Comparative analysis of proteins in the culture supernatants of human intestinal epithelial cells infected with the wild-type and *rtxE* mutant of *Vibrio vulnificus*

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Abstract. Bacterial virulence factors and secreted extracellular proteins from damaged host cells following infection have been recognized as key mediators in the pathophysiological alterations observed in septic shock, and have also been shown to have a synergistic influence on bacterial pathogenicity. We hypothesized that during infections, virulence factors as well as host-shed proteins may synergistically influence aspects of the pathogenicity of *V. vulnificus*, such as primary septic shock and overproduction of proinflammatory cytokines. However, virulence factors and host-derived proteins have yet to be clearly evaluated during *V. vulnificus* infection. In this study, we analyzed and compared the proteins in conditioned supernatants generated from co-cultures of host cells and either wild-type or *rtxE* mutant *V. vulnificus* using LC-QTOF-MS/MS analysis. In a previous study, we determined that the culture supernatants of the *rtxE* mutant *V. vulnificus*-infected INT-407 cells induced significantly lower levels of IL-8 production from human intestinal epithelial cells than did the culture supernatants of wild-type *V. vulnificus*-infected INT-407 cells. LC-QTOF-MS/MS analysis results demonstrated that levels of proteins such as HSP90 α/β, 14-3-3 γ, PRX II, hnRNP K, β-actin, α-tubulin and *V. vulnificus* flagellin were significantly lower in the culture supernatants of *rtxE* mutant *V. vulnificus*-infected INT-407 cells than in the culture supernatants of wild-type *V. vulnificus*-infected INT-407 cells. These results demonstrate that *V. vulnificus* RTX toxins acting via rtxE, a transporter of virulence factors, play a very important role in the pathogenesis of *V. vulnificus*, as well as in its initial role in inducing pathogenic mediators from host cells.

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Introduction

Vibrio vulnificus is a gram-negative bacterium that causes severe septicemia in humans. *V. vulnificus* infection disease is usually caused by the consumption of seafood containing *V. vulnificus*. *V. vulnificus* induces fever, septic shock, and the formation of secondary lesions, accompanied in many cases by the emergence of ulcer and edema in patients (1). The mortality of primary septicemia exceeds 50% in septic patients, and death may occur within 1-2 days after the initial signs of illness. The majority of fatal cases are caused by septic shock (2), which results from a variety of virulence factors of *V. vulnificus*, including capsular polysaccharides (CPS) (3,4), siderophores (5), hemolysin (6), and repeat in toxin (RTX) (7).

Septic shock or septicemia results from bacterial virulence factors or the overproduction and dysregulation of the host factors in response to bacterial infections. *V. vulnificus* is severely acutely cytotoxic to host cells and generates a variety of virulence factors. Excessive cell damage by *V. vulnificus* infection from tissues undergoing necrosis, apoptosis, or severe inflammation can result in the release of relatively large quantities of host proteins, such as actin, into the extracellular space and peripheral circulation (8). An accumulation of secreted extracellular proteins from the host can contribute to increased blood viscosity, endothelial damage, and microvascular thrombosis (9).

These host- or bacteria-derived factors may persistently activate the generation of proinflammatory mediators such as tumor necrosis factor-α, IL-1β, IL-6, IL-8, and nitric oxide from affected hosts (10,11). The majority of these mediators display multiple biological effects, and have been identified as key mediators in the pathophysiological alterations associated with septic shock. Potential roles for these mediators have been suggested by the results of *in vivo* animal studies (12,13). Thus, we hypothesized that during infections, virulence factors, as well as host proteins, may exert a synergistic influence on *V. vulnificus* pathogenicity.

Gram-negative bacterial pathogens have been shown to generate structurally similar RTX toxins, including *V. cholerae* (14), *A. actinomycetemcomitans* (15), *P. baemolytica*, *A. pleuropneumoniae*, *P. vulgaris* and *M. morganii* (16-18). The RTX

toxin induces leakage of the cytoplasmic membrane, osmotic swelling, and cell lysis (19). Recently, the *V. cholerae* rtxA toxin has been recognized as one the most potent cytotoxic toxins, which display actin cross-linking activities and are secreted to the bacterial exterior by the type I secretion systems (TISS) consisting of rtxB, rtxD and rtxE (14,20,21). Another group has shown *V. vulnificus* RTX toxin to be a multifunctional cytotoxin, which performs a central role in the pathogenesis of *V. vulnificus* infectious disease. We previously demonstrated that the *rtxA* and *rtxE* mutants of *V. vulnificus* significantly impaired the cell rounding effect and cell viability in intestinal epithelial cells (22,23). The *V. vulnificus* RTX toxin performs an important role in inducing cytotoxicity in human intestinal epithelial cells. This cytotoxicity is correlated closely with apoptotic cell death (24).

The induction of pathological responses including the expression of proinflammatory cytokines is most likely due to *V. vulnificus* virulence factors such as RTX toxins, as well as endogenous proteins secreted from the host. In particular, endogenous proteins secreted by the host play a very important role in the expression of proinflammatory cytokines and inflammatory mediators. These and other related studies have provided evidence suggesting that immunotherapeutic intervention strategies that abrogate the biological activities of these mediators may exert a significant protective effect against the lethal effects of LPS (25). We believed that the secreted endogenous proteins from host cells could be increased significantly in *V. vulnificus*-associated septicemia. Recently, other groups have determined proinflammatory cytokine profiles in the sera of *V. vulnificus* septicemia patients (26). However, the identification of secreted host proteins from epithelial cells infected with *V. vulnificus* and their effects on pathological responses have yet to be investigated. In this study, we identified and compared proteins found in the culture supernatants of human intestinal epithelial INT-407 cells infected with the wild-type and *rtxE* mutant *V. vulnificus* via LC-QTOF-MS/MS analysis.

Materials and methods

Cell cultures. INT-407 cells, a human intestinal epithelial cell-line, were purchased from the American Type Culture Collection (ATCC, Manassas, VA), and maintained at 37˚C in 5% CO_2 in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (Gibco-BRL, Grand Island, NY) and antibiotics (10 U/ml penicillin G and 10 μg/ml streptomycin) (growth medium).

Bacterial strain and growth conditions. Wild-type MO6-24/O *V. vulnificus* used in this study was isolated from clinical patients. *rtxE* mutant and *rtxE*-complemented *V. vulnificus* were generated as previously described (22,23). For the infection experiments, the bacteria were grown at 30˚C in Luria-Bertani medium supplemented with 2.0% NaCl (LBS medium), and diluted to approximately $6x10^8$ CFU/ml in LBS, then centrifuged and resuspended in antibiotic-free growth medium prior to infection into human intestinal epithelial cells. Bacterial concentrations were confirmed via viable cell counts on LBS agar.

Infection protocol. INT-407 epithelial cells were infected with *V. vulnificus*, as previously described (22,23). In brief, INT-407 cells ($3x10^6$) were seeded and cultured for 24 h in antibiotic-free growth medium at 37˚C in a 5% CO_2 incubator. Prior to infection, the bacteria were centrifuged for 3 min at 5,000 x g, resuspended, and adjusted to approximately $6x10^8$ CFU/ml in antibiotic and phenol red-free MEM. The bacterial suspensions were added to INT-407 cells at a multiplicity of infection (MOI, ratio of bacteria no. to epithelial cell no.), after which the infected cells were incubated for 2 h in a 5% CO_2 incubator at 37˚C in antibiotic and phenol red-free growth medium.

Purification of proteins in the culture supernatants of V. vulnificus-infected intestinal epithelial cells. The culture supernatants from *V. vulnificus*-infected INT-407 cells were harvested by removing pellet cells via centrifugation at 4,500 x g for 10 min at 4˚C. The clarified supernatants were filtered through a 0.22 μM membrane filter (Millipore, Bedford, MA) to ensure removal of any remaining bacterial cells and debris. The supernatants were cooled and then 3.25 g $(NH_4)_2SO_4$ were added per 10 ml volume. This mixture was gently rocked at 4˚C for overnight. The proteins were then collected by centrifugation at 4,500 x g for 30 min at 4˚C. The proteins was resuspended in dialysis buffer (20 mM Tris-HCl. pH 7.0, 400 mM NaCl, 1 mM EDTA and 0.2 mM $CaCl_2$) to 1/50 the original volume. The samples were dialyzed for 12 h in 10 kDa molecular weight cut-off membranes (Pierce, Rockford, IL) against 1 liter dialysis buffer, with at least three changes in fresh dialysis buffer. Afterwards, the mixture was concentrated 10- to 15-fold using Amicon Ultra-4 (Millipore).

Western blot analysis. Protein concentrations were determined by using the BCA™ Protein assay reagent A (Pierce). Equal amounts (20 μg) of proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred onto a polyvinylidene fluoride membrane using a Semi-Phor (Hoefer Sci., Holliston, MA). The membrane was then incubated with washing buffer (PBS solution containing 0.1% Tween-20) containing 1% bovine serum albumin for at least 1 h to block nonspecific protein binding. Afterwards, the membrane was, respectively, treated with anti-β-actin, anti-α-tubulin, anti-HSP90 α/β, anti-PRX II, anti-14-3-3 γ, anti-hnRNP K, and anti-flagellin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). After incubation with HRP-conjugated secondary antibody, immunoreactive proteins were detected with the ECL system (Amersham Biosciences, UK).

Trypsin digestion of proteins. The proteins isolated from the culture supernatants of INT-407 cells and *V. vulnificus* were incubated overnight with 50 μl of 50 mM ammonium bicarbonate with trypsin: proteins (1:50) at 37˚C. Twenty micrograms of lyophilized trypsin (Promega, Madison, WI) was reconstituted in 100 μl of 50 mM acetic acid to form the stock solution which was diluted in 50 mM ammonium bicarbonate [1:10 (v/v)] and incubated for 15 min at 37˚C.

LC-QTOF MS/MS analysis. The enzymatically digested and extracted peptides from co-culture shed proteins were injected onto the column of LC-quadruple TOF MS on the Agilent 1100 system (Agilent, Palo Alto, CA) coupled to a QSTAR-XL mass spectrometer instruments (MDS Sci., Toronto, Canada).

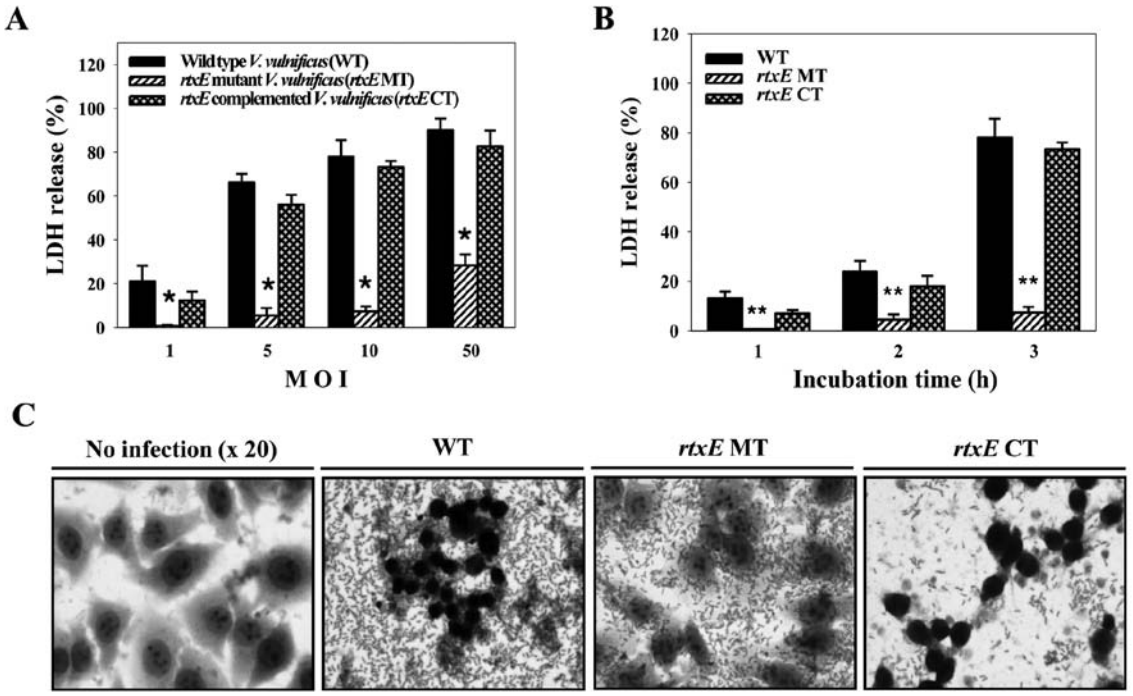

Figure 1. Effects of the *rtxE* gene mutation on cell cytotoxicity of *V. vulnificus* against intestinal epithelial cells. (A) INT-407 cells were infected for 3 h with wild-type (WT), *rtxE* mutant (*rtxE* MT), or *rtxE* complemented (*rtxE CT) V. vulnificus* at various MOIs, after which cell cytotoxicity was assessed by an LDH release assay, as described in Materials and methods. The data are expressed as means ± standard errors (n=3). *P<0.01, relative to groups infected with the wild-type at each MOI. (B) INT-407 cells were infected with wild-type, *rtxE* mutant, or *rtxE*-complemented *V. vulnificus* at a MOI of 10 for various times. The data are expressed as means ± standard errors (n=3). **P<0.01, relative to a group infected with the wild-type for each time. (C) INT-407 ($2x10^4$ cells/well) cells were incubated for 3 h with *V. vulnificus* in 24-well plates at an MOI of 10. The culture plates were centrifuged and washed twice with pre-warmed PBS (pH 7.4), and fixed with 4% paraformaldehyde. The cells were washed twice with PBS, and stained with Giemsa solution. Images of specimens were acquired with a microscope.

Both of a 0.3x5 mm trapping and a 0.1x150 mm resolving column packed with 3.5 μm, 300Å pore size (Agilent) were used for LC to fractionate prior to MS/MS analysis. The samples were separated using mobile phases A and B with a four-step linear gradient of 3 to 12.5% B in the first 15 min, followed by 12.5-32.5% B in the next 95 min and 32.5-45% B in the next 110 min and 35-90% in the last 15 min (mobile phase A, 0.1% formic acid-water: mobile phase B, 0.1% formic acid in acetonitrile). The peptides eluted at 0.3 μl/min were introduced into the mass spectrometer and MS, and MS/MS spectra were obtained in the information-dependent acquisition mode. The instrument cycled through acquisition of a full-scan MS spectrum, followed by three MS/MS scans of the most abundant ions from the MS scans obtained by collision with helium gas and only multiple charged ions were chosen for MS/MS. Each cycle was composed of 1 sec MS and 3 sec MS/MS. The instrument was programmed to utilize an exclusion list so as not to perform redundant MS/MS of peptide molecular ions. For the MS/MS analysis the collision energy was set to a rolling collision energy and the excluded former target ion was 90 sec.

Database searches. The search engine MASCOT (Matrix Science, London, UK) was used to analyze the MS/MS data by searching the Swiss-Prot human and eubacteria protein sequence database. The protein matches were considered valid if MS/MS data for multiple unique peptides were matched. The search parameters were: MS accuracy 0.5 Da, MS/MS accuracy 0.2 Da, allowed one miscleavage, fixed modification of cysteine in carbamidomethylated form, variable modification of oxidized methionine, deamidation (NQ) and formylation for only eubateria and acetylation for only human.

Statistical analyses. Student's t-tests and one-way analysis of variance (ANOVA), followed by the Bonferroni method, were employed in order to determine statistical differences between the values of the various experimental and control groups. P-values <0.05 were considered to be statistically significant.

Results

rtxE mutant V. vulnificus significantly reduces cytotoxicity against human intestinal epithelial cells. We previously reported that the *V. vulnificus* rtxA toxin secreted through the rtxE transporter induced significant cytotoxicity against human intestinal epithelial cells (22,23), and that the *V. vulnificus* rtxA toxin is associated with IL-8 production in human intestinal epithelial cells (27). In an effort to determine the role of the *V. vulnificus rtxE* gene in this cytotoxicity, human intestinal INT-407 epithelial cells were infected with wild-type (WT), *rtxE* mutant (*rtxE* MT) and *rtxE*-complemented (*rtxE* CT) *V. vulnificus*. As shown in Fig. 1A and B, the cytotoxicity induced by infection with *rtxE* MT *V. vulnificus* was significantly lower in INT-407 cells than in cells infected with WT *V. vulnificus*. However, these levels were restored in INT-407 cells infected with *rtxE* CT *V. vulnificus*. Furthermore, we noted that WT and *rtxE* CT *V. vulnificus* cause severe damage to intestinal epithelial cells (Fig. 1C). The INT-407

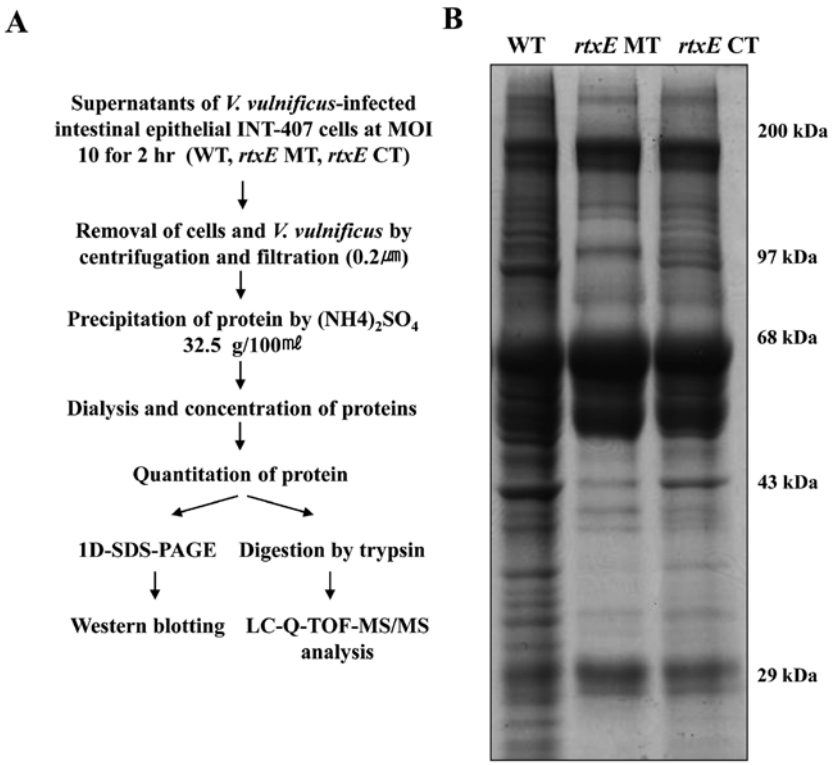

Figure 2. Schematic diagram of an experimental procedure for proteome research on the supernatants from *V. vulnificus*-infected intestinal epithelial INT-407 cells. (A) Preparation of the supernatants from wild-type (WT), *rtxE* mutant (*rtxE* MT), and *rtxE*-complemented (*rtxE* CT) *V. vulnificus*-infected intestinal epithelial INT-407 cells. (B) The supernatants were separated on a 6-16% gradient 1D-SDS PAGE gel and stained with Coomassie Brilliant Blue R250.

cells stained with Giemsa displayed marked cellular damage and cytoplasmic loss, whereas the cells infected with *rtxE* MT *V. vulnificus* displayed less-damaged surfaces and less profound cytoplasmic losses (Fig. 1C). These results demonstrate that the *rtxE* gene of *V. vulnificus* performs an important role in *V. vulnificus* infectious disease.

Comparison of proteins isolated from the culture supernatants of WT or rtxE MT V. vulnificus-infected INT-407 cells by 1D-SDS PAGE. We hypothesized that during infections, virulence factors as well as host proteins may be released into the culture supernatants. These factors may synergistically affect the pathogenicity of *V. vulnificus*. In a previous report, we demonstrated that the co-culture supernatants from rtxE MT *V. vulnificus*-infected INT-407 cells induced significantly lower levels of IL-8 production via NF-κB activation in human intestinal epithelial cells than the co-culture supernatants from WT *V. vulnificus*-infected INT-407 cells (27). Therefore, we anticipate that the culture supernatants from WT *V. vulnificus*-infected intestinal epithelial cells harbor a variety of inflammatory mediators from hosts and *V. vulnificus*.

To identify proteins shed from *V. vulnificus*-infected host cells, the culture supernatants were collected from WT, *rtxE* MT, or *rtxE* CT *V. vulnificus*-infected intestinal epithelial cells (Fig. 2A). The proteins were separated via 1D-SDS PAGE, followed by staining with Coomassie Brilliant Blue R250. The separated proteins in-gel image of the differently expressed bands is shown in Fig. 2B. In particular, the 30-150 kDa of shed proteins were dramatically different from the co-culture supernatants obtained from the WT and *rtxE* MT *V. vulnificus*-infected intestinal epithelial cells.

Identification of co-culture supernatants from WT or rtxE MT V. vulnificus-infected human intestinal epithelial cells. To completely identify shed proteins from WT or *rtxE* MT *V. vulnificus*-infected host cells, the proteins were first digested with trypsin, after which the tryptic digests were analyzed via LC-Q-TOF-MS/MS analysis. This method is more sensitive and accurate than traditional methods such as silver staining. In this study, 86 proteins were isolated from the WT *V. vulnificus*-infected intestinal epithelial cells (Table I), whereas 16 proteins were obtained from the *rtxE* MT *V. vulnificus*-infected intestinal epithelial cells (Table II). Forteen of the 86 proteins were derived only from WT *V. vulnificus* (Table III). Interestingly, bacterially-derived proteins were not detected in the cultures of the *rtxE* MT *V. vulnificus*-infected intestinal epithelial cells.

The identified proteins from the intestinal epithelial cells are involved in a variety of functions including the cytoskeleton, actin-associated network, metabolic enzymes, ribosomal proteins, and signal transduction (Table I and II). By way of contrast, the proteins identified from *V. vulnificus* are involved in cell motility, metabolic enzymes, and chaperone proteins (heat shock proteins) (Table III).

Subcellular location and functional classification of the identified proteins. These proteins were further classified according to their subcellular locations (Fig. 3). Interestingly, the highly detected proteins of host cells in the supernatants from the WT (39%) or *rtxE* MT (81%) *V. vulnificus*-infected intestinal epithelial cells were located primarily within the host cytoplasm (Fig. 3A and B). Additionally, *V. vulnificus*-derived proteins were located in the cytoplasm of *V. vulnificus* (65%) (Fig. 3C).

Table I. Host cell-derived proteins in the co-culture supernatants of wild type *V. vulnificus* and intestinal epithelial INT-407 cells.

Protein description	Swiss-Prot accession number	Cellular location	Experimental MW (Da)/pI
Actin network protein			
A-actinin-1	P12814	CP	103563/5.25
A-actinin-4	O43707	CP, N	105245/5.27
Actin, cytoplasmic1(β-actin)	P60709	CP	42052/5.29
Actin-related protein 2/3 complex subunit 4	P59998	CS	19637/8.53
Calponin-3	Q15417	NA	36562/5.69
Filamin-A (A-filamin)	P21333	CS	283192/5.73
Filamin-B (FLN-B)	O75369	CS	280188/5.49
Keratin, type I cytoskeletal 18	P05783	CS	47897/5.34
Keratin, type II cytoskeletal 7	P08729	CS	51312/5.50
Keratin, type II cytoskeletal 8	P05787	CS	53540/5.52
Microtubule-actin cross-linking factor 1,	Q96PK2	CP	673727/5.20
Profilin-1 (Profilin I)	P07737	CS	15085/8.48
Talin-1	Q9Y490	CS	271766/5.77
Tubulin α-ubiquitous chain	P68363	CS	50804/4.94
Tubulin β-2 chain	P07437	CS	50095/4.78
Tubulin β-2C chain	P68371	CS	50255/4.79
Vimentin	P08670	CS	53545/5.06
Vinculin (metavinculin)	P18206	CS	124161/5.51
Heat shock protein			
Heat-shock protein β-1	P04792	CP, N	22826/5.98
Heat shock cognate 71 kDa protein	P11142	CP	71082/5.37
Heat shock protein HSP 90-α (HSP 86)	P07900	CP	84875/4.94
Heat shock protein HSP 90-β	P08238	CP	83423/4.97
Stress-induced-phosphoprotein1	P31948	NA	63227/6.40
T-complex protein 1 subunit α	P17987	CP	60819/5.8
T-complex protein 1 subunit β	P78371	CP	57663/6.02
T-complex protein 1 subunit γ	P49368	CP	61066/6.10
T-complex protein 1 subunit δ	P50991	CP	58270/8.13
T-complex protein 1 subunit θ	P50990	CP	60022/5.42
T-complex protein 1 subunit ε	P48643	CP	60089/5.45
Metabolic enzyme			
Pyruvate kinase isozymes M1/M2	P14618	NA	58339/7.95
Transitional endoplasmic reticulum ATPase	P55072	CP, N	89819/5.14
Fructose-bisphosphate aldolase A	P04075	NA	39720/8.39
Fatty acid synthase	P49327	CP	275850/5.99
ATP-citrate synthase	P53396	CP	121660/6.95
D-3-phosphoglycerate dehydrogenase	O43175	NA	57225/6.31
Glucose-6-phosphate 1-dehydrogenase	P11413	NA	59553/6.44
FK506-binding protein 4	Q02790	CP, N	51926/5.35
Peptidyl-prolyl cis-trans isomerase A	P62937	CP	18098/7.82
UDP-glucose 6-dehydrogenase	O60701	NA	55674/6.73
L-lactate dehydrogenase A chain	P00338	CP	36819/8.46
Inorganic pyrophosphatase	Q15181	CP	33095/5.54
Serine hydroxymethyltransferase	P34897	Mit	56414/8.76
Asparagine synthetase	P08243	NA	64768/6.40
Protein arginine N-methyltransferase 1	Q99873	N	42029/5.31
Ribosomal protein			
40S ribosomal protein SA (p40)	P08865	CP	32816/4.79
40S ribosomal protein S3	P23396	CP	26842/9.68
40S ribosomal protein S5	P46782	CP, R	22902/9.73
40S ribosomal protein S7	P62081	CP, R	22113/10.09

Table I. Continued.

Protein description	Swiss-Prot accession number	Cellular location	Experimental MW (Da)/pI
40S ribosomal protein S8	P62241	CP, R	24344/10.32
40S ribosomal protein S11	P62280	CP, R	18459/10.31
40S ribosomal protein S15a	P62244	CP, R	14813/10.14
60S ribosomal protein L7	P18124	CP, R	29264/10.66
60S ribosomal protein L8	P62917	CP, R	28104/11.03
60S ribosomal protein L30	P62888	CP, R	12816/9.65
60S acidic ribosomal protein P0	P05388	CP, R	34423/5.71
60S acidic ribosomal protein P2	P05387	CP	11658/4.42
Protein degradation			
Proteasome activator complex subunit 1	Q06323	NA	28876/5.78
Ubiquitin-activating enzyme E1	P22314	NA	118858/5.49
Heterogeneous nuclear ribonucleoprotein			
hnRNP K	P61978	CP, N	51230/5.39
hnRNP H	P31943	CP, N	49352/5.89
Poly(rC)-binding protein 1	Q15365	CP, N	37987/6.66
Signaling protein			
14-3-3 protein γ	P61981	CP	28325/4.80
14-3-3 protein ζ/δ	P63104	CP	27899/4.73
14-3-3 protein θ	P27348	CP	28032/4.68
14-3-3 protein ε	P62258	CP	29326/4.63
14-3-3 protein σ	P31947	CP	27871/4.68
Ras GTPase-activating-like protein IQGAP1	P46940	M	189761/6.08
AP-2 complex subunit β-1	P63010	M	105398/5.22
Galectin-3	P17931	N	26098/8.61
Transcription or replication factor			
ATP-dependent DNA helicase 2 subunit 1	P12956	N	69953/6.23
ATP-dependent DNA helicase 2 subunit 2	P13010	N	83091/5.55
ATP-dependent RNA helicase DDX3X	O00571	N	73466/6.73
DNA replication licensing factor MCM3	P25205	N	91551/5.53
DNA-dependent protein kinase catalytic subunit	P78527	N	473749/6.75
DNA replication licensing factor MCM5	P33992	N	83031/8.64
Alanyl-tRNA synthetase	P49588	CP	107476/5.31
Threonyl-tRNA synthetase	P26639	CP	84294/6.23
Valyl-tRNA synthetase	P26640	NA	141642/7.53
Ribonucleoside-diphosphate reductase large subunit	P23921	CP	90925/6.76
Polypyrimidine tract-binding protein 1	P26599	N	57357/9.22
Polyadenylate-binding protein 1	P11940	CP, N	70854/9.52
Splicing factor	P23246	N	76216/9.45
Elongation factor 1-α 1	P68104	CP	50451/9.10
Elongation factor 1-γ	P26641	CP	50298/6.27
Elongation factor 2 (EF-2)	P13639	CP	96115/6.42
Translation factor			
Eukaryotic initiation factor 4A-I	Q14240	NA	46601/5.33
Eukaryotic translation initiation factor 3 subunit 5	O00303	CP	37654/5.24
Eukaryotic translation initiation factor 3 subunit 9	P55884	CP	92833/4.89
Eukaryotic translation initiation factor 3 subunit 10	Q14152	CP	166867/6.83
RNA-binding protein FUS	P35637	N	53622/9.40
Other function protein			
Clathrin heavy chain 1	Q00610	CP	193129/5.48
Coatomer subunit β	P53618	CP, GA	108211/5.64
Eukaryotic peptide chain release factor subunit 1	P62495	CP	49097/5.51

Table I. Continued.

Protein description	Swiss-Prot accession number	Cellular location	Experimental MW (Da)/pI
Ezrin (p81) (cytovillin) (villin-2)	P15311	M	69339/5.9
Multifunctional protein ADE2	P22234	NA	47659/7.09
Neuroblast differentiation-associated protein AHNA	Q09666	N	312580/6.29
Niban-like protein (Meg-3)	Q96TA1	NA	83144/5.81
Non-POU domain-containing octamer-binding protein	Q15233	N	54311/9.01
Peroxiredoxin-2 (EC 1.11.1.15)	P32119	CP	21918/5.67
Tropomyosin α-3 chain	P06753	CS	32856/4.68
Serum albumin precursor	P02768		71317/5.92
Channel or transporter protein			
Exportin-1 (Exp1)	O14980	CP, N	124447/5.71
Exportin-2 (Exp2)	P55060	CP, N	111145/5.51
Importin β-1 subunit	Q14974	CP, N	98420/4.68
Importin β-3	O00410	CP, N	124901/4.83
Importin-7 (Imp7)	O95373	CP, N	120751/4.70

CP, cytoplasm; CS, cytoskeleton; GA, Golgi apparatus; M, membrane; Mit, mitochondrion; N, nucleus; R, ribosomal; V, vesicle; NA, no annotation

Table II. Host cell-derived proteins in the co-culture supernatants of *rtxE* mutant *V. vulnificus* and intestinal epithelial INT-407 cells.

Protein description	Swiss-Prot accession number	Cellular location	Experimental MW (Da)/pI
Actin network protein			
Actin, cytoplasmic 2 (G-actin)	P63261	CP	42108/5.31
Actin-depolymerizing factor	P06396	CP	86043/5.90
α-2-macroglobulin precursor	P01023	CP	164600/6.00
α-cardiac actin	P68032	CP	42334/5.23
Fibulin-1 precursor	P23142	CP	81315/5.11
Acute phasge protein			
Complement C3 precursor	P01024	CP	188585/6.02
Complement C4-A precursor	P0C0L4	CP	194247/6.65
Complement C5 precursor	P01031	CP	189923/6.11
ITI heavy chain H2	P19823	CP	106826/6.40
ITI heavy chain H3	Q06033	CP	99401/5.61
Transcription factor			
Zinc finger protein 690	Q8IWY8	CP, N	98010/6.55
Transport protein			
Vitamin D-binding protein precursor	P02774	CP	54526/5.4
Hemoglobin subunit α	P69905	CP	15174/8.73
Metabolic enzyme			
Heparan-sulfate 6-O-sulfotransferase 1	O60243	V	48879/9.04
Other protein			
Poly(rC)-binding protein 1	Q15365	CP, N	37987/6.66
Plasminogen precursor	P00747	CP	93247/7.04
Synaptic vesicle membrane protein VAT-1 homolog	Q99536	V	42122/5.88

CP, cytoplasm; CS, cytoskeleton; GA, Golgi apparatus; M, membrane; Mit, mitochondrion; N, nucleus; R, ribosomal; V, vesicle; NA, no annotation.

Table III. Bacterial proteins in the co-culture supernatants of wild type *V. vulnificus* and intestinal epithelial INT-407 cells.

Protein description	Swiss-Prot accession number	Cellular location	Experimental MW (Da)/pI
Chaperones			
Chaperone protein htpG	Q82TV8	CP	69482/4.98
Chaperone protein dnaK	Q7N8Y4	CP	68875/4.75
Chaperone protein dnaK2	Q7V9G2	CP	68357/4.78
Chaperone protein dnaK3	Q8DH10	CP	75990/5.11
Cell motility and secretion			
Flagellin D	Q9KQ61	M	39749/4.87
Polar flagellin B/D	Q56702	M	40149/4.89
Metabolism			
Dephospho-CoA kinase	Q74IB6	CP	22298/9.04
S-adenosylmethionine synthetase	Q81KI0	CP	44075/4.92
Fumarate hydratase class II	Q7VKC9	CP	50679/5.72
Argininosuccinate lyase	Q9K821	CP	51186/5.37
6-phosphofructokinase	Q92BE4	CP	34368/5.57
Methyltransferase gidB	Q9RYD6	NA	27506/6.55
Replication			
DNA topoisomerase 3	Q9KQF5	CP, N	73178/9.17
Ribosomal protein			
30S ribosomal protein S8	Q9RSL4	R	15096/10.06

CP, cytoplasm; CS, cytoskeleton; GA, Golgi apparatus; M, membrane; Mit, mitochondrion; N, nucleus; R, ribosomal; V, vesicle; NA, no annotation.

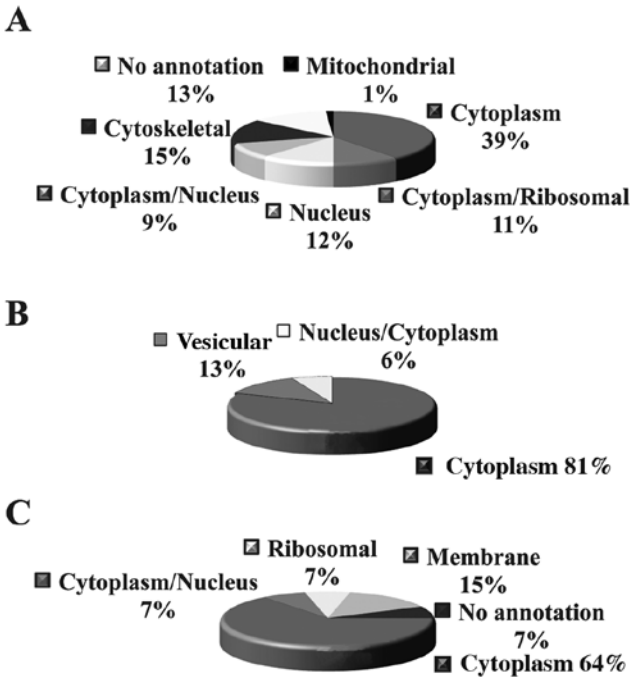

Figure 3. Subcellular location of identified proteins in the culture supernatants from wild-type or *rtxE* mutant *V. vulnificus*-infected INT-407 cells. (A) Subcellular location of host cell-related proteins in the supernatant from wild-type *V. vulnificus*-infected intestinal epithelial INT-407 cells. (B) Subcellular location of host cells-related proteins in the supernatant from *rtxE* mutant *V. vulnificus*-infected intestinal epithelial INT-407 cells. (C) Subcellular location of *V. vulnificus*-related proteins in the supernatant from wild *V. vulnificus*-infected intestinal epithelial INT-407 cells.

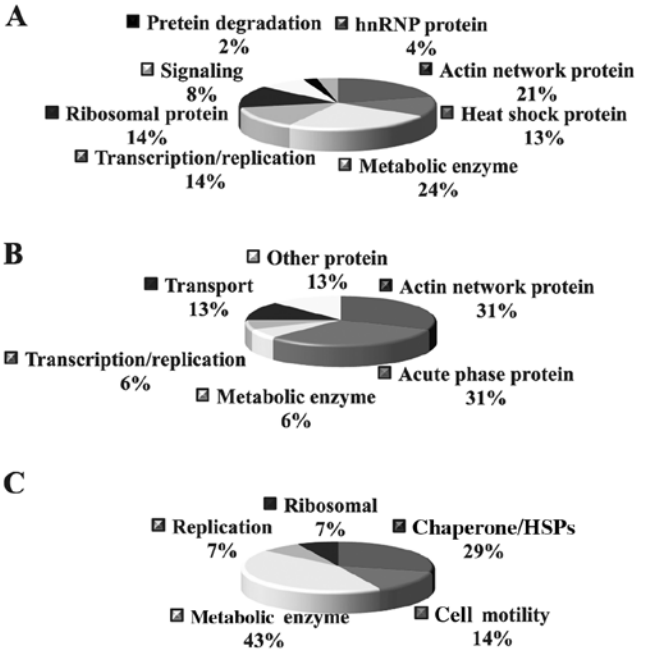

Figure 4. Functional classification of identified proteins in the culture supernatants from wild-type or *rtxE* mutant *V. vulnificus*-infected intestinal epithelial INT-407 cells. (A) Functional classification of host cell-related proteins in the supernatant from wild-type *V. vulnificus*-infected intestinal epithelial INT-407 cells. (B) Functional classification of host cell-related proteins in the supernatant from *rtxE* mutant *V. vulnificus*-infected intestinal epithelial INT-407 cells. (C) Functional classification of *V. vulnificus*-related proteins in the supernatant from wild-type *V. vulnificus*-infected intestinal epithelial INT-407 cells.

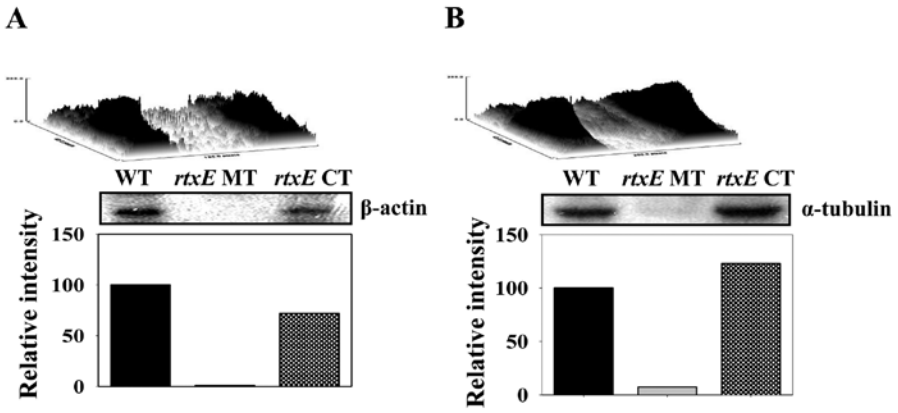

Figure 5. Quantitative confirmation of host cell-derived proteins via Western blot analysis. Proteins from the co-culture supernatant prepared as described in Materials and methods were analyzed via Western blot analysis using anti-β-actin (A) and anti-α-tubulin antibodies (B). The intensity of each band was densitometrically determined and expressed as intensity relative to the corresponding control from wild-type *V. vulnificus*-infected intestinal epithelial INT-407 cells (100%).

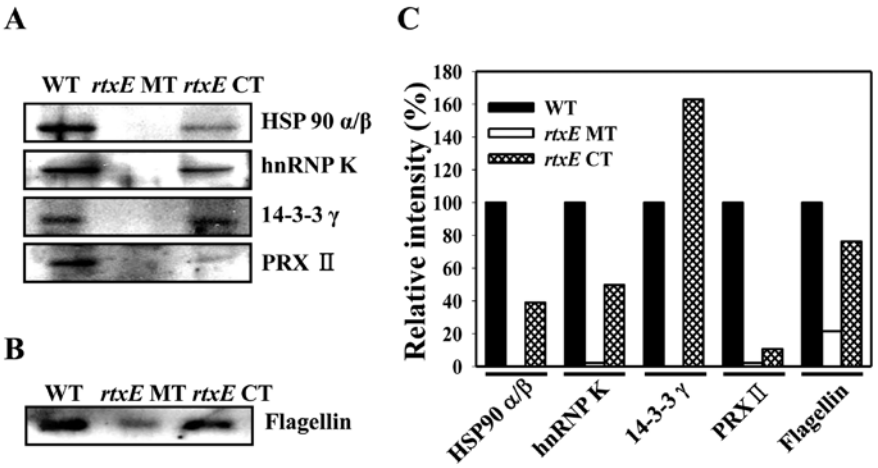

Figure 6. Quantitative confirmation of proteins derived from host cells or *V. vulnificus* by Western blot analysis. (A) Host cell-related proteins in the culture supernatants were analyzed via Western blot analysis using anti-HSP90 α/β, hnRNP K, anti-14-3-3 γ, and anti-peroxiredoxin II antibody. (B) Proteins prepared from the culture supernatants of INT-407 cells and *V. vulnificus* were analyzed via Western blot analysis using anti-flagellin antibody. (C) The intensity of each protein band was densitometrically determined and expressed as an intensity relative to the corresponding control from wild-type *V. vulnificus*-intestinal epithelial INT-407 cells (100%).

These 86, 16 and 14 identified proteins were then classified further according to function. As shown in Fig. 4, the classified host cell derived-proteins from WT *V. vulnificus*-infected intestinal epithelial cells included actin network proteins (21%), metabolic enzymes (24%), ribosomal proteins (14%), heat shock proteins (13%) and heterogeneous nuclear ribonucleoproteins (hnRNPs) (4%); together these accounted for approximately 86% of the total proteins (Fig. 4A). On the other hand, classified host cell-derived proteins from *rtxE* MT *V. vulnificus*-infected intestinal epithelial cells included actin network proteins (31%) and acute phase proteins (31%), which accounted for approximately 62% of the total proteins (Fig. 4B). The *V. vulnificus*-derived proteins were classified as metabolic enzymes (43%), chaperone proteins (heat shock proteins) (29%) and cell mobility proteins (14%) (Fig. 4C).

Comparison and validation of the quantitation of identified proteins. We further confirmed the differential expression of the proteins identified by mass spectrometry via a Western blotting assay. As demonstrated in Figs. 5 and 6, the relative expression of seven representative proteins such as β-actin, α-tubulin, HSP90 α/β, hnRNP K, 14-3-3 γ, peroxiredoxin II (PRX II), and flagellin in the culture supernatants of INT-407 cells infected with either the WT or *rtxE* MT *V. vulnificus* were consistent with those obtained via mass spectrometry approaches (Figs. 5 and 6, and Tables I-III).

Discussion

The majority of *V. vulnificus* studies have focused on the bacterial factors involved in the disease (2). While a great deal is currently known regarding some of the bacterial virulence factors involved in the disease process, little is known about the host cellular or endogenous factors relevant to *V. vulnificus*-induced primary septicemia. Furthermore, no studies have yet been carried out to analyze the culture supernatants from *V. vulnificus*-infected host cells via proteomic approaches.

In this study, we conducted experiments to identify factors in the culture supernatants from *V. vulnificus*-infected intestinal epithelial cells, using LC-QTOF-MS/MS analysis. This proteomic technique has been validated as a method appropriate for application to studies of protein shedding. We expected that the co-culture supernatant from *V. vulnificus*-infected intestinal epithelial INT-407 cells would include proteins from both *V. vulnificus* and host cells. As anticipated, many of the proteins originating from the host cells that were identified in the co-culture supernatants were known to be involved in a variety of functions including cytoskeleton, actin-associated networks, metabolic enzymes, and signal transduction; these proteins belonged to classes including the actins, tubulins, hnRNPs, heat shock proteins, 14-3-3 proteins, and peroxiredoxin II (Table I). A few proteins of *V. vulnificus* origin were metabolic enzymes, chaperones (heat shock proteins), and or motility proteins, such as argininosuccinate lyase, phosphoenol pyruvate-protein phosphotransferase, or flagellin B/D (Table III).

In this study, we determined that the majority of the co-culture supernatant proteins from the *V. vulnificus*-infected intestinal epithelial INT-407 cells had originated from the host cells. These proteins included human β-actin, α-tubulin, HSP90 α/β, hnRNP K, 14-3-3 γ and peroxiredoxin II. Importantly, these proteins were not detected in the co-culture supernatant from the *rtxE* mutant *V. vulnificus*-infected intestinal epithelial INT-407 cells. Additionally, *V. vulnificus* flagellin was also not detected, or was detected at significantly lower quantities in the co-culture supernatant from *rtxE* mutant *V. vulnificus*-infected intestinal epithelial INT-407 cells than in the co-culture supernatants from wild-type *V. vulnificus*-infected intestinal epithelial INT-407 cells (Fig. 6).

hnRNPs are described as a major group of nuclear RNA binding proteins that carry out transcription, RNA processing, mRNA translation, and turnover-associated functions (28). In the present study, a total of two types of hnRNP factors were detected at significant quantities in the co-culture supernatants from wild *V. vulnificus*-infected intestinal epithelial INT-407 cells. The hnRNP K and poly(rC)-binding proteins participate in positive- strand virus genome replication. The 14-3-3 protein is externalized to the medium by epithelial cell types, although the function of this protein in *V. vulnificus* infectious disease remains to be clarified. PRX II belongs to a ubiquitous PRX family, which has been associated with multiple functions, including enhancing natural killer cell activity (29), increasing cell resistance to oxidative stress (30), protecting erythrocytes against oxidative stress (31), and anti-HIV activity (32). However, the function of PRX II in the context of *V. vulnificus* infectious disease also requires clarification. Heat shock proteins (HSP) are highly conserved proteins detected in all prokaryotes and eukaryotes. Under normal physiological conditions HSPs are expressed at low levels (33). However, a broad variety of stressful stimuli, including environmental, pathological (viral, bacterial, parasitic infections), or physiological stimuli, have been shown to induce marked increases in intracellular HSP synthesis (34). Recent findings have demonstrated that both HSP60 and HSP70 can employ CD14 to induce the generation of proinflammatory cytokines, thereby suggesting that HSPs represent a novel class of putative endogenous ligands for TLRs (35). TLRs are known to be involved in the induction of IL-8 production. LPS and heat shock proteins are also well known to induce IL-8 production via TLR4 (35,36). Therefore, we anticipate that the heat shock proteins may play important roles in *V. vulnificus* septicemia via the induction of inflammatory cytokines such as IL-8.

On the other hand, proteins originating from *V. vulnificus* constitute a very small portion of the total proteins identified by mass spectrometry. Interestingly, the flagellins of *V. vulnificus*, known as TLR5 ligands, were present in abundance in the co-culture supernatants. Recently, uropathogenic *E. coli* has been shown to activate IL-8 production via P-fimbriae, and the flagellins of different enteropathogenic bacteria and *V. vulnificus* flagellin have been determined to activate IL-8 production via TLR5 (37-39).

Collectively, the findings of this study show that *V. vulnificus rtxE* performs an important role in inducing cell cytotoxicity in human intestinal epithelial cells. The co-culture supernatants from *V. vulnificus*-infected INT-407 cells harbored a variety of bacterial toxins and inflammatory mediators from host cells and *V. vulnificus*, which are critically important to *V. vulnificus* septicemia. Additionally, these endogenous proteins and virulence factors may increase IL-8 production via the NF-κB pathway. It is probable that *V. vulnificus* rtxE toxin secretes the rtxA toxin and initiates the release of the endogenous mediators from host cells, thereby resulting in the induction of *V. vulnificus* infectious disease.

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