activates *tdh2* transcription through binding to the sequence around the 144 bp nucleotide upstream of the coding region [54]. Moreover, the activation of *tdh2* by ToxR was culture medium dependent, and the most effective condition was the bacteria cultured in KP broth (2% peptone, 0.5% NaCl, 0.03 M KH_2PO_4, pH 6.2) [54]. Two other novel ToxR-like regulatory proteins VtrA and VtrB were reported to regulate the transcription of the genes encoded within the Vp-PAI including *tdh* genes [55,56]. Both *vtrA* and *vtrB* are located within the Vp-PAI region, and transcription of *vtrB* was under positive control of VtrA. Deletion of *vtrA* or *vtrB* downregulates the expression levels of over 60 genes, which were almost exclusively encoded in the Vp-PAI region. Thus, the regulatory proteins VtrA and VtrB are the two master regulators for the gene expression in the Vp-PAI. *V. parahaemolyticus* CalR, the LysR-type transcriptional regulatory protein, was also involved in the regulation of *tdh2* transcription [57]. CalR inhibits the transcription of *tdh2* through binding to the DNA region located from 224 to 318 bp upstream of *tdh2*, and thereby inhibits hemolytic activity of *V. parahaemolyticus*. The transcription of *calR* itself was directly activated by ToxR, and a feedback CalR repressed *toxR* and