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

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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 causing gastroenteritis, it also can cause wound infections and septicemia (5, 23, 36).

Most clinical isolates of *V. parahaemolyticus* from patients with diarrhea show β-hemolysis on Wagatsuma agar (37). This phenomenon is known as the Kanagawa phenomenon (KP) and is considered a good marker to distinguish between pathogenic and nonpathogenic *V. parahaemolyticus* strains (25, 37). Thermostable direct hemolysin (TDH), which is responsible for the KP (28, 43), has multiple biological activities, including hemolysis, enterotoxicity, cytotoxicity, and cardiotoxicity (11–13, 26, 29, 32, 34, 38). For this reason, TDH has been considered a major virulence factor of *V. parahaemolyticus*.

Whole-genome sequencing of a KP-positive *V. parahaemolyticus* strain revealed that this strain contains two sets of gene clusters for the type III secretion system (T3SS), T3SS1 and T3SS2, one on each of its two chromosomes (21). T3SS gene clusters have been detected in numerous Gram-negative animal and plant pathogens, where they enable delivery of virulence factor proteins (effectors) into the cytosol of eukaryotic cells (4, 8, 15). *V. parahaemolyticus* T3SS1 is reportedly involved in cytotoxic activity against a variety of cell lines (1, 2, 19, 31), while T3SS2 has been demonstrated to be involved in enterotoxicity in the rabbit ileal loop test, as well as in cytotoxic activity against Caco-2 and HCT-8 cells (18, 19, 33). However, these studies used a *tdhAS* deletion mutant strain of *V. parahaemolyticus* (POR-1) as the parent for construction of the T3SS deletion mutations to exclude any biological activities of TDH. It is therefore still not clear what the contribution of T3SSs to these activities is under conditions in which TDH is produced.

V. parahaemolyticus sometimes also causes wound infections and septicemia (5, 23, 36). Unlike the information for gastrointestinal illness, there is no epidemiological information that indicates a correlation between any known virulence factor of *V. parahaemolyticus* and septicemia. The results of an intraperitoneal challenge experiment indicated that the lethality in mice due to *V. parahaemolyticus* infection occurred irrespective of the production of TDH, suggesting that another virulence factor(s) besides TDH may contribute to *V. parahaemolyticus*-induced septicemia (10).

In this study, we constructed mutant strains with nonfunctional T3SSs from a *V. parahaemolyticus* strain possessing *tdh* to investigate the roles and contributions of the two T3SSs and TDH to the pathogenicity of *V. parahaemolyticus* (i.e., cytotoxic activity against several cell lines, enterotoxicity in the rabbit ileal loop test, and lethality in mice).

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
V. parahaemolyticus strains		
RIMD2210633 (WT)	Clinical isolate; KP positive; serotype O3:K6	27
Δ*vscN1*	T3SS1-deficient strain; *vscN1* deletion mutant (deletion from nt −225 to 1068 of the gene) derived from WT	This study
Δ*vscN2*	T3SS2-deficient strain; in-frame *vscN2* deletion mutant (deletion from nt 160 to 978 of the gene) derived from WT	This study
Δ*vscN1* Δ*vscN2*	T3SS1- and T3SS2-deficient strain; *vscN1* (deletion from nt −225 to 1068 of the gene) and *vscN2* (deletion from nt 160 to 978 of the gene) double-deletion mutant derived from WT	This study
POR-1	*tdhAS* null mutant strain; *tdhAS* deletion mutant derived from WT	32
POR-2	TDH- and T3SS1-deficient strain; in-frame *vcrD1* deletion mutant (deletion from nt 640 to 1731 of the gene) derived from POR-1	33
POR-3	TDH- and T3SS2-deficient strain; in-frame *vcrD2* deletion mutant (deletion from nt 315 to 761 of the gene) derived from POR-1	33
Δ*vcrD1* Δ*vcrD2*	TDH-, T3SS1-, and T3SS2-deficient strain; *vcrD1* (deletion from nt 640 to 1731 of the gene) and *vcrD2* (deletion from nt 315 to 761 of the gene) double-deletion mutant derived from POR-1	19
E. coli strains		
DH5α	F$^-$ ϕ80*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17 phoA supE44 thi-1 gyrA96 relA1* λ$^-$	Novagen
SM10 λ*pir*	*thi thr leu tonA lacY supE recA*::RP4-2Tc::Mu λ*pir* R6K	24
Plasmids		
pT7bule	Multicopy (ColE1 *ori*) TA cloning vector, Amp^r	Novagen
pYAK1	R6K-ori suicide vector for gene replacement, Cm^r	17
pYAK1-Δ*vscN1*	Derivative of suicide vector pYAK1 for generating the *vscN1* deletion mutant	33
pYAK-Δ*vscN2*	Derivative of suicide vector pYAK1 for generating the *vscN2* deletion mutant	33
pSA19Cm-MCS	Complement vector for *V. parahaemolyticus*, Cm^r	18
pSACm-1	pSA19Cm-MCS containing promoter of *vopN1-vopD1* operon in EcoRI-BamHI site	31
pSA-tdhP	pSA19Cm-MCS containing promoter of *tdhA* in EcoRI-SmaI site	18
p*tdhA*	Derivative of pSACm-MCS containing *tdhA* loci	This study
p*vcrD1*	Derivative of pSACm-1 containing *vcrD1* loci	This study
p*vscN2*	Derivative of pSA-tdhP containing *vscN2* loci	This study

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 *sacB* gene conferring sensitivity to sucrose, were introduced into *Escherichia coli* SM10 λ*pir* and transferred to *V. parahaemolyticus* strain RIMD2210633 by conjugation. The mutant strains were selected using resistance to 10% sucrose and sensitivity to chloramphenicol.

Complementation of deleted genes in the mutant strains. Complementation of deleted genes was carried out follows. The DNA region including a deleted gene was amplified by PCR using the following primers: for complementation of *tdhA*, tdhA-comp-F (5′-GGATCCGGCCATGTTACCGCTTGAGG-3′) and tdhA-comp-R (5′-GTCGACGCCTTATCGCTTGGTCGATAGCTGG-3′); for complementation of *vcrD1*, vcrD1-comp-F (5′-GGATCCACAACTG GACCAGCGTTACCT-3′) and vcrD1-comp-R (5′-GTCGACCGTCACTTCG ATCCCCGTTTG-3′); and for complementation of *vscN2*, vscN2-comp-F (5′-GGATCCTTCATTTCAGGTGATTGAATAATGCTCAAG-3′) and vscN2-comp-R (5′-GTCGACGCTTCCTTGCTTGCTTTTCTATCC-3′). Each amplified fragment was cloned, and its sequence was ascertained. The insert was excised by restriction digestion with BamHI and SalI (at the sites underlined above) and cloned into the appropriate vector as described in Table 1. Each plasmid was introduced into *V. parahaemolyticus* mutant strains by electroporation (1.5 kV, 100 Ω, 25 μF).

Cytotoxicity assays. The cytotoxic assays were performed as described previously (19). Briefly, eukaryotic cells (HeLa, Caco-2, and RAW 264 cells) were infected at a multiplicity of infection (MOI) of 10. After infection, the release of lactate dehydrogenase (LDH) into the medium was quantified with a CytoTox96 kit (Promega, Madison, WI) used 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* (10^9 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 (10^8 CFU) were inoculated intraperitoneally into female C3H/HeN 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 using an experimental 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, NY).

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).

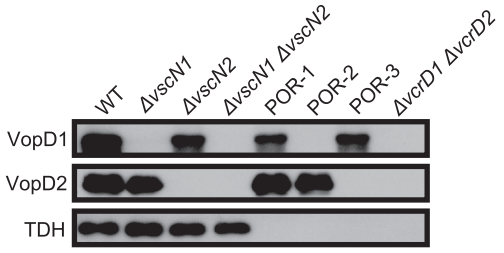

FIG. 1. TDH is secreted in a T3SS-independent manner, as shown by immunoblot analysis of bacterial supernatants from isogenic derivatives of the *V. parahaemolyticus* RIMD2210633 strain grown for 6 h in LB broth containing 0.5% NaCl. The lanes contained wild-type strain RIMD2210633 (WT), a T3SS1-deficient strain derived from the WT strain (the Δ*vscN1* strain), a T3SS2-deficient strain derived from the WT strain (the Δ*vscN2* strain), a T3SS1- and T3SS2-deficient strain derived from the WT strain (the Δ*vscN1* Δ*vscN2* 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 Δ*vcrD1* Δ*vcrD2* 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 Δ*vscN1* and Δ*vscN1* Δ*vscN2* strains) or from the *tdhAS* gene mutant strain POR-1 (the POR-2 and Δ*vcrD1* Δ*vcrD2* 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 Δ*vscN2* and Δ*vscN1* Δ*vscN2* strains) or from the *tdhAS* gene mutant strain POR-1 (the POR-3 and Δ*vcrD1* Δ*vcrD2* 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 Δ*vscN1* and Δ*vscN1* Δ*vscN2* strains) or from the *tdhAS* deletion mutant strain POR-1 (the POR-2 and Δ*vcrD1* Δ*vcrD2* 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 Δ*vscN1* and POR-2 strains, between the Δ*vscN2* and POR-3 strains, and between the Δ*vscN1* Δ*vscN2* and Δ*vcrD1* Δ*vcrD2* 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* (p*vcrD1*) gene, but not by in *trans* complementation with the *tdh* (p*tdhA*) gene (Fig. 2B). In the same way, when Caco-2 cells were infected with T3SS1-deficient strains derived from the WT strain (the Δ*vscN1* 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 Δ*vscN1* Δ*vscN2* strain) and from the POR-1 strain with *tdhAS* deleted (the Δ*vcrD1* Δ*vcrD2* strain). The cytotoxicities of the Δ*vcrD1* Δ*vcrD2* and Δ*vscN1* Δ*vscN2* strains were fully restored by in *trans* complementation with the *vcrD1* (p*vcrD1*) and *vcsN2* (p*vscN2*) 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 Δ*vscN1* strain), as well as with the T3SS1- and T3SS2-deficient strain derived from the WT strain (the Δ*vscN1* Δ*vscN2* 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 Δ*vcrD1* Δ*vcrD2* strain) (Fig. 3A). The cytotoxicity of the Δ*vcrD1* Δ*vcrD2* strain was fully restored by in *trans* complementation with the *vcrD1* (p*vcrD1*) and *tdh* (p*tdhA*) 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

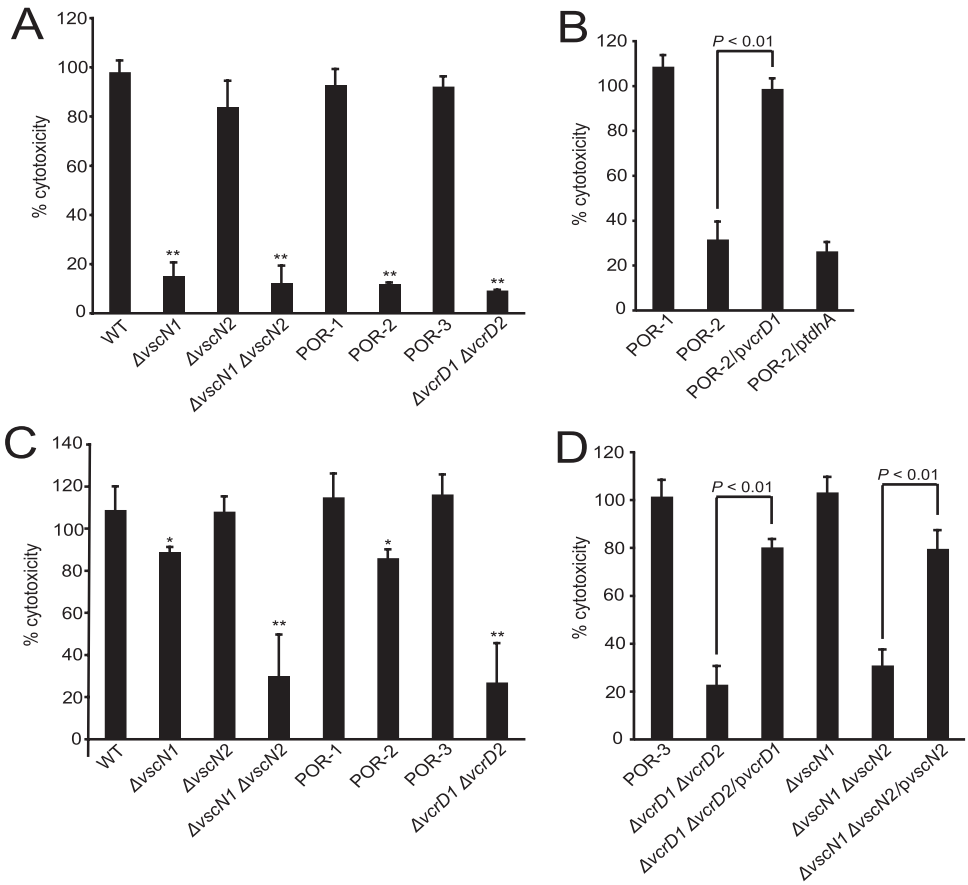

FIG. 2. T3SS1- and T3SS2-dependent cytotoxic activity. HeLa cells (A and B) and Caco-2 cells (C and D) were infected with the RIMD2210633 (WT) strain or the isogenic mutants indicated at a multiplicity of infection (MOI) of 10. At 4.5 h (HeLa cells) and 6 h (Caco-2 cells) after infection, cytotoxic activity 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 Δ*vscN1* strain (T3SS1 deficient), the Δ*vscN2* strain (T3SS2 deficient), the Δ*vscN1* Δ*vscN2* strain (T3SS1 and T3SS2 deficient), POR-1 (*tdhAS* mutant), POR-2 (*tdhAS* and T3SS1 deficient), POR-3 (*tdhAS* and T3SS2 deficient), the Δ*vcrD1* Δ*vcrD2* strain (*tdhAS*, T3SS1, and T3SS2 deficient), POR-2/p*vcrD1* (POR-2 complemented with *vcrD1*), POR-2/p*tdhA* (POR-2 complemented with *tdhA*), the Δ*vcrD1* Δ*vcrD2*/p*vcrD1* strain (the Δ*vcrD1* Δ*vcrD2* strain complemented with *vcrD1*), and the Δ*vscN1* Δ*vscN2*/p*vscN2* strain (the Δ*vscN1* Δ*vscN2* 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 (*, $P < 0.05$; **, $P < 0.01$).

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 Δ*vscN1* strain) and with the T3SS1- and T3SS2-deficient strain derived from the WT (the Δ*vscN1* Δ*vscN2* strain) were neutralized by both MAb 1-24 and anti-TDH serum. These results strongly indicate that not only T3SS1 but also TDH secreted by bacteria is responsible for the cytotoxic activity against RAW 264 cells.

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 *tdh*-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 Δ*vscN1* 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-

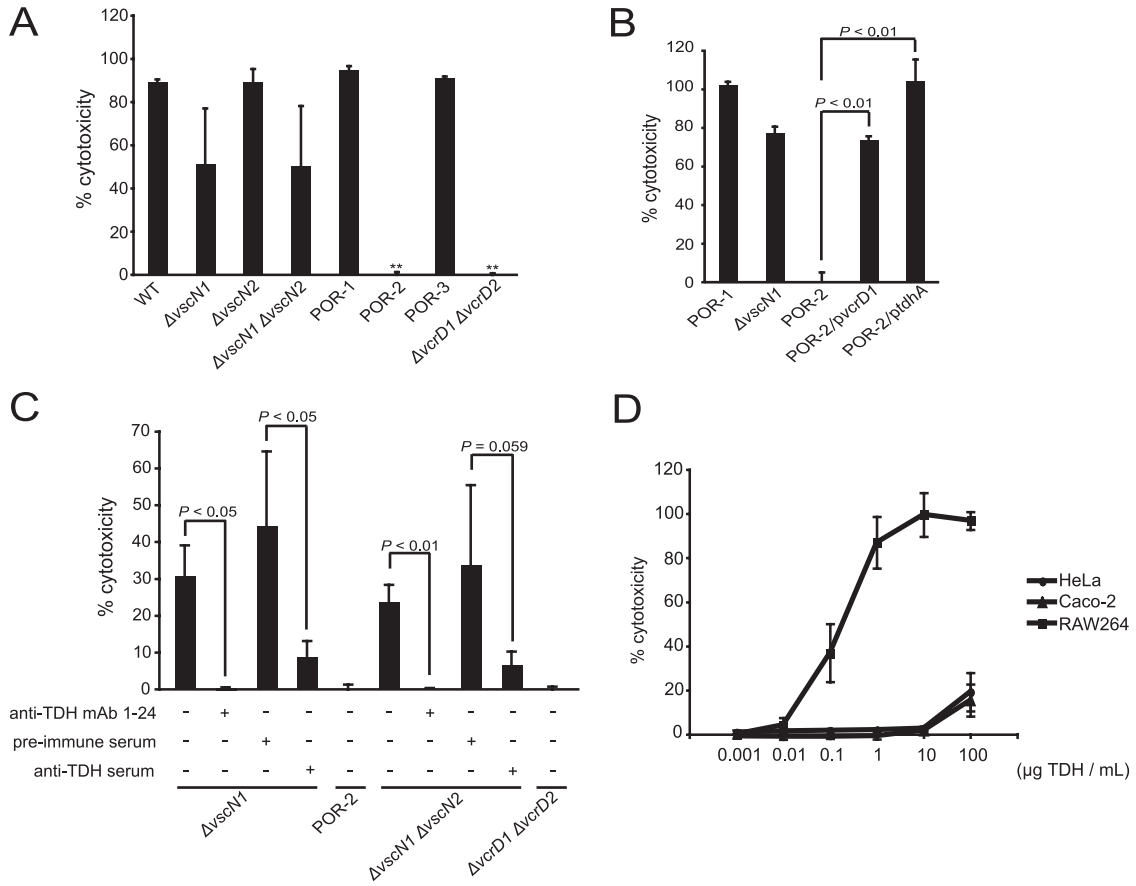

FIG. 3. T3SS1- and TDH-dependent cytotoxic activity against RAW 264 cells. (A and B) RAW 264 cells were infected with the RIMD2210633 (WT) strain or the isogenic mutants indicated at a multiplicity of infection (MOI) of 10. At 3 h after infection, cytotoxic activity was evaluated by determining the amount 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 Δ*vscN1* strain (T3SS1 deficient), the Δ*vscN2* strain (T3SS2 deficient), the Δ*vscN1* Δ*vscN2* strain (T3SS1 and T3SS2 deficient), POR-1 (*tdhAS* mutant), POR-2 (*tdhAS* and T3SS1 deficient), POR-3 (*tdhAS* and T3SS2 deficient), the Δ*vcrD1* Δ*vcrD2* strain (*tdhAS*, T3SS1, and T3SS2 deficient), POR-2/p*vcrD1* (POR-2 complemented with *vcrD1*), and POR-2/p*tdhA* (POR-2 complemented with *tdhA*). (C) RAW 264 cells with anti-TDH neutralizing monoclonal antibody (MAb 1-24), preimmne serum, or anti-TDH serum were infected with the Δ*vscN1* and Δ*vscN1* Δ*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 μg/ml) were used to challenge HeLa, 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 ($P < 0.01$).

and T3SS2-deficient strains derived from the WT strain (the Δ*vscN2* and Δ*vscN1* Δ*vscN2* strains) and from POR-1 (the POR-3 and Δ*vcrD1* Δ*vcrD2* strains), 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 Δ*vscN2* strain was restored by transcomplementation with the *vscN2* gene (p*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 *V. parahaemolyticus*-induced enterotoxicity in the present rabbit model.

Effect of anti-TDH serum on enterotoxicity induced by *V. parahaemolyticus*. The effect of anti-TDH serum on purified-TDH-induced or *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 *V. parahaemolyticus* infection.

TDH and T3SS1 are responsible for lethality in mice. Although *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 *V. parahaemolyticus* strain and the lethality in mice induced by intraperitoneal infection with a KP-negative *V. parahaemolyticus* strain was not significant, suggesting that another virulence factor(s) besides TDH could

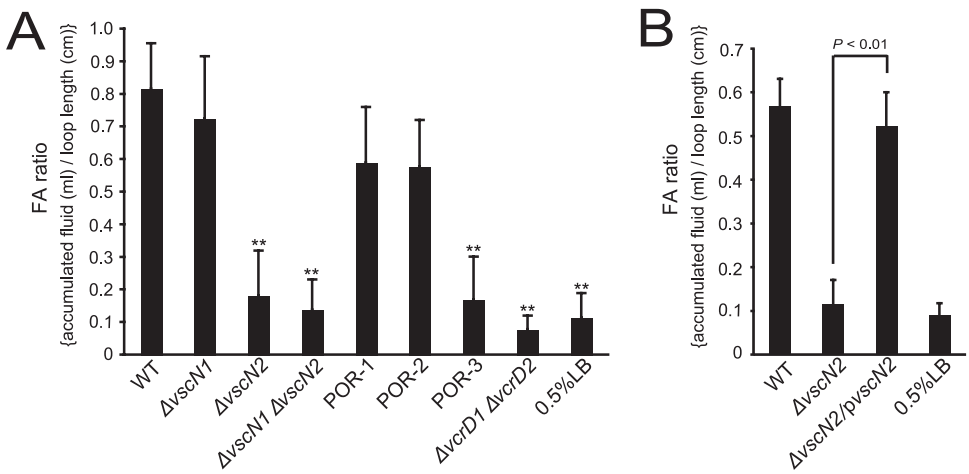

FIG. 4. T3SS2 is necessary for enterotoxicity. (A and B) Enterotoxicity induced by *V. parahaemolyticus* as evaluated by fluid accumulation in the rabbit ileal loop test. *V. parahaemolyticus* RIMD2210633 (10^9 CFU), the isogenic mutants indicated (10^9 CFU), and LB broth containing 0.5% NaCl (noninfected control) were injected into ligated rabbit ileal loops. The mutants tested were the Δ*vscN1* strain (T3SS1 deficient), the Δ*vscN2* strain (T3SS2 deficient), the Δ*vscN1* Δ*vscN2* strain (T3SS1 and T3SS2 deficient), POR-1 (*tdhAS* mutant), POR-2 (*tdhAS* and T3SS1 deficient), POR-3 (*tdhAS* and T3SS2 deficient), the Δ*vcrD1* Δ*vcrD2* strain (*tdhAS*, T3SS1, and T3SS2 deficient), and the Δ*vscN2*/p*vscN2* strain (the Δ*vscN2* strain complemented with *vscN2*). Eighteen hours after injection, the fluid accumulation (FA) ratio in each loop was determined. FA is the amount of accumulated fluid (in milliliters) per centimeter of ligated rabbit small intestine. The error bars indicate standard deviations for the results from triplicate experiments. Two asterisks indicate that the results are significantly different from the results obtained with the WT strain ($P < 0.01$).

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 (LD_{50}) of the WT strain was compared with that of *tdhAS*-deficient strain POR-1, and no significant difference in LD_{50} was observed between the WT and POR-1 strains (7.0 logs and 7.6 logs, respectively); the LD_{50}s obtained were in the same range as the LD_{50}s 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 Δ*vcrD1* Δ*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

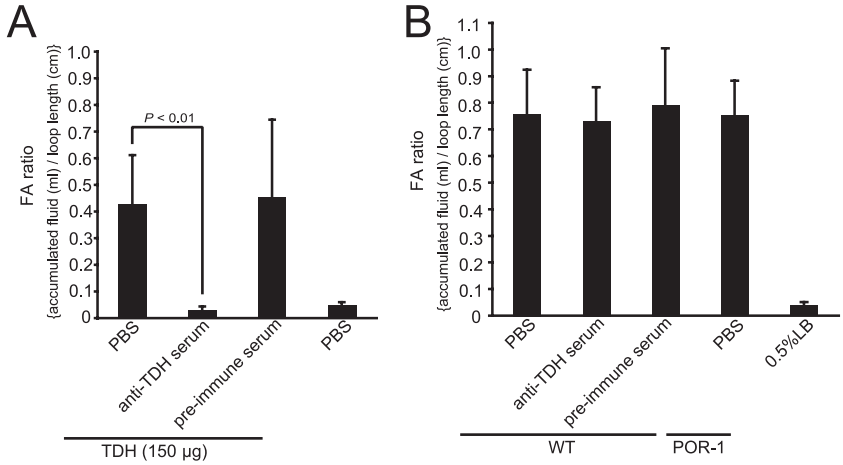

FIG. 5. Effect of anti-TDH polyclonal serum on the accumulation of fluid in ileal loops induced by purified TDH or *V. parahaemolyticus*. (A) Purified TDH was mixed with either PBS, anti-TDH serum, or preimmune serum, after which the mixture was injected into a ligated rabbit ileal loop. PBS alone was used as a negative control without TDH. (B) A suspension of wild-type *V. parahaemolyticus* was mixed with either PBS, anti-TDH serum, or preimmune serum, after which the mixture was injected into a ligated rabbit ileal loop. POR-1 (*tdhAS* deficient) was mixed with PBS, and 0.5% LB (LB broth containing 0.5% NaCl) was used as a noninfected control. Eighteen hours after infection, the FA ratio in each loop was determined. The error bars indicate the standard deviations for the results from triplicate experiments.

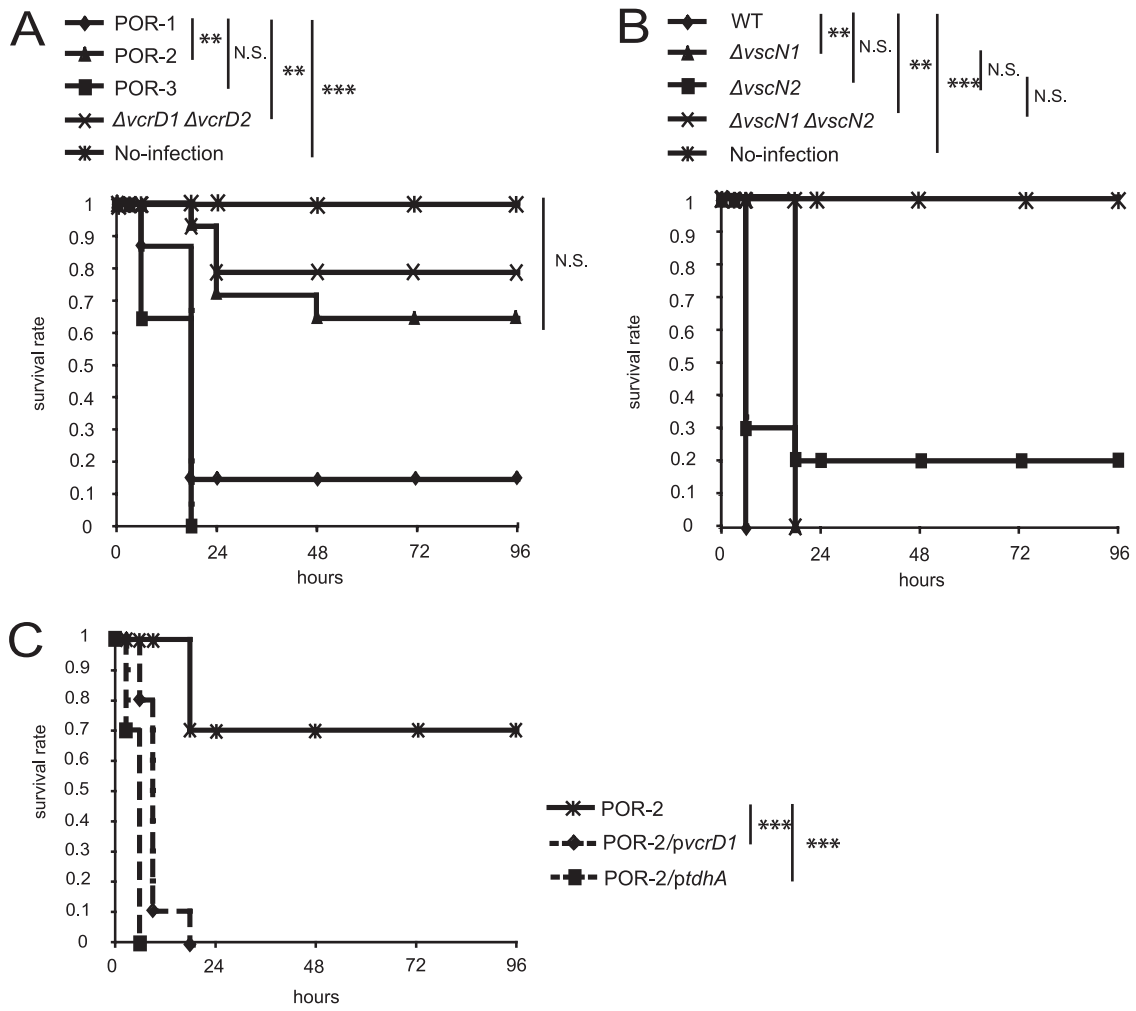

FIG. 6. TDH and T3SS1 are responsible for mouse lethality. (A) C3H/HeN female mice ($n = 15$) were infected intraperitoneally with *tdhAS*-deficient strain POR-1 and T3SS-deficient mutant strains derived from POR-1. The strains used were POR-1 (*tdhAS* mutant), POR-2 (*tdhAS* and T3SS1 deficient), POR-3 (*tdhAS* and T3SS2 deficient), and the Δ*vcrD1* Δ*vcrD2* strain (*tdhAS*, T3SS1, and T3SS2 deficient); a no-infection control was also included. (B) CH3/HeN female mice ($n = 10$) were infected intraperitoneally with WT and T3SS-deficient mutant strains derived from the WT strain. The strains used were the WT strain, the Δ*vscN1* strain (T3SS1 deficient), the Δ*vscN2* strain (T3SS2 deficient), and the Δ*vscN1* Δ*vscN2* strain (T3SS1 and T3SS2 deficient); a no-infection control was also included. (C) C3H/HeN female mice ($n = 10$) were infected intraperitoneally with POR-2, POR-2/p*vcrD1* (POR-2 complemented with *vcrD1*), and POR-2/p*tdhA* (POR-2 complemented with *tdhA*). The lethality in mice was recorded at the times indicated. The murine survival rate was analyzed with Kaplan-Meier and log rank tests (**, $P < 0.01$; ***, $P < 0.001$; N.S., not significant).

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*vcrD1* or POR-2/p*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 *V. parahaemolyticu*s.

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 *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 *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 *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 10^9 cells/loop, while previous studies used 10^8 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 LD_{50} 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, genotype 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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