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Functional Characterization of EpsC, a Component of the Type II Secretion System, in the Pathogenicity of *Vibrio vulnificus*[▽]

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EpsC, one of the components comprising the type II secretion system (T2SS), was isolated from a human-pathogenic bacterium, *Vibrio vulnificus*, to evaluate its role in eliciting virulence. An *espC*-deleted mutant of *V. vulnificus* displayed a reduced cytotoxicity to the human cell line HEp-2 and an attenuated virulence in a mouse model. This mutant exhibited dramatic defects in the secretion of diverse extracellular proteins, such as outer membrane proteins, transporters, and the known secreted factors, notably, a hemolysin (VvhA) and an elastase (VvpE). A defect in its secretion of proteins was restored by in *trans* complementation of the intact *epsC* gene. Analyses of cellular fractions revealed that VvhA and VvpE of the Δ*epsC* mutant were not excreted outside the cell but were present mainly in the periplasmic space. Examination of a *V. vulnificus* mutant deficient in TolC, a component of the T1SS, showed that it is not involved in the secretion of VvhA and VvpE but that it is necessary for the secretion of another major toxin of *V. vulnificus*, RtxA. Therefore, the T2SS is required for *V. vulnificus* pathogenicity, which is mediated by at least two secreted factors, VvhA and VvpE, via facilitating the secretion and exposure of these factors to host cells.

Seven distinct pathways for the secretion of extracellular proteins have been identified in Gram-negative bacteria to date (1, 40). These include the signal sequence-independent pathway (type I), the main terminal branch of the general secretion pathway (type II), the contact-dependent pathways (type III and type IV), the filamentous hemagglutinin secretion pathway, and the autotransporter pathway. For many pathogens, the type II secretion system (T2SS) is involved in the secretion of diverse virulence factors, including proteases (38), lipases (32, 44), cellulases (39), pectinases (3), phospholipases (58), and toxins (18, 46, 56). Secretion via the T2SS occurs in two distinct steps. First, the proteins to be secreted are expressed with signal peptides that target them to either the Sec or Tat machinery for transport across the cytoplasmic membrane into the periplasm (14). Once the signal peptides of the target proteins are removed, the processed proteins are translocated across the outer membrane.

In *Vibrio cholerae*, the T2SS apparatus is encoded by at least 12 genes, including a cluster of genes encompassing the *epsC* gene to the *epsN* gene and a separate locus encoding PilD (14). Inactivation or removal of the T2SS machinery in *V. cholerae* results in a defect in the extracellular release of its periplasmic content and an increased sensitivity to bile salt and polymyxin B (53). The structures of some components of the T2SS, i.e., EpsM, EpsE, and EpsC, of *V. cholerae* have been characterized (2, 15, 24, 42). EpsC protein is one of the key components connecting the inner and outer membrane secretion system, and its role in the secretion system has been experimentally verified in *V. cholerae* (24).

Vibrio vulnificus is a Gram-negative pathogenic bacterium that is frequently associated with primary septicemia following consumption of contaminated shellfish (12, 16). Among secreted proteins from *V. vulnificus*, a cytolytic hemolysin (VvhA) (61), an elastase (VvpE) (35), and a contact-dependent killing toxin (RtxA) (22, 28) have been proven to directly cause severe damage to human tissues. The role of the T2SS in the pathogenesis of *V. vulnificus* has been implied by evaluating the phenotype of a mutant with a knockout of the *pilD* gene, coding for the type IV leader peptidase/*N*-methyltransferase, which is also involved in the biogenesis of type IV pili (37, 38). Thus, the *pilD* mutant was not only defective in the extracellular activities of some proteins but also unable to form type IV pili, which are important for the pathogenesis of *V. vulnificus* (36, 37). It suggests that the reduced virulence of the *pilD* mutant might be caused by both a defect in the T2SS and a defect of pilus formation. In addition, RtxA secretion has not been thoroughly examined in *V. vulnificus* to determine whether it is mediated by the T2SS and/or the type I secretion system (T1SS) (29), although *V. cholerae* RtxA was reported to be secreted by the T1SS (6). Therefore, we investigated the roles of the T2SS in *V. vulnificus* without affecting the formation of type IV pili by constructing a mutant with the *epsC* gene, encoding an essential structural component of the T2SS, knocked out and by comparing its phenotypes with those of the T1SS mutant of *V. vulnificus*.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains used for plasmid DNA preparation and the conjugational transfer of plasmids were grown at 37°C

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
E. coli		
DH5α	*supE44* Δ*lacU169* (ϕ80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Laboratory collection
SM10 λ*pir*	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu λ*pir*, OriT of RP4, Km^r, conjugational donor	54
BL21(DE3)	*E. coli* B F^- *dcm ompT hsdS* (r_B^- m_B^-) *gal* λ (DE3)	Invitrogen
V. vulnificus		
MO6-24/O	Clinical isolate	61
HW1	Δ*epsC* mutant of MO6-24/O	This study
HW2	Δ*vvhA* mutant of MO6-24/O	This study
ML06	Δ*vvpE* mutant of MO6-24/O	This study
HW4	Δ*epsC* Δ*rtxA* mutant of MO6-24/O	This study
HW3	Δ*tolC* mutant of MO6-24/O	This study
MW064	Δ*rtxA* mutant of MO6-24/O	28
Plasmids		
pBluescript (II) SK (+)	Cloning vector, Ap^r, *lac* promoter (*lacZ*), f1 ColE1	Stratagene
pSKepsCup	pBluescript (II) SK (+), 520 bp upstream of *epsC*	This study
pSKepsCupdown	pSKepsCup, 506 bp downstream of *epsC*	This study
pSKvvhAup	pBluescript (II) SK (+), 1,037 bp upstream of *vvhA*	This study
pSKvvhAupdown	pSKvvhAup, 469 bp downstream of *vvhA*	This study
pMLE01	pBluescript (II) SK (+), 798 bp upstream of *vvpE*	This study
pMLE02	pMLE01, 1,031 bp downstream of *vvpE*	This study
pMLE03	pMLE02, 1,258-bp *nptI* gene, Kan^r	This study
pUC4K	pUC4 with *nptI*, Ap^r Km^r	Pharmacia Biotech
pSKtolCup	pBluescript (II) SK (+), 530 bp upstream of *tolC*	This study
pSKtolCupdown	pSKtolCup, 545 bp downstream of *tolC*	This study
pDM4	Suicide vector, OriR6K Cm^r	33
pDMΔepsC	pDM4, 1,026-bp Δ*epsC* DNA fragment	This study
pDMΔvvhA	pDM4, 1,506-bp Δ*vvhA* DNA fragment	This study
pMLE04	pDM4, 3,087-bp Δ*vvpE-nptI* DNA fragment	This study
pDMΔtolC	pDM4, 1,075-bp Δ*tolC* DNA fragment	This study
pRK415	Broad-host-range plasmid, Tc^r	19
pRKepsC	pRK415, 1,035-bp $epsC^+$	This study
pET21b (+)	Expression vector for a histidine-tagged protein	Novagen
pETrepsC	pET21b (+), 776-bp $epsC^+$	This study
pETrvvpE	pET21b (+), 1,826-bp $vvpE^+$	This study
pET28b (+)	Expression vector for a histidine-tagged protein	Novagen
pETrvvhA	pET28b (+), 1,411-bp $vvhA^+$	This study

in Luria-Bertani (LB) broth or on LB broth containing 1.5% agar. *V. vulnificus* strains were cultured at 30°C in LB medium supplemented with an additional 2% NaCl (LBS). The *V. vulnificus* Δ*rtxA* mutant was a gift from S. H. Choi (Seoul National University) (28).

The following antibiotics were used at the indicated concentrations: ampicillin (100 μg/ml), chloramphenicol (25 μg/ml), kanamycin (50 μg/ml), and tetracycline (10 μg/ml) for *E. coli* and ampicillin (500 μg/ml), chloramphenicol (2 μg/ml), kanamycin (100 μg/ml), and tetracycline (3 μg/ml) for *V. vulnificus*. All medium components were purchased from Difco, and the chemicals and antibiotics were obtained from Sigma.

Construction of the mutant strains of *V. vulnificus*. For construction of the *epsC* mutant, the upstream region of the *epsC* gene was amplified from the genomic DNA of *V. vulnificus* MO6-24/O with the primers epsCupF and epsCupR (Table 2). The resultant 520-bp DNA fragment was then digested with ApaI and PstI and ligated into pBlueScript (II) SK (+), to produce pSKepsCup. The downstream region of the *epsC* gene was amplified using the primers epsCdownF and epsCdownR (Table 2). The resultant DNA fragment of 506 bp was treated with PstI and SacI and ligated into pSKepsCup to yield pSKepsCupdown. The 1,026-bp ApaI-SacI DNA fragment of pSKepsCupdown was transferred into a suicide vector, pDM4 (33), resulting in the formation of pDMΔepsC. The plasmid pDMΔepsC in SM10 λ*pir* (54) was mobilized to *V. vulnificus* MO6-24/O, and the conjugants were selected by plating the conjugation mixture of *E. coli* and *V. vulnificus* on LBS plates supplemented with 2 μg/ml chloramphenicol. A colony with characteristics indicating a double-homologous-recombination event (resistance to 5% sucrose and sensitivity to chloramphenicol) was further confirmed by PCR using the primers epsCupF and epsCdownR and then named HW1. For complementation of the mutant, a 1,035-bp DNA fragment was amplified using epsCcomF and epsCcomR (Table 2), which contains an *epsC* open reading frame (ORF) and a 136-bp region upstream of the *epsC* gene. This DNA fragment was then cloned into the broad-host-range vector pRK415 (19) to produce pRKepsC. This $epsC^+$-containing plasmid was mobilized to the Δ*epsC* strain via conjugation. The wild type carrying pRK415 and the Δ*epsC* strain carrying pRK415 were also constructed in the same manner to serve as controls. For construction of the *vvhA* mutant, the upstream (1,037-bp) and downstream (469-bp) regions of the *vvhA* gene were amplified using the primer sets vvhAupF/vvhAupR and vvhAdownF/vvhAdownR, respectively (Table 2). The ApaI-SacI DNA fragment of pSKvvhAupdown was transferred into pDM4 to produce pDMΔvvhA, which was then used to generate a *V. vulnificus* Δ*vvhA* mutant, as described above. Similarly, a plasmid (pMLE02) including the upstream (798-bp) and downstream (1,031-bp) regions of the *vvpE* gene were amplified by the following primer sets: vvpE-upF/vvpE-upR and vvpE-downF/vvpE-downR (Table 2). A gene, *nptI*, encoding a kanamycin resistance enzyme isolated from pUC4K (Pharmacia), was inserted into pMLE02 to generate pMLE03. The ApaI-XbaI DNA fragment of pMLE03 was ligated into pDM4 to produce pMLE04, which was used to make the *V. vulnificus* Δ*vvpE* mutant, as described above. To inactivate the *tolC* gene, the primer sets of tolCupF/tolCupR and tolCdownF/tolCdownR (Table 2) were utilized to produce the 530-bp upstream and the 545-bp downstream regions of *tolC* gene. A 1,075-bp DNA fragment of pSKtolCupdown was cloned into pDM4 to make pDMΔtolC, which was used to generate the *V. vulnificus* Δ*tolC* mutant. A *V. vulnificus* strain defective in both the *rtxA* and *epsC* genes were constructed by allelic exchange via a conjugal transfer of pDMΔepsC into the *V. vulnificus* Δ*rtxA* mutant (28).

Preparation of polyclonal of antibodies. The 795-bp DNA encompassing the *epsC* ORF was amplified using two primers, repsCF and repsCR (Table 2), and

TABLE 2. Oligonucleotide primers used in this study

Primer	Nucleotide sequence (5′–3′)[a]	Enzyme site
epsCupF	GATCGGGCCCTGCACGTTGGCCATTATAGTGGACT	ApaI
epsCupR	GATCCTGCAGAATCATCCGCGTCAGCAATGGGCTA	PstI
epsCdownF	GATCCTGCAGTAACACTTAGGCTCAAAGCGTAAG	PstI
epsCdownR	GATCGAGCTCCACGTTACCAGCGCCCGCATTATCAATC	SacI
epsCcomF	GATCCTGCAGTCGAATAAAGAAAGATAAATC	PstI
epsCcomR	GATCGAATTCACGCTTTGAGCCTAAGTGTTA	EcoRI
vvhAupF	ATCGGGGCCCATTTCGATTTTTCATTGAATAAA	ApaI
vvhAupR	CAGCGGATCCCATCTTATTTTCCCTCAGATT	BamHI
vvhAdownF	CAGCGGATCCGTCGGTGGTCGTATGTT	BamHI
vvhAdownR	ATCGGAGCTCGGTAATCAGGAACTCATTTCG	SacI
vvpE-upF	CCGCTCGAGGGAGAGAGTCATGTACAGTGAGG	PstI
vvpE-upR	CGGGATCCGCAAACCGACCACAAGTTCTTGG	BamHI
vvpE-downF	CGGGATCCCGAGACTACGAGCTTGAACGGA	BamHI
vvpE-downR	GCTCTAGACCATCACTTGAAGACCAAAGGC	XbaI
tolCupF	ATGCGGGCCCTTAAACAATTCATTTATTGCACAACTG	ApaI
tolCupR	ATGCCTGCAGGATCAAACCCTGTTGCGTCGCTGCGTAT	PstI
tolCdownF	GATCCTGCAGGTTGATGCTGGTTTGATTGCGAA	PstI
tolCdownR	GATCGAGCTCTGGCTGCTTCGACATCCTTCATGC	SacI
repsCF	GATCCATATGACAGGCTGGACTTTAGGGCAAGTC	NdeI
repsCR	GATCCTCGAGGTAAATATCATGCTGCTGTCCGT	XhoI
rvvhAF	GATCCCATGGAGATGAAAAAAATGACTCTGTTTACC	NcoI
rvvhAR	GAGCCTCGAGTTTGACTTGTTGTAATGTGG	XhoI
rvvpEF	GATCCATATGAAACACAACCAACGTCATCGT	NdeI
rvvpER	GATCCTCGAGCTTTAACGTCACACCGTTGTA	XhoI

[a] The indicated restriction site is underlined.

then cloned into the pET21b (+) expression vector (Novagen). The recombinant EpsC (rEpsC) protein was overexpressed by adding isopropyl-β-D-thiogalactoside (IPTG) at a concentration of 1 mM and then separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The induced protein band was excised, resuspended with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 7.4). This protein was confirmed to be *V. vulnificus* EpsC by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis as described below, and then it was used as an antigen to produce polyclonal antibodies (Ab) via intraperitoneal injection into pathogen-free rats (CrjBgi; CD [SD] IGS, 6-week-old female rats). After three injections at 2-week intervals, sera were obtained from the immunized rats, and the titer of the resultant Ab was tested. The *vvhA*$^+$ DNA fragment of 1,411 bp was cloned into the pET28b (+) expression vector to produce pETrvvhA. The rVvhA protein was used as an antigen to prepare Ab specific to the *V. vulnificus* VvhA protein by immunization of rats as described above. A *vvpE*$^+$-containing DNA fragment of 1,826 kb was also amplified by PCR using the primers rvvpEF and rvvpER (Table 2) and then cloned into pET21b (+) to produce pETrvvpE. The recombinant protein was overexpressed in *E. coli* BL21 cells harboring the expression plasmid with 1 mM IPTG and separated by 12% SDS-PAGE to immunize a pathogen-free rat. The identities of all the recombinant proteins used for immunization were confirmed by in-gel digestion with trypsin (Sigma) and subsequent MS analyses. MS analyses were performed using a MALDI-TOF Voyager DETM STR biospectrometry workstation (Applied Biosystems). The resultant trypsin peptide profiles were used to search the Swiss-Prot protein (www.expasy.ch) and NCBI (www.ncbi.nlm.nih.gov) databases, primarily with the MASCOT (Matrix Science) search engine.

Western blot analyses. Bacterial extracts were prepared by sonication of bacterial cells in TNT buffer (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, and 0.05% [vol/vol] Tween 20). The protein pool in the culture supernatants was concentrated 200-fold by precipitation with trichloroacetic acid (TCA) or filtration through Amicon/Centricon YM-10 columns (Millipore). Cell lysates and concentrated supernatants prepared in sample buffer (50 mM Tris-HCl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, and 20% glycerol) were separated by SDS-PAGE and transferred onto nitrocellulose membranes (Millipore). Membranes were blocked with 3% skim milk in Tris-buffered saline with Tween 20 (TBST; 150 mM NaCl, 50 mM Tris-HCl, and 0.1% Tween 20) and then incubated overnight at 4°C with the polyclonal Ab (1:2,000 dilution). After incubation with alkaline phosphate (AP)-conjugated secondary Ab, immunoreactive bands were visualized using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. For analysis of RtxA, a contact-dependent exoprotein, *V. vulnificus* strains were incubated with HEp-2 cells for 2 h at a multiplicity of infection (MOI) of 20, as previously described (22, 28, 29). The resultant secretomes were concentrated by using Amicon/Centricon YM-10 columns (Millipore) and analyzed by Western blotting using anti-RtxA Ab (1:2,000 dilution), which was a gift from S. H. Choi.

Secretome analyses. The wild-type and Δ*epsC* mutant strains were cultured to the exponential phase (optical density at 600 nm [OD_{600}] = 0.8) or the stationary phase (OD_{600} = 4.0), and their culture supernatants were then collected by passing cells through 0.22-μm-pore-size filters. Filtered supernatants were mixed with the same volume of prechilled 10% TCA and incubated on ice for 30 min. After a 30-min centrifugation at 9,000 rpm and 4°C, the pellets were washed with acetone three times, dried, and then resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole, pH 8.0). The protein contents in the secretome preparations were determined by a Bio-Rad protein assay kit (Bio-Rad). One hundred nanograms or 50 μg of proteins was separated by 5-to-20% gradient SDS-PAGE and visualized by silver nitrate or Coomassie blue staining, respectively. Protein bands showing differential intensity between the wild-type and Δ*epsC* strains were excised and treated by in-gel digestion with trypsin (Sigma). MS analyses of these trypsin-treated proteins were performed as described above. Some proteins presented in Table 2 were further analyzed by quadrupole TOF (Q-TOF) in addition to MALDI-TOF MS. Product ion spectra were collected in the information-dependent acquisition (IDA) mode and were analyzed with an Agilent 6530 accurate-mass Q-TOF MS. For the Q-TOF liquid chromatography-tandem MS (LC-MS/MS) data sets, tandem mass spectra were submitted to our MASCOT in-house database search engine (NCBI NR database downloaded on 31 July 2009). Mass tolerances of 100 ppm and 0.2 Da were used for precursor and fragment ions, respectively. For protein identification, a MASCOT ion score of >26 was used as the criterion.

Cytotoxicity assay. HEp-2 cells were seeded at 2×10^5 cells per well into 12-well culture plates and grown overnight at 37°C in the presence of 5% CO_2. *V. vulnificus* cells harvested at an OD_{600} of 0.8 or 4.0 were added to HEp-2 cells at an MOI of 20 and then incubated at 37°C for an additional hour in the presence of 5% CO_2. Otherwise, HEp-2 cells (2×10^5) were treated with the concentrated proteins in the culture supernatants (4 or 8 μg protein/ml), followed by 4 h of incubation at 37°C. The cells were detached from the culture plates by a 10-min treatment with 0.025% trypsin-0.02% EDTA solution and stained with propidium iodide (PI; BD Pharmingen) at a final concentration of 1 μg/ml. The degree of cell death was assessed using fluorescence-activated cell sorting (FACS) analysis of PI-stained cells. At least 10,000 events were collected per sample with a FACScan (Becton Dickinson).

Virulence in mice. At an OD_{600} of 0.8, bacterial cells were harvested and washed twice with PBS. Equal numbers (6×10^5) of bacteria were intraperitoneally injected into specific-pathogen-free, 7-week-old, female ICR mice (10 mice per group; Orient Bio Inc.), and the number of live mice was monitored for 25 h after the injection.

Estimation of hemolytic activity. Bacterial growth was monitored by measuring the OD_{600} of cultures, and the supernatants of cultures were prepared by centrifugation and filtration. Serial 2-fold dilutions of culture supernatants in PBS including bovine serum albumin (1 mg/ml) were incubated with equal volumes of PBS containing 1% human red blood cells (RBCs) at 37°C for 1 h. After unlysed RBCs were removed by centrifugation, the lysed RBCs were quantitatively measured by determining spectrometry at an OD_{540}. Hemolysin activity was expressed as (OD_{540} of sample)/(OD_{540} of hemolysis induced by 0.02% Triton X-100) (4, 51).

Detection and quantification of exoprotease activity. For detection of the exoprotease activities of bacterial cells, bacterial cells or culture supernatants harvested at various time points were used to assay proteolytic activity as described previously (11, 43, 59). Briefly, filtered supernatants were incubated at 30°C for 1 h with equal volumes of azocasein solution (5 mg/ml in 50 mM Tris-HCl, pH 8.0, and 0.04% NaN_3). Upon the termination of the reactions with the addition of 6.7% (wt/vol) TCA and subsequent centrifugation (12,000 rpm, 4 min) to remove unreacted azocasein, the absorbance of the supernatant containing azopeptides was measured at 442 nm. To visualize the proteolytic band derived from elastase activity in a polyacrylamide gel, a zymographic analysis was performed as described previously (43).

Fractionation of bacterial cells and measurement of alkaline phosphatase activity. Various *V. vulnificus* strains, the wild-type, Δ*epsC*, and Δ*vvhA* strains, were freshly grown to an OD_{600} of 1 at 30°C in LBS broth. Through centrifugation, the culture supernatant was separated from the bacterial cells. The harvested cells were then divided into cytoplasmic and periplasmic fractions as described by Manoil and Beckwith (31). Harvested cells were resuspended in a spheroplast buffer (100 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 0.5 mM sucrose, 1 mM $MgCl_2$, and 20 μg/ml phenylmethylsulfonyl fluoride [PMSF]) and subjected to centrifugation (3 min, 13,000 rpm, 4°C). The pellets were warmed, resuspended in ice-cold water, and treated with 20 mM $MgCl_2$. Then, osmotically shocked cells were centrifuged (5 min, 13,000 rpm, 4°C), the supernatants were saved as periplasmic fractions, and the pellets were further resuspended in a solution containing 10 mM Tris-HCl, pH 8.0, and 20 μg/ml PMSF. The cells in the pellet fractions were lysed by a treatment with lysozyme (20 μg/ml) and a series of freezing/thawing. After centrifugation, the resultant supernatants were saved as cytoplasmic fractions.

Three fractions, i.e., a culture supernatant, a periplasmic fraction, and a cytoplasmic extract, were assayed for their alkaline phosphatase activity in a reaction mixture (50 mM Tris-HCl, pH 8.0, and 1 mM $MgCl_2$) including 0.2 mg/ml *p*-nitrophenylphosphate as described previously (53, 62). After incubation at 37°C for 40 min, the reactions were terminated by adding 50 mM EDTA and monitored for their absorbance at 405 nm. Specific activity was defined by the formula (OD_{420}/reaction time [in minutes] × milligrams of protein) × 1,000.

Statistical analyses. Results are expressed as the averages ± standard deviations of results from at least three independent experiments. Statistical analysis was performed using analysis of variance (ANOVA). Differences were judged as statistically significant when the *P* value was less than 0.05. Statistical analysis for pairwise comparison was performed using Student's *t* test (SYSTAT program, SigmaPlot version 9; Systat Software Inc.). Differences were considered significant if the *P* values were <0.05. A log rank test (http://bioinf.wehi.edu.au/software/russell/logrank/) was performed to estimate the statistical significance of the mouse lethality experiments.

RESULTS

***In silico* analysis of the T2SS in *V. vulnificus*.** Using the amino acid sequences for T2SS members of *V. cholerae*, the *V. vulnificus* CMCP6 genomic database (GenBank accession no. AE016795 and AE016796) was screened for the putative T2SS components. Genes for proteins EpsC to -N (from VV1_0878 to VV1_0867) were clustered in chromosome 1 of *V. vulnificus*. The *pilD* gene for type IV leader peptidase/*N*-methyltransferase, which is involved in both the T2SS and type IV pilus biogenesis in *V. vulnificus*, was located at a separate locus (VV1_1623). An *epsA*-homologous ORF (VV1_0621) with 50% similarity to the *V. cholerae* EpsA protein was found at a site distal from the *eps* cluster. We could not identify any gene homologous to *epsB* in the *V. vulnificus* genome. Each component of the T2SS in the other *Vibrio* spp. (*V. parahaemolyticus*, *V. cholerae*, *V. harveyi*, and *V. fischeri*) showed homology with that of *V. vulnificus*, ranging from 61 to 96%, and no distinct difference was observed in the degrees of homology in Eps proteins among pathogenic and nonpathogenic vibrios.

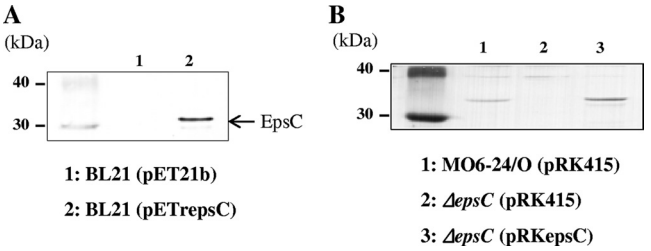

FIG. 1. Confirmation of the deletion of *epsC* from *V. vulnificus* and the complementation strain using anti-EspC antibodies. (A) Western blot of extracts of *E. coli* BL21 carrying the expression vector pET21b (lane 1) and pET21b pETrepsC (lane 2) with anti-EpsC Ab; (B) Western blot analysis using anti-EpsC Ab on extracts of wild-type *V. vulnificus* harboring the broad-host-range vector pRK415 (lane 1), the Δ*epsC* mutant harboring pRK415 (lane 2), and the Δ*epsC* mutant carrying pRKepsC, a complementation plasmid of the wild-type *epsC* gene (lane 3).

In *V. cholerae*, EpsE, EpsL, EpsM, and EpsA comprise the secretion machinery in the inner membrane, and EpsD is a member of a secretory apparatus in the outer membrane (14, 47, 53). As a component connecting the inner and outer membrane secretion apparatus, EpsC has been shown to be crucial for successful secretion in *V. cholerae* (24). Thus, in this study, we generated a *V. vulnificus* mutant deficient in the *epsC* gene to define the role of the T2SS in the pathogenesis of this bacterium.

Construction and confirmation of the Δ*epsC* mutant of *V. vulnificus*. Deletion of the putative *epsC* ORF from the genomic DNA of *V. vulnificus* was confirmed by PCR using the primers epsCupF and epsCdownR (Table 2), which were specific to the upstream and downstream regions of the deleted ORF, respectively (data not shown). To confirm that the phenotypes of the resultant mutant were derived from the *epsC* gene knockout, a complementation plasmid containing the intact *epsC* gene was transformed into the *V. vulnificus* Δ*epsC* mutant. Because the overexpressed protein band in *E. coli* extracts was used to make anti-EpsC antibodies without purification, its identity was analyzed by MALDI-TOF, which showed that it is the recombinant EpsC protein, as expected (data not shown). Once the specificity of anti-EpsC Ab was confirmed by Western blotting of *E. coli* expressing rEpsC (Fig. 1A), the cellular levels of the EpsC protein in various *V. vulnificus* strains were examined by Western blot analysis using anti-EpsC Ab (Fig. 1B). An immunoreactive protein was present in the extract of the wild type carrying the vector plasmid pRK415 and the *V. vulnificus* Δ*epsC* mutant with the complementation plasmid, but it was absent in the extract of the *V. vulnificus* Δ*epsC* mutant carrying pRK415.

Deletion of the *epsC* gene results in a dramatic defect in secretion of various proteins from *V. vulnificus*. The role of the

T2SS in the secretion of extracellular proteins was examined by comparing the secretome profile of wild-type *V. vulnificus* with that of the Δ*epsC* mutant, which were harvested at the exponential (OD_{600} = 0.8) and stationary (OD_{600} = 4.0) phases. The patterns of secreted proteins from both phases were significantly altered in the *V. vulnificus* Δ*epsC* mutant (Fig. 2A and B). Identification of the secreted proteins (Table 3) revealed that transport proteins (a long-chain fatty acid transport protein and an ABC-type amino acid transporter), metabolic enzymes (UDP-sugar hydrolase and peptidyl-prolyl *cis-trans* isomerase), an adhesin (*N*-acetylglucosamine-binding protein A), outer membrane proteins (outer membrane protein OmpU and a putative outer membrane protein), proteases (elastase and chitinase), and cytolysin. All of the identified proteins were shown to contain putative signal sequences in their N termini. Interestingly, some exoproteins were shown to be excreted more often from the mutant than from the wild type, but their identities were not examined further.

To verify that the altered secretion in this mutant was due to the deletion of the *epsC* gene, the mutant was complemented with the intact *epsC* gene in pRK415 and its secretome was compared with the secretomes of *V. vulnificus* strains containing pRK415. The wild-type and mutant cells at stationary phase repeatedly showed similar patterns in their secretomes (Fig. 2C). Limited identification of secreted proteins revealed that at least three exoproteins, hemolysin, elastase, and *N*-acetylglucosamine-binding protein A, were recovered to wild-type levels when the mutant was complemented.

Comparative secretome analysis of the wild type and the Δ*epsC* mutant suggested that the T2SS was responsible for the secretion of proteins involved in various functions of *V. vulnificus*, including elastase (VvpE) and cytolysin (VvhA). In subsequent experiments, we confirmed whether the T2SS was directly involved in the secretion of these factors of *V. vulnificus*.

Deletion of the *epsC* gene results in a dramatic defect in *V. vulnificus* cytotoxicity to HEp-2 cells. Using PI staining and FACS analysis, we asked whether a defective T2SS affected the virulence of *V. vulnificus* to host cells (Fig. 3A). About 5% of HEp-2 cells without exposure to *V. vulnificus* were stained with PI, whereas 96% of HEp-2 cells exposed to exponential-phase wild-type *V. vulnificus* (at an MOI of 20) were stained with PI. When HEp-2 cells were incubated with the exponential-phase Δ*epsC* mutant, only 27% of HEp-2 cells were stained with PI ($P = 3.46E^{-6}$). Infection of HEp-2 cells with the same *V. vulnificus* strains harvested at the stationary phase also resulted in similar patterns of cytotoxicity exerted by exponential-phase cells of *V. vulnificus* (data not shown). In addition, complementation of the intact *espC* gene into the *V. vulnificus* Δ*epsC* mutant increased bacterial cytotoxicity from 11% to 58% (P = 0.017), which is almost the level of cytotoxicity shown by the wild type carrying pRK415 (67%) (P = 0.66).

Since the *epsC* gene encodes an essential component of the T2SS, we attributed the reduced cytotoxicity of the Δ*espC* mutant to its defect in the secretion of extracellular cytotoxic factors of *V. vulnificus*. Accordingly, we compared the cytotoxic effects on HEp-2 cells by secreted bacterial proteins of various *V. vulnificus* strains (Fig. 3B). The percentages of PI-stained cells were 43 and 59% upon incubation with 4 and 8 μg/ml of secreted proteins of wild-type *V. vulnificus*, respectively. Cytotoxicity was dramatically decreased to 8.0% ($P = 8.09E^{-7}$) and

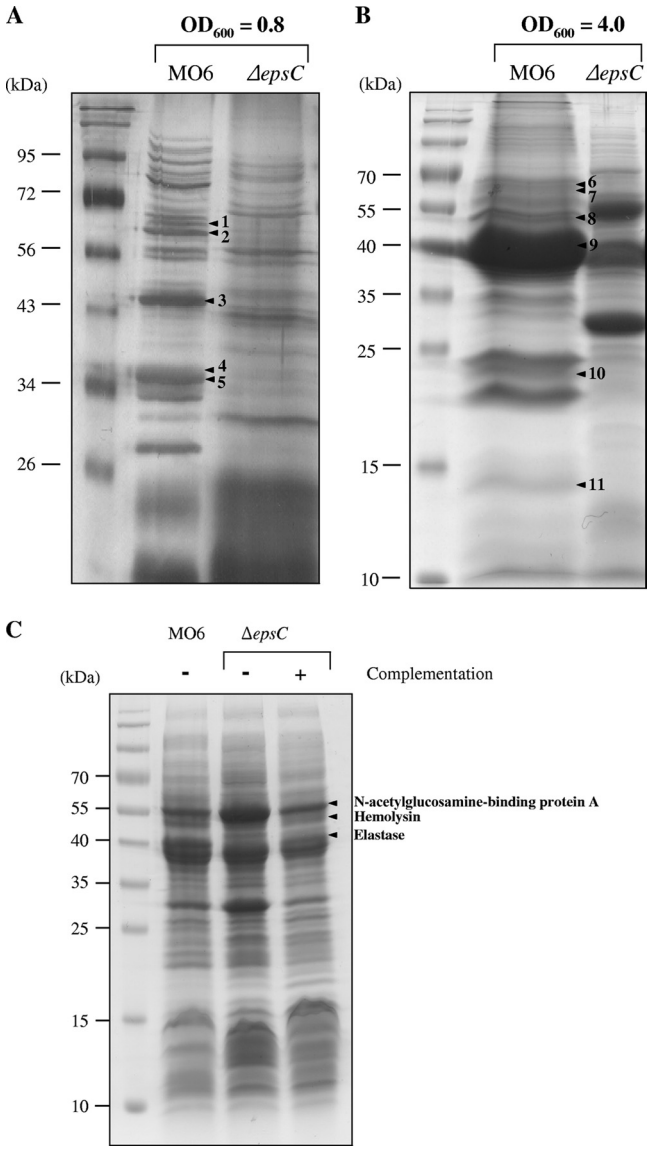

FIG. 2. Comparative secretome analyses of the wild-type and *V. vulnificus* Δ*epsC* strains. (A, B) The wild-type and Δ*epsC* strains were cultured to the exponential (OD_{600} = 0.8) or the stationary (OD_{600} = 4.0) phase. The collected spent medium was precipitated with equal volumes of 10% TCA and washed three times with acetone. One hundred nanograms (OD_{600} = 0.8) (A) or 50 μg (OD_{600} = 4.0) of proteins (B) was separated by SDS-PAGE and visualized by silver staining or Coomassie blue staining, respectively. Reduced-secretion proteins in the Δ*epsC* mutant were identified by trypsin treatment and subsequent MALDI/TOF or Q-TOF analysis (Table 3). (C) Comparative secretome analysis of the wild type carrying pRK415, the Δ*epsC* mutant carrying pRK415, and the Δ*epsC* mutant carrying pRKepsC. *V. vulnificus* strains were cultured to the stationary phase (OD_{600} = 4.0) in LBS supplemented with tetracycline. One-hundred micrograms of proteins in each spent medium was separated by SDS-PAGE and visualized by Coomassie blue staining. Three representative reduced-secretion protein bands, *N*-acetylglucosamine-binding protein A, hemolysin, and elastase, in the Δ*epsC* mutant carrying pRK415 were identified by MALDI/TOF or Q-TOF analysis as described in Materials and Methods.

TABLE 3. Proteins of which secretion was reduced in the *V. vulnificus* Δ*epsC* mutant

Band(s)[a]	Method[b]	Protein name	Molecular mass (kDa)[c]	ORF[d]	Coverage (%)	Score (valid score)[e]
1, 2	Q-TOF	UDP-sugar hydrolase	62 (58)	VV1_0248	5	183 (52)
3	MS	Long-chain fatty acid transport protein	44 (42)	VV1_1972	38	152 (81)
4	MS	Outer membrane protein OmpU	37 (35)	VV1_1686	30	100 (68)
5	Q-TOF	ABC-type amino acid transporter	37 (34)	VV1_2703	14	43 (26)
6	MS	Chitinase	62 (60)	VV1_0417	26	60 (56)
7	Q-TOF	*N*-Acetylglucosamine-binding protein A	53 (50)	VV2_0044	11	62 (26)
8	Q-TOF	Hemolysin	53 (51)	VV2_0404	77	218 (26)
9	MS	Elastase	66 (63)[f]	VV2_0974	32	152 (56)
10	Q-TOF	Outer membrane protein	20 (18)	VV2_0807	9	62 (27)
11	Q-TOF	Peptidyl-prolyl *cis-trans* isomerase	20 (18)[f]	VV1_1817	6	28 (27)

[a] Numbers are derived from the protein bands shown in Fig. 2.
[b] The identities of proteins treated with trypsin were determined by mass spectrometry (MS) or Q-TOF.
[c] The molecular mass of each protein before processing of the putative leader peptide, which was predicted by the SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/), is presented in parentheses (after processing).
[d] Protein annotation and GenBank ORF designation of *V. vulnificus* strain CMCP6.
[e] Cutoff values for validation are in parentheses.
[f] The predicted size of this protein is higher than the apparent size of the protein band (Fig. 2), which is presumed to be further processed.

8.6% ($P = 1.40E^{-4}$) when two concentrations of secreted proteins of the Δ*epsC* mutant were challenged. We then examined whether the attenuated cytotoxicity of secreted proteins of the Δ*epsC* mutant could recover to wild-type levels by introducing the complementation plasmid pRKepsC (Fig. 3C). With 4 μg/ml of secreted proteins of the wild type carrying pRK415, 19% of the cells stained with PI, and the portion of dead cells was increased to 95% at 8 μg/ml of the same secreted proteins. In contrast, the control strain (Δ*epsC* mutant with the vector pRK415) showed cytotoxicities of 7.4% ($P = 1.80E^{-5}$) and 9.1% ($P = 1.86E^{-6}$). When HEp-2 cells were incubated with the secretome of the Δ*epsC* mutant strain carrying pRKepsC, 11% of the HEp-2 cells were stained by PI with 4 μg/ml of the secretome ($P = 0.00126$) and PI-stained HEp-2 cells were increased to 74% in the presence of 8 μg/ml of secreted protein ($P = 0.25$).

In this experiment, the cytotoxicity of the Δ*rtxA* mutant was also measured, since RtxA is the most potent toxin of *V. vulnificus* known so far and it functions in a contact-dependent manner (22, 28). The cytotoxicity of the Δ*rtxA* mutant was dramatically attenuated to 7.0% (Fig. 3A) ($P = 1.44E-07$), as shown previously (22, 28). The *V. vulnificus* Δ*rtxA* Δ*epsC* double mutant demonstrated a slightly lower level of cytotoxicity to HEp-2 cells than the Δ*rtxA* mutant. In contrast, the cytotoxicity of the secretome of the Δ*rtxA* mutant (95 to 98%) was higher than that of the wild type (Fig. 3B). The secretome derived from the Δ*epsC* Δ*rtxA* double mutant demonstrated a lower level of cytotoxicity than the *V. vulnificus* Δ*epsC* mutant ($P = 3.03E^{-7}$ and $1.70E^{-4}$, respectively), indicating that the mode of cytotoxicity exerted by the Δ*epsC* mutant is not caused by RtxA, which is presumed to be secreted by the T1SS.

Deletion of the *epsC* gene results in a dramatic defect in mouse lethality by *V. vulnificus*. We then examined the role of the T2SS in the pathogenesis of *V. vulnificus* using a mouse infection model with the wild type or Δ*epsC* mutant. Mice received an intraperitoneal injection of bacterial cells, and their survival was monitored (Fig. 4A). All of the mice injected with wild-type *V. vulnificus* were dead by 6 h postinjection, while 8 of 10 mice injected with the Δ*epsC* mutant were alive by 25 h postinjection. Therefore, the Δ*epsC* mutant showed significant attenuation in mouse lethality (P values = $2.7E^{-6}$, log rank test).

The complemented and control *V. vulnificus* strains were also tested for their virulence in a mouse model (Fig. 4B). The Δ*epsC* mutant carrying pRK415 demonstrated significantly reduced mouse lethality compared to the wild type carrying pRK415 (P values = $4.3E^{-6}$, log rank test) and the Δ*epsC* mutant carrying pRKepsC (P values = $3.9E^{-6}$, log rank test). Despite the recovered mouse lethality of the complemented strain, it was slightly less effective than wild-type *V. vulnificus* carrying pRK415, suggesting that introduction of the plasmid containing the wild-type *epsC* gene into the Δ*epsC* mutant caused a partial, but significant, recovery of bacterial virulence in mouse model experiments (P values = 0.0023, log rank test).

The T2SS is involved in the secretion of hemolysin(s) in *V. vulnificus*. Based on secretome analyses, VvhA secretion was mediated by the T2SS, and thus we examined whether EpsC was indeed involved in the secretion of hemolysin in *V. vulnificus* by estimating hemolytic activity and VvhA protein levels in culture supernatants (Fig. 5). Culture media grown by various *V. vulnificus* strains at various growth phases were tested for their ability to lyse human RBCs. Hemolytic activity was maximal when the wild type carrying pRK415 entered the stationary phase and then declined during the late stationary phase (Fig. 5A). The level of hemolytic activity in the supernatant from the Δ*epsC* mutant carrying pRK415 was too low to be detected in this assay, which was similar to that in the culture supernatants of Δ*vvhA* mutant *V. vulnificus* (Fig. 5B and C, respectively). The *V. vulnificus* Δ*epsC* mutant carrying pRKepsC recovered hemolysin activity to the level of wild-type *V. vulnificus* carrying the vector plasmid pRK415 (Fig. 5D).

In this hemolytic assay, the supernatant of the Δ*rtxA* mutant was also included, since *V. vulnificus* RtxA toxin has a hemolytic activity but it functions in a contact-dependent manner (22, 28). The hemolytic activity of the supernatant of the Δ*rtxA* mutant was similar to that of its isogenic wild type, MO6-24/O, which was maximal at early stationary phase (Fig. 5E and F). In the case of the *V. vulnificus* Δ*rtxA* Δ*epsC* double mutant, we could not detect any hemolytic activities from the culture supernatant at any growth phase (Fig. 5G). Therefore, VvhA

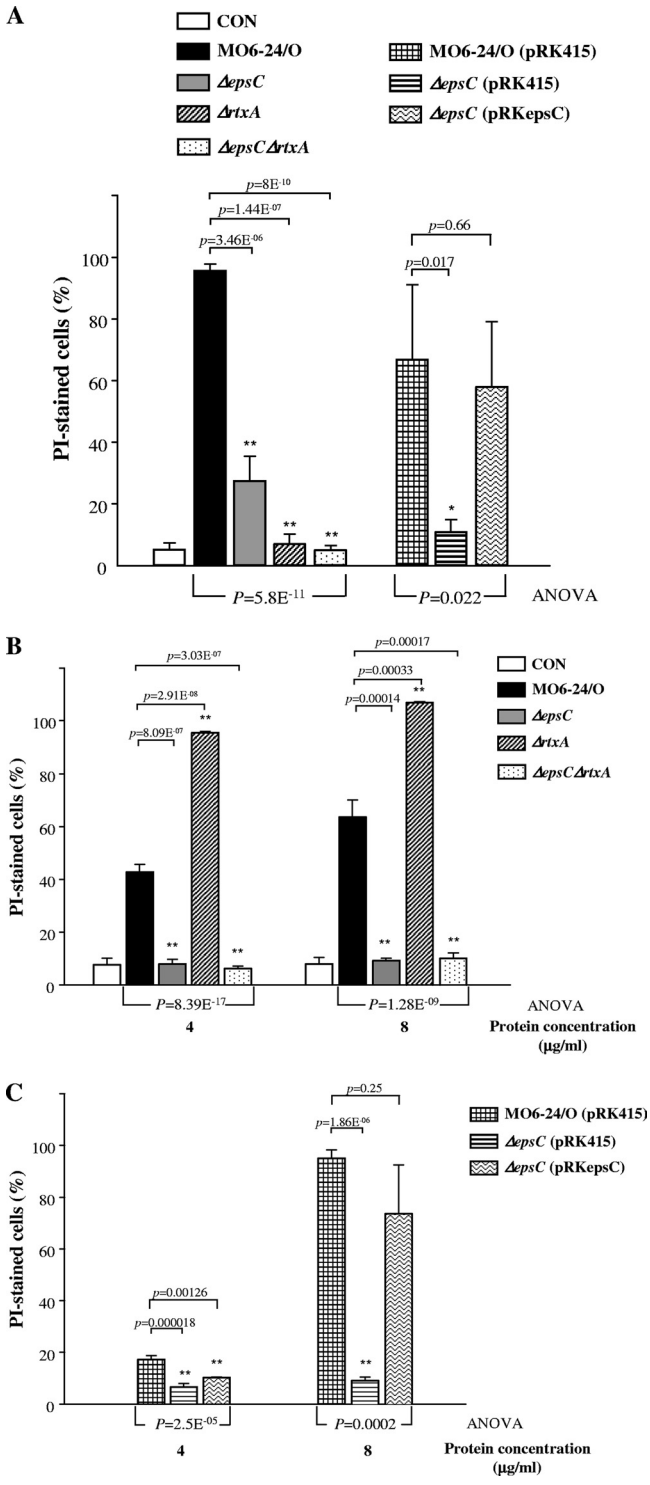

FIG. 3. Role of EpsC in *V. vulnificus*'s cytotoxicity to HEp-2 cells. Determination of cytotoxicities of various *V. vulnificus* strains (A) and their secreted protein pools (B and C) against HEp-2 cells by propidium iodide (PI) staining. (A) Percentages of the dead HEp-2 cells were measured after 1 h of incubation with the wild-type, Δ*epsC*, Δ*rtxA*, and Δ*epsC* Δ*rtxA* mutant *V. vulnificus* strains at an MOI of 20. (B) Percentages of dead HEp-2 cells were measured after 4 h of incubation with 4- or 8-μg/ml protein pools secreted by the wild-type, Δ*epsC*, Δ*rtxA*, and Δ*epsC* Δ*rtxA* mutant *V. vulnificus* strains. (C) Percentages of dead HEp-2 cells were measured after treatment of secreted proteins of the wild type carrying pRK415, the Δ*epsC* mutant harboring pRK415, and the Δ*epsC* mutant carrying pRKepsC. Unexposed HEp-2 cells were also stained with PI as a control (CON; open bars). Error bars represent the means ± standard deviations of results from three independent experiments. Single asterisks indicate percentages of PI-stained cells that were significantly different ($0.01 < P < 0.05$) from wild-type *V. vulnificus* cells by Student's *t* test, while double asterisks indicate a *P* of <0.01. *P* values from an ANOVA analysis are also presented in the graph for each set of data.

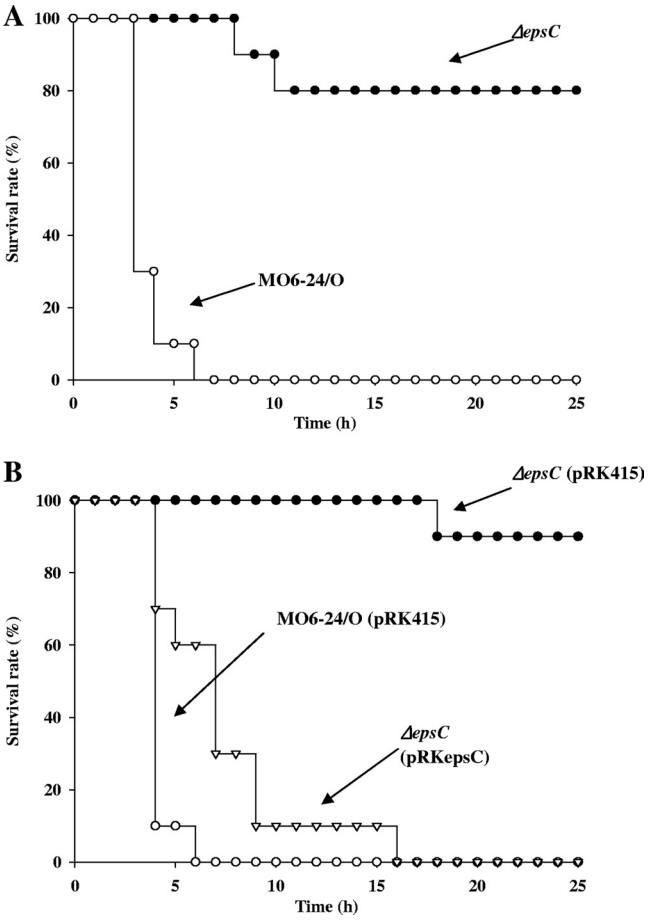

FIG. 4. Mouse lethality of the T2SS-defective *V. vulnificus* mutant. Seven-week-old specific-pathogen-free female ICR mice were intraperitoneally infected with wild-type and Δ*epsC* mutant *V. vulnificus* strains (A). Equal numbers of bacteria (6×10^5) were injected into 10 mice per group, and the survival of the mice was monitored for 25 h after injection. Open and filled circles represent the number of live mice infected with the wild type and Δ*epsC* mutant, respectively. (B) The numbers of mice infected with the wild type carrying pRK415, the Δ*epsC* mutant carrying pRK415, and the Δ*epsC* mutant carrying pRKespC are presented as open circles, filled circles, and open inverted triangles, respectively.

activity plays a major role in the hemolytic assay using cell-free culture supernatants.

With polyclonal Ab specific to recombinant VvhA (rVvhA), the secretion of VvhA was specifically examined in the *V. vulnificus* wild type carrying pRK415, the Δ*epsC* mutant carrying pRK415, the Δ*epsC* mutant carrying pRKepsC, and the Δ*vvhA* mutant (Fig. 6A). The identity of the overexpressed protein was confirmed to be VvhA by MALDI-TOF analysis

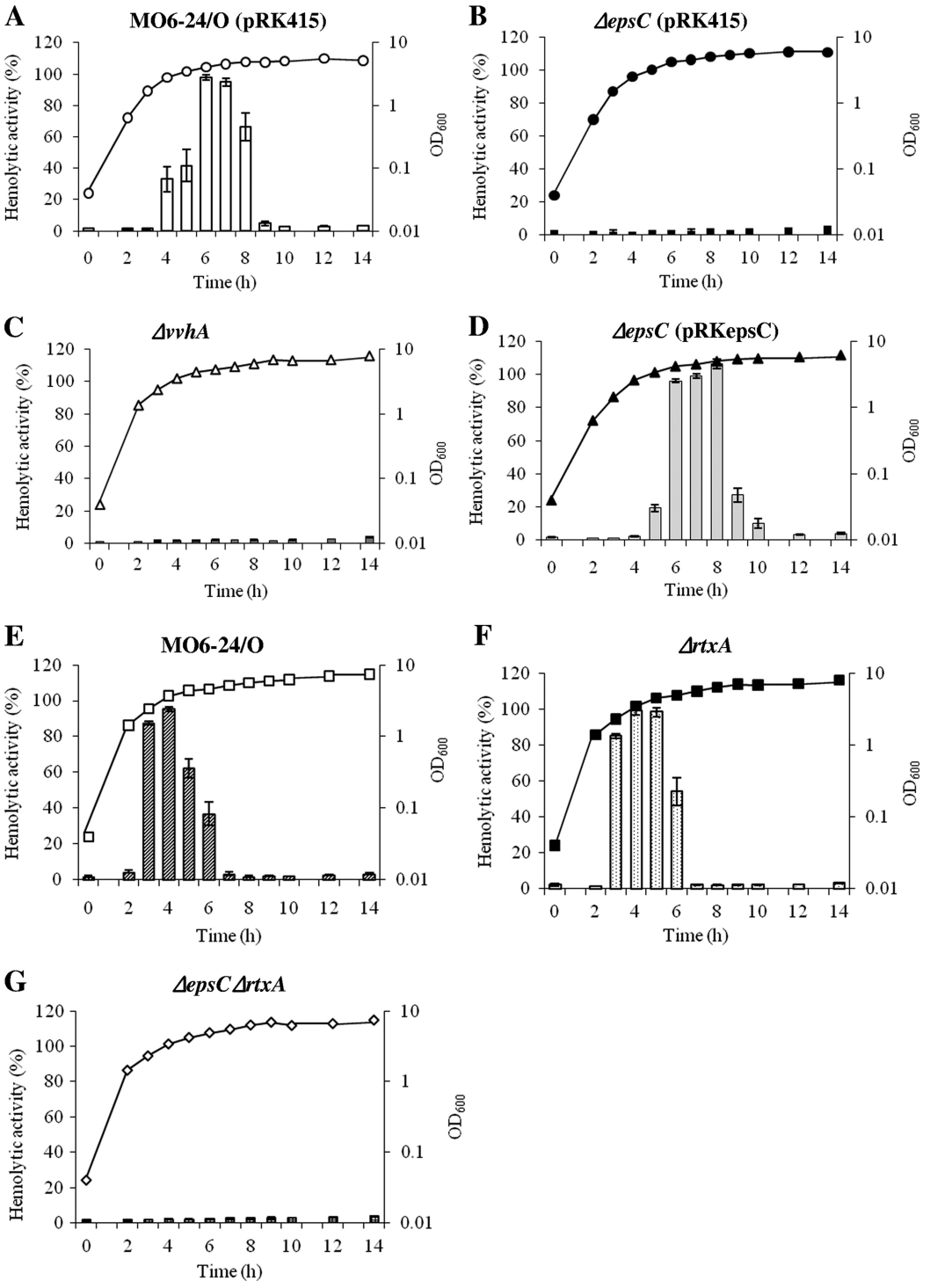

FIG. 5. Hemolytic activities of secreted proteins of various *V. vulnificus* strains. Hemolytic activities in the culture supernatants of wild-type MO6-24/O carrying pRK415 (A), the Δ*epsC* mutant carrying pRK415 (B), the Δ*vvhA* mutant (C), the Δ*epsC* mutant carrying pRKepsC (D), wild-type MO6-24/O (E), the Δ*rtxA* mutant (F), and the Δ*epsC* Δ*rtxA* mutant (G) throughout the bacterial growth period designated by the curves for OD_{600}s of cultures. Serial 2-fold dilutions of supernatants were incubated with equal volumes of a 1% suspension of human RBCs for 1 h at 37°C. Hemolysin activity was expressed as a percentage (the OD_{540} of the sample over the OD_{540} of RBCs lysed by treatment with Triton X-100). Data are presented as averages of results from three independent experiments, with error bars.

prior to Western blot analysis (data not shown). An immunoreactive band of ~50 kDa was detected in the culture supernatant of the *V. vulnificus* wild type carrying pRK415, and it was absent in the culture supernatants of the Δ*epsC* mutant carrying pRK415 and the Δ*vvhA* mutant. As expected, the immunoreactive band reappeared in the Δ*epsC* mutant carrying the complementation plasmid pRKepsC.

In the next experiment, we examined whether the secretion

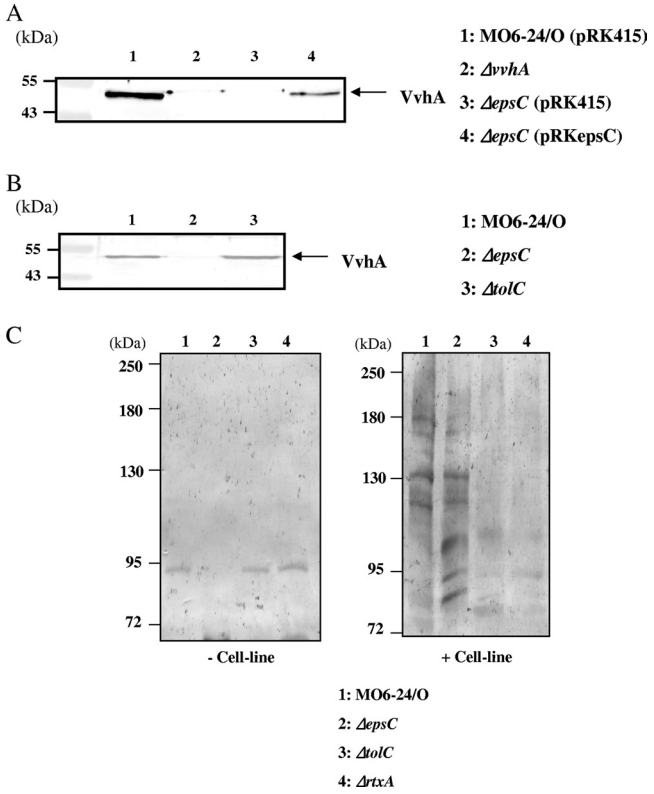

FIG. 6. Secretion of hemolysins in various *V. vulnificus* strains. (A, B) Secretion of VvhA hemolysin in various *V. vulnificus* strains. (A) The spent media after growth by the wild type carrying pRK415 (lane 1), the Δ*vvhA* mutant (lane 2), the Δ*epsC* mutant carrying pRK415 (lane 3), or the Δ*epsC* mutant carrying pRKepsC (lane 4) were analyzed by Western blot analysis using anti-VvhA Ab. (B) Western blot analysis of secretomes of wild-type *V. vulnificus* MO6-24/O (lane 1), the Δ*epsC* mutant (lane 2), and the Δ*tolC* mutant (lane 3). (C) Secretion of RtxA in various *V. vulnificus* strains. Culture supernatants were prepared from the *V. vulnificus* wild type (lanes 1), Δ*epsC* mutant (lanes 2), Δ*tolC* mutant (lanes 3), and Δ*rtxA* mutant (lanes 4) without (left) or with (right) preincubation with human cells as described in Materials and Methods. Concentrated supernatants were analyzed by Western blotting using anti-RtxA Ab.

of VvhA is mediated only by the T2SS and not by the T1SS (Fig. 6B). Secretomes of the wild type, Δ*epsC* mutant, and Δ*tolC* mutant were analyzed for the presence of VvhA, indicating that secreted proteins of the Δ*tolC* mutant contained VvhA, as the wild-type secretome did. We examined the secretion of RtxA, in addition to VvhA, in the culture supernatants of Δ*epsC* and Δ*tolC* mutants by Western blotting using anti-RtxA Ab (Fig. 6C). Western blot analysis of culture supernatants of *V. vulnificus* cells grown in the absence of the host cells did not show any immunoreactive protein band corresponding to the RtxA polypeptides. However, when culture supernatant derived from *V. vulnificus* cells grown in the presence of the host cell line were analyzed, several immunoreactive bands were observed in the *V. vulnificus* wild type and Δ*epsC* mutant, which were absent in the supernatant of the Δ*tolC* mutant as well as in that of the Δ*rtxA* mutant. This suggests that RtxA is secreted via the T1SS but not via the T2SS.

The T2SS is involved in the secretion of exoproteases in *V. vulnificus*. VvpE, an elastase, was found to be a dominant protein among the *V. vulnificus* secretomes, the secretion of which was abolished by deletion of the *epsC* gene (Fig. 2). Therefore, we directly examined the dependence of VvpE secretion on the presence of functional EpsC. Most of all, wild-type MO6-24/O carrying pRK415, the Δ*epsC* mutant with pRK415, and the Δ*epsC* mutant with pRKepsC were evaluated for their total extracellular protease activity by monitoring for their ability to degrade azocasein (Fig. 7A). The culture supernatant of the wild type carrying pRK415 efficiently degraded azocasein. In contrast, the supernatant of the Δ*epsC* mutant carrying pRK415 demonstrated an undetectable level of azocasein degradation. Culture supernatants from the *V. vulnificus* Δ*epsC* mutant with the complementation plasmid pRKepsC regained the ability to degrade azocasein. The supernatant of the Δ*vvpE* mutant demonstrated a low level of azocasein-degrading activity, but it was higher than that of the Δ*epsC* mutant carrying pRK415. The higher proteolytic activity of the Δ*vvpE* mutant than of the Δ*epsC* mutant was also demonstrated in the assay using a skim milk-agar plate (Fig. 7B). Observation of the lower proteolytic activity of the Δ*epsC* mutant carrying pRK415 or of the Δ*epsC* mutant than of the Δ*vvpE* mutant suggests the presence of an additional protease(s) secreted via the T2SS in *V. vulnificus*.

Using polyclonal Ab specific to recombinant rVvpE, the secretion of elastase was specifically monitored in the culture supernatants grown by these strains (Fig. 7C). Prior to Western blot analysis, the recombinant protein used for immunization was also examined by MALDI-TOF analysis, indicating that VvpE is a recombinant elastase, as expected (data not shown). An immunoreactive band was detected as a protein of 45 kDa only in the supernatant of the wild type carrying pRK415 and the Δ*epsC* mutant carrying pRKepsC and not in the culture supernatant of the Δ*epsC* mutant carrying pRK415 and the Δ*vvpE* mutant of *V. vulnificus*. We further examined the involvement of the T1SS in VvpE secretion. The activities of VvpE in the secretomes of the wild-type, Δ*epsC*, and Δ*tolC* strains were monitored by a zymographic analysis (Fig. 7D), indicating that secreted proteins of the Δ*tolC* mutant contained VvpE activity, like the wild type (43), but that the Δ*epsC* mutant did not contain VvpE activity. It suggests that VvpE is secreted via the T2SS but not via the T1SS.

Trapping of VvhA and VvpE in the periplasm of the *V. vulnificus* Δ*epsC* mutant. Since both VvhA and VvpE were not detected in the culture supernatant of the Δ*epsC* mutant, we examined the cellular fractions containing hemolytic activity and VvpE in the *V. vulnificus* Δ*epsC* mutant. Specifically, we asked if synthesized VvhA or VvpE in the cytoplasm of the Δ*epsC* mutant was not successfully secreted into the medium through the outer membrane. Extracts of various *V. vulnificus* strains, including the wild-type, Δ*epsC*, and Δ*vvhA* mutant *V. vulnificus* strains, were divided into three fractions (cytoplasmic, periplasmic, and culture supernatant) and assayed for hemolysin activity (Table 4). Hemolysin activity was present mainly in the culture supernatant of the wild type but barely detected in the cytoplasmic and periplasmic fractions. In contrast, hemolysin activity was found mainly in the periplasmic fraction of the *V. vulnificus* Δ*epsC* mutant, while little or no activity was present in the cytoplasmic fraction or in the culture

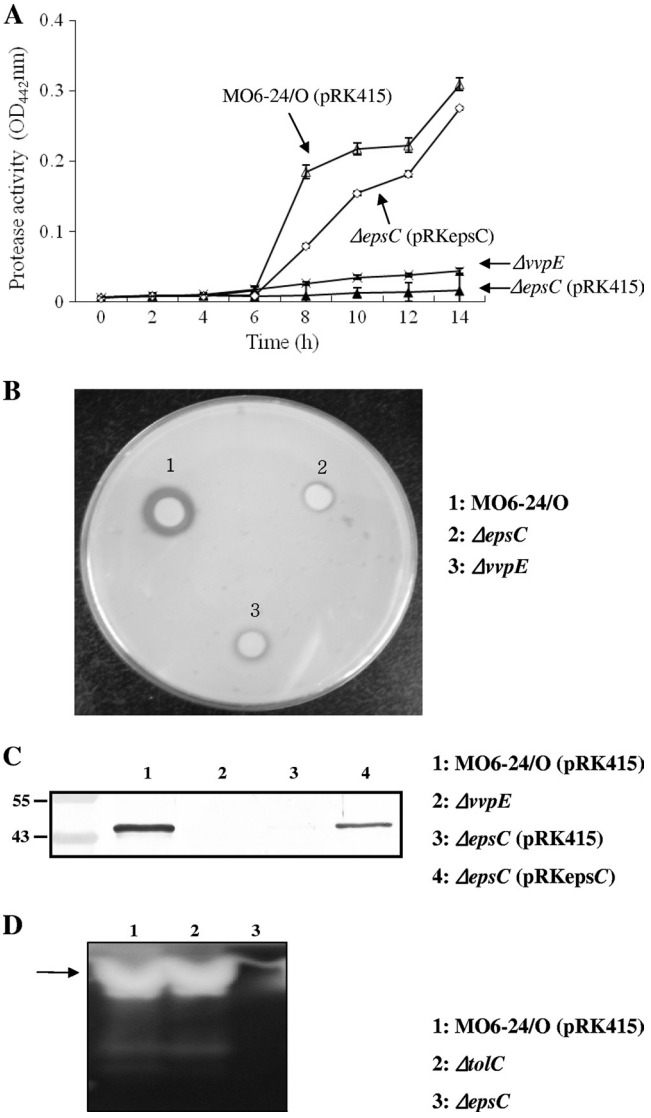

FIG. 7. Role of EpsC in the secretion of exoprotease(s) from *V. vulnificus*. (A) Proteolytic activities in the culture supernatants obtained from the *V. vulnificus* wild type carrying pRK415, the Δ*epsC* mutant carrying pRK415, the Δ*epsC* mutant carrying pRKepsC, and the Δ*vvpE* mutant. The total proteolytic activity was assayed after a 1-h incubation of the cell-free supernatant with an equal volume of azocasein. Upon termination of the reaction, the level of the produced azopeptide was determined by OD_{442} determination. (B) Comparison of the total protease activity shown by *V. vulnificus* strains, namely, the wild type (spot 1), Δ*epsC* mutant (spot 2), and Δ*vvpE* mutant (spot 3). *V. vulnificus* cells were spotted on a 1.5% skim milk-agar plate, and the cleared zones around bacterial colonies were monitored after 16 h of incubation at 30°C. (C) Secretion of VvpE in various *V. vulnificus* strains. The spent media after growth by the wild type carrying pRK415 (lane 1), the Δ*vvpE* mutant (lane 2), the Δ*epsC* mutant carrying pRK415 (lane 3), and the Δ*epsC* mutant carrying the complementation plasmid pRKepsC (lane 4) were analyzed by Western blotting using anti-VvpE Ab. Numbers at the left of the gel are molecular masses (in kilodaltons). (D) Zymographic analysis of VvpE activity. Cell-free supernatants of wild-type *V. vulnificus* MO6-24/O (lane 1), the Δ*tolC* mutant (lane 2), and the Δ*epsC* mutant (lane 3) collected at the stationary phase (OD_{600} of ~4.0) were loaded on a 12% polyacrylamide gel copolymerized with 0.3% gelatin. In-gel proteolytic activities were visualized as clear zones in a gelatin-containing gel upon Coomassie brilliant blue R staining. A clear zone produced by the elastase (VvpE) is indicated with an arrow.

supernatant, respectively. Hemolysin was barely detected in any of the fractions of the *V. vulnificus* Δ*vvhA* mutant. As a control for preparation of the periplasmic fraction, we monitored these fractions for their alkaline phosphatase activity, which is well known to be a periplasmic enzyme. Only for periplasmic fractions of these strains could we detect alkaline phosphatase activity.

In the case of VvpE, the periplasmic fractions were prepared from the wild-type, Δ*vvpE*, Δ*epsC*, and Δ*vvhA* mutant *V. vulnificus* strains and were analyzed by Western blotting using anti-VvpE Ab (Fig. 8). Only the periplasmic fraction of the Δ*epsC* mutant demonstrated an immunoreactive protein of 45 kDa. All these results suggest that a deficiency in EpsC function resulted in a defect of the transport of target proteins from the periplasmic space to the outer membrane.

Differential effects of VvhA and VvpE in the cytotoxicity of *V. vulnificus*. This study provides evidence that cytotoxic factors are secreted via the T2SS in *V. vulnificus*. We examined the possibility that VvhA and/or VvpE is the critical factor in the T2SS-mediated cytotoxicity of *V. vulnificus* to HEp-2 cells. The same amounts of secreted proteins of the Δ*vvhA* mutant, Δ*vvpE* mutant, and its isogenic strain, the wild type, were assayed for their cytotoxicity against HEp-2 cells by PI staining (Fig. 9). While 59% of the HEp-2 cells treated with wild-type *V. vulnificus* ($P = 0.00019$) were stained with PI, 9.5% of HEp-2 cells were stained with PI when they were treated with the supernatant of the Δ*vvhA* strain (8.0 μg/ml). In contrast, the culture supernatant of the Δ*vvpE* mutant strain showed slightly higher cytotoxicity (79%) than that of wild-type *V. vulnificus*, although their difference was not statistically significant ($P = 0.28$).

DISCUSSION

V. vulnificus is a Gram-negative pathogenic bacterium that causes primary septicemia due to multiorgan failure as a result of rapidly progressive shock syndrome (5, 12, 23). *In vitro* studies using human peripheral blood mononuclear cells (PBMC) and human Jurkat T-cell lines clearly demonstrated that *V. vulnificus* can induce the death of human cells at a dramatic rate (20, 21). In addition to the direct interaction of live *V. vulnificus* with human cells (22, 27, 28, 29, 57), incubation of human cell lines with the culture supernatant of *V. vulnificus* triggers the death of human cells, indicating a potential role of extracellular compounds secreted from *V. vulnificus* in pathogenicity (Fig. 3B and C). We searched the proteins secreted by the T2SS in order to better understand the participation of bacterial secretion systems in bacterial cytotoxicity to human cells.

The T2SS is one of the protein secretion systems responsible for the secretion of virulence factors in diverse Gram-negative bacterial pathogens (7). Heat-labile enterotoxin, a main toxin produced by enterotoxigenic *E. coli*, is secreted via the T2SS (56). In *Legionella pneumophila*, which causes a pneumonia known as Legionnaires' disease, the T2SS helps the bacterium grow and survive within various host environments, including amoebae, macrophages, and lungs (8, 55). In this microorganism, diverse extracellular proteins are translocated via the T2SS, including endoglucanase, aminopeptidases, a metalloprotease, and acid phosphatases (8, 39, 45). The T2SS plays an

TABLE 4. Localization of hemolytic and alkaline phosphatase activities in fractions of various *V. vulnificus* strains

Strain	Hemolytic activity (U/ng protein) in indicated cell fraction[a]			Alkaline phosphatase activity in indicated cell fraction[c]		
	Cytoplasm	Periplasm	Supernatant	Cytoplasm	Periplasm	Supernatant
Wild type	ND[b]	ND	1,640	ND	74	ND
Δ*epsC* mutant	0.735	118	ND	ND	48	ND
Δ*vvhA* mutant	ND	ND	ND	ND	43	ND

[a] Hemolytic activity was calculated by Shinoda et al. (51).
[b] ND, not detectable (<0.5 U/ng protein).
[c] Alkaline phosphatase activity was calculated by Yim and Villarejo as (OD_{420}/reaction time [in minutes] × milligrams of protein) × 1,000 (62).

important role in the pathogenesis of *V. cholerae* by secreting cholera toxin (48), which is responsible for the symptoms of the disease (17). The role of the T2SS in *V. vulnificus*, however, has been studied by using a *pilD* mutant strain defective in both the T2SS and type IV pili (36, 37). Therefore, in the present study, we investigated the roles of the T2SS in *V. vulnificus* by using a mutant defective in *epsC*, encoding an essential structural component of the T2SS, and found that its T2SS is involved in the secretion of diverse exoproteins (Fig. 2 and Table 3), including known secreted factors such as VvhA and VvpE (Fig. 5 to 7), and that its deletion results in a significant attenuation in mouse lethality (Fig. 4).

VvhA is a representative exotoxin produced by *V. vulnificus* (41). Although the cytotoxic mechanism of purified VvhA has been extensively studied in various cell lines (26, 30, 51, 63), the pathological significance of VvhA remains controversial. Depletion of VvhA in *V. vulnificus* through a deletion of the *vvhA* gene resulted in the same 50% lethal dose (LD_{50}) as that of the wild-type strain in mice mortality assays (60). However, an intraperitoneal injection of the Δ*vvhA* mutant resulted in a 3-fold-increased LD_{50}, and an oral inoculation of the Δ*vvhA* mutant further increased its LD_{50} value by 5-fold compared to that of the wild type (9). In addition to the effects of bacterial cells on mice, the cytotoxic effects of the secreted protein pools from each bacterial strain have been studied by estimating the lactate dehydrogenase (LDH) release from and observing the morphological change of human cell lines, showing contradictory results. Secreted proteins prepared from the Δ*vvhA* mutant showed decreased cytotoxicity against CHO cells when they were scored by microscopic observation (60). On the other hand, treatment of HEp-2 cells with the Δ*vvhA* secretome resulted in a release of LDH equal to that with the wild-type secretome (9). In our study, the cytotoxicity of the Δ*vvhA* secretome was measured by PI staining and subsequent FACS analysis of treated HEp-2 cells (Fig. 9), which clearly indicated that the mutant secretome shows significantly attenuated cytotoxicity.

The *V. vulnificus* Δ*epsC* mutant showed reduced exoprotease activity (Fig. 7A and B), in which the mainly reduced exoprotease was identified as VvpE (Fig. 7C and D). VvpE is an exoprotease responsible for the pathology caused by *V. vulnificus*, which was confirmed by using a purified enzyme (25, 34). However, deletion of *vvpE* in *V. vulnificus* does not alter the cytotoxicity against INT407 cells (13) or HEp-2 cells (9). In a mouse model, the Δ*vvpE* mutant demonstrated a lower LD_{50} if the mutant cells were administrated via the oral route (9, 49), but it showed an LD_{50} similar to that of the wild type if the bacteria were injected via an intraperitoneal route (9, 13, 49). A cytotoxicity assay using the secreted proteins of the Δ*vvpE* mutant demonstrated higher LDH release from HEp-2 cells

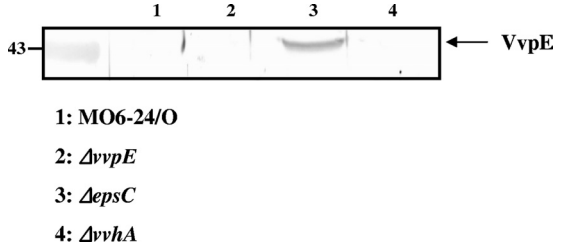

FIG. 8. Western blot analysis of periplasmic fractions using anti-VvpE Ab. The bacterial cells of the wild type (lane 1), the Δ*vvpE* mutant (lane 2), the Δ*epsC* mutant (lane 3), and the Δ*vvhA* mutant (lane 4) were grown to an OD_{600} of 1.0, and then each periplasm fraction was prepared as described in Materials and Methods. The resultant periplasmic fractions were analyzed by Western blot analysis using anti-VvpE Ab. The number at the left of the gel is the molecular mass (in kilodaltons).

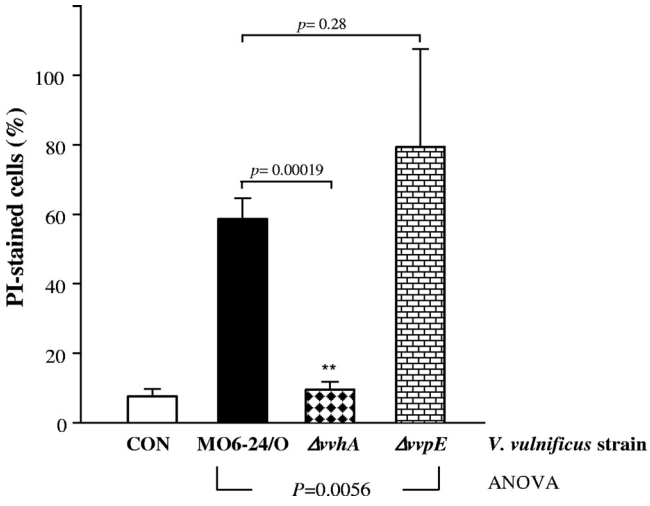

FIG. 9. Differential roles of VvhA and VvpE in cytotoxicity to HEp-2 cells. HEp-2 cells were treated with 8 μg/ml of secreted proteins prepared from *V. vulnificus* MO6-24/O, the Δ*vvhA* mutant, or the Δ*vvpE* mutant and then incubated for 4 h. Percentages of dead HEp-2 cells were determined by PI staining and subsequent FACS analysis. HEp-2 cells unexposed to the secreted proteins of *V. vulnificus* were also stained with PI (open bar). Error bars represent the means ± standard deviations of results from three independent experiments. Double asterisks indicate that percentages of PI-stained cells were significantly different ($P < 0.01$) from that of wild-type *V. vulnificus* PI-stained cells by Student's *t* test. *P* values of ANOVA analysis are also presented in the graph.

than in the wild type (9), which is in accordance with our observation determined by PI staining and the FACS method (Fig. 9). Therefore, VvhA is a direct cytotoxic factor, whereas VvpE may function indirectly to modulate other cytotoxic factors in a negative mode. The higher cytotoxicity of the Δ*vvpE* strain has been attributed to the increased level of VvhA in the Δ*vvpE* mutant, raising the possibility that VvhA is one of the target proteins inactivated by VvpE (48; H.-J. Lee, M.-A. Lee, and K.-H. Lee, unpublished result). Thus, the differential contributions of VvhA and VvpE and their interactive characteristics in the pathogenesis of *V. vulnificus* need further investigation.

The *V. vulnificus* Δ*epsC* mutant showed no apparent hemolytic activity (Fig. 5) and an absence of VvhA in its secreted protein pool (Fig. 6A). Thus, no apparent hemolytic activity in the Δ*epsC* culture supernatant was assumed to be caused mainly by its defects in the secretion of VvhA. Through analyses of cellular localization of VvhA among the separated fractions of the mutant, we showed hemolytic activity entrapped in the periplasmic space of this mutant (Table 4). These results indicate that translocation of VvhA from the periplasm to outside the cell is required for EpsC to be functional and that its transport from the cytoplasm to the periplasmic space is independent of EpsC but may occur via the Tat or Sec secretion system.

We also examined the role of RtxA, since it is also involved in the hemolytic function shown by *V. vulnificus* (22). The *V. vulnificus* Δ*rtxA* mutant was dramatically defective in cytotoxicity against HEp-2 cells (Fig. 3A), whereas its culture supernatant was still proficient in killing HEp-2 cells with a higher degree of cytotoxicity than the wild type by a yet-unknown mechanism (Fig. 3B). These results indicate that *V. vulnificus* needs to directly contact host cells to exhibit the extracellular function of RtxA, as previously shown (22, 28). In addition, RtxA secretion was not mediated by the T2SS but rather happened through the T1SS, as shown in the experiment using the *V. vulnificus* Δ*tolC* mutant (Fig. 6C). Thus, the defect in hemolytic activity and the reduced virulence of the Δ*epsC* mutant were not derived from altered secretion of RtxA.

We extended the list of proteins secreted by the T2SS, in addition to VvhA and VvpE, in *V. vulnificus* through comparative secretome analysis between the wild type and the Δ*epsC* mutant (Fig. 2). All the identified proteins, whose secretion was abolished or highly reduced in the T2SS-defective mutant, were shown to have the leader peptide sequences (10), as predicted by the SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/). It strongly suggests that the identified proteins in this study are targets for the *V. vulnificus* T2SS (Table 3).

Several putative membrane-bound proteins were found to be secreted at basal levels in the T2SS-defective strain. In *V. cholerae*, loss of extracellular secretion due to a T2SS defect results in a broad range of alterations in its outer membrane integrity, which causes interference in its barrier function and induction of the extracytoplasmic stress sigma factor RpoE (53). Cell envelope perturbation due to a T2SS defect induces oxidative stress and misregulation of iron homeostasis in *V. cholerae* (52). In *Shewanella oneidensis*, the T2SS is also involved in the extracellular secretion of two outer membrane lipoproteins, MtrC and OmcA, which are important for reducing metal oxide (50). We observed that the loss of T2SS function resulted in the increased sensitivity of *V. vulnificus* to bile salt (data not shown), indicating that the outer membrane integrity was altered by the loss of the T2SS in *V. vulnificus*. Therefore, the specific role of the T2SS in stress responses of *V. vulnificus* under host environments will be another interesting aspect to be investigated.

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