Bacteria in the genus Vibrio produce extracellular proteolytic enzymes to obtain nutrients via digestion of various protein substrates. However, the enzymes secreted by human pathogenic species have been documented to modulate the bacterial virulence. Several species including Vibrio cholerae and V. vulnificus are known to produce thermolysin-like metalloproteases termed vibriolysin. The vibriolysin from V. vulnificus, a causative agent of serious systemic infection, is a major toxic factor eliciting the secondary skin damage characterized by formation of the hemorrhagic blisters. The vibriolysin from intestinal pathogens may play indirect roles in pathogenicity because it can activate protein toxins and hemagglutinin by the limited proteolysis and can affect the bacterial attachment to or detachment from the intestinal surface by degradation of the mucus layer. Two species causing wound infections, V. alginolyticus and V. parahaemolyticus, produce another metalloproteases so-called collagenases. Although the detailed pathological roles have not been studied, the collagenase is potent to accelerate the bacterial dissemination through digestion of the protein components of the extracellular matrix. Some species produce cymotrypsin-like serine proteases, which may also affect the bacterial virulence potential. The intestinal pathogens produce sufficient amounts of the metalloprotease at the small intestinal temperature; however, the metalloprotease production by extra-intestinal pathogens is much higher around the body surface temperature. On the other hand, the serine protease is expressed only in the absence of the metalloprotease.

Keywords: Vibrio, vibriolysin, thermolysin, collagenase, serine protease
Table 1 | Infectious diseases caused by Vibrio species.

| Species       | Intestinal diseases | Extra-intestinal diseases |
|---------------|---------------------|---------------------------|
|               | Cholera | Diarrhea | Sepsis | Wound-infection | Others* |
| V. alginolyticus | ±      | +       | ±      | ±              |         |
| V. cholerae O1/O139 | ++     | +       | +      | ±              |         |
| V. cholerae non O1/ non O139 | ++     | +       | +      | ±              |         |
| V. cincinnatiensis | ±     |         | ±      | ±              |         |
| V. damselae | ±      |         | ±      | ±              |         |
| V. fluvialis | ±      |         | ±      | ±              |         |
| V. furnissii | ±      |         | ±      | ±              |         |
| V. hollisae | ±      |         | ±      | ±              |         |
| V. metschnikovii | ±    |         | ±      | ±              |         |
| V. mimicus | ±      |         | ±      | ±              |         |
| V. parahaemolyticus | ±  | +       | ++     | ±              |         |
| V. vulnificus | ±      | ±       | +      | ±              |         |

++, Major disease; +, Minor disease; ±, Rare disease.

*Include otitis media, choleystitis, meningitis.

Human pathogenic vibrios produce various extracellular factors including enterotoxin, hemolysin, cytotoxin, protease, collagenease, phospholipase, siderophore, and hemagglutinin (Janda et al., 1988). Of these factors, enterotoxin, hemolysin, and cytotoxin are directly related to the clinical symptoms; however, siderophore and hemagglutinin may play roles in the establishment of the infection.

Bacterial extracellular proteolytic enzymes

Human pathogenic vibrios produce various extracellular factors including enterotoxin, hemolysin, cytotoxin, protease, collagenease, phospholipase, siderophore, and hemagglutinin (Janda et al., 1988). Of these factors, enterotoxin, hemolysin, and cytotoxin are directly related to the clinical symptoms; however, siderophore and hemagglutinin may play roles in the establishment of the infection.

Proteolytic enzymes hydrolyzing a peptide bond in proteins and peptides are essential for the homeostatic control in both eukaryotes and prokaryotes, and thus, the bacterial enzymes also have various physiological roles in the life cycle of the microorganisms. However, the enzymes produced by pathogenic species occasionally act as toxic factors to the infected host (Hase and Finkelstein, 1993; Harrington, 1996). Proteolytic enzymes are classified into several groups, such as aprotic, cysteine, and serine protease, and metalloprotease; however, many of the bacterial toxic proteases are in the metalloprotease group, which often contains a zinc (II) ion in the catalytic center (Hase and Finkelstein, 1993; Hooper, 1994).

As shown in Table 2, human pathogenic Vibrio species also produce and secrete proteolytic enzymes, and several enzymes have been extensively characterized as direct toxic factors causing skin damage or indirect virulence factors processing other protein toxins. The enzymes produced by vibrios are in two metalloprotease groups (vibriolysin and collageneases) or one serine protease group (chymotrypsin-like proteases).

The progress in the molecular biology has provided much information on the DNA-derived amino acid sequences of metalloproteases and has shown the presence of the consensus sequence HEXXH as the zinc-binding motif. This motif was also found in some bacterial protein toxins including clostridial neurotoxins.

### Table 2 | Extracellular proteolytic enzymes produced by pathogenic Vibrio species.

| Species       | Metalloprotease | Serine protease |
|---------------|-----------------|----------------|
|               | Vibriolysin | Collagenease | Chymotrypsin-like protease |
| V. alginolyticus | +          | +             | +                         |
| V. cholerae   | +          |               | +                         |
| V. fluvialis  | +          |               | +                         |
| V. metschnikovii | +       |               | +                         |
| V. meningitidis | +        |               | +                         |
| V. parahaemolyticus | +      |               | +                         |
| V. vulnificus | +          |               | +                         |
**Bacteroides fragilis** enterotoxin, and **Bacillus anthracis** lethal factor. Indeed, these bacterial toxins were verified to show the remarkably specific proteolytic action toward a target host protein (Miyoshi and Shinoda, 2000). For instance, clostridial neurotoxins can cleave the restricted protein components of the neurotranscno-tosis machinery, which leads to the blockade of neurotransmitter release and consequent muscle paralysis (Schaivo et al., 1993). In addition, a novel cytotoxin that consists of one A subunit and five B subunits was isolated from some enterohemorrhagic *Escherichia coli* strains, and the A subunit was indicated to be a subtilase-like serine protease (Paton and Paton, 2010). The RTX (repeated-in-toxin) toxins are large multifunctional cytotoxins and are possible to modulate the virulence of a number of gram-negative bacterial pathogens including *V. cholerae* and *V. vulnificus* (Satchell, 2007; Prochazkova et al., 2009; Roig et al., 2011). In **V. cholerae** RTX toxin, the cysteine protease domain was reported to mediate autoprocessing of the toxin (Sheahan et al., 2007; Shen et al., 2009).

**VIBRIOLYSIN**

**BIOCHEMICAL PROPERTIES**

Zinc-containing metalloproteases consist of four superfamilies based on the amino acid residues in the zinc-binding site, and the zincins superfamily is characterized by the possessing of the HEXXH motif (Hooper, 1994). The thermolysin family, in which prototype enzyme is thermolysin from *Bacillus thermo-proteolyticus*, is a one of major members of this superfamily. The enzymes in this family are commonly characterized by the presence of Glu at the 25th position from the first His of the above motif. *V. proteolyticus* is a marine microorganism that was first isolated from the intestine of a small, wood-boring isopod crustacean (Merkel et al., 1964). Griffin and Prescott (1970) purified a highly active metalloprotease from the bacterial culture supernatant. Durham (1989) first disclosed the designation of this enzyme as vibriolysin in the patent literature. Thereafter, highly homologous metalloproteases have been isolated from other *Vibri* species including human pathogens *V. cholerae*, *V. fluvialis*, *V. mimicus*, and *V. vulnificus*. Therefore, the name of vibriolysin is currently applicable to all of these proteolytic enzymes (Miyoshi et al., 2012a).

Vibriolysin hydrolyzes specifically the peptide bond at the amino group side of the P1 amino acid residue, which is usually a hydrophobic amino acid residue (e.g., Phe, Tyr, or Leu; Narukawa et al., 1993). Synthetic oligopeptides, such as carboben-sonate (Z-Gly-Phe-NH$_2$) and Z-Gly-Leu-NH$_2$, are thus commonly used as the suitable substrate. On the other hand, phosphoramido-don [N-[(α-aminomethyl)oxy-α-methoxyphosphanyl]-Leu-Trp] and zinc-[Z-(N-hydroxy carbamido)-4-methylpentanoyl-Ala-Gly amide] are well-known competitive inhibitors. In addition, phenylalanylbenzyloxycarbonyl-Pro-Leu-Gly-Pro-Arg, the substrate developed for bacterial collagenases, is significantly hydrolyzed by the enzyme (Miyoshi et al., 1997). Vibriolysin is also highly active on a wide variety of protein substrates. Namely, the enzyme exhibits significant proteolytic activity of casein, albumin, hemoglobin, type I and IV collagen, gelatin, elastin, fibrin, and fibrinogen (Miyoshi et al., 1995; Miyoshi and Shinoda, 2000).

Like thermolysin, vibriolysin is synthesized as an inactive precursor, and maturation of the precursor is achieved by several processing stages (Chang et al., 2007). In the case of the enzyme from *V. vulnificus* (Figure 1), it is initially synthesized as the preproenzyme (609 aa, 65,964 Da) with a typical signal peptide (Cheng et al., 1996). The signal peptide is cleaved during its passage through the inner membrane in the signal peptidase-dependent manner. In the periplasm, the propeptide that may function as an intramolecular chaperone mediating maturation of the enzyme and/or a specific inhibitor to protect autodigestion of the enzyme (Chang et al., 2007) is then cleaved by an autocatalytic mechanism, and finally, the mature vibriolysin (413 aa, 44,648 Da) is generated. The vibriolysin matured consists of two functional domains: the N-terminal domain (314 aa, 34,049 Da) mediating the catalytic action, and the C-terminal domain (99 aa, 10,656 Da) essential for efficient attachment to protein substrates (Yun et al., 2012). The N-terminal domain is easily obtained by autocatalytic limited-digestion of the C-terminal domain (Miyoshi et al., 1997). The N-terminal domain alone possesses sufficient proteolytic activity toward oligopeptides or soluble proteins, while it shows markedly reduced activity toward insoluble proteins such as type I collagen and elastin.

The N-terminal domain of vibriolysin is significantly related to other enzymes in the thermolysin family; however, its C-terminal domain may be unique. The feature of the proteolytic action, which is mediated by the N-terminal domain, is considerably similar to other enzymes. However, neither suitable specific peptide substrate nor competitive inhibitor for vibriolysin has been developed. On the other hand, the hemagglutinating action on rabbit erythrocytes, which is due to association of both N-terminal and C-terminal domains with the erythrocyte membrane, is a distinctive feature of vibriolysin so far as reported (Alam et al., 1995).

**PATHOLOGICAL ROLES**

As summarized in Figure 2, a variety of pathological roles of vibriolysin have been documented (Shinoda and Miyoshi, 2012). In the local infections such as the wound-infection, vibriolysin is thought to be a direct toxic factor that causes hemorrhagic tissue...
Miyoshi Proteolytic enzymes of vibrios

FIGURE 2 | Pathological roles of extracellular proteolytic enzymes in infectious diseases caused by Vibrio species.

Damage through digestion of the basement membrane around vascular endothelial cells, and that forms edematous lesions through generation of inflammatory mediators (Figure 1). The enzyme from *V. vulnificus* can enhance the vascular permeability when injected into the mammalian dorsal skin. In rat skin, this reaction is due to the release of histamine from mast cells, because the vascular enhancement was abolished by simultaneous injection of diphenhydramine, an anti-histaminic agent (Miyoshi et al., 1987c). In guinea pig skin, however, the permeability enhancement is most likely due to activation of the factor XII-plasma kallikrein-kinin cascade (Miyoshi et al., 1987a). Namely, the skin reaction was not blocked by diphenhydramine, but it was modulated by *in situ* administration of the specific inhibitors affecting the cascade activation. Bradykinin, a well-known *in vivo* mediator of inflammation, is finally generated through activation of the cascade. Further studies to clarify the activation mechanism of the human cascade have been carried out, and the results demonstrated that vibriolysin could generate the active forms via limited proteolysis of the inactivezymogen (Miyoshi et al., 2004). Plasma prekallikrein was converted to the active kallikrein, which can liberate bradykinin from kininogen, by cleavage of the Arg371-Ile372 bond. On the other hand, factor XII was activated by hydrolysis of the Arg211-Val214 or Gly357-Leu358 bond, and activated factor XII could convert plasma prekallikrein to kallikrein. Vibriolysin also induces the hemorrhagic reaction in the mammalian dorsal skin. The enzyme from *V. vulnificus* showed the greatest hemorrhagic activity compared with some bacterial metalloproteases, thermolysin from *B. thermoproteolyticus*, serralysin from *Serratia* species, and collagenase from *Clostridium histolyticum* (Miyoshi et al., 1998). The levels of the *in vivo* hemorrhagic activities of these proteases were correlated with those of the *in vitro* proteolytic activities toward the reconstituted basement membrane gel. Of two major basement components, laminin and type IV collagen, only the latter was easily digested by vibriolysin. This indicates that type IV collagen forming the framework of the basement membrane is the target protein. Therefore, specific degradation of type IV collagen causes destruction of the basement membrane, breakdown of capillary vessels, and finally the leakage of blood components including erythrocytes.

In the systemic infections including septicemia, vibriolysin may act as a synergistic pathogenic factor through disordered proteolysis of various plasma proteins, which in turn disturbs the physiological homeostasis, and eventually elicits an immunocompromised state in the infected host. For instances, vibriolysin has been documented to facilitate the bacterial infection by disturbance of the plasma protease-protease inhibitor systems (Miyoshi et al., 1995), and to interfere with the blood homeostasis through prothrombin activation and fibrinolysis (Chang et al., 2005; Kwon et al., 2007). Acceleration of the heme utilization was also reported as the pathogenic role of vibriolysin (Nishina et al., 1992). Ferric/ferrous ion is essential for *in vivo* growth of pathogenic microorganisms. However, the concentration of free ferric/ferrous ion in human body is very low (10^{-15} to 10^{-18} M) because of the presence of heme-proteins including hemoglobin and iron-binding proteins. Therefore, pathogenic microorganisms invaded into human body must operate the systems to acquire ferric/ferrous ion. Heme, a complex of porphyrin with ferric/ferrous ion and a prosthetic group in hemoglobin or other heme-proteins, is often used as an iron source by human pathogenic bacterial species (Stojiljkovic and Perkins-Balding,
Yu and Lee (1999) and Kim et al. (2002) carried out cloning of α2 that human plasma contains a broad-range protease inhibitor et al., 2004; Sun et al., 2006). Incidentally, it should be noted V. vulnificus that the vibriolysin-deficient mutants of V. vulnificus also inactivated with 2 M) as a primary inhibitor for exogenous α2. The vibriolysin from V. cholerae O1 can activate CT through nicking of the A subunit of CT (Booth et al., 1984). Vibriolysin also converts the precursor of the entero- toxic hemolysin produced by V. cholerae to the mature toxin through removal of the 15 kDa N-terminal propeptide (Nagamune et al., 1996). Although the results shown above suggest indirect pathogenic roles of vibriolysin, the possibility of the direct roles has also been reported. Ghosh et al. (2006) purified vibriolysin from a CT gene-negative strain of V. cholerae non-O1/non-O139 and measured the enterotoxic activity. The purified enzyme caused accumulation of the hemorrhagic fluid in the rabbit ileal loop assay and increase in the intestinal short-circuit current in the Using chamber assay. Additionally, through the analysis with sev- eral mutants genetically constructed, Silva et al. (2006) showed that vibriolysin was necessary for full expression of enterotoxocity of V. cholerae O1.

COLLAGENASES

Yu and Lee (1999) and Kim et al. (2002) carried out cloning of the V. parahaemolyticus gene encoding a collagenase, which was designated as PrtV/PrtVp (562 aa, 63,156 Da) and VppC (814 aa, 89,833 Da), respectively. These enzymes were revealed to be metalloproteases in the zinc superfamily having the consensus zinc-binding HEXXH motif, but neither of the collagenases was in the thermolysin family. Miyoshi et al. (2008) showed that, only when V. parahaemolyticus was cultivated at 26°C, the vppC gene was sufficiently expressed, and VppC was secreted from the bacterial cell after removal of the N-terminal 72 amino acid residues. In contrast, expression of the prtV/prtVp gene was negligible in the wild type strain. The gene was significantly expressed by dis- ruption of the vppC gene; however, the product PrtV/PrtVp was not secreted into the cultivation broth (Miyoshi et al., 2008), sug- gesting PrtV/PrtVp is a cell-associated enzyme. VppC purified showed the steady activity to hydrolyze Z-Gly-Pro-Gly-Gly-Pro- Ala, the specific substrate for bacterial collagenases, and to digest gelatin. This indicates that VppC may contribute to the wound-infection by V. parahaemolyticus because putative digestion of the components of the extracellular matrix by VppC may accel- erate the bacterial dissemination and may form cellu lite skin damage. V. algolysin, another species causing the wound-infection, is known to produce a VppC homolog (Takeuchi et al., 1992).

CHYMOTRYPSIN-LIKE PROTEASES

In 2002, two research groups reported individually purification of a serine protease, which was termed protease A and VPP1 respectively, from the culture supernatant of V. parahaemolyti- cus (Ishihara et al., 2002; Lee et al., 2002). These proteases were identical and corresponded to the VPM0227 protein (677 aa, 71,638 Da) of strain RIMD 2210663 (GenBank accession number: BA000032). However, the amino acid sequencing of the purified enzyme indicated that the N-terminal 121 amino acid residues were removed during the maturating process. This serine protease designated herein as protease A/VPP1 showed the immunological cross-reactivity with serine proteases from V. metchnikovii and V. algolysin (Ishihara et al., 2002). Indeed, protease A/VPP1 revealed highly similarity of the amino acid sequence to the enzyme from V. metchnikovii (Kwon et al., 1995). Production of protease A/VPP1 was much higher at 25 than at 37°C and was induced by the addition of gelatin to the cultivation broth. Miyoshi et al. (2008) reported that production of this serine protease was remarkably increased by disruption of the vppC gene, suggest- ing that protease A/VPP1 is a substitutive enzyme of VppC. The purified protease was found to be sensitive to chymostatin, the well-known competitive inhibitor for chymotrypsin, and to hydrolyze the specific peptide-I4-methyl-coumaryl-7-amide (MCA) substrates for chymotrypsin, such as Glutaryl-Ala-Ala- Phe-MCA and Succinyl-Ala-Ala-Pro-Phe-MCA. Therefore, it may be concluded that protease A/VPP1 is a chymotrypsin-like ser- ine protease. The cytotoxicity against CHO, HeLa, or Vero cells and the mouse lethal toxicity of purified protease A/VPP1 was demonstrated by Lee et al. (2002). Our preliminary study showed the proteolytic activity of the purified enzyme toward extracel- lular matrix components, laminin and type I collagen. These results suggest that protease A/VPP1 also modulate the bacterial pathogenicity.

Vibrio vulnificus sometime causes severe hemorrhagic sep- ticism called vibriosis in eels of the culture farms (Tison et al., 1982, Biosa et al., 1991). A few strains isolated from diseases eels were recently found to have lost the 80 kb genomic region includ- ing the gene encoding vibriolysin, but instead of vibriolysin, these strains secrete a serine protease termed VvsA, which is an ortholog of protease A/VPP1 from V. parahaemolyticus (Miyoshi et al., 2012b). As protease A/VPP1, production of VvsA was extremely increased in the absence of the functional gene encoding vibri- oliysin (Wang et al., 2008). The vvsA gene constitutes an operon with a downstream gene vvsR, of which product VvsR may act as the chaperon supporting the maturating process of VvsA. The database analysis showed that several Vibrio species including V. parahaemolyticus have the homologous genes to vvsAR, indicating...
widely distribution of the chymotrypsin-like serine protease in Vibrio species.

REGULATION OF PRODUCTION OF PROTEOLYTIC ENZYMES

Vibrio species are ubiquitous microorganisms in aquatic environments, but 11 species cause intestinal or extra-intestinal infections to humans. During the infection process, the bacterial cells must sense the change of environmental factors, such as temperature, pH, salinity, and osmolality, and then, the bacterium must transmit the signals into the cells through the specific signal-transduction systems, which result in the change of expression of the genes. Especially, the genes encoding the toxic or virulence factors, which may be required for in vivo survival and growth, must be expressed at an appropriate place and time in a tightly regulated fashion (Heithoff et al., 1997; Lee et al., 1999). Amongst the environmental factors, temperature is thought to be the most important. The virulence genes of the bacterium causing intestinal infections are expressed sufficiently at 37°C, while the genes of the bacterium causing extra-intestinal infections are expressed more effectively around the body surface temperature (Watanabe et al., 2004; Miyoshi et al., 2006). The gene expression is also often affected by the salinity. For instance, the expression level of the pvvG gene in V. parahaemolyticus is higher at 3% NaCl than 0.9% NaCl (Miyoshi et al., 2008). Although the intracellular second messengers, such as cyclic AMP and cyclic di-GMP, and the global general regulators including RpoS and histone-like nucleotide structuring protein are also involved in the gene regulation (Benitez et al., 2011; Wang et al., 2011, 2012), the molecular mechanisms how the bacterium senses the environmental signals and transmits the signals into the cells are not clarified.

Production of proteolytic enzymes is tightly dependent on the growth phase and reaches to the maximum level at the early stationary phase. Many pathogenic bacteria coordinate expression of the virulence genes in response to the bacterial cell density. This regulation system is termed the quorum sensing (QS) and is controlled by the small diffusible signal molecule called autoinducer (AI; Whitehead et al., 2001; Henke and Bassler, 2004). At the low cell density, the QS system cannot modulate the gene expression because the concentration of AI is too low. However, at the high cell density, the AI concentration around the bacterial cell reaches the threshold level, the AI molecule is sensed by the sensor protein, the signals are transmitted into the cell, and finally, the expression of a set of genes is started or stopped.

In Vibrio species including V. cholerae, V. mimicus, and V. vulnificus (Miller et al., 2002; Milton, 2006; Sultan et al., 2006), the AI molecule is detected by the specific membrane-bound sensor protein, which causes conversion of the sensor protein from kinase to phosphatase. Subsequently, the sensor protein/phosphatase mediates dephosphorylation of LuxU-LuxO, the response regulator proteins. The dephosphorylated LuxU has no activity to inhibit LuxO or its homolog, the master transcriptional regulator for the genes under the control of the QS system. Therefore, at the high cell density, the transcriptional status of the target genes is changed by the function of LuxU or its homologue. Production of the proteolytic enzymes by pathogenic vibrios is closely related with the extracellular AI level (Kim et al., 2003; Kawasaki et al., 2004; Raychaudhuri et al., 2006). For instance, the mutant of the AI synthetase showed apparently reduced production of vibriolysin. Therefore, the QS system markedly controls the expression of the proteolytic genes in Vibrio species. However, it should be noted that the QS system of V. cholerae or V. mimicus is operated sufficiently at 37°C (Sultan et al., 2006), whereas, the system of V. vulnificus is operated effectively at 26°C but not at 37°C (Miyoshi et al., 2006).

CONCLUSION

The proteolytic enzymes produced by human pathogenic Vibrio species may play a variety of pathological roles: direct roles by digesting many kinds of host proteins or indirect roles by producing other toxic protein factors. Especially, vibriolysin from V. vulnificus is thought to be a major virulence factor. However, some contradictions of the pathogenic roles were also reported (Shao and Hor, 2006; Sun et al., 2006). It must be mentioned that the purified enzymes from V. vulnificus and V. proteolyticus, a non-pathogenic species, are difficult to distinguish in the in vivo actions, because both enzymes are members of vibriolysin and have comparative biochemical and toxic activities. However, it has been demonstrated that the high growing ability of V. vulnificus in the mammal host is important for the pathogenicity of the bacterium (Watanabe et al., 2004). In addition, production of the toxic or virulence factors including proteolytic enzymes is tightly regulated by environmental factors, the bacterial cell density and so on. Therefore, the overall experiments from various approaches are necessary for evaluation of the extracellular proteolytic enzymes as the virulence factors.

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