Contribution of *Vibrio parahaemolyticus* Virulence Factors to Cytotoxicity, Enterotoxicity, and Lethality in Mice

Hirotaka Hiyoshi,1 Toshio Kodama,2 Tetsuya Iida,1* and Takeshi Honda2

Laboratory of Genomic Research on Pathogenic Bacteria, International Research Center for Infectious Diseases,1 and Department of Bacterial Infections,2 Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565-0871, Japan

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*Vibrio parahaemolyticus*, one of the human-pathogenic vibrios, causes three major types of clinical illness: gastroenteritis, wound infections, and septicemia. Thermostable direct hemolysin (TDH) secreted by this bacterium has been considered a major virulence factor of gastroenteritis because it has biological activities, including cytotoxic and enterotoxic activities. Previous reports revealed that *V. parahaemolyticus* strain RIMD2210633, which contains *tdh*, has two sets of type III secretion system (T3SS) genes on chromosomes 1 and 2 (T3SS1 and T3SS2, respectively) and that T3SS1 is responsible for cytotoxicity and T3SS2 is involved in enterotoxicity, as well as in cytotoxic activity. However, the relative importance and contributions of TDH and the two T3SSs to *V. parahaemolyticus* pathogenicity are not well understood. In this study, we constructed mutant strains with nonfunctional T3SSs from the *V. parahaemolyticus* strain containing *tdh*, and then the pathogenicities of the wild-type and mutant strains were evaluated by assessing their cytotoxic activities against HeLa, Caco-2, and RAW 264 cells, their enterotoxic activities in rabbit ileal loops, and their lethality in a murine infection model. We demonstrated that T3SS1 was involved in cytotoxic activities against all cell lines used in this study, while T3SS2 and TDH had cytotoxic effects on a limited number of cell lines. T3SS2 was the major contributor to *V. parahaemolyticus*-induced enterotoxicity. Interestingly, we found that both T3SS1 and TDH played a significant role in lethal activity in a murine infection model. Our findings provide new indications that these virulence factors contribute to and orchestrate each distinct aspect of the pathogenicity of *V. parahaemolyticus*.

*Vibrio parahaemolyticus* is a Gram-negative halophilic bacterium that inhabits estuarine and coastal waters and can be isolated from seafood (6, 7, 9). It causes acute gastroenteritis in humans after they consume contaminated raw or undercooked seafood. Although this microorganism is better known for seafood. Although this microorganism is better known for

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TABLE 1. Bacterial strains and plasmids used in this study

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E. coli strains

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MATERIALS AND METHODS

Bacterial strains and plasmids. V. parahaemolyticus RIMD2210633 (KP positive, serotype O3:K6) (27) was used for construction of deletion mutants and functional studies. All bacterial strains and plasmids used in this study are listed in Table 1.

Construction of the T3SS deletion mutants. The vscN1 deletion (nucleotides [nt] -225 to 1068) and in-frame vscN2 deletion (nt 160 to 978) strains of V. parahaemolyticus RIMD2210633 were constructed using deletion vectors (33) (Table 1) by introducing homologous recombination as previously described (33). Briefly, deletion vectors, which contained the sucB gene conferring sensitivity to sucrose, were introduced into Escherichia coli SM10 xpir and transferred to V. parahaemolyticus strain RIMD2210633 by conjugation. The mutant strains were selected using 10% sucrose and sensitivity to chloramphenicol.

Complementation of deleted genes in the mutant strains. Complementation of deleted genes was carried out according to the manufacturer’s instructions. To evaluate the neutralizing effect of anti-TDH antibodies (39) on cytotoxicity, bacterial suspensions were mixed with anti-TDH antibodies before infection.

Rabbit ileal loop test. The rabbit ileal loop test was performed as previously described, with slight modifications, and six rabbits were used in each experiment (33). The isogenic mutant strains of V. parahaemolyticus (106 CFU loop) or purified TDH (150 µg loop) was injected into the ligated ileal loop of a rabbit, which was followed by measurement of the fluid accumulation in each loop at 18 h after injection. To evaluate the effect of anti-TDH serum on enterotoxicity, the serum was mixed with a bacterial suspension or purified TDH before injection. Fluid accumulation (FA) ratios were calculated by determining the amount of accumulated fluid (in milliliters) per centimeter of ligated rabbit small intestine.

Murine infection model. Bacterial suspensions (106 CFU) were inoculated intraperitoneally into female C57/He/N mice that were 4 to 5 weeks old, after which we examined the symptoms and calculated the numbers of mice killed at specified times. All animal experiments were performed according to an institutional protocol approved by the Ethics Review Committee for Animal Experimentation of the Research Institute for Microbial Diseases (Osaka University, Osaka, Japan).

Western blot analysis. Secreted proteins were prepared as described previously (18). Samples used for Western blot analysis were separated by SDS-PAGE. Transferred membranes (Millipore, Bedford, MA) were probed with anti-VopD1 (33), anti-VopD2 polyclonal antibody (18), or anti-TDH monoclonal antibody (39) and then with horseradish peroxidase-conjugated goat anti-rabbit antibody (ZYMED) or rabbit anti-mouse antibody (ZYMED). The blots were developed with an ECL Western blot kit (Amersham Biosciences, Piscataway, NJ).

RPLA assay. The reverse passive latex agglutination (RPLA) assay was performed according to the manufacturer’s instructions (Denka Seiken Co. Ltd.).

Statistical analysis. Student’s t tests assuming unequal variances were used for statistical analyses (P values of <0.05 were considered statistically significant). An analysis of the murine survival ratio was performed with Kaplan-Meier and log rank tests (P values of <0.01 were considered statistically significant).
FIG. 1. TDH is secreted in a T3SS-independent manner, as shown by immunoblot analysis of bacterial supernatants from isogenic deriv-
atives of the *V. parahaemolyticus* RIMD2210633 strain grown for 6 h in LB broth containing 0.5% NaCl. The lanes contained wild-type strain RIMD2210633 (WT), a T3SS1-deficient strain derived from the WT strain (the ΔvscN1 strain), a T3SS2-deficient strain derived from the WT strain (the ΔvscN2 strain), a T3SS1- and T3SS2-deficient strain derived from the WT strain (the ΔvscN1 ΔvscN2 strain), a tdhAS mutant strain derived from the WT strain (POR-1), a T3SS1-deficient strain derived from POR-1 (POR-2), a T3SS2-deficient strain derived from POR-1 (POR-3), and a T3SS1- and T3SS2-deficient strain derived from POR-1 (the ΔvcrD1 ΔvcrD2 strain). Blots were probed with anti-VopD1 (top panel), anti-VopD2 polyclonal antibodies (middle panel), and anti-TDH monoclonal antibodies (bottom panel).

RESULTS

TDH is secreted in a T3SS-independent manner. In most previous studies examining the cytotoxicity and enterotoxicity of *V. parahaemolyticus* T3SSs, mutant strains derived from a tdhAS gene deletion mutant of *V. parahaemolyticus* (POR-1) were used in order to exclude the possibility of involvement of TDH in the phenotypes (1–3, 18, 19, 31, 33). It is therefore still not clear to what extents each of the T3SSs and TDH contribute to the cytotoxic and enterotoxic activities induced by wild-type *V. parahaemolyticus*. To address this issue, we constructed mutant strains with nonfunctional T3SSs from a *V. parahaemolyticus* wild-type strain (WT) possessing tdh. We used these strains, together with previously constructed mutant strains (Table 1), for subsequent studies. First, the capacities of these strains to secrete TDH, as well as T3SS1- and T3SS2-dependent proteins, were assessed by Western blotting. As shown in Fig. 1, VopD1, which is a T3SS1-secreted protein (33), was not detected in the supernatants of nonfunctional T3SS1 mutant strains derived from the WT strain (the ΔvscN1 and ΔvscN1 ΔvscN2 strains) or from the tdhAS gene mutant strain POR-1 (the POR-2 and ΔvcrD1 ΔvcrD2 strains) (Fig. 1, top panel). Similarly, VopD2, which is a T3SS2-secreted protein (18), was not detected in the supernatants of the T3SS2-deficient mutant strains derived from the WT strain (the ΔvscN2 and ΔvscN1 ΔvscN2 strains) or from the tdhAS gene mutant strain POR-1 (the POR-3 and ΔvcrD1 ΔvcrD2 strains) (Fig. 1, middle panel). Unlike secretion of the VopD1 and VopD2 proteins, secretion of TDH was not affected by the presence of T3SSs (Fig. 1, bottom panel). In addition, RPLA measurement with a KP reverse passive latex agglutination kit (Denka Seiken Co. Ltd.) (42) revealed that there were not significant differences in the amounts of TDH secreted regardless of the presence of the T3SSs (data not shown). These results suggest that neither the T3SS1 apparatus nor the T3SS2 apparatus is involved in TDH secretion.

T3SS1- and T3SS2-dependent cytotoxic effects. The cytotoxic activities of the tdh and T3SS deletion mutant strains were evaluated in terms of the release of cytosolic lactate dehydrogenase (LDH) from cultured cells. As shown in Fig. 2A, when HeLa cells were infected with the T3SS1-deficient mutant strains derived from the WT strain (the ΔvscN1 and ΔvscN1 ΔvscN2 strains) or from the tdhAS deletion mutant strain POR-1 (the POR-2 and ΔvcrD1 ΔvcrD2 strains), their cytotoxicities were dramatically less than the corresponding cytotoxicities resulting from WT and POR-1 infection, respectively. There was not a significant difference in cytotoxicity between the strains expressing TDH and the strains not expressing TDH (that is, between the WT and POR-1 strains, between the ΔvscN1 and POR-2 strains, between the ΔvscN2 and POR-3 strains, and between the ΔvscN1 ΔvscN2 and ΔvcrD1 ΔvcrD2 strains). Thus, T3SS1, but not T3SS2 and TDH, was a major contributor to the cytotoxic activity against HeLa cells observed. The cytotoxicity of POR-2 was fully restored by in trans complementation with the vcrD1 (pvdD1) gene, but not by in trans complementation with the tdh (pvdD4) gene (Fig. 2B). In the same way, when Caco-2 cells were infected with T3SS1-deficient strains derived from the WT strain (the ΔvscN1 strain) and from the POR-1 strain (strain POR-2), the cytotoxicities were significantly reduced compared with the corresponding cytotoxic activities observed in WT and POR-1 infections, respectively (Fig. 2C). However, the T3SS1-deficient strains showed partial cytotoxicity, and this cytotoxicity was dramatically reduced when the cells were infected with both T3SS1- and T3SS2-deficient strains derived from the WT strain (the ΔvscN1 ΔvscN2 strain) and from the POR-1 strain with tdhAS deleted (the ΔvcrD1 ΔvcrD2 strain). The cytotoxicities of the ΔvcrD1 ΔvcrD2 and ΔvscN1 ΔvscN2 strains were fully restored by in trans complementation with the vcrD1 (pvdD1) and vscN2 (pvdD2) genes, respectively (Fig. 2D). As determined for HeLa cells, no participation of TDH in cytotoxic activity against Caco-2 cells was observed. These results indicate that T3SS1 exhibits cytotoxic activity against both cell lines, whereas T3SS2 exhibits cytotoxic activity only against Caco-2 cells, as previously reported (19). Moreover, TDH secreted by bacteria had little effect on the cytotoxic activity against these cell lines.

T3SS1- and TDH-dependent cytotoxic activities against RAW 264 cells. We next examined the cytotoxic activities against cells of the nonepithelial, macrophage-like cell line RAW 264. When RAW 264 cells were infected with the T3SS1-deficient strain derived from the WT strain (the ΔvscN1 strain), as well as with the T3SS1- and T3SS2-deficient strain derived from the WT strain (the ΔvscN1 ΔvscN2 strain), the cytotoxicities were apparently reduced compared with the cytotoxicity of the WT strain, although the difference was not statistically significant (Fig. 3A). However, cytotoxicity was almost absent when the cells were infected with the T3SS1-deficient strain derived from the tdhAS mutant strain POR-1 (POR-2) or with the T3SS1- and T3SS2-deficient strain derived from POR-1 (the ΔvcrD1 ΔvcrD2 strain) (Fig. 3A). The cytotoxicity of the ΔvcrD1 ΔvcrD2 strain was fully restored by in trans complementation with the vcrD1 (pvdD1) and tdh (pvdD4) genes (Fig. 3B). Thus, not only T3SS1-dependent cytotoxic activity but also TDH-dependent cytotoxic activity against RAW 264 cells was observed. The latter activity was confirmed by using both a TDH-neutralizing monoclonal antibody (MAB 1-24) and anti-TDH serum (Fig. 3C). MAB 1-24
is known to be capable of neutralizing hemolysis induced by purified TDH by inhibiting it in the postbinding process (39). The cytotoxic activities observed with the T3SS1-deficient strain derived from the WT strain (the ΔvscN1 strain), the ΔvscN2 strain (T3SS2 deficient), the ΔvscN1 ΔvscN2 strain (T3SS1 and T3SS2 deficient), POR-1 (tdhAS mutant), POR-2 (tdhAS and T3SS1 deficient), POR-3 (tdhAS and T3SS2 deficient), the ΔvcrD1 ΔvcrD2 strain (tdhAS, T3SS1, and T3SS2 deficient), POR-2/ΔvcrD1 (POR-2 complemented with vcrD1), POR-2/ΔtdhA (POR-2 complemented with tdhA), the ΔvcrD1 ΔvcrD2/ΔvcrD1 strain (the ΔvcrD1 ΔvcrD2 strain complemented with vcrD1), and the ΔvscN1 ΔvscN2/pvscN2 strain (the ΔvscN1 ΔvscN2 strain complemented with vscN2). The error bars indicate standard deviations for results from triplicate (A and C) or quadruplicate (B and D) independent experiments. The asterisks indicate that results were significantly different from the results obtained with the WT strain (*, P < 0.05; **, P < 0.01).

**RAW 264 cells are very sensitive to TDH.** As shown above, TDH secreted by bacteria could induce cytotoxic activity only against RAW 264 cells in the present study. We therefore evaluated the sensitivities of the cell lines to purified TDH. As shown in Fig. 3D, the cytotoxic activity of purified TDH against RAW 264 cells was saturated at a concentration of 1 μg/ml. For HeLa and Caco-2 cells, on the other hand, the cytotoxic activities of TDH at a concentration of 100 μg/ml were only 19.2% ± 8.7% and 15.4% ± 7.3%, respectively. These results show that RAW 264 cells were much more sensitive to purified TDH than HeLa and Caco-2 cells were.

**T3SS2 is necessary for enterotoxicity.** It was thought until recently that TDH is the major contributor to enterotoxicity since purified TDH exhibits enterotoxic activity (12, 26, 38). Recent studies using TDH-deficient strains, however, showed that T3SS2 is also involved in enterotoxicity (18, 33). However, the relative contributions of these two virulence factors to enterotoxicity have not been examined yet. To address this issue, we used the rabbit ileal loop test to evaluate the enterotoxic activities of T3SS-deficient strains derived from the WT strain or from the strain with tdhAS deleted. As shown in Fig. 4A, although the enterotoxic activities of a tdhAS-deficient strain (POR-1) and a tdh- and T3SS1-deficient strain (POR-2) appeared to be slightly reduced compared with that of the WT or ΔvscN1 strain, there was no significant difference between these strains. However, the levels of enterotoxic activity of T3SS2-deficient mutant strains, as well as those of the T3SS1-
and T3SS2-deficient strains derived from the WT strain (the \( \Delta \text{vscN2} \) and \( \Delta \text{vscN1} \Delta \text{vscN2} \) strains) and from POR-1 (the \( \text{POR-2} \) strain), were the same as the level of enterotoxic activity of the noninfected control (0.5% NaCl in LB broth). No difference in enterotoxicity due to the presence of T3SS1 was observed. The decrease in the enterotoxicity of the \( \Delta \text{vscN2} \) strain was restored by transcomplementation with the \( \text{vscN2} \) gene \((\text{p} \text{vscN2})\) at the same level that is present in the WT strain (Fig. 4B). These findings strongly suggest that T3SS2, but not TDH and T3SS1, is a major contributor to \( \text{V. parahaemolyticus} \)-induced enterotoxicity in the present rabbit model.

**Effect of anti-TDH serum on enterotoxicity induced by \( \text{V. parahaemolyticus} \).** The effect of anti-TDH serum on purified-TDH-induced or \( \text{V. parahaemolyticus} \)-induced enterotoxicity was also examined. The fluid accumulation was pronounced as a result of injection of purified TDH, but it was completely inhibited by the anti-TDH serum (Fig. 5A). Although this anti-TDH serum could also neutralize the cytotoxicity caused by TDH secreted by bacteria observed in RAW 264 cells (Fig. 3C), the enterotoxicity induced by the WT strain was not inhibited at all (Fig. 5B). These results indicate that TDH secreted by bacteria is not responsible for the enterotoxicity caused by \( \text{V. parahaemolyticus} \) infection.

**TDH and T3SS1 are responsible for lethality in mice.** Although \( \text{V. parahaemolyticus} \) can cause not only gastroenteritis but also wound infections and septicemia, there is little experimental or epidemiological information about these effects. A previous study found that the difference between the lethality in mice induced by intraperitoneal infection with a KP-positive \( \text{V. parahaemolyticus} \) strain and the lethality in mice induced by intraperitoneal infection with a KP-negative \( \text{V. parahaemolyticus} \) strain was not significant, suggesting that another virulence factor(s) besides TDH could inhibit the anti-TDH serum (Fig. 5A). Although this anti-TDH serum could also neutralize the cytotoxicity caused by TDH secreted by bacteria observed in RAW 264 cells (Fig. 3C), the enterotoxicity induced by the WT strain was not inhibited at all (Fig. 5B). These results indicate that TDH secreted by bacteria is not responsible for the enterotoxicity caused by \( \text{V. parahaemolyticus} \) infection.

**FIG. 3.** T3SS1- and TDH-dependent cytotoxic activity against RAW 264 cells. (A and B) RAW 264 cells were infected with the RIMD2210633 (WT) strain or the isogenic mutants indicated at a multiplicity of infection (MOI) of 10. At 3 h after infection, cytotoxic activity was evaluated by determining the amount of LDH released (relative to the amount of LDH released from uninfected cells treated with detergent, which was defined as 100%). The mutants tested were the \( \Delta \text{vscN1} \) strain (T3SS1 deficient), the \( \Delta \text{vscN2} \) strain (T3SS2 deficient), the \( \Delta \text{vscN1} \Delta \text{vscN2} \) strain (T3SS1 and T3SS2 deficient), \( \text{POR-1} \) (\( \text{vscN1} \) mutant), \( \text{POR-2} \) (\( \text{vscN2} \) and T3SS1 deficient), \( \text{POR-3} \) (\( \text{vscN2} \) and T3SS2 deficient), the \( \Delta \text{vcrD1} \Delta \text{vcrD2} \) strain (\( \text{vscN2} \), T3SS1, and T3SS2 deficient), \( \text{POR-2/pvcrD1} \) (\( \text{POR-2} \) complemented with \( \text{vcrD1} \)), and \( \text{POR-2/tdHA} \) (\( \text{POR-2} \) complemented with \( \text{tdHA} \)). (C) RAW 264 cells with anti-TDH neutralizing monoclonal antibody (MAb 1-24), preimmune serum, or anti-TDH serum were infected with the \( \Delta \text{vscN1} \) and \( \Delta \text{vscN1} \Delta \text{vscN2} \) strains. Three hours after infection, cytotoxic activity was evaluated by determining the amount of LDH released. (D) Various concentrations of purified TDH (0.001 to 100 \( \mu \text{g/ml} \)) were used to challenge \( \text{HeLa} \), \( \text{Caco-2} \), and RAW 264 cells for 1 h. Cytotoxic activity was assayed by measuring the total amount of cellular LDH released into the culture supernatant. The error bars indicate standard deviations for results from triplicate (A, C, and D) or quadruplicate (B) independent experiments. Two asterisks indicate that the results are significantly different from the results obtained with the WT strain (\( P < 0.01 \)).
We therefore examined the possible involvement of TDH and T3SSs in a murine infection model. The murine infection model is used as an animal sepsis model in studies of *Vibrio vulnificus*, which causes primary septicemia (41). To determine whether TDH is responsible for lethality in mice, the 50% lethal dose (LD50) of the WT strain was compared with that of *tdhAS*-deficient strain POR-1, and no significant difference in LD50 was observed between the WT and POR-1 strains (7.0 logs and 7.6 logs, respectively); the LD50s obtained were in the same range as the LD50s reported previously (10). Next, the lethal effects of T3SS-deficient strains derived from POR-1 were determined. Surprisingly, when T3SS1-deficient mutant strains, such as POR-2 (*tdhAS* and T3SS1-deficient strain) and the *vcrD1*/*vcrD2* strain (*tdhAS*, T3SS1, and T3SS2-deficient strain), were used, the lethality in mice was significantly reduced compared with that of the parent strain POR-1 and the *tdhAS* and T3SS2-deficient strain POR-3 (Fig. 6A), while no contribution of T3SS2 to this lethal activity was observed (P values were not statistically significant as determined by a log rank test). We next examined whether T3SS1 is the sole virulence factor of *V. parahaemolyticus* in this murine infection model by using T3SS-deficient mutants derived from the TDH-expressing

be involved in these pathogenic activities (10). We therefore examined the possible involvement of TDH and T3SSs in a murine infection model. The murine infection model is used as an animal sepsis model in studies of *Vibrio vulnificus*, which causes primary septicemia (41). To determine whether TDH is responsible for lethality in mice, the 50% lethal dose (LD50) of the WT strain was compared with that of *tdhAS*-deficient strain POR-1, and no significant difference in LD50 was observed between the WT and POR-1 strains (7.0 logs and 7.6 logs, respectively); the LD50s obtained were in the same range as the LD50s reported previously (10). Next, the lethal effects of T3SS-deficient strains derived from POR-1 were determined. Surprisingly, when T3SS1-deficient mutant strains, such as POR-2 (*tdhAS* and T3SS1-deficient strain) and the *ΔvcrD1 ΔvcrD2* strain (*tdhAS*, T3SS1, and T3SS2-deficient strain), were used, the lethality in mice was significantly reduced compared with that of the parent strain POR-1 and the *tdhAS* and T3SS2-deficient strain POR-3 (Fig. 6A), while no contribution of T3SS2 to this lethal activity was observed (P values were not statistically significant as determined by a log rank test). We next examined whether T3SS1 is the sole virulence factor of *V. parahaemolyticus* in this murine infection model by using T3SS-deficient mutants derived from the TDH-expressing
strain (WT). Unexpectedly, all mutant strains showed lethal activity against the mice in spite of the absence of T3SSs (Fig. 6B). Moreover, when mice were infected intraperitoneally with POR-2/p\textit{vcrD1} or POR-2/p\textit{tdhA}, lethality was restored (Fig. 6C). This indicates that TDH and T3SS1 may have additive effects on virulence against mice, suggesting that, in addition to T3SS1, TDH may also be responsible for lethality in mice infected with \textit{V. parahaemolyticus}.

**DISCUSSION**

In this study, our aim was to determine the roles of TDH and T3SSs in the three distinct aspects of the pathogenicity (cytotoxicity, enterotoxicity, and septicemia) of \textit{V. parahaemolyticus} infection.

First, using an immunoblotting assay, we found that TDH-, T3SS1-, and T3SS2-dependent proteins were secreted separately via their own secretion systems (Fig. 1). Our previous studies demonstrated that secretion of the T3SS1 and T3SS2 effector proteins is correlated with the translocation of these proteins into host cells (19, 33). Although TDH possesses a putative signal peptide sequence which is essential for secretion by the sec secretory pathway (30, 35) (i.e., a type II secretion system), it was possible that TDH is also secreted via the T3SS2 apparatus because the \textit{tdh} genes are located proximate to the T3SS2 region in chromosome 2 (21). In the present study, we could not obtain positive evidence showing that TDH is secreted via the T3SSs (Fig. 1).

Our investigation demonstrated that T3SS1 is the dominant contributor to \textit{V. parahaemolyticus} cytotoxicity, while T3SS2 and TDH can induce cytotoxic activity against only a limited number of cell lines (Fig. 2 and 3), even though there are no
apparent differences in adherence to the host cells among mutant strains (data not shown). TDH-dependent cytotoxic activity was observed with RAW 264 cells (Fig. 3A) and was completely inhibited by addition of TDH-neutralizing monoclonal antibody MAb 1-24 and anti-TDH serum (Fig. 3B). In addition, we showed that RAW 264 cells are more sensitive than other cell lines to purified TDH (Fig. 3C), which suggests that this is the most likely reason that TDH-dependent cytotoxicity is observed only with RAW 264 cells.

Analysis and determination of virulence factors involved in enterotoxicity are important for elucidating the pathogenicity of *V. parahaemolyticus*, since the most common symptom of infection by this bacterium is gastroenteritis. We showed that there was no difference in enterotoxicity due to the presence of TDH or T3SS1 under our experimental conditions (Fig. 4). Furthermore, anti-TDH serum could not suppress the fluid accumulation caused by wild-type *V. parahaemolyticus* (Fig. 5B), even though the same concentration of the anti-TDH serum could inhibit bacterially secreted TDH-dependent cytotoxicity observed with RAW 264 cells (Fig. 3C), as well as purified TDH-induced enterotoxicity (Fig. 5A). These results agree with those of a previous study, which suggested that accumulation of fluid induced by *V. parahaemolyticus* is not directly related to TDH but may be caused by another factor(s) (14). It is possible that the predicted but as-yet-unknown factor(s) responsible for *V. parahaemolyticus*-induced enterotoxicity is an effector(s) injected by T3SS2. In contrast to our results, it was reported previously that the tdhAS deletion mutant strain did not exhibit enterotoxicity or exhibited partially reduced enterotoxicity in the rabbit ileal loop test (29, 32). Although the reason for this difference is not clear, it may be due to experimental differences in (i) bacterial strains (RIMD2210633 was used the present study, while in a previous study [29] AQ3815 was used), (ii) the number of bacteria used for infection (we used 10⁹ cells/loop, while previous studies used 10⁷ cells/loop [29, 32]), and (iii) growth media (we used LB broth supplemented with 0.5% NaCl, and other investigators used brain heart infusion broth supplemented with 2% NaCl [29] or LB broth supplemented with 3% NaCl [32]). We believe that especially the difference in the salt concentrations of the culture media may be the cause of the difference since T3SS2 genes tend to be expressed in media with low salt concentrations (unpublished data). It stands to the reason that the conditions of T3SS2 gene expression were not taken into consideration in previous studies since T3SS2 of *V. parahaemolyticus* had not been identified at that time. The culture conditions used in our study can evaluate enterotoxic activity more accurately since they allow expression of not only TDH but also both T3SSs (Fig. 1 to 3). We therefore concluded that T3SS2 is the dominant contributor to the enterotoxicity of *V. parahaemolyticus*. Although there have been several reports about T3SS2 effectors (19, 20, 40), the relationship between these effectors and T3SS2-dependent enterotoxicity remains unknown. Future studies need to explore whether any of these effectors are involved in T3SS2-dependent enterotoxicity.

Although it is known that *V. parahaemolyticus* can cause wound infections and septicemia, there is little experimental or epidemiological information about the symptoms, except for one previous study which reported that the difference in mouse lethality between KP-positive and KP-negative strains was not significant (10). In our study, we demonstrated the importance of T3SS1 and TDH in virulence in the murine infection model. As shown in Fig. 6A, we found that a TDH null mutant strain (POR-1) could have a lethal effect on mice when they were infected intraperitoneally and that T3SS1 made a major contribution to the lethal activity. These results can account for the previously reported finding that the difference in mouse lethality between KP-positive and KP-negative strains was not significant, because T3SS1-related genes were conserved in all *V. parahaemolyticus* strains regardless of whether they were KP positive or KP negative (16, 22). Although there was no significant difference in LD₅₀ between WT and the tdhAS deletion mutant strain POR-1 (7.0 logs and 7.6 logs, respectively), TDH seemed to be partially involved in lethality. All mice died when they were infected by *V. parahaemolyticus* containing tdhAS regardless of the presence of T3SSs (Fig. 6B). In addition, we showed that both TDH and T3SS1 contributed to lethality in mice by performing a complementation study (Fig. 6C). The findings suggest that TDH and T3SS1 have additive effects on virulence for mice. In the case of *V. vulnificus*, a previous study determined that a high level of cytotoxic activity against macrophages is responsible for lethality in mice (41). Because TDH and T3SS1 contribute to cytotoxic activity against macrophage-like cell line RAW 264 (Fig. 3), this may be the reason why TDH and T3SS1, but not T3SS2, are responsible for lethality in mice. Although there have been no epidemiological studies examining whether *V. parahaemolyticus* strains isolated from wound infections and septicemia patients are KP positive or KP negative, the prevalence of the T3SS1 genes in *V. parahaemolyticus* strains makes it reasonable to assume that not only KP-positive strains but also KP-negative strains are isolated from patients with such infections. Since there is no clinical evidence, genotypic analysis of strains isolated from patients with wound infections and septicemia may be needed.

In this study, we determined the roles of TDH and T3SSs in the pathogenicity of *V. parahaemolyticus*. It is interesting that each virulence factor appears to have a specific role in a distinct aspect of each pathogenic mechanism. Although the question of which effector protein(s) is responsible for T3SS2-dependent enterotoxicity and which effector protein(s) is responsible for T3SS1-dependent mouse lethality remains to be answered, we expect that our findings will be a stepping stone toward understanding the pathogenic mechanism of *V. parahaemolyticus*.

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**REFERENCES**


