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Two Forms of *Vibrio vulnificus* Metalloprotease VvpE are Secreted via the Type II General Secretion System

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Vibrio vulnificus **has been known to secrete one form of metalloprotease VvpE (45 kDa) that is cleaved to 34 kDa-VvpE and 11 kDa-C-terminal propeptide via extracellular autoproteolysis. However, we found that extracellular secretion of both the 34 and 45 kDa forms of VvpE began in the early growth phase; moreover, 34 kDa-VvpE existed as the major form in *V. vulnificus* cell lysates and culture supernatants. In addition, extracellular secretion of both 34 and 45 kDa-VvpE was blocked by mutation of the *pilD* gene, which encodes for the type IV leader peptidase/*N*-methyltransferase of the type II general secretion system, and the blocked VvpE secretion was recovered by *in trans*-complementation of the wild-type *pilD* gene. These results indicate that 34 kDa-VvpE is the major form secreted along with 45 kDa-VvpE from the early growth phase via the PilD-mediated type II general secretion system.**

Keywords: *Vibrio vulnificus*, metalloprotease, general secretion system

Vibrio vulnificus is a Gram-negative halophilic bacterium that causes fatal septicemia in patients with underlying hepatic diseases, long-term heavy consumption of alcohol, or other immunocompromised conditions (Strom and Paranjpye, 2000; Gulig *et al*., 2005). Among several potential virulence factors of *V. vulnificus*, a metalloprotease VvpE has been most extensively studied. Purified VvpE exhibits a variety of biological activities including induction of hemorrhagic damage and dermonecrosis, enhancement of vascular permeability and formation of edema (Kothary and Kreger, 1987; Miyoshi *et al.*, 1987, 1995, 1997, 2001, 2003), and functions as a prothrombin activator and fibrionolytic enzyme (Chang *et al*., 2005; Kwon *et al.*, 2007). Recently, we also found that VvpE is essentially required for the swarming of *V. vulnificus*, which is thought to be a good model for bacterial surface adherence and colonization (Kim *et al*., 2007b).

Metalloproteases belonging to the thermolysin family are commonly synthesized as pro-forms with several domains consisting of a signal peptide, an N-terminal propeptide acting as an intramolecular chaperone, and a mature region having protease activity, and are secreted after the proteolytic cleavage of the signal peptides and N-terminal propeptides (Kessler and Safrain, 1988, 1994; O'Donohue and Beaumont, 1996; Chang *et al.*, 2007). Vibrio species also produce metalloproteases belonging to the thermolysin family. These metalloproteases have noticeably additional C-terminal propeptides, but nevertheless, their main secreted forms do not have the C-terminal propeptides (Häse and Finkelstein, 1990, 1991; David *et al.*, 1992; Milton *et al.*, 1992). The latter are thought to be cleaved via extracellular autoproteolysis.

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V. vulnificus metalloprotease VvpE also belongs to the thermolysin family, and is structurally and functionally similar to other Vibrio metalloproteases. However, 45 kDa-VvpE with C-terminal propeptide is known to be mainly secreted into media (Kothary and Kreger, 1987; Miyoshi *et al.*, 1987, 1997; Jeong *et al.*, 2000) via the type II general secretion system (Paranjpye *et al*., 1998; Kim *et al.*, 2007a) and converted to 34 kDa-VvpE via the autoproteolysis of 11 kDa-C-terminal propeptide after extracellular secretion (Miyoshi *et al.*, 1997). Actually, it has been verified that a small portion of 45 kDa-VvpE is converted into 34 kDa-VvpE via extracellular autoproteolysis. However, unlike an *in vitro* system, an *in vivo* system is not a closed culture system, and so VvpE produced *in vivo* must be immediately diffused out. Accordingly, extracellular autoproteolysis of 45 kDa-VvpE can be a rare and insignificant phenomenon in an *in vivo* system. Moreover, 34 and 45 kDa-VvpE with the same N-terminal amino acid sequence were recently co-purified from culture supernatants, with the 34 kDa-VvpE demonstrated to be the major secreted form (Chang *et al.*, 2005; Kwon *et al.*, 2007). This finding suggests that 45 kDa-VvpE may be cleaved into 35 kDa-VvpE within bacterial cells and then secreted, rather than cleaved outside bacterial cells after being secreted.

The 34 kDa form of VvpE has sufficient proteolytic activity toward soluble proteins but reduced activity toward insoluble proteins. In contrast, the 45 kDa form of VvpE has sufficient proteolytic activity toward insoluble proteins as well as soluble proteins, and exhibits a hemagglutinating activity (Miyoshi *et al.*, 1997, 1998, 2001, 2003). Accordingly, it is of significance to determine which form of VvpE is the main secreted form. In this study, we determined whether or not both 34 and 45 kDa-VvpE are produced from the early growth phase within bacterial cells and secreted via

the type II general secretion system, and which of the two forms is the major secreted form.

Materials and Methods

Bacterial strains, plasmids, primers, media, and reagents

Bacterial strains, plasmids, and primers used in this study are listed in Table 1. Heart Infusion (HI) broth and agar (BD, USA) containing 2% NaCl were used as basal media for cultivating *V. vulnificus* strains. Luria-Bertani (LB) agar (BD, USA) and broth were used to cultivate *Escherichia coli* strains. When required, antibiotics were included in the following concentrations: 20 μg/ml ampicillin and 2 μg/ml tetracycline for *V. vulnificus*, and 50 μg/ml ampicillin for *E. coli*. Unless otherwise noted, all reagents were purchased from Sigma-Aldrich (USA).

Construction of a *pilD*-deletion mutant and an *in trans pilD*-complemented strain

A *V. vulnificus pilD*-deletion mutant (RC104) was constructed by allelic exchange using R6K origin suicide vector pDM4, as previously described (Kim *et al.*, 2007b). To restore the wild type *pilD* gene in *V. vulnificus* RC104, a PCR product containing the wild type *pilD* gene was subcloned into the broad host range vector pLAFR3II and transferred to *V. vulnificus* RC104 by triparental mating as previously described (Kim *et al.*, 2007b). The *pilD*-complemented strain was designated as RC108.

Production of recombinant VvpE protein and rabbit polyclonal anti-VvpE-body

The Glutathione S-Transferase (GST) Gene Fusion System (Amersham Pharmacia Biotech, Sweden) was used for overexpression and purification of a GST-VvpE fusion protein. The region of the *vvpE* gene encoding the 45 kDa VvpE was amplified using the PCR primers, GST-vvpE for with a *Bam*HI overhang and Int-vvpE-rev with an *Eco*RI overhang. The resulting 1,242 kb *Bam*HI-*Eco*RI fragment was cloned into pGEX-4T-1. The resulting plasmid pRC144 was transformed into *E. coli* DH5α and BL21. The overexpression of GST-VvpE fusion protein in *E. coli* BL21 was induced at 28°C for 6 h by 0.2 mM isopropyl-1-thio-β-D-galactopyranoside. The bacterial pellet was suspended in lysis buffer (10 mM Tris-HCl; pH 8.0, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 0.2 mM phenylmethylsulfonylfluoride, 5 mM dithiothreitol, 1.5% Sarkosyl, 50 μg/ml lysozyme, and 2% Triton X-100). After incubation for 15 min on ice, the cell suspension was sonicated on an ice bath with a Vibra Cell™ microtip sonicator (Sonics and Materials, USA) at amplitude of 30%. After centrifugation at 11,000 rpm and 4°C for 30 min, the recovered supernatant was loaded onto the washed Glutathione Sepharose 4B column. The resulting matrix was thoroughly washed with phosphate-buffered saline (PBS, pH 7.2). The GST-VvpE fusion protein was eluted with glutathione elution buffer (100 mM Tris-HCl; pH 8.0, 120 mM NaCl, and 20 mM reduced glutathione) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The GST-VvpE fusion protein was injected intradermally to a New Zealand White rabbit for immunization. After four injections, blood was collected via cardiac puncture and serum was obtained after centrifugating the blood. The specificity of polyclonal anti-VvpE-body produced was previously tested by Western blot (Kim *et al*., 2007a, 2007b).

Culture condition, growth monitoring, and preparation of culture supernatants and whole cell lysates

V. vulnificus strains were inoculated into fresh HI broth at 1×10^6 cfu/ml, and cultured with vigorous shaking (220 rpm) at 37°C for 24 h. During culture, culture aliquots were with-

Table 1. Bacterial strains, plasmids, and primers used in this study

Bacterial strains, plasmids, and primers	Relative characteristics	Sources and references
V. vulnificus		
MO6-24/O	highly virulent clinical isolate	Lab collection
CMM1049	MO6-24/O with *vvpE* deletion	Kim *et al*. (2006)
CMM1502	CMM1049 with *in trans vvpE* complementation	Kim *et al*. (2006)
RC104	MO6-24/O with *pilD* deletion	Kim *et al.* (2007a)
RC108	RC104 with *in trans pilD* complementation	Kim *et al.* (2007a)
E. coli		
DH5α	F- *recA1*; restriction negative	Lab collection
BL21	F-, *ompT*, *hsdS* (r_B-, m_B-), *gal*	Lab collection
Plasmids		
pGEX-4T-1	An expression vector for a GST-fusion protein	Amer. Pharm. Biotech.
pRC144	pGEX-4T-1 with 1,242 kb *Bam*HI-*Eco*RI fragment containing *vvpE* gene	This study
Primers		
GST-vvpE-for	5'-cg-[ggatcc][a]-gcgcaagcaggcggtactggac-3'	This study
Int-vvpE-rev	5'-cg-[gaattc][b]-tcaatattgtagctttaacgtcacacc-3'	This study

[a, b] the recognition sequences of *Bam*HI and *Eco*RI, respectively.

drawn to monitor bacterial growth. Bacterial growth was monitored by measuring the optical densities of culture aliquots at 600 nm. Culture supernatants and bacterial pellets were obtained by centrifugation of culture aliquots at 10,000 rpm for 5 min. Culture supernatants were immediately stored at -20°C to minimize the breakdown of secreted proteins, especially VvpE. The bacterial pellets were washed three times with phosphate-buffered saline (PBS, pH 7.2), mixed with lysis buffer (60 mM Tris-HCl; pH 6.8, 10% glycerol, 1% SDS), boiled for 10 min, and centrifuged at 10,000 rpm for 5 min to remove cell debris. The resulting supernatants were used as whole cell lysates. Total protein concentration in the

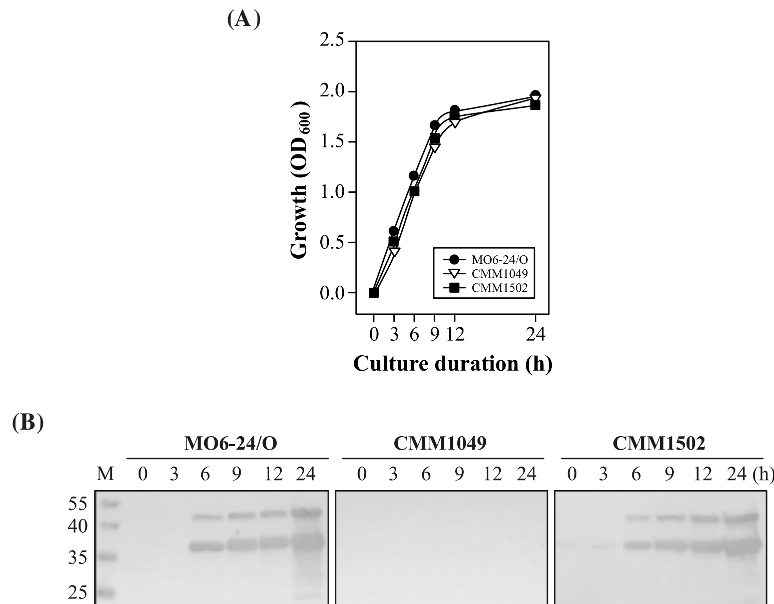

Fig. 1. Extracellular secretion of VvpE. *V. vulnificus* MO6-24/O, *vvpE*-deleted CMM1049, and *vvpE*-complemented CMM1502 strains were cultured with vigorous shaking (220 rpm) in HI broth at 37°C for 24 h. During culture, culture aliquots were obtained at the indicated times in order to monitor bacterial growth, and culture supernatants were obtained by centrifugation of the culture aliquots at 10,000 rpm for 5 min in order to observe extracellular VvpE production on Western blot. (A) Bacterial growth was monitored by the measurement of the optical density at 600 nm (OD_{600}) of the culture aliquots. (B) Western blot using rabbit polyclonal anti-VvpE antibody was conducted after electrophoresis of equal volumes (10 μl) of the culture supernatants using 10% SDS-PAGE gels under denaturing conditions.

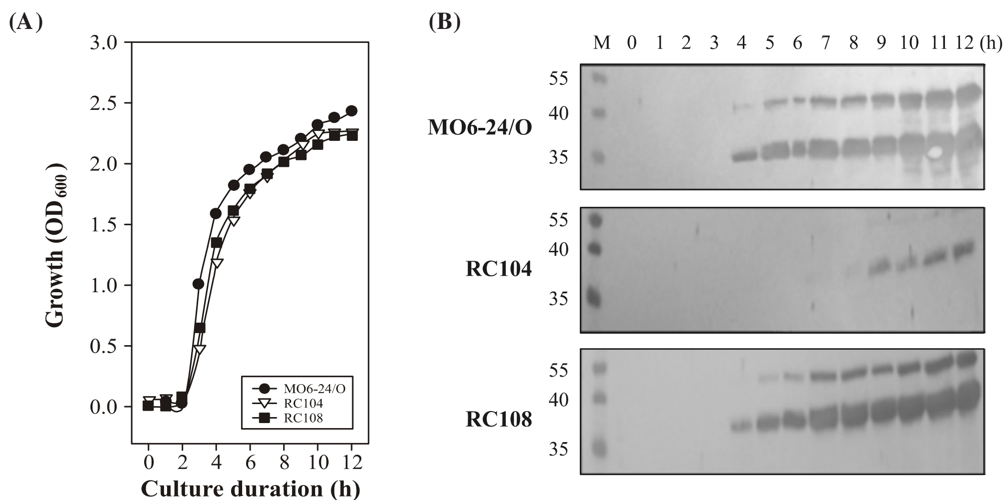

Fig. 2. Effect of the *pilD* mutation on extracellular secretion of VvpE. *V. vulnificus* MO6-24/O, *pilD*-deleted RC104 and *pilD*-complemented RC108 strains were cultured with vigorous shaking (220 rpm) in HI broth at 37°C for 24 h. During culture, culture aliquots were obtained at the indicated times in order to monitor bacterial growth, and culture supernatants were obtained by centrifugation of the culture aliquots at 10,000 rpm for 5 min to observe extracellular VvpE production on Western blot. (A) Bacterial growth was monitored by the measurement of the optical density at 600 nm (OD_{600}) of the culture aliquots. (B) Western blot using rabbit polyclonal anti-VvpE antibody was conducted after electrophoresis of equal volumes (10 μl) of the culture supernatants using 10% SDS-PAGE gels under denaturing conditions.

whole cell lysates was measured using the Bradford Reagent.

SDS-PAGE and Western blot

To observe the pattern of extracellular VvpE, equal volumes (10 μl) of culture supernatants were electrophoresed on 10% SDS-PAGE gels after denaturation by heating and β-mercaptoethanol. To observe the pattern of intracellular VvpE, whole cell lysates including 10 μg of total protein were electrophoresed on the same gels. After electrophoresis, proteins were transferred to PROTRAN nitrocellulose membranes (Whatman GmbH, Germany). The membranes were incubated with blocking solution (0.2% Tween 20 and 5% Skim milk in PBS) at 4°C overnight, allowed to react with rabbit polyclonal anti-VvpE-body [1:1,000 diluted in washing buffer consisting of 0.1% Tween 20 and 1% Skim milk in PBS] and with anti-rabbit-IgG-body conjugated with alkaline phosphatase (Sigma; 1:15,000 diluted in washing buffer), and finally visualized with 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium (Sigma) substrate solution.

Results

Synchronous extracellular production of both 34 and 45 kDa-VvpE

To observe the pattern of extracellular VvpE, we cultured wild-type MO6-24/O, *vvpE*-deleted CMM1049, and *vvpE*-complemented CMM1502 in HI broth, and obtained culture aliquots and supernatants at appropriate times during culture. No growth differences were observed among the three strains (Fig. 1A). When Western blot was conducted using culture supernatants, MO6-24/O was shown to produce both 34 and 45 kDa-VvpE, but neither form was detected for CMM1049. CMM1502 produced the same forms of VvpE as MO6-24/O. Moreover, 34 kDa-VvpE was produced to higher levels than 45 kDa-VvpE throughout the 24 h culture period (Fig. 1B). Small VvpE products (probably 11 kDa-C-terminal propeptides) were sometimes observed, but in insignificant levels in MO6-24/O and CMM1502 (data not shown).

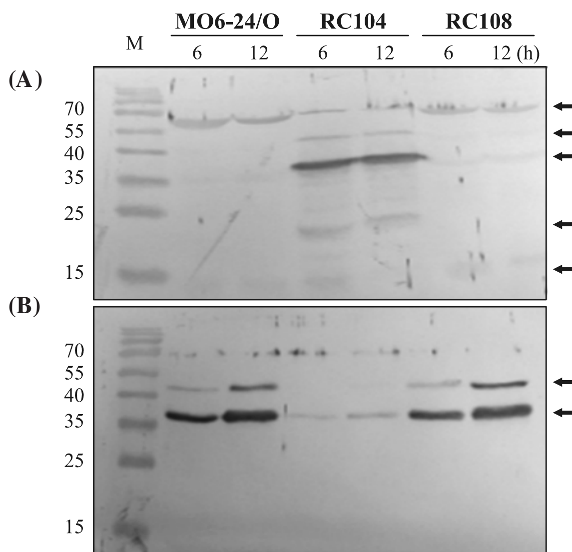

Fig. 3. Intracellular and extracellular VvpE production. *V. vulnificus* MO6-24/O, *pilD*-deleted RC104, and *pilD*-complemented RC108 strains were cultured with vigorous shaking (220 rpm) in HI broth at 37°C for 24 h. During culture, culture aliquots were obtained at the indicated times, and then bacterial pellets and culture supernatants were separated by centrifugation of the culture aliquots at 10,000 rpm for 5 min. Whole cell lysates were obtained after the bacterial pellets were lyzed with lysis buffer, boiled for 10 min, and then centrifuged at 10,000 rpm for 5 min to remove cell debris. Western blot using rabbit polyclonal anti-VvpE antibody was conducted after electrophoresis of the whole cell lysates (10 μg of total protein; (A) and equal volumes (10 μl) of the culture supernatants (B) using 10% SDS-PAGE gels under denaturing conditions. The arrows indicate VvpE derivatives.

Secretion of both 34 and 45 kDa-VvpE via the PilD-mediated type II general secretion system

To determine if both 34 and 45 kDa-VvpE were secreted through the PilD-mediated type II general secretion system, we cultured wild-type MO6-24/O, *pilD*-deleted RC104, and *pilD*-complemented RC108, and obtained culture aliquots and supernatants at every hour during culture. No growth difference was observed among the three strains (Fig. 2A). When Western blot was conducted using culture supernatants, MO6-24/O and RC108 began to produce both forms of VvpE from 4 h after culture initiation and, thereafter, production levels gradually increased (Fig. 2B). As shown in Fig. 1B, 34 kDa-VvpE was produced to higher levels than 45 kDa-VvpE throughout the culture period. In contrast, RC104 began to produce only a small amount of 34 kDa-VvpE beginning at 9 h after culture initiation.

Presence of both 34 and 45 kDa-VvpE within bacterial cells

To observe the pattern of intracellular VvpE under conditions that delayed or blocked extracellular secretion of VvpE via the disruption of the type II general secretion system, we cultured wild type MO6-24/O, *pilD*-deleted RC104 and *pilD*-complemented RC108, obtained both bacterial pellets and culture supernatants at appropriate times during culture, and prepared whole cell lysates from the bacterial pellets. Western blot was performed using the whole cell lysates and culture supernatants. The 66 kDa-pro-VvpE was readily detected, while the 34 and 45 kDa-VvpE were observed in trace amounts in the whole cell lysates from MO6-24/O and RC108, whereas the 34 and 45 kDa-VvpE, as well as 66 kDa-pro-VvpE, were readily observed in the whole cell lysates from RC104. Moreover, 34 kDa-VvpE was observed as a major form compared to 45 kDa-VvpE (Fig. 3A). Small VvpE products expected to be 11 kDa-C-terminal propeptides were also observed. In contrast, 34 and 45 kDa-VvpE were observed in the culture supernatants obtained from MO6-24/O and RC108, whereas only a small amount of 34 kDa-VvpE was observed in the culture supernatants obtained from RC104. The 34 kDa-VvpE was also observed as a major form compared to the 45 kDa-VvpE in culture supernatants (Fig. 3B). Small VvpE products expected to be 11 kDa-C-terminal propeptides were not observed in culture supernatants.

Discussion

Some serious doubts have been raised with regard to its roles in the pathogenesis of *V. vulnificus* infections (Jeong *et al.*, 2000; Shao and Hor, 2000; Fan *et al.*, 2001; Kim *et al.*, 2006). Nevertheless, it is undeniable that purified VvpE exhibits a variety of biological activities as described above (Kothary and Kreger, 1987; Miyoshi *et al.*, 1987, 1995, 1997, 2001, 2003; Chang *et al.*, 2005; Kwon *et al.*, 2007; Kim *et al.*, 2007b). Accordingly, new paradigms will be required to elucidate the yet-ill-defined roles of VvpE.

It has been known that 45 kDa-VvpE is mainly secreted into media (Kothary and Kreger, 1987; Miyoshi *et al.*, 1987, 1998; Jeong *et al.*, 2000), and then is converted to 34 kDa-VvpE through the autoproteolytic removal of C-terminal propeptide when incubated at 37°C (Miyoshi *et al.*, 1997). However, according to our results, both 34 and 45 kDa-VvpE begin to be produced from the early growth phase; moreover, 34 kDa-VvpE is a major form compared to the 45 kDa-VvpE within bacterial cells, as well as in culture supernatants. The intracellular 34 and 45 kDa-VvpE could not be observed in wild-type MO6-24/O and *pilD*-complemented RC108, but were clearly evident in *pilD*-mutated RC104. These are thought to be because both VvpE forms are rapidly secreted out via the type II general secretion system either simultaneously with or immediately after *vvpE* gene expression. Overall, these findings indicate that a major portion of 45 kDa-VvpE is processed to form 34 kDa-VvpE within bacterial cells and, thereafter, is secreted, whereas only a minor portion of 45 kDa-VvpE is secreted without the proteolytic cleavage of C-terminal propeptide. Our results are supported by the recent demonstration that 34 and 45 kDa forms of VvpE with the same N-terminal sequence can be co-purified from culture supernatants, with the 34 kDa-VvpE being the major secreted form (Chang *et al.*, 2005; Kwon *et al.*, 2007).

According to our results, 45 kDa-VvpE with C-terminal propeptide is secreted at considerable levels, even though it is a minor form. However, other Vibrio proteases including HA/protease are secreted without C-terminal propeptides (Häse and Finkelstein, 1990, 1991). Accordingly, the C-terminal propeptide of VvpE is thought to be more resistant to proteolytic action than those of other Vibrio proteases (Miyoshi *et al.*, 1997), but the reason remains to be clarified. In addition, small derivatives considered to be the deleted C-terminal propeptides were sometimes observed in culture supernatants (data not shown), which indicates that a small portion of the 45 kDa-VvpE is also converted to the 34 kDa-VvpE after extracellular secretion, as described by others (Miyoshi *et al.*, 1997).

It is known that 34 kDa-VvpE has sufficient and similar proteolytic activity toward soluble proteins such as azocasein and plasminogen but displays reduced activity toward insoluble proteins such as fibrin and elastin (Miyoshi *et al.*, 1995; Chang *et al.*, 2005; Kwon *et al.*, 2007). In contrast, 45 KDa-VvpE has sufficient proteolytic activity toward insoluble proteins as well as soluble proteins, and agglutinates rabbit erythrocytes, binds to the erythrocyte ghosts, and digests the ghost membrane proteins. Hemorrhagic skin damages induced by 34 kDa-VvpE are weaker and slower than the damages induced by 45 kDa-VvpE (Miyoshi *et al.*, 1997, 1998, 2001, 2003). These findings indicate that the 11 kDa C-terminal propeptide of VvpE acts as a substrate-binding domain, and that the 45 kDa-VvpE is a crucial factor eliciting hemorrhagic skin damage through digestion of the vascular basement membrane. However, as shown in this study, 45 kDa-VvpE is a minor secreted form as compared to 34 kDa-VvpE; moreover, some of it is converted to 34 kDa-VvpE after extracellular secretion. These findings suggest that the pathogenetic role of 45 kDa-VvpE may not be as significant as that of 34 kDa-VvpE.

VvpE is secreted through the PilD-mediated type II general secretion system mainly during the late growth phase (Kothary and Kreger, 1987; Miyoshi *et al.*, 1987; Paranjpye *et al.*, 1998; Jeong *et al.*, 2000). Recent studies have presented a new paradigm with regard to production of VvpE. Transcription of the *vvpE* gene begins from the early growth phase through the promoter L (logarithmic) albeit a low level, and then is profoundly induced during the late growth phase through the promoter S (stationary) (Jeong *et al.*, 2001, 2003). Our previous study using non-denaturing native-PAGE proved that extracellular production of VvpE via the PilD-mediated type II general secretion system also begins from the early growth phase, consistent with transcription of the *vvpE* gene (Kim *et al.*, 2007a, 2007b). In the present study, using denaturing SDS-PAGE, a mutation of the *pilD* gene completely blocked extracellular secretion of 45 kDa-VvpE and significantly reduced or delayed extracellular secretion of 34 kDa-VvpE, indicating that both VvpE are secreted through the PilD-mediated type II general secretion system from the early growth phase. The reason why a small amount of 34 kDa-VvpE was detected in the culture supernatants obtained from the PilD-deficient mutant only after bacterial growth had reached the stationary growth phase might be because intracellular 34 kDa-VvpE was released from autolyzed cells.

PilD is a type IV leader peptidase/N-methyltransferase, a bi-functional enzyme that proteolytically cleaves the specialized leader sequence of type IV pilin precursors and induces *N*-methylation of the newly exposed N-terminal amino acid before assembly into the pilus structure (Nunn, 1999). However, the actual role of PilD in *V. vulnificus* is not known except that it is required for the secretion of VvpE via the type II general secretion system. According to our results, mature 34 and 45 kDa-VvpE were still observed within bacterial cells in the PilD-deficient background (Fig. 3A), and were observed as a single proteolytic band on zymograms using Skim milk or gelatin, akin to a single protein on native gel (Kim *et al.*, 2007a, 2007b), indicating that 34 and 45 kDa-VvpE have the same electrical charge. These findings suggest that PilD may not be essentially required for the proteolytical cleavage of the N-terminal leader peptide and/or the methylation of VvpE precursor. Accordingly, the actual role of PilD in the maturation or secretion of VvpE remains to be clarified.

In conclusion, *V. vulnificus* produces and secretes both 34 and 45 kDa-VvpE from the early growth phase via the PilD-mediated type II general secretion system; moreover, 34 kDa-VvpE was the major secreted form compared to 45 kDa-VvpE.

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