ORIGINAL

Characterization of Outer Membrane Vesicles Produced by *Vibrio vulnificus*

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Abstract : *Vibrio vulnificus* (*V. vulnificus*) is a halophilic gram-negative bacterium that inhabits coastal warm water and induce severe diseases such as primary septicemia. To investigate the mechanisms of rapid bacterial translocation on intestinal infection, we focused on outer membrane vesicles (OMVs), which are extracellular vesicles produced by Gram-negative bacteria and deliver virulence factors. However, there are very few studies on the pathogenicity or contents of *V. vulnificus* OMVs (Vv-OMVs). In this study, we investigated the effects of Vv-OMVs on host cells. Epithelial cells INT407 were stimulated with purified OMVs and morphological alterations and levels of lactate dehydrogenase (LDH) release were observed. In cells treated with OMVs, cell detachment without LDH release was observed, which exhibited different characteristics from cytotoxic cell detachment observed in *V. vulnificus* infection. Interestingly, OMVs from a *Vibrio Vulnificus* Hemolysin (VVH) and Multifunctional-autoprocessing repeats-in -toxin (MARTX) double-deletion mutant strain also caused cell detachment without LDH release. Our results suggested that the proteolytic function of a serine protease contained in Vv-OMVs may contribute to pathogenicity of *V. vulnificus* by assisting bacterial translocation. This study reveals a new pathogenic mechanism during *V. vulnificus* infections. J. Med. Invest. 71 : 102-112, February, 2024

Keywords **:** *Infection, Outer Membrane Vesicle, Pathogenicity, Serine Protease, Vibrio Vulnificus*

INTRODUCTION

Vibrio vulnificus (*V. vulnificus*) is a halophilic gram-negative bacterium that inhabits coastal warm water. *V. vulnificus* causes food-borne disease when raw or undercooked seafood is consumed and infects wounds following seawater exposure (1). Food-borne *V. vulnificus* infections exhibit several symptoms, such as diarrhea, vomiting, and abdominal pain, in otherwise healthy people. However, when immunocompromised patients with underlying diseases such as chronic liver diseases, diabetes mellitus, and hemochromatosis are infected by *V. vulnificus*, they often develop severe necrotizing fasciitis and primary septicemia (2). Most of these patients die within 48 hours of the infection, and the mortality rate is extremely high, in excess of 50% (3). This is why *V. vulnificus* is considered deadly. Due to its rapid disease progression, antibiotic treatment is extremely ineffective. Therefore, it is important to elucidate pathogenic mechanisms in *V. vulnificus*.

Previous studies have identified some main *V. vulnificus* virulence factors, such as a cytolysin-hemolysin (VvhA), multifunctional cytotoxin (RtxA1), and metalloprotease (VvpE). VvhA induces autophagy-related cell death (4), RtxA1 causes programmed necrotic cell death (5), and VvpE mediates the intestinal colonization by disrupting tight junctions (6). However, the links between the mechanism by which these pathogenic factors are transported and rapid sepsis are not clear.

Gram-negative bacteria produce extracellular vesicles, called outer membrane vesicles (OMVs), which are typically 20-200 nm in diameter. OMVs are produced when a portion of the outer membrane protrudes from the cell surface and pinches off. Therefore, various bacterial components such as lipopolysaccharide (LPS), phospholipids, outer membrane proteins, and entrapped periplasmic proteins are included in OMVs (7). With this biogenetic background, OMVs are known to harbor a wide range of function, such as antimicrobial resistance (8), bacteria-host lipid exchange (9), and others. Moreover, OMVs enable the delivery of pathogenic factors to various tissues via the blood stream (10).

Previous studies have shown that many gram-negative bacteria deliver virulence factors using OMVs. OMVs of Enterohemorrhagic *Escherichia coli* O157 carry a cocktail of virulence factors that cause cell death, such as Stx2a, CdtV, hemolysin, and flagellin (11). Furthermore, OMVs transport LPS to the host cells more effectively than bacterial cells alone and activate caspase-11 *in vitro* and *in vivo* (12). *V. cholerae* OMVs deliver Zn-dependent hemagglutinin protease (HAP) and calcium-dependent trypsin-like serine protease (VesC), which induce cytotoxicity and inflammatory responses in host cells (13). A previous report indicated that *V. vulnificus* OMVs deliver cytolysin-hemolysin (VvhA) into epithelial cells to induce cytotoxicity (14). However, there are very few studies on pathogenicity or contents of *V. vulnificus* OMVs (Vv-OMVs).

In this study, we investigated the biological effects of Vv-OMVs on host cells and found that Vv-OMVs induce cell detachment without causing lactate dehydrogenase (LDH) release. This study is the first report showing that Vv-OMVs cause cell detachment independently of VvhA and RtxA1.

Received for publication August 7, 2023 ; accepted November 30, 2023.

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MATERIALS AND METHODS

Bacterial strains and culture conditions

V. vulnificus strains–wild-type, VVH deletion mutant (ΔVvhA), MARTX deletion mutant (ΔRtxA1), and double knockout mutant (DKO) strains–of CMCP6 were provided by Dr. Takashige Kashimoto at Kitasato University (15, 16). *V. parahaemolyticus*, *V. cholerae*, and *V. mimicus* strains were obtained from Research Institute for Microbial Diseases (RIMD), Osaka University (Table 1). *V. vulnificus*, *V. cholera*, and *V. mimicus* strains were cultured in 2% NaCl-LB broth (1% tryptone, 0.5% yeast extract), and *V. parahaemolyticus* strains were cultured in 3% NaCl-LB broth overnight at 37°C with shaking (170 rpm). Upon receipt, bacterial culture medium was centrifuged at 12,000 rpm for 1.5 minutes, concentrated and grown on thiosulfate-citrate-bile salts-sucrose (TCBS) agar overnight. A single colony was picked up from the TCBS agar and cultured into LB broth overnight. The resulting bacterial suspension was mixed with a sterile 50% glycerol solution (final 15% glycerol) and stored at -80°C. For experiments, bacterial strains were picked from the stock and were cultured in 2% or 3% LB broth overnight.

Isolation of bacterial OMVs

Bacteria were cultured on an LB agar plate overnight that was then overlaid with 10 ml of 2% or 3% NaCl-LB broth and incubated for an additional 7 hours. The liquid portion of the culture was centrifuged at 5,000 x g for 30 minutes at 4°C. The supernatant was treated with two-step filtration : 0.20 μm filter (Corning, NY) to remove remaining bacteria and a 100-kDa MWCO Amicon Ultra-4 centrifugal filter unit (Merck Millipore, MA) to remove proteins below 100 kDa. The supernatant was then ultra-centrifuged at 150,000 x g for 15 hours at 4°C. The supernatant was removed carefully and the resultant OMV pellet was dissolved in PBS buffer and stored at -80°C. A diagram is shown in Figure S1A. The protein content of the purified OMVs were lysed with RIPA buffer (pH 7.4, 50 mM Tris HCl, 150 mM NaCl, 1 mM EDTA, 1% Sodium deoxycholate, 0.1% SDS, 1% Triton-X) and determined using a Protein Assay BCA Kit (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). Hereinafter, protein concentration was used to substitute the concentration of OMVs. The quality of OMVs were checked by measuring the diameter size of the particles and inclusion of multiple proteins (Figure S1B and Figure S1C).

Cell culture and OMV treatment

The human epithelial cell line INT407 and the human epithelial colorectal adenocarcinoma cell line Caco-2 were cultured in DMEM (Sigma-Aldrich, MO) supplemented with 10% FBS (Gibco BRL, MD) and 50 μg/ml gentamicin (Sigma-Aldrich) under 5% CO_2 at 37°C for 3-4 days. The cells were seeded in 12- or 24-well culture plates at a density of 1×10^5 and 5×10^4 cells/well, respectively. After 4 days, the cells were washed twice with PBS, replaced with serum-free DMEM, and treated with 5 μg/ml OMVs in serum-free media.

V. vulnificus infections

The culture medium for human cell lines was replaced with serum-free DMEM at least 3 hours before infection. *V. vulnificus* was centrifuged at 12,000 rpm for 1.5 min, washed with PBS twice, adjusted to an optical density at 600 nm of 1.0 with PBS, diluted 10 times with PBS, and applied at 1/10 volume of the culture medium. The cells were infected with bacteria at a multiplicity of infection (MOI) of 1-10 under 5% CO_2 at 37°C.

Microscopy

Cells seeded in microwell plates were observed by the optical microscope BX50 (Olympus Corporation, Tokyo, Japan) and pictures were taken. The scale bars in each picture depicts 100 μm.

Cytotoxicity LDH assay

Cells seeded in 24-well plates were treated with *V. vulnificus* or OMVs. After 3 hours, supernatants were transferred to 96-well plates and the extracellular lactate dehydrogenase (LDH) release level was measured with the Cytotoxicity Detection Kit (Promega Corporation, WI) per manufacturer instructions. The maximum release (100% release) was estimated using 1% Triton-treated cells.

Crystal-violet staining

Cells seeded in 24-well plates were treated with *V. vulnificus* or OMVs. After 3 hours, the medium was removed from the wells and 200 μl crystal-violet staining solution (Becton, Dickinson and Company, NJ) was added to each well. After a 30 minute incubation at room temperature, the plates were washed in a stream of water, and the remaining liquid was removed. The plates were air-dried for 30 minutes at room temperature, and the optical density at 590 nm was measured.

Table 1. Bacterial strains used in this study.

Species	Bacteria	Description	Source
Vibrio vulnificus	CMCP6	*V. vulnificus* wild type, clinical isolate	15, 16
	ΔVvhA, RtxA1	VV2_0404 and VV2_0479 deletion mutant derived from CMCP6	
	ΔVllY	VV1_2768 deletion mutant derived from CMCP6	This study
	ΔPepA	VV1_1476 deletion mutant derived from CMCP6	
	ΔSecB	VV1_1278 deletion mutant derived from CMCP6	
Vibrio parahaemolyticus	RIMD2210633	*V. parahaemolyticus* wild type, clinical isolate	Research Institute for Microbial Diseases (RIMD), Osaka University
Vibrio cholerae	RIMD2203102	*V. choelare* wild type, clinical isolate	
Vibrio mimicus	RIMD2218070	*V. mimicus* wild type, clinical isolate	

Reagents

The Protease Inhibitor Cocktail Set Ⅲ DMSO Solution (EDTA Free), 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), aprotinin bovine lung, and leupeptin hemisulfate monohydrate were obtained from Wako. The stocks were dissolved in water. Bestatin and pepstatin A were also obtained from FUJIFILM Wako Pure Chemical Corporation, and the stocks were dissolved in DMSO. E-64 was obtained from Sigma-Aldrich, and the stock was dissolved in water. Each protease was used with the following final concentration : AEBSF 1 mmol/L, aprotinin bovine lung 0.8 μmol/L, E-64 15 μmol/L, leupeptin hemisulfate 20 μmol/L, bestatin 50 μmol/L, pepstatin A 10 μmol/L.

Construction of mutant strains

The mutant strains in this study are listed in Table 1. Knockout mutants of VV1-2768 (VllY), VV1_1476 (PepA), and VV1_1278 (SecB) were constructed by homologous recombination using the suicide vector pYAK1 (R6K*ori*, *sacB*, Cm^r). Oligonucleotide primers for PCR are listed in Table 2. Two DNA fragments, 500-bp upstream and downstream regions of targeted gene, were amplified from genomic DNA by PCR. For the PCR reagents, we used Tks Gflex DNA polymerase and 2×Gflex PCR Buffer (Takara Bio Inc., Shiga, Japan). Overlap PCR was carried out using two DNA fragments as templates with forward-1 and reverse-2 primers and cloned into PCR Blunt II-TOPO® plasmid vector (Invitrogen, MA). After the PCR products containing deletion fragment were verified by sequencing analysis, the PCR products were digested with restriction enzymes, ligated into pYAK1, and then heat-shock transformed into DH5α-λpir strain. pYAK1 was extracted from the DH5α-λpir and transformed into *E.coli* BW19851 strain. The pYAK1-transformed *E.coli* BW19851 strain and *V. vulnificus* were conjugated on 0.5% NaCl-LB plate and then screened on TCBS agar with 5 μg/ml chloramphenicol. *V. vulnificus* with pYAK1 was negatively selected on an LB plate containing 10% sucrose by incorporation of the *Bacillus subtilis sacB* gene. Knockout mutants were selected and confirmed by PCR.

RESULTS

Vv-OMVs induce cell detachment associated with cell aggregation without causing cell death

To examine the effects of Vv-OMVs on host cells, INT407 cells were treated with purified Vv-OMVs and their morphological alteration observed. Two to three hours after Vv-OMV stimulation, the cells aggregated and detached from the bottom of the culture dish (Figure 1A). The observation was quantified by crystal-violet staining in which only live and attached cells are stained (Figure 1B). Similar result was obtained using Vv-OMVs from other *V. vulnificus* strains as well (Figure S2A). We observed that *V. vulnificus*-infected cells showed cell detachment as well, but it was not associated with cell aggregation (Figure 1A). Similar results were obtained in Caco-2 cells (Figure 1C). These results showed that Vv-OMVs induce cell detachment associated with cell aggregation whereas *V. vulnificus* infection causes cell detachment without cell aggregation.

To better characterize the difference of cell detachment between *V. vulnificus* and Vv-OMVs, cytotoxicity was estimated by measuring LDH release from cells. LDH release was significantly high in *V. vulnificus*-infected cells compared to PBS-treated cells, while LDH release from Vv-OMVs-treated cells was similar to that of PBS-treated cells (Figure 1D). Additionally, we estimated the cell viability in Vv-OMV-treated cells by re-culturing the collected cells. Interestingly, cells treated with Vv-OMVs showed regrowth and reached 100% confluence after 3 days of incubation. However, *V. vulnificus*-infected cells did not grow within the 3-day re-culture period (Figures 1E and 1F). These data indicated that Vv-OMVs induce cell detachment without causing cell death.

Cell detachment is caused by the OMVs of another Vibrio species

To understand the specificity of cell detachment with aggregation induced by Vv-OMVs, we purified OMVs from other *Vibrio* species (*V. parahaemolyticus*, *V. cholera*, *and V. mimicus*). OMVs of *V. parahaemolyticus* and *V. cholera* did not induce cell detachment. In contrast, cellular detachment was caused by OMVs of *V. mimicus* (Figures 2A and 2B). Surprisingly, *V. mimicus* OMVs also induced

Table 2. Primers used in this study.

Primer name	Sequence(5'-3')	Description
dVV1_2768-1-BamHI	GGATCCCTGGAATCATTCGGCTGATCTCTC	deletion mutant of VV1_2768
dVV1_2768-2	GAAGTTACCTTCGCCAAGCTGCTCGATACC	
dVV1_2768-3	GGTATCGAGCAGCTTGGCGAAGGTAACTTC	
dVV1_2768-4-PstI	CTGCAGACTGACAAATGGCCATAGTCACAG	
dVV1_1476-1-BamHI	GGATCCCAATGAGTTGAACGCAGCTACGCC	deletion mutant of VV1_1476
dVV1_1476-2	GACCAGCATAGAGACAGAAAGGCGACGTGG	
dVV1_1476-3	CCACGTCGCCTTTCTGTCTCTATGCTGGTC	
dVV1_1476-4-SphI	GCATGCAAGTAGCCTTGCTCTTCCCAAGTC	
dVV1_1278-1-BamHI	GGATCCATCGCCGTGATAATGAGCTTCGTG	deletion mutant of VV1_1278
dVV1_1278-2	CGCTAGGTTCAGTTGTGGTGAGTTTGGCGC	
dVV1_1278-3	GCGCCAAACTCACCACAACTGAACCTAGCG	
dVV1_1278-4-SphI	GCATGCGCAGATACGAGAATCATCACGCAG	

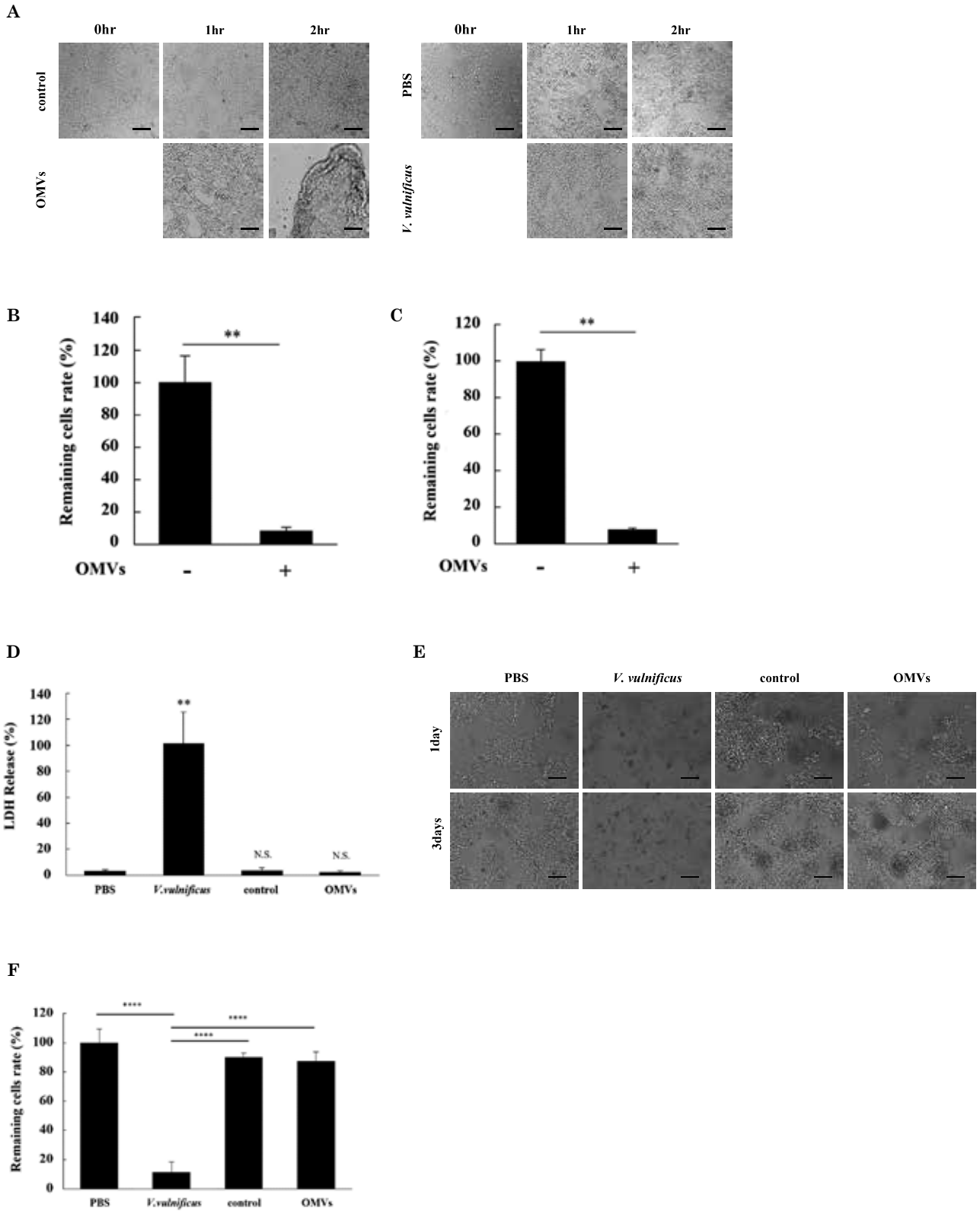

Figure 1. Vv-OMVs induce cell detachment associated with cell aggregation without causing cell death. INT407 cells were treated with 5 μg/ml Vv-OMVs or infected with *V. vulnificus* at a 1-10 MOI at 37 ℃ for 3 hours, and (A) the morphological alterations were observed (each scale bar indicates 100 μm). The remaining cells in the site of wells were quantified by crystal-violet staining in (B) INT 407 cells and (C) Caco-2 cells treated with or without Vv-OMVs. The results are expressed as a percentage relative to the control (** : $p<0.01$, by t-test, n=4). (D) LDH-releasing levels from Vv-OMV-treated or *V. vulnificus*-infected cells were measured by a cytotoxicity LDH assay. OMVs fraction obtained from *V. vulnificus*-free LB plate was used as control. (N.S. : not significant, ** : $p<0.01$, by t-test, n=4). Cells detached by Vv-OMV treatment or *V. vulnificus* infection were re-cultured and the morphology was observed (E), then the remaining cells rates were quantified (F). OMVs fraction obtained from *V. vulnificus*-free LB plate was used as control. The results are expressed as percentages relative to PBS-treated cells (**** : $p<0.0001$, by ANOVA, n=4).

cell cytotoxicity, which differed from the OMVs obtained from other *Vibrio* species (Figure 2C). These data indicated that the effects of OMVs on host cells depend on the *Vibrio* species.

Heat treatment and protease-inhibitor treatment attenuated cell detachment

In general, OMVs include several endotoxins such as flagellin and lipopolysaccharide (LPS). LPS is a heat-stable component of the gram-negative bacterial cell wall (17) that induces production of proinflammatory cytokines in several tissues (18). To test the contribution of LPS in cell detachment, we heat-treated Vv-OMVs at 95°C for 5 minutes prior to stimulating INT407 cells. Treatment with heated Vv-OMVs did not induce cell detachment (Figures 3A and 3B), suggesting that cell detachment by OMVs was not caused by LPS but induced by other heat-labile components.

Another study reported that *V. cholerae* OMVs deliver unique proteases that induce cytotoxic and inflammatory responses (13). Next we examined the association of proteases in cell detachment by using a protease-inhibitor (PI) cocktail. Interestingly, PI-cocktail treatment attenuated the cell detachment caused by Vv-OMVs (Figure 3B). As six protease inhibitors, AEBSF hydrochloride, aprotinin, E-64, leupeptin hemisulfate, bestatin, and pepstatin were included in this PI cocktail, we next treated Vv-OMVs with each individual PI. We found that AEBSF hydrochloride, which targets serine proteases, showed a strong attenuation of Vv-OMV-induced cell detachment (Figures 3C and 3D). Similar result was obtained using Vv-OMVs from other *V. vulnificus* strains as well (Figure S2B). These results suggested that serine proteases are instrumental in Vv-OMV-induced cell detachment.

Factors other than VvhA and RtxA1 cause cell detachment

VvhA and RtxA1, which are well characterized toxins in *V. vulnificus*, are known to induce cell death in host cells (4, 5). To investigate whether cell detachment was associated with VvhA or RtxA1, we purified OMVs from a VvhA and RtxA1 double-deletion mutant *V. vulnificus* strain (ΔVvhA/RtxA1) and treated INT407 or Caco-2 cells with the obtained OMVs. Interestingly, ΔVvhA/RtxA1 OMVs caused cell detachment at similar levels to wild-type Vv-OMVs (Figures 4A, 4B and 4C). Similarly, LDH release was not observed following treatment with ΔVvhA/RtxA1 OMVs (Figure 4D). Our data suggested that Vv-OMVs contain factors other than VvhA and RtxA1 that play a major role in inducing cell detachment.

Several proteins are unique to V. vulnificus OMVs

To identify proteins included in Vv-OMVs, we conducted a LC/MS/MS analysis, using *V. parahaemolyticus* OMVs (Vp-OMVs) as a comparative control that identified 34 and 54 proteins from Vp-OMVs and Vv-OMVs, respectively. While 12 proteins were common to both Vp-OMVs and Vv-OMVs, 42 proteins were specific to Vv-OMVs (Figure S3A). We hypothesized that these 42 proteins might be related to the specific effects of Vv-OMVs on host cells. Putative functions and subcellular localizations of the 42 identified proteins in Vv-OMVs were summarized in Figures S3B and S3C, respectively. Among these 42 proteins, we identified VllY, PepA, and SecB as putative candidates for proteins that induce cell detachment and constructed corresponding deletion-mutant strains for each protein. Cell detachment was not altered from wild-type by any of the OMVs obtained from these deletion-mutant strains (Figure 5), which indicates that these proteins do not serve as cell detachment factors.

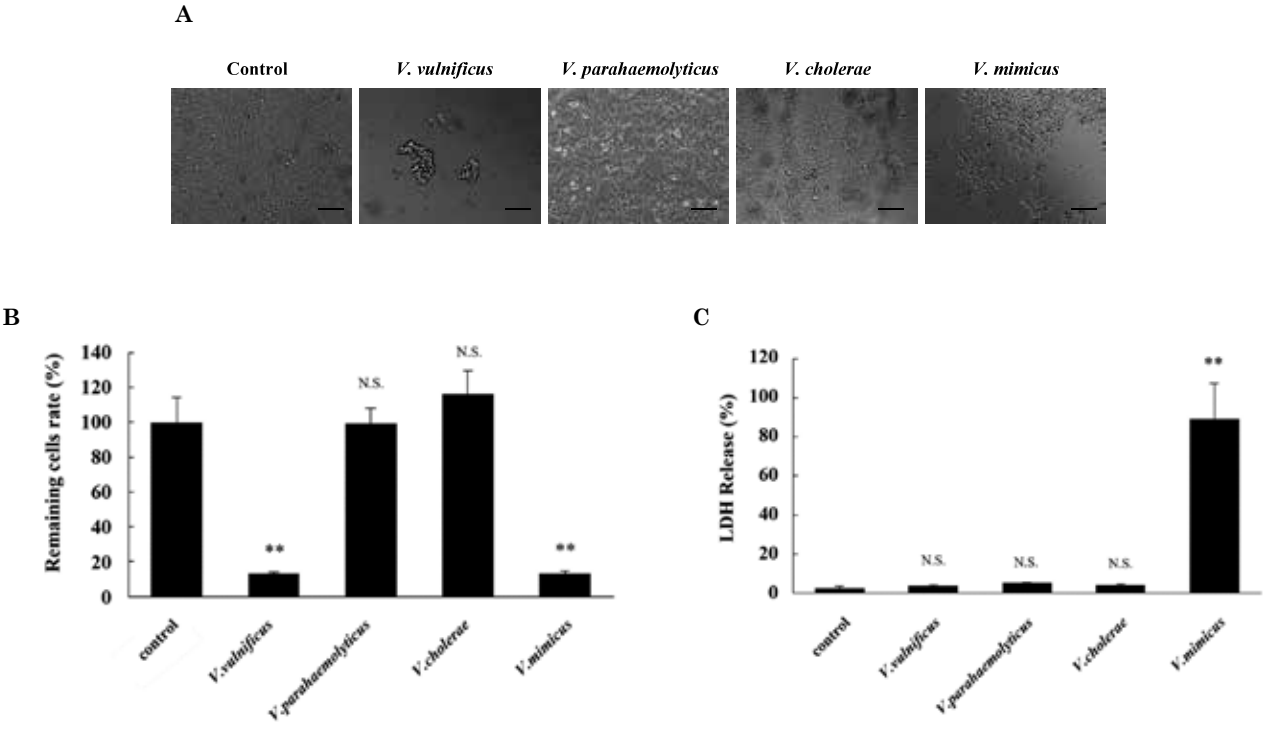

Figure 2. Cell detachment by OMVs of *Vibrio* species. INT407 cells were treated with 5 μg/ml OMVs purified from each *Vibrio* species at 37 ºC for 3 hours. The morphological alterations were observed (A), then the remaining cell rates (B) and LDH-releasing levels (C) were quantified (N.S. : not significant, ** : $p<0.01$, by t-test, n=4).

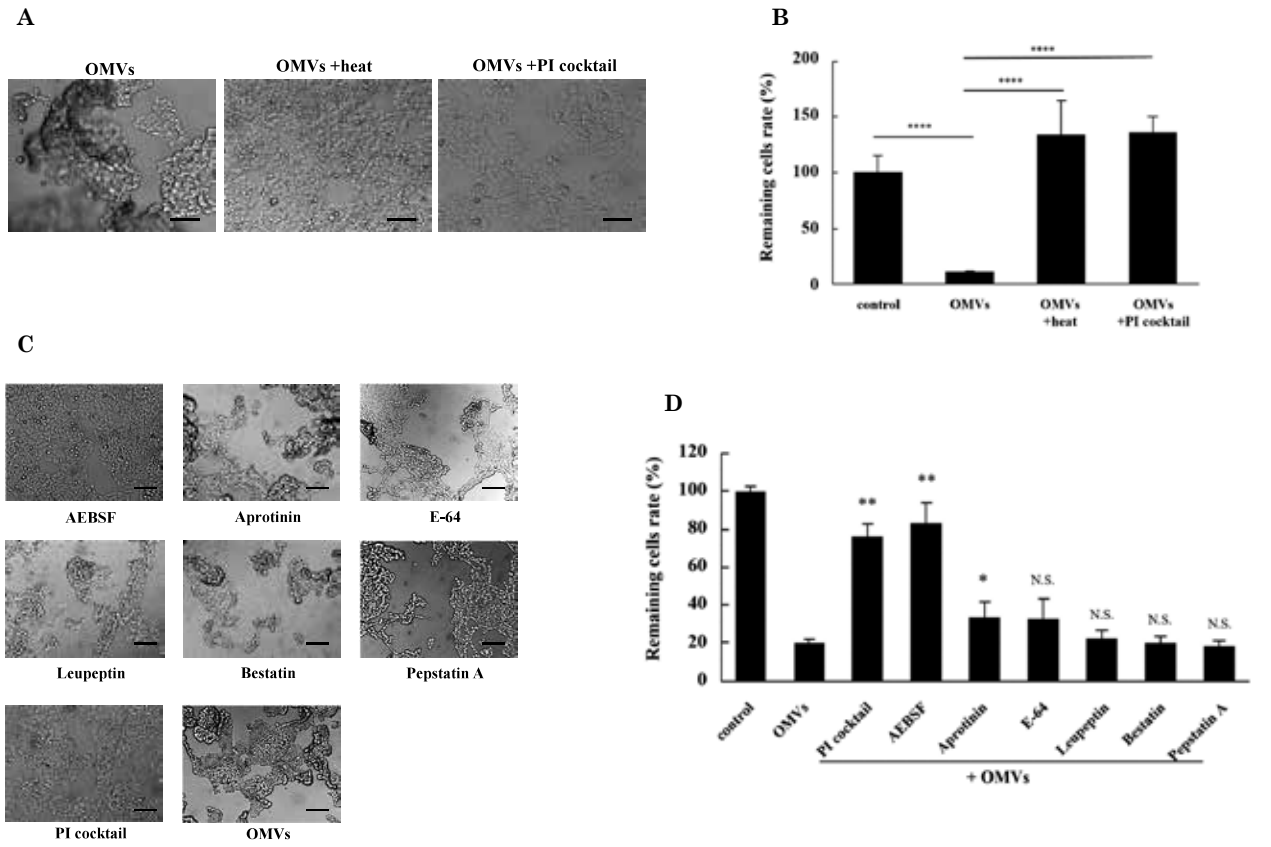

Figure 3. Cell detachment was prevented by heat treatment and protease inhibitors. Vv-OMVs were heated at 95 ºC for 5 minutes and used to treat INT407 cells. The protease inhibitor was mixed with Vv-OMVs, which were then incubated with INT407 cells at 37 ºC for 1 hour. The morphological alterations were observed (A and C), then the remaining cells rates were quantified (B) (**** : $p<0.0001$, by ANOVA, n=4) and (D) (** : $p<0.01$, * : $p<0.05$, N.S. : not significant, by t-test, n=4).

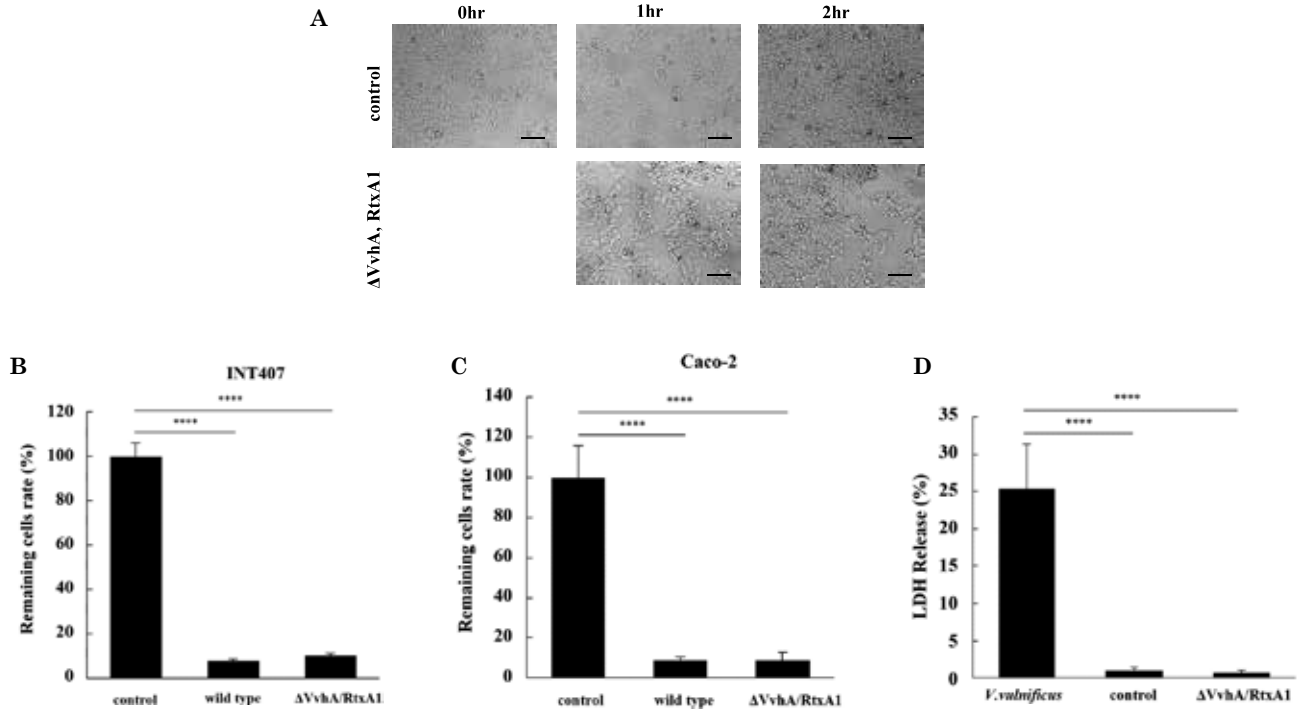

Figure 4. Cell detachment is caused by factors other than VvhA and RtxA1. INT407 and Caco-2 cells were treated with 5 μg/ml OMVs purified from the VvhA/RtxA1 double-deletion mutant *V. vulnificus* strain at 37ºC for 3 hours. The morphological alterations were observed (A), then the remaining cells rates were quantified in INT 407 (B) and Caco-2 (C) cells. The LDH-releasing levels were also quantified (D) (**** : $p<0.0001$, by ANOVA, n=4).

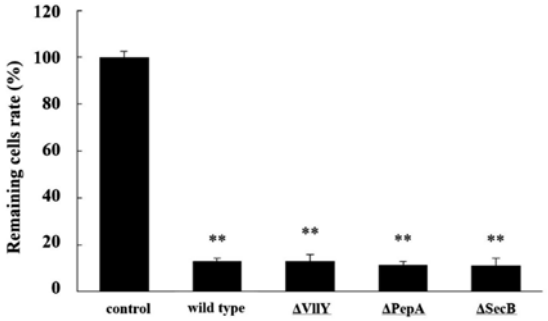

Figure 5. Evaluation of *V. vulnificus* OMV-protein deletion-mutant strains. Proteins contained in OMVs were identified by LC/MS/MS. OMVs were isolated from the indicated deletion-mutant strains. INT407 cells were treated with 5 μg/ml of OMVs from each of three deletion-mutant strains at 37 ºC for 3 hours, and the remaining cells rates were quantified (** : $p<0.01$, by t-test, n=4).

DISCUSSION

In this study, we revealed that Vv-OMVs cause cell detachment without LDH release (Figure 1) and that this phenotype is specific to *V. vulnificus* (Figure 2). In particular, cell detachment was induced independently of VvhA and RtxA1 (Figure 4) and might be caused by serine protease (Figure 3). Interestingly, we found that the inhibitory effect of Vv-OMVs cell detachment activity differed between the serine protease inhibitors AEBSF and aprotinin, which we speculate that aprotinin has low cell permeability and could not efficiently infiltrate the cell membrane and led to inefficient inhibition of cell detachment activity. These data suggest that serin protease included in OMV induces cell detachment.

Serine proteases have numerous biological roles in gram-negative bacteria (19). In *Campylobacter jejuni*, it is reported that the serine protease HtrA is included in OMVs and related to pathogenicity (20, 21). In another report, the Enteropathogenic *E.coli* serine protease EspC is indicated to be a pathogenic factor transported by the type-3 secretion system (T3SS), which transports virulence effector proteins to host epithelial cells and induces cell death or activates inflammatory cell signaling (22). Many virulent gram-negative bacteria, including most *Vibrio* species have a T3SS (23, 24). However, the *V. vulnificus* genome does not include any T3SS-associated genes (25). As we described in Figure 3, cell detachment without LDH release was specific to Vv-OMVs among *Vibrio* species, which means that there is a diversity in OMV's roles depending on the species of *Vibrio*. Thus, we hypothesized that Vv-OMVs have a role as a delivery system of virulence factors in *V. vulnificus* instead of T3SS.

Next, we considered if there is relationship between the Vv-OMVs and the fact that *V. vulnificus* infection has a rapid disease progression. To deliver bacterial components to places far from the infected sites, bacteria use tiny particles such as exosomes produced by host cells (26) or microvesicles that bud from the bacterial body itself (13). Additionally, it is reported that some serine proteases cleave adherent junction proteins, which helps bacteria access deeper tissues (27, 28). We found that under the presence of abundant Vv-OMVs, more *V. vulnificus* bacterial body tend to pass through the intestinal cell-to-cell junction from apical side to basolateral side, which might indicate that Vv-OMVs has a role to support the bacterial translocation of *V.vulnificus* (Figure S4). Moreover, since we revealed that Vv-OMVs could induce cell-detachment only two to three hours after infection (Figure 1), we hypothesize that *V. vulnificus* initiates the production of functional Vv-OMVs as soon as infection is established. Those OMVs circulate through the blood stream, spreading an unknown serine protease that attenuates the binding of surrounding cells, leading to systemic and rapid disease progression.

In this study we identified 42 specific proteins in Vv-OMVs compared to Vp-OMVs using LC/MS/MS (Figure S3). Since Vp-OMVs did not cause cell detachment (Figure 2), we hypothesized that the responsible virulence factors were included in the 42 proteins. The 42 proteins showed a wide diversity in their function and localization, but there were no hypothetical serine proteases included in Vv-OMVs. We therefore targeted and knocked out several genes which could be predicted to be associated with virulence to investigate serine protease mediated cell detachment, but in this study we were not able to identify a putative serine protease from LC/MS/MS analysis. Further studies are required at this point.

Here, we described the Vv-OMV-specific cell detachment and how it may support the bacterial translocation in *V. vulnificus* infection. Further study of OMVs can help to reveal new pathogenic mechanisms in *V. vulnificus* infection.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

FUNDING

This work was financially supported by JSPS KAKENHI Grant Number 20H01616, Japan.

ACKNOWLEDGMENTS

This study was supported by the Support Center for Advanced Medical Sciences, Tokushima University Graduate School of Biomedical Sciences.

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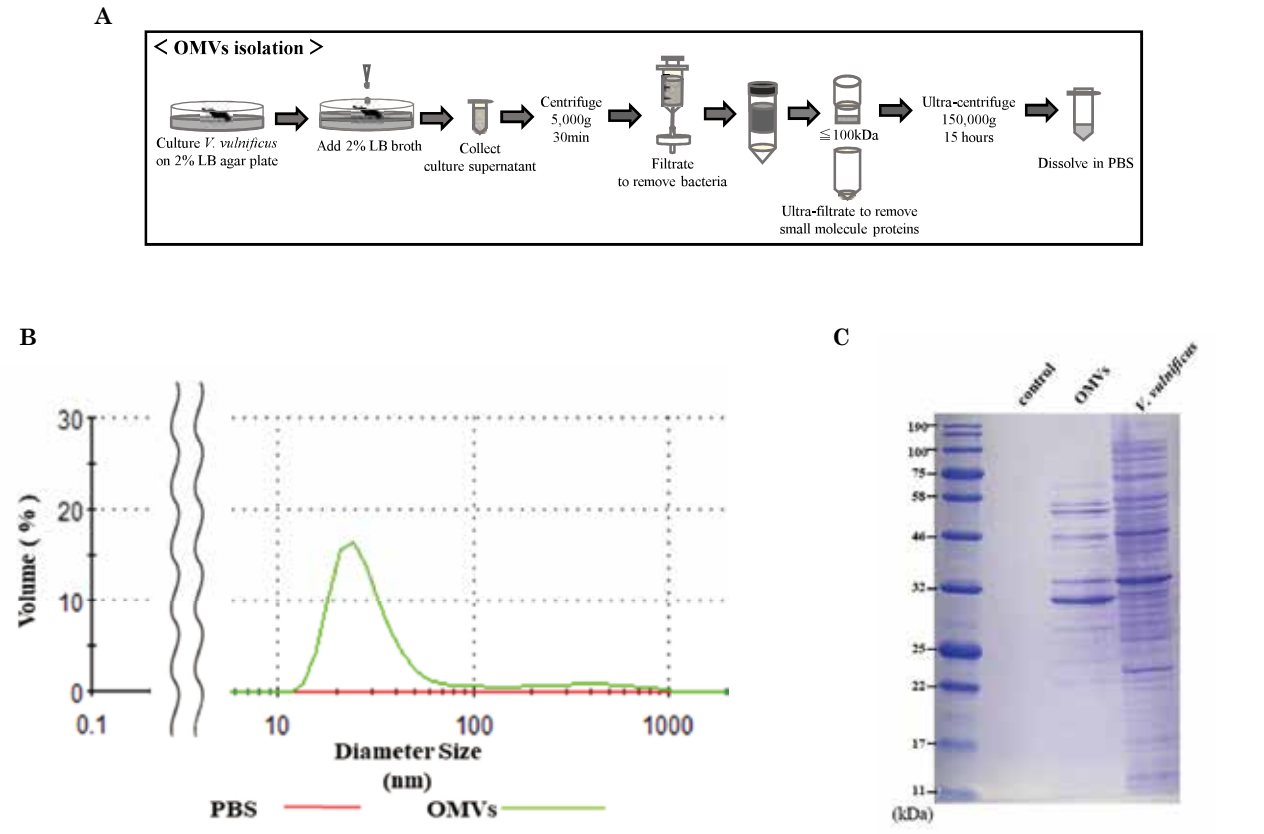


Figure S1. Isolation of Vv-OMVs. OMVs were isolated from *V. vulnificus* by combining ultracentrifugation and filtering methods (A). The size of OMVs were measured by Zetasizer Nano which can measure particle size (0.3 nm-10 μm) by using dynamic light scattering (B). Bacteria cells and OMVs were lysed with RIPA buffer and the presence of proteins were confirmed by CBB staining after SDS-PAGE (C).

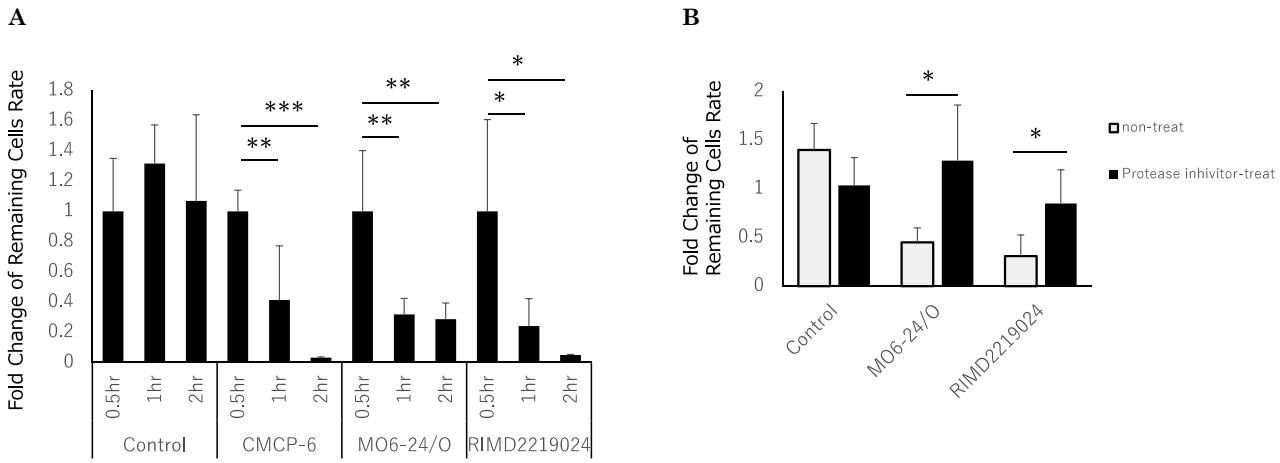
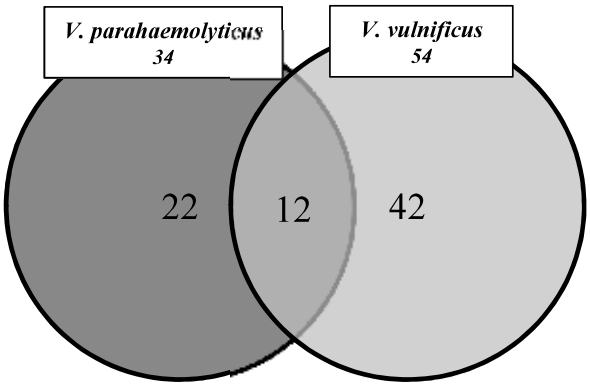


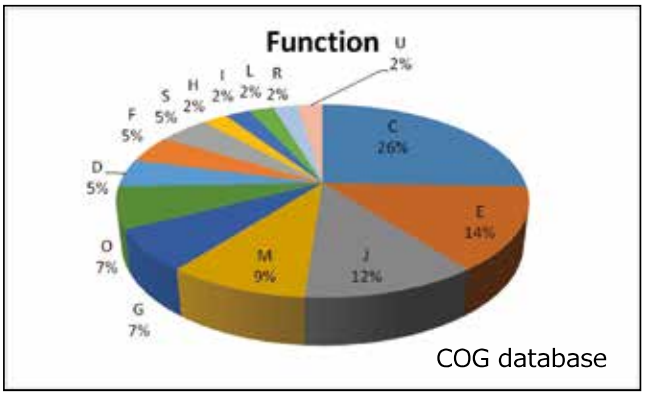
Figure S2. OMVs from three different V.vulnificus strains show similar features. OMVs from three different *V.vulnificus* strains were treated on cultured INT407 cells and remaining cells rate data was obtained by Crystal-violet staining (A) (*** : $p<0.005$, ** : $p<0.01$, * : $p<0.05$, by t-test, n=4). The same strains were treated with serine protease inhibitor AEBSF and remaining cells rate data was obtained by Crystal-violet staining (B) (* : $p<0.05$, by t-test, n=4).

A



Accession	Score	Mass	pI	Sequence coverage	emPAI	Description	COG	Function	Subcellular localization
PEPA_VIBVU	219	46641	5.15	0.44	0.89	Peptidase A	E	Amino acid transport and metabolism	Cytoplasmic
SECB_VIBVU	62	17246	4.57	0.34	0.28	Protein-export protein SecB	U	Intracellular trafficking, secretion, and vesicular transport	Cytoplasmic
VLLY_VIBVU	30	40255	5.01	0.08	0.11	Hemolysin VIIY	R	General function prediction only	Cytoplasmic

B



C	Energy production and conversion
E	Amino acid transport and metabolism
J	Translation, ribosomal structure, and biogenesis
M	Cell wall/ membrane/ envelope biogenesis
G	Carbohydrate transport and metabolism
O	Posttranslational modification, protein turnover, chaperones
D	Cell cycle control, cell division, chromosome partitioning
F	Nucleotide transport and mechanism
S	Function unknown
H	Coenzyme transport and metabolism
I	Lipid transport and mechanism
L	Replication, recombination and repair
R	General function prediction only
U	Intracellular trafficking, secretion, and vesicular transport

C

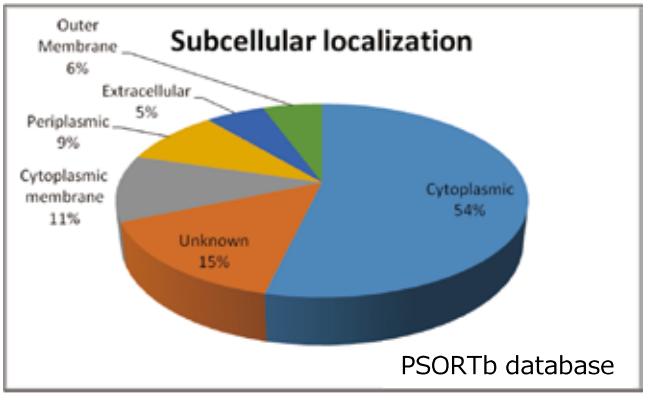


Figure S3. LC/MS/MS and related analysis. Proteins contained in OMVs were identified by LC/MS/MS. Among 54 proteins included in Vv-OMVs, 42 was specific to Vv-OMVs, which included PepA, SecB and VllY (A). The functions of proteins specific to Vv-OMVs were classified using COG database (available at : https://www.ncbi.nlm.nih.gov/research/cog) (B). The subcellular localization of proteins specific to Vv-OMVs were classified using PSORTb database (available at : https://www.psort.org/psortb/) (C).

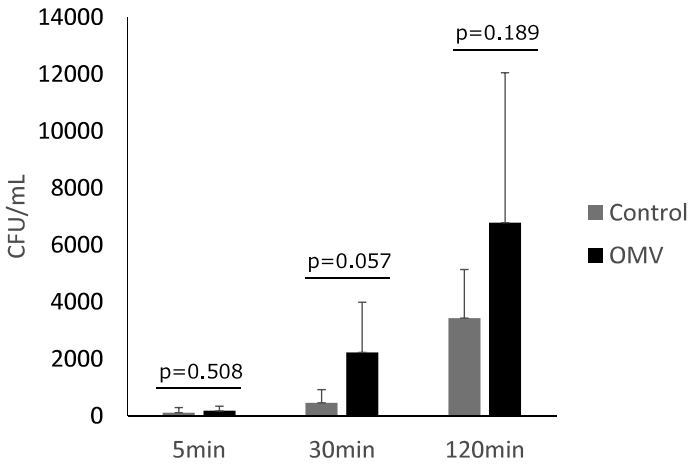


Figure S4. Evaluation of bacterial translocation of V.vulnificus. Caco-2 cells were plated to a transwell plate, and *V.vulnificus* was added on apical compartment with or without Vv-OMVs. After 30 minutes or 2 hours post-infection, basolateral compartment was collected and the CFU of *V.vulnificus* was measured.