Regular Article

Investigation of the Expression of Serine Protease in *Vibrio vulnificus*

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***Vibrio vulnificus* is a Gram-negative estuarine bacterium that causes infection in immuno-compromised patients, eels, and shrimp. *V. vulnificus* NCIMB2137, a metalloprotease-negative strain isolated from a diseased eel, produces a 45-kDa chymotrypsin-like alkaline serine protease known as VvsA. The gene encoding *vvsA* also includes another gene, *vvsB* with an unknown function; however, it is assumed to be an essential molecular chaperone for the maturation of VvsA. In the present study, we used an *in vitro* cell-free translation system to examine the maturation pathway of VvsA. We individually expressed the *vvsA* and *vvsB* genes and detected their mRNAs. However, the sample produced from *vvsA* did not exhibit protease activity. A sodium dodecyl sulfate (SDS) analysis detected the VvsB protein, but not the VvsA protein. A Western blotting analysis using a histidine (His)-tag at the amino terminus of proteins also showed no protein production by *vvsA*. These results suggested the translation, but not the transcription of *vvsA*. Factors derived from *Escherichia coli* were used in the *in vitro* cell-free translation system employed in the present study. The operon of the serine protease gene containing *vvsA* and vvsB was expressed in *E. coli*. Although serine proteases were produced, they were cleaved at different sites and no active mature forms were detected. These results indicate that the operon encoding *vvsA* and *vvsB* is a gene constructed to be specifically expressed in *V. vulnificus*.**

Key words *Vibrio vulnificus* serine protease; intermolecular chaperone; cell-free translation system

INTRODUCTION

Vibrio vulnificus is a common Gram-negative halophilic estuarine bacterium that is the leading cause of seafood-related deaths in many countries.[1)] Septicemia is the most lethal infection caused by *V. vulnificus*, with an average mortality rate >50%. A serine protease called VvsA was previously suggested to be the virulence factor in *V. vulnificus* strains isolated from diseased eels.[2)] These strains were responsible for severe systemic infections in cultured eels.[3,4)]

VvsA is a 45-kDa chymotrypsin-like protease derived from the 59-kDa intermediate product remaining after the removal of the C-terminal 14-kDa polypeptide (Fig. 1). VvsA is an orthologue of the extracellular protease produced by *V. parahaemolyticus*, the causative agent of wound infection and gastroenteritis in humans.[5)] VvsA is involved in characteristic skin damage during infection in humans and may cause external and internal hemorrhaging in eel vibriosis.[6)] The gene *vvsA* and downstream gene *vvsB* constitute an operon, with VvsB functioning as the molecular chaperone for VvsA.[7)] Similar operons have been found in *V. parahaemolyticus*, *V. alginolyticus*, *V. harveyi*, and *Photobacterium profundum*. VvsA shares a high homology of 63–76% with all homologues, whereas that of VvsB is 29–56%.

Cell-free protein synthesis systems have recently emerged as a powerful technological platform for rapid, efficient, and cost-effective production. Difficulties are sometimes associated with the expression of bacterial proteins due to their potent toxicity towards the producer. Therefore, the *in vitro* protein synthesis machinery is a technical boon. Senoh *et al.* identified the crucial amino acid residues leading to the inactivation of *V. vulnificus* hemolysin (VVH) using an *in vitro* cell-free translation system.[8)]

In the present study, we attempted to synthesize the active form of serine protease using an *in vitro* cell-free translation system for the functional characterization of VvsA.[8,9)]

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions *V. vulnificus* NCIMB2137 isolated from eels was used in the present study. Bacterial strains were grown in Luria–Bertani medium at 37 °C with appropriate antibiotics.

Generation of the DNA Template to Clone *vvsA* and *vvsB* Genes Encoding Serine Proteases The rapid translation system (RTS100 *E. coli* HY Kit, 5PRIME) is an *in vitro* protein synthesis system that uses *Escherichia* (*E.*) *coli* lysates. In the present study, DNA in a linear form or cloned in the expression vector pIVEX2.4d was used for RTS. Linear DNA fragments (N-terminal histidine (His)-tag) were used as substrates for RTS of *vvsA*. Two types of *vvsA*, *vvsA* (GenBank accession number, AB509375, wild-type *vvsA*) and the *vvsA*-S369A mutant were used. Circular DNA was used for RTS of *vvsB* (GenBank accession number, AB509375, wild-type *vvsB*) and *vvsAB* (wild-type).

The sequences of primers used to prepare these gene fragments are shown in Table 1. Genomic DNA (200 ng) was used for the full-length amplification of the *vvsA* gene (1999 bp) using vvsA-f and vvsA-r (Table 1). To prepare the gene fragment of *vvsA*-S369A, two-step PCR was used. One fragment of 1388 bp was amplified with the primers vvsA-f and vvsA-R1 and another fragment of 565bp with vvsA-F2 and vvsA-r. These fragments were fused to generate the *vvsA*-S369A product.

In the second PCR, 100 ng of the PCR product from the first PCR was directly used. During this reaction, adapter primers

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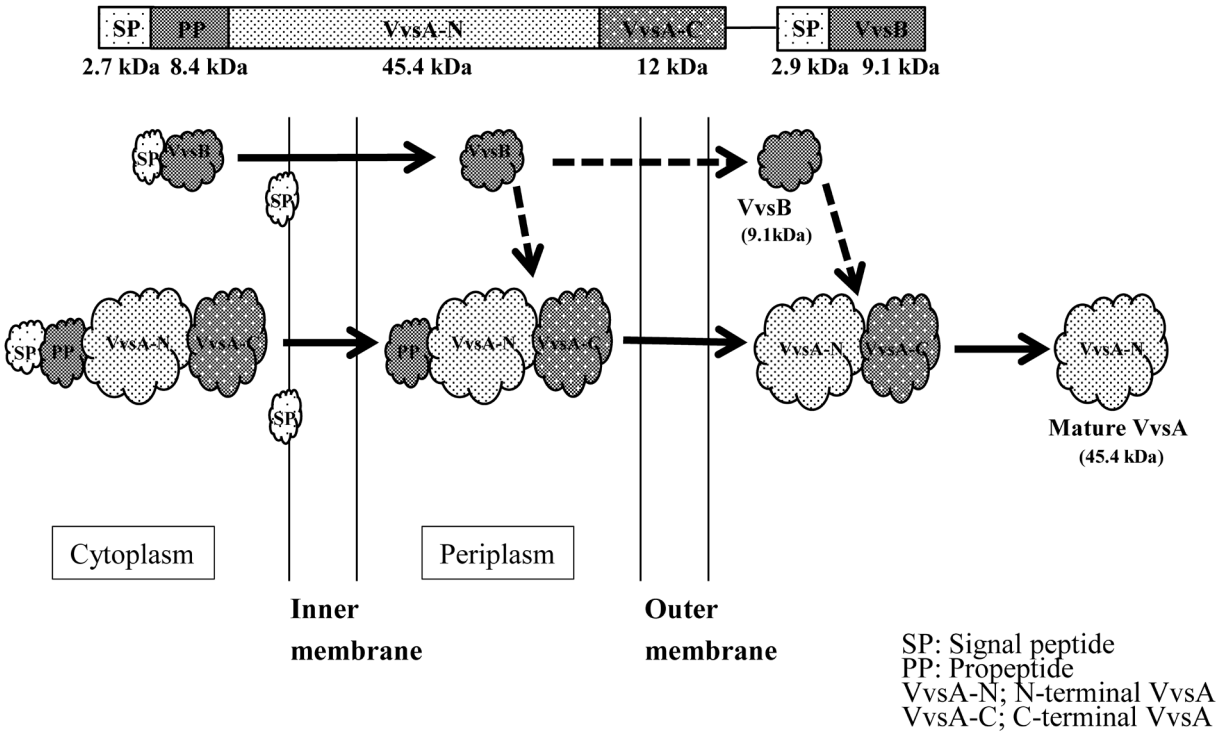

Fig. 1. Schematic Representation of the Maturation Process of VvsA

Table 1. Primers Used in This Study

Primer	Sequence (5′→3′)
vvsA-f	CGCTTAATTAAACATATGACCTTCCGCCGATGGCCTTCGACGA
vvsA-r	TTAGTTAGTTACCGGATCCCTTACTGGAAGGTTAATGTCCAGCTA
vvsA-S	GC**GCGGCCGC**TTCCGCCGATGGC
vvsA-F2	CGATTACGAGTACTACAATG**GCA**CGGCAATGGCGAC
vvsA-R1	GTCGCCAT**TGC**CGTGCCATTGTAGTACTCGTAATCG
vvsB-F	CTTTAAGAAGGAGATATACCATGTTCCGCCGATGGCCTTCGACGA
vvsB-r	TGATGATGAGAACCCCCCCCTTACTGGGTGTACAGTTGCCTAAC
vvsA-SalI	GGGCGAATTG**GAGCTC**TTCATAGAACTATCTTCTTATGTTTTG
pBlue-kpnI-*vvsA*-R	ACAAAAGCTG**GGTAC**TTACTGGAAGGTTAATGTCCAG
pBlue-kpnI-*vvsB*-termR	ACAAAAGCTG**CCATGG**AATCGCAAGGCAGCTCCTCTTAAG

Underlined: Overlapping regions. Bold: *vvsA*-F2 and *vvsA*-R1 were changed amino acids. The restriction enzyme site on the vector multicloning site (MCS).

were used to add the His_6-Tag along with the upstream regulatory elements at the 5′ end and downstream regulatory elements at the 3′ end of the final PCR product.

The genomic DNA of *V. vulnificus* NCIMB2137 was used to amplify the *vvsB* gene without a signal peptide sequence. This construct was cloned into the expression vector pIVEX2.4d, followed by transformation into *E. coli* DH5*α*. The positive transformant was cultivated at 37 °C overnight, and the recombinant plasmid was purified for subsequent use in RTS.

Sequencing of the Cloned Mutant Gene (*vvsA*-S369A) After clones had been amplified by PCR, the nucleotide sequence of the amplicon of *vvsA* (S369A) was elucidated using the Brilliant Dye™ Terminator Cycle Sequencing Kit and ABI PRISM™ 3500 Genetic Analyzer.

RT-PCR Total RNA was extracted from RTS products using the RNeasy Mini Kit and treated with deoxyribonuclease (DNase)I. Two hundred nanograms was added to the Ready-To-Go RT-PCR Kit and incubated at 42 °C for 30 min for cDNA synthesis. PCR amplification was performed using appropriate primers and confirmed by electrophoresis.

***In Vitro* Synthesis of Proteins Using RTS** The *in vitro* expression of the serine protease gene was performed using the cell-free system of RTS ProteoMaster (Roche, Switzerland). The morphology of templates was linear DNA (0.1 μg) or circular DNA (0.5 μg). A reaction volume of 50 μL was prepared according to the manufacturer's protocol. The reaction was performed at 400 rpm at 25 °C for 6 h. A protease inhibitor was added during metalloprotease expression only.

Purification of His-Tagged Proteins and the Protease Assay The Capturem His-tagged Purification Mini-prep Kit (TaKaRa Bio, Otsu, Japan) was used to purify RTS products. After the protein synthesis reaction, 200 μL of the reaction solution was purified according to the manufacturer's protocol. The protease activities of RTS products were measured according to the method described by Miyoshi *et al.*[10]

Sodium Dodecyl Sulfate (SDS)-Polyacrylamide Gel Electrophoresis (PAGE) Analysis and N-Terminal Amino Acid Sequencing RTS products (10 μL) and the periplasmic

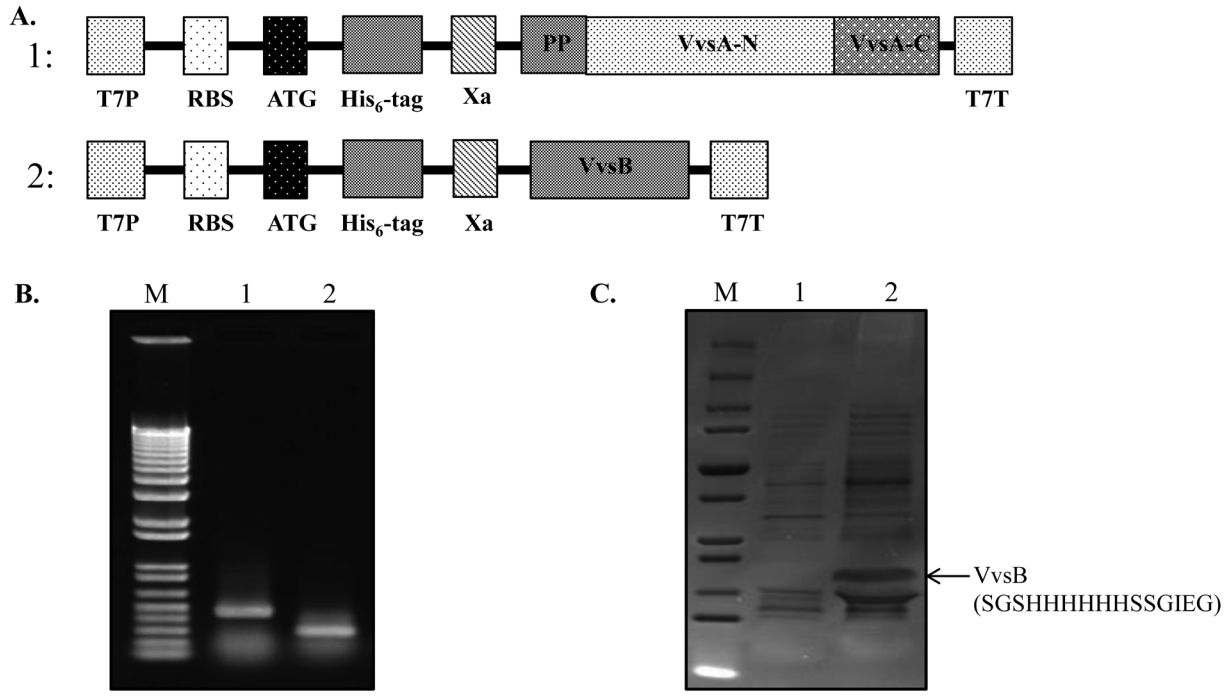

Fig. 2. Gene Arrangement Map Used and Expression in the Rapid Translation System (RTS)

(A) 1 and 2 are the gene arrangements of *vvsA* and *vvsB*, respectively, inserted into the pIVEX2.4d expression vector. T7P; T7 Promoter, RBS; Ribosome-binding site, ATG; Start codon, His_6-tag; gene for the tag of His_6, Xa; Factor Xa restriction protease cleavage site, T7T; T7 Terminator. (B) mRNA expression from linear *vvsA* and circular *vvsB* DNA in RTS. DNA fragments 1 and 2 shown in A were inserted into the pIVEX2.4d expression vector. At the time of use, the vector into which gene fragment 1 was inserted was linearized by cleavage. The genes prepared were incubated at 30 °C for 6 h in RTS (RTS100 *E. coli* HY Kit, Roche Molecular Biochemicals). After the incubation, total RNA was isolated and RT-PCR was performed to detect the mRNAs of *vvsA* and *vvsB*. The products were electrophoresed on a 1.0% agarose gel. Lane 1, RT-PCR of *vvsA* (446 bp); lane 2, RT-PCR of *vvsB* (325 bp), lane M, size markers (1-kb Plus DNA Ladder; 100–12000 bp). (C) Protein expression from *vvsA* and *vvsB* DNA in RTS. The reaction mixtures obtained by the incubation in the *in vitro* protein synthesis system were separated by SDS-PAGE followed by Coomassie brilliant blue staining of the gel. Lanes 1 and 2 are samples recovered from solutions containing *vvsA* and *vvsB*, respectively. Lane M, molecular weight markers (10, 15, 20, 25, 37, 50, 75, 100, 150, and 250 kDa).

fraction isolated from *E. coli* were subjected to SDS-PAGE followed by staining with Coomassie brilliant blue R-250. Images were captured with the Molecular Imager ChemiDoc™ XRS+ Imaging System (BIO-RAD, U.S.A.).

The polyvinylidene difluoride (PVDF) membrane (BIO-RAD) was stained with Amido Black T. The N-terminal amino acid sequence was elucidated using an Applied Biosystem Precise Sequencer (Applied Biosystem, Foster city, CA, U.S.A.)

Folding of the Urea-Denatured Supernatant through Stepwise Dialysis in the Presence of Purified VvsB We mixed 40 μL of purified VvsB (0.293 μg/μL) with 40 μL of the culture supernatant of *V. vulnificus* NCIMB2137. The culture supernatant was prepared from a centrifugal culture solution incubated at 26 °C overnight. Ten microliters of this mixture were stored for later use as a positive control to measure azocasein activity. This mixture was placed on a dialysis membrane (Spectra/Por® Dialysis membrane Standard RC Trial kit MWCO: 6–8 kDa, SPECTRUM LABS. COM., U.S.A.) and immersed in 7 M urea solution overnight. Ten microliters of this denatured mixture were stored for later use as a negative control in the azocasein assay. The remaining mixture was immersed in 5 M urea solution followed by 3 M and then 1 M urea. After the mixture was dialyzed against 20 mM Tris–HCl solution (pH 8.0), azocasein activity of the re-folded mixture was measured along with positive and negative controls. The data is the mean + standard error (S.E.) of three experiments. It indicates the significant difference ($p < 0.05$) by T TEST.

Preparation of the Cell Membrane Fraction Log phase bacteria were collected and washed twice with 2% NaCl solution. The bacterial pellet was suspended in pure water to allow osmotic shock lysis. After centrifugation, the bacterial pellet was dissolved in 50 mM Tris–HCl buffer (pH 8.0) and used as the cell membrane fraction.

Preparation of *E. coli* Recombinants and the Extracted Protein Fraction The 2123 bp of *vvsA* and 2553 bp fragments of the *vvsAB* genes including operator/promoter (O/P) and Shine–Dalgarno sequence (SD) derived from *V. vulnificus* NCIMB2137. These fragments were amplified using the primer sets Prom-vvsA-salI (*SalI*): pBlue-kpnI-vvsA-R (*KpnI*) for *vvsA* and Prom-vvsA-salI (*SalI*): pBlue-kpnI-vvsB-termR (*KpnI*) for *vvsAB* (Table 1). These PCR products were cloned into the pBluescript IIKS+ vector containing the Amp^r marker by the HiFi DNA Assembly Master Mix kit (NEW ENGLAND BioLabs). These recombinants were transformed into *E. coli* HB101 (TaKaRa). The periplasmic fraction was collected by the osmotic shock method.[11]

RESULTS AND DISCUSSION

Serine protease has been shown to play a significant role as a virulence factor in *Vibrionaceae*.[2,6,7] In the present study, we used RTS to express *V. vulnificus* serine protease, VvsA, a 45-kDa toxic factor (Fig. 1), which may be responsible for eel vibrios,[6] and attempted to identify essential factors for the construction of functional VvsA.

Since we used a cell-free system, each target gene was cloned without a signal peptide (Fig. 1).

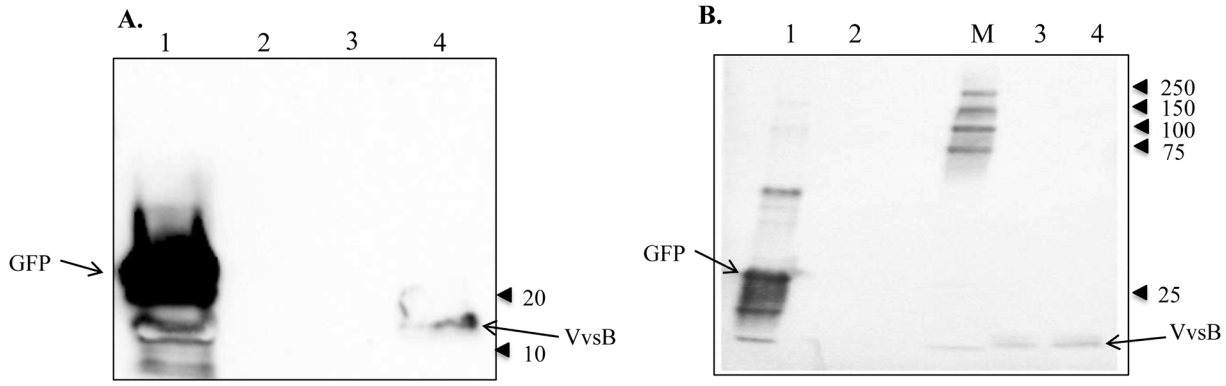

Fig. 3. Detection of Gene Products by a Western Blot Analysis

Genes were incubated in RTS at 25 °C for 6 h, and the products were treated with 5% SDS at 100 °C for 5 min and subjected to SDS-PAGE. Thereafter, the proteins were transferred to a PVDF membrane and detected using anti-His-tag serum, and the proteins possessing the His-tag were detected with anti-His-tag serum. (A) Protein detection from linear *vvsA* and circular *vvsB* DNA in RTS. Samples 2, 3, and 4 shown in the figures were prepared from reaction mixtures containing pIVEX2.4d (vector), the linear *vvsA* (wild-type), and the vector inserted with *vvsB*, respectively. (B) Protein detection from each mixture genes in RTS. Sample 3 was prepared from the reaction mixture containing two genes: the linear *vvsA* (wild-type) and the vector inserted with *vvsB*. Sample 4 was also prepared from reaction mixtures containing two genes: the linear *vvsA* mutant (VvsA-S369A) and the vector inserted with *vvsB*. These genes were mixed in samples 3 and 4 at *vvsA* : *vvsB* = 1 : 1.6 as the DNA ratio. The sample in lane 1 is GFP.

DNA fragments (*vvsA* or *vvsB*) were inserted into the pIVEX2.4d expression vector and used to synthesize proteins in RTS at 30 °C for 6 h. Total RNA was isolated from each sample and RT-PCR was performed to detect the expression of mRNA. Detectable levels of the mRNAs of these two genes were noted after 6 h (Fig. 2B). However, expression at the protein level was not detected for VvsA, whereas a band was observed at approximately 11.7 kDa for VvsB. To confirm that the protein band at 11.7 kDa in the gel was VvsB, it was extracted and its N-terminal amino acid sequence was elucidated. The results obtained showed that the band was VvsB (Fig. 2C).

The serine protease from *Aeromonas sobria* (ASP) is dependent on another downstream protein called ORF2 for proper folding. The maturation of ASP with ORF2 was previously shown to be initiated in the periplasmic space and ASP became active after dissociating from ORF2.[12,13] We considered VvsB to also act as a molecular chaperone during the synthesis of VvsA. Since VvsA and VvsB were expressed separately in RTS, VvsB did not associate with VvsA or contribute to the construction of the active form of VvsA.

In many serine proteases, a chaperone facilitates the construction of the active form. These chaperones are often encoded as an intramolecular chaperone in the sequence.[14–18] The nascent protein containing serine protease and the chaperone often make contact with the cell membrane and the nascent serine protease becomes the active form. In RTS, there were no interactions with the cell membrane. This may be another reason for the unsuccessful production of VvsA in RTS.

A previous study reported that the serine protease of *V. vulnificus* underwent autolysis.[19] A possible reason why we did not detect the expression of VvsA was self-catalyzed proteolysis before folding. Therefore, we produced a variant gene of vvsA in which serine at the catalytic center was replaced with alanine to produce the inactive mutant VvsA-S369A.

The Western blot using the His-tag antibody only detected the expression of positive control GFP and VvsB. As a result of the N-terminal amino acid sequence of the protein corresponding to VvsB detected by a Western blotting, it was confirmed to the sequence of VvsB, as indicated in Fig. 3A. However, there was still no detectable mutant VvsA produced. This result eliminated the possibility of VvsA degradation due to autolysis. We then attempted to express VvsA in the presence of VvsB by mixing the linear DNA product of *vvsA* and circular *vvsB* at a 1 : 1.6 ratio. The expression of VvsB, but not VvsA was noted (Fig. 3B).

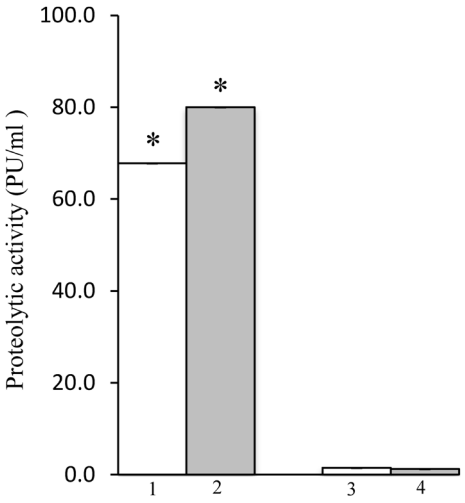

Fig. 4. Effect of VvsB on the Protease Activity Expression

Azocasein (5 mg/mL) was used as a substrate for the measurement of protease activity. One protease unit (PU) was defined as the amount of enzyme hydrolyzing 1.0 μg of azocasein in 1 min. Lanes 1, 2: the white bar indicates a mixture of the His-tag purification buffer 10 μL and *V. vulnificus* culture supernatants 40 μL; the gray bar represents the mixture of purified VvsB 10 μL (0.293 μg/μL) and 40 μL of *V. vulnificus* culture supernatants; lanes 3, 4: the samples obtained after the removal of urea after slow dialysis. The data are the mean + S.E. of three experiments. The asterisk (*) indicates the significant difference ($p < 0.05$) by T TEST.

Additionally, there was no protease or peptidase (Synthetic substrate: succinyl (Suc)-alanine (Ala)-Ala-proline (Pro)-phenylalanine (Phe)-4-methylcoumaryl-7-amide) activity by any RTS products.

The effective renaturation of reduced lysozyme was previously achieved by the gentle removal of urea by dialysis.[20] To define the role of VvsB, we performed the *in vitro* refolding process on the urea-denatured culture supernatant (containing VvsA) by slow dialysis in the presence of purified VvsB, and measured protease activity after refolding. The results obtained showed the loss of proteolytic activity in the culture supernatant after dialysis (Fig. 4 lanes 3 and 4). This result

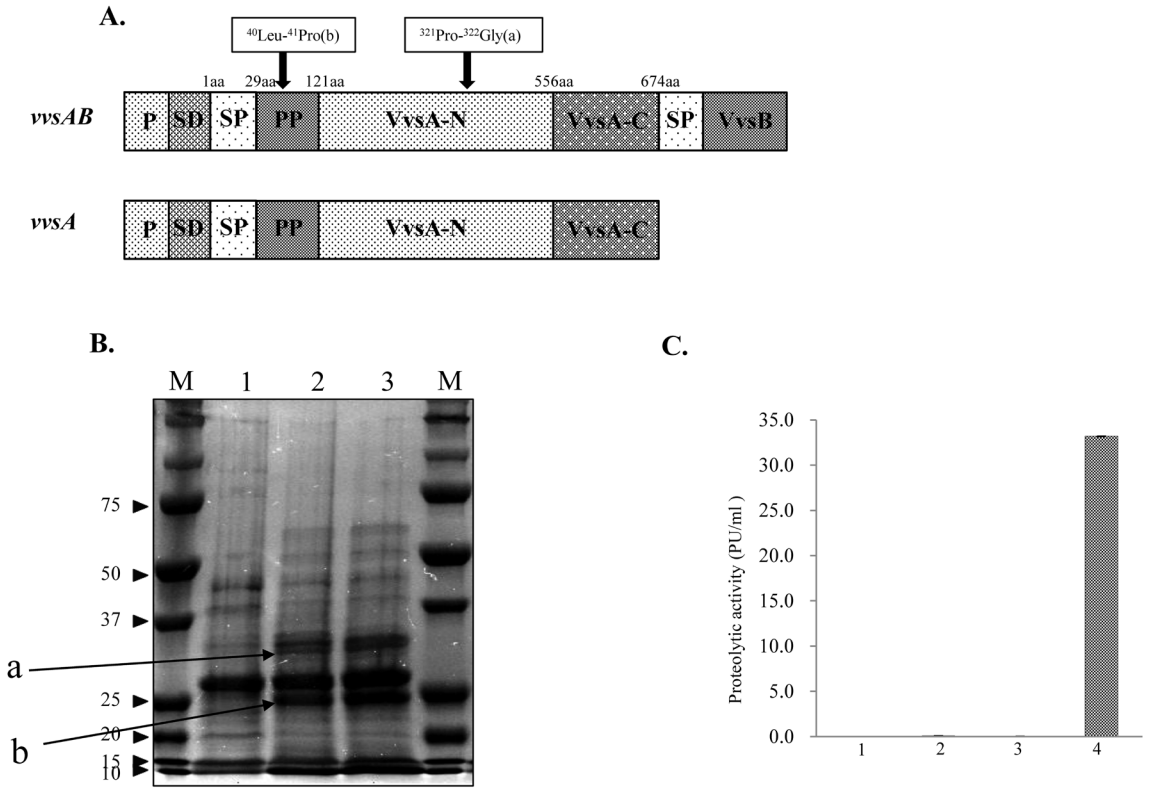

Fig. 5. Analysis of Proteins Released into the Periplasmic Space of *E. coli* from the Operon of vvsA and the Proteolytic Activity of Recovered Samples

(A) Arrangement of the functional regions of two gene fragments inserted into the vector. The numbers shown in the upper part of *vvsAB* are the amino acid numbers of the precursor of VvsA synthesized in *V. vulnificus*. P; Promoter, SD; Shine–Dalgarno sequence, SP; Signal peptide, PP; Propeptide. Amino acid number of the precursor of VvsA. (B) SDS-PAGE analysis of samples obtained from the periplasmic space. The periplasmic fraction obtained by the osmotic shock method from *E. coli* recombinants harboring the pBluescript IIKS+ vector with the insertion of *vvsA* or *vvsAB*. The periplasmic fraction was precipitated by 10% trichloroacetic acid, treated with 5% SDS at 100 °C for 5 min, and loaded onto 10% SDS-PAGE. Lane M, Protein molecular weight marker (250, 150, 100, 75, 50, 37, 25, 20, 15, and 10 kDa); lane 1, pBluescript IIKS+ vector; lane 2, *vvsA* inserted into the pBluescript IIKS+ vector; lane 3, *vvsAB* inserted into the pBluescript IIKS+ vector. (C) Proteolytic activity of samples. Azocasein (5 mg/mL) was used as a substrate for the measurement of protease activity in the periplasmic fraction obtained from recombinants. One protease unit (PU) was defined as the amount of enzyme hydrolyzing 1.0 μg of azocasein in 1 min. Lane 1, the pBluescript IIKS+ vector; lane 2, *vvsA* inserted into the pBluescript IIKS+ vector; lane 3, *vvsAB* inserted into the pBluescript IIKS+ vector; lane 4, the *V. vulnificus* NCIMB2137 culture supernatant collected from the stationary phase at 30 °C.

suggests three scenarios: the *in vitro* refolding process did not generate properly folded VvsA; factors other than VvsB may contribute to the stable and active conformation of VvsA; and VvsA was only expressed in a properly folded structure using bacterial machinery. Moreover, the protease activity of the culture supernatant was elevated in the presence of purified VvsB (Fig. 4). This result indicates that VvsB suppressed the autolysis of VvsA and, thus, warrants further investigation.

Since none of the protocols described above led to the production of the active form of VvsA using RTS, we employed the conventional method of protein expression using the bacterial system. We cloned the *vvsA* and *vvsAB* operon in the pBluescript IIKS+ vector and transformed it into *E. coli* HB101. Figure 5 shows the results of the SDS-PAGE analysis of the periplasmic fraction extracted from each of the recombinants.

Figure 5A shows a map of the genes introduced into this vector that were expressed in the *E. coli* system. Figure 5B shows the results of SDS-PAGE on the expressed proteins. Two protein bands were characteristically detected in two samples containing different vectors, which were inserted with the *vvsA* and *vvsAB* operon, respectively (protein bands a and b in Fig. 5B). These two bands were not in the sample containing the vector (native) (Fig. 5B, lane 1).

Bands a and b were excised, and their amino terminal sequences were elucidated. The amino terminal sequences of a and b were GVAILSTVTVGEGVL and PTKYMVKFKEGSNAR, respectively. Based on these sequences, a was the region covering from glycine (Gly)-322 of VvsA and b was that from Pro-41 of VvsA (Fig. 5A).

A previous study reported that VvsA was synthesized as a 57-kDa precursor (pro-VvsA), which was later converted into the 45-kDa mature form after the removal of the N-terminal signal peptide and propeptide in *V. vulnificus*.[7] Cleavage at the site of VvsA in *E. coli* (between the 40th and 41st and between the 321st and 322nd) did not occur in *V. vulnificus* (Fig. 5A). Cleavage at this site interfered with the production of active VvsA.

Cleavage in *E. coli* was observed in samples with and without *vvsB* (Fig. 5B, lanes 2, 3). The sample in lane 3 in Fig. 5B was recovered from the solution containing a vector possessing *vvsB*. However, there was no evidence that the protein of VvsB was produced from *E. coli* in the sample. These results indicated that cleavage in *E. coli* was independent of VvsB.

In the absence of the synthesis of VvsB, this cleavage may occur, even in *V. vulnificus*. The present results showed that active VvsA was not produced in the absence of VvsB. VvsB did not appear to be expressed from the operon encoding *vvsA* in *E. coli* based on the result of lane 3 in Fig. 5B. However, active VvsA was produced in *V. vulnificus*. Therefore, the ex-

pression of *vvsA* is a characteristic event of *V. vulnificus* that does not occur in other bacteria.

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Conflict of Interest The authors declare no conflict of interest.

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