



# VvpE mediates the intestinal colonization of *Vibrio vulnificus* by the disruption of tight junctions

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## ABSTRACT

The disruption of gastrointestinal tight junctions and their colonization evoked by enteric pathogens are hallmarks of the pathogenesis. *Vibrio* (*V.*) *vulnificus*, VvpE, is an elastase which is responsible for host surface adherence and vascular permeability; however, the functional roles of VvpE in the pathogenesis of *V. vulnificus* (WT) are poorly understood. In the present study, we have investigated the role of VvpE in regulation of intestinal tight junctions and the colonization of WT. We found that mutation of the *vvpE* gene from *V. vulnificus* (*vvpE* mutant) prevents intestinal tight/adherens junction dysregulation due to a WT infection and maintains the physiological level of the epithelial paracellular permeability. Interestingly, the *vvpE* mutant exhibited defective intestinal colonization abilities, whereas WT colonization was significantly elevated in the ileum in a time-dependent manner. Finally, the *vvpE* mutant negated the enterotoxicity, the breakdown of red blood cells, and pro-inflammatory responses, all of which are induced by the WT infection. In addition, the results of a LC-MS/MS analysis showed that VvpE contributes to WT pathogenesis in multiple ways by interacting with intestinal proteins, including  $\beta$ -globin, Annexin A2, Annexin A4, F-actin, and intelectin-1b. These results demonstrate that VvpE plays important role in promoting the tight junction disruption and intestinal colonization of *V. vulnificus* and that it also has the ability to interact with the intestinal proteins responsible for microbial pathogenesis.

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## 1. Introduction

Enteric bacterial pathogens have various bacterial infectious stratagems to circumvent the epithelial barrier of the gut (Ashida et al., 2012). Specifically, impairment of the intestinal tight/adherens junction (TAJ) during bacterial infection

**Abbreviations:** AST, aspartate aminotransferase; ALT, alanine aminotransferase; CFU, colony-forming unit; Cont, control; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; FITC, Fluorescein isothiocyanate; H&E, hematoxylin and eosin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Ig, immunoglobulin; LB, Luria Bertani; LC-MS/MS, liquid chromatography–tandem mass spectrometry; PBS, phosphate-buffered saline; PVDF, polyvinylidene fluoride; ROD, relative optical density; ROS, reactive oxygen species; rVvpE, recombinant protein VvpE; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SD, standard deviation; TBST, tris-buffer solution–tween 20; TAJ, tight/adherens junction; TLRs, toll-like receptors; Veh, vehicle.

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facilitates the invasion of bacteria to promote colonization and enhance epithelial cell-microbiota interactions, evoking host protective/stress responses such as multiple pro-inflammatory responses (Ashida et al., 2012). *Vibrio* (*V.*) *vulnificus* is an anaerobic Gram-negative bacterium which often causes lethal infections in humans who are immunocompromised or who have underlying diseases such as cirrhosis of the liver (Blake et al., 1979; Park et al., 1991). The most prominent aspect of *V. vulnificus* pathogenesis is its ability to infect a host via the gastrointestinal tract, after which it rapidly spreads from the small intestine to the blood stream (Jeong and Satchell, 2012). Thus, studies regarding the factors leading to the dysregulation of TAJ are likely to be critical for uncovering the mechanisms of the pathogenesis of *V. vulnificus* in the intestine. Many pathogens have been found to cause TAJ dysregulation either as a consequence of host infections or by producing toxic products (Berkes et al., 2003). Fragilysin is an example of toxin produced by *Bacteroides fragilis* that disrupts the TAJ barrier by the proteolytic degradation of E-cadherin (Wu et al., 1998). The pathogenic proteins known as toxins A and B of *Clostridium difficile* have also been shown to regulate protein

kinase C and the Rho GTPase pathway, respectively, in promoting TAJ dysregulation (Berkes et al., 2003). Therefore, the identification of functional toxins of bacterial pathogens which alter the structure and function of the intestinal TAJ barrier could provide an important therapeutic strategy for bacterial infections.

Although the majority of the virulence effects of *V. vulnificus* are known to derive from secreted toxins encoded by cytolytic pore-forming hemolysin (VvhA) (Jeong and Satchell, 2012) and multifunctional autoprocessing repeats in the toxin (MARTXVv) (Jeong and Satchell, 2012; Lee et al., 2007a), several other secreted and cell-associated factors of *V. vulnificus* have also been proposed as potential virulence determinants which are also involved in the pathogenesis of *V. vulnificus*. A 45 kDa elastase designated as *V. vulnificus* VvpE is considered to be another possible virulence factor of *V. vulnificus* (Kothary and Kreger, 1987; Miyoshi, 2006). Purified VvpE was proven to induce hemorrhagic damage and dermonecrosis (Kothary and Kreger, 1987; Miyoshi and Shinoda, 1992; Molla et al., 1989) and was shown to cause tissue necrosis and increased vascular permeability, thus facilitating the invasion of this bacterium (Jones and Oliver, 2009; Miyoshi, 2006). While VvpE is the major elastase produced by *V. vulnificus*, there are no previous reports related to the functional role of VvpE in the regulation of intestinal TAJ dysregulation and the colonization of *V. vulnificus*.

On the other hand, many types of pathogenic bacteria mimic host cell ligands to transduce signals into host cellular responses. For example, internalin, a bacterial surface protein of *Listeria monocytogenes*, is known to interact with E-cadherin to invade hepatocytes (Mengaud et al., 1996). Invasin, a pathogenic protein which is the major adhesion and invasion factor of *Yersinia enterocolitica* (Isberg and Leong, 1990), along with IpaB of *Shigella flexneri* (Skoudy et al., 2000), have also been shown to interact with the mammalian receptors  $\alpha 5 \beta 1$  integrin and CD44, respectively. By binding to the host receptors, these pathogens have profound effects on host cellular processes. Interestingly, VvpE plays important roles in the surface adherence of *V. vulnificus* by facilitating swarming, and it regulates the invasiveness of *V. vulnificus* by cleaving the host IgA and lactoferrin (Kim et al., 2007). Thus, it is important to determine whether VvpE interacts with the essential host cell surface proteins responsible for the adherence and virulence effect of *V. vulnificus* within cells.

In this study, therefore, we investigate the essential role of VvpE in the regulation of the intestinal TAJ dysregulation and the colonization of *V. vulnificus* and further identify the potential intestinal proteins interacting with VvpE which are responsible for the activation of multiple cellular signaling pathways.

## 2. Material and methods

### 2.1. Chemicals

Fluorescein isothiocyanate (FITC)-labeled 4-kDa dextran was purchased from Sigma Chemical Company (St. Louis, MO, USA). The following antibodies were purchased: Claudin 1/2, Occludin, E-cadherin,  $\beta$ -actin and Connexin43 antibodies (Santa Cruz Biotechnology, Paso Robles, CA, USA); intelectin-1b antibody (Abcam Cambridge, MA, USA); F-actin antibody (Cell Signaling Technology, Danvers, MA, USA); Annexin A2 and Annexin A4 antibodies (BD Biosciences, Franklin Lakes, NJ, USA); Horseradish peroxidase (HRP)-conjugated goat anti-rabbit and goat anti-mouse IgG antibodies (Jackson ImmunoResearch, West Grove, PA, USA); Rabbit anti-VvpE antibody was kindly provided by Prof. Sang Ho Choi (Seoul National University, Korea). All other reagents were of the highest purity commercially available and were used as received.

### 2.2. Ethics statement

All animal procedures were performed following the National Institutes of Health Guidelines for the Humane Treatment of Animals, with approval from the Institutional Animal Care and Use Committee of Seoul National University (SNU-140108-4). Animals of only male were used in this study. All surgery was performed under zoletil-xylazine, and all efforts were made to minimize suffering.

### 2.3. Bacterial strains, plasmids, and culture media

All *V. vulnificus* strains (M06-24/O WT and M06-24/O *vvpE*) are isogenic and naturally resistant to polymyxin B (Table 1). Unless otherwise noted, *V. vulnificus* strains were grown in Luria Bertani (LB) medium supplemented with 2.0% (wt/vol) NaCl (LBS) at 30 °C. All media components were purchased from Difco (Difco Laboratories Inc, Detroit, MI). *V. vulnificus* were grown to mid-log phase ( $A_{600} = 0.500$ ) corresponding to  $2 \times 10^8$  CFU/mL and centrifuged at  $6000 \times g$  for 5 min. The pellet was washed with phosphate buffered saline (PBS) and adjusted to desired colony-forming unit (CFU)/mL based on the  $A_{600}$  determined using a UV-VIS spectrophotometer (UV-1800, Shimadzu, Japan) to estimate culture density.

### 2.4. Intestinal paracellular permeability and colonization assay

The intestinal paracellular flux was determined by examining the apical-to-basolateral flux of fluorescein isothiocyanate-labeled 4-kDa dextran (FITC-dextran, 1 mg/mL). Seven-week-old ICR mice ( $n = 10$ ) inoculated intragastrically (i.g.) with 100  $\mu$ L of the boiled *V. vulnificus* at 100 °C for 30 min (Cont), *V. vulnificus* (WT), and a mutant deficient in *vvpE* gene in *V. vulnificus* (*vvpE* mutant) at  $1.1 \times 10^9$  CFU/mL, and killed at 4 h, 8 h, and 16 h later. The immunogenicity of Cont in TAJ disruption, bacterial colonization, enterotoxicity, and intestinal inflammatory responses was not found for 16 h, compared to the non-treated mice (data not shown). It is noted that the mice were given FITC-labeled 4 kDa dextran in PBS (20 mg/100  $\mu$ L) by oral injection for 4 h prior to the sacrifice. A blood sample was collected from caudal vena cava, and the plasma was taken for measuring the concentration of FITC-dextran. The fluorescence intensity of the plasma was examined with a Victor3 luminometer (Perkin-Ehmer Inc., Waltham, MA, USA) using 488 nm excitation and 515 nm emission. Standard curves were generated by serial dilution of FITC-dextran in PBS. On the other hand, mouse colonization assays were performed essentially as described in earlier work (Kim et al., 2013). Mice given an i.g. inoculation of WT, boiled WT (Cont), and *vvpE* mutant were sacrificed and intestine, colon, spleen, and liver of each mouse were collected, washed, and homogenized. The homogenates of each organ were serially diluted and spread on LB agar containing polymyxin B (100 U/mL). CFUs were normalized to grams of intestinal tissues (CFU/g) to represent superficial bacterial counts.

### 2.5. Complementation of the *vvpE* mutant

To complement the *vvpE* mutation, an open reading frame (ORF) of *vvpE* was amplified from the genomic DNA of *V. vulnificus* M06-24/O by PCR with the primer pair VVPE001F and VVPE001R (Supplementary Table 1) and then digested with BamHI. The amplified *vvpE* ORF was subcloned into the broad-host-range vector pRK415 (Keen et al., 1988) linearized with the same enzyme to result in pKK1450 (Table 1). *Escherichia coli* S17-1 $\lambda$ pir, tra strain (Simon et al., 1983) containing pKK1450 was used as a conjugal donor to *vvpE* mutant. The plasmid pKK1450 was delivered into

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