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An extracellular serine protease produced by *Vibrio vulnificus* NCIMB 2137, a metalloprotease-gene negative strain isolated from a diseased eel

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V. vulnificus proteolytic enzymes

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Abstract

Vibrio vulnificus is a ubiquitous estuarine microorganism but causes fatal systemic infections in immunocompromised humans, cultured eels or shrimps. An extracellular metalloprotease VVP has been reported to be a potential virulence factor of the bacterium; however, a few strains isolated from a diseased eel or shrimp were recently found to produce a serine protease termed VvsA, but not VVP. In the present study, we found that these strains had lost the 80 kb genomic region including the gene encoding VVP. We also purified VvsA from the culture supernatant through ammonium sulfate fractionation, gel filtration and ion-exchange column chromatography, and the enzyme was demonstrated to be a chymotrypsin-like protease, as well as those from some vibrios. The gene *vvsA* was shown to constitute an operon with a downstream gene *vvsB*, and several *Vibrio* species were found to have orthologues of *vvsAB*. These findings indicate that the genes *vvp/vvpE* and *vvsAB* might be mobile genetic elements.

Keywords

Polymerase chain reaction; Purification; Serine protease; metalloprotease; *Vibrio vulnificus*

Abbreviations

Boc, *t*-butyloxycarbonyl; CBB, Coomassie brilliant blue R-250; LB, Luria-Bertani; MCA, 4-methylylcoumaryl-7-amide; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PU, proteinase unit; RT, reverse transcription; Suc, succinyl; VVH, *Vibrio vulnificus* hemolysin; VVP/VvpE, *Vibrio vulnificus* protease; VvsA, *Vibrio vulnificus* serine protease

Introduction

Vibrio vulnificus is a motile and facultative anaerobic gram-negative bacterium and inhabits ubiquitously at estuarine and marine environments (Janda et al., 1988; Chakraborty *et al*., 1997). However, after consumption of contaminated seafood, this species causes fatal septicemia in humans who have underlying diseases such as hepatic cirrhosis, hepatitis or diabetes (Strom and Paranjpye, 2000; Miyoshi 2006). Although *V. vulnificus* infections have been documented in many countries such as the United States, Japan and South Korea, no epidemic case is reported. In addition, this species causes epidemic and serious systemic infections called vibriosis in cultured eels (Tison et al., 1982). Currently, the genomic nucleotide sequences of some human clinical isolates including strain YJ016 (accession numbers, BA000037 for chromosome I, and BA000038 for chromosome II) are available on the database.

An extracellular metalloprotease, VVP (*V. vulnificus* protease) or VvpE, is a potential virulence factor of the bacterium (Miyoshi and Shinoda, 1997). The purified enzyme has been reported to enhance vascular permeability due to exocytotic histamine release from mast cells and/or due to activation of the bradykinin-generating cascade, and to evoke the hemorrhagic skin damage through digestion of the vascular basement membrane. Additionally, VVP can disturb the host's physiological homeostasis through disordered proteolysis of many kinds of plasma proteins, which may elicit eventually an immunocompromised state in the host. The experiments using the VVP/VvpE mutants or the protease inhibiters have also indicated significant roles of the metalloprotease in the bacterial pathogenicity (Miyoshi and Shinoda, 1988; Miyoshi et al., 1994; Valiente et al., 2008).

Wang et al. (2008) recently carried out the genotyping of *V. vulnificus* clinical strains

based on the *vvp/vvpE* gene. Most strains were apparently classified into the genotype A or B by the PCR (polymerase chain reaction) method. However, a few strains from diseased eel or shrimp could not be categorized because no PCR product was obtained, which suggested the absence of the *vvp/vvpE* gene. Interestingly, theses strains were found to produce a serine protease (VVA0302 of strain YJ016) instead of VVP/VvpE (VVA1465 of strain YJ016) (Wang et al., 2008). Several *Vibrio* species including human or fish pathogens have been documented to secrete a serine protease (Shinoda and Miyoshi 2011). For instance, *Vibrio parahaemolyticus*, a causative agent of human wound infections or gastroenteritis, produces an extracellular serine protease termed VPP1 or protease A (Ishihara et al., 2002; Lee et al., 2002). This serine protease is also a putative virulence factor. Lee et al. (2002) reported that the purified enzyme had the cytotoxic activity to CHO, HeLa and Vero cells and the lethal activity to mice.

In the present study, we first clarified whether the non-classified strains had lost only the *vvp/vvpE* gene or the larger genomic region including the metalloprotease gene. Next, we carried out purification and characterization of *V. vulnificus* serine protease that was designated as VvsA. In addition, we undertook the sequencing and analysis of the *vvsA* gene.

Materials and methods

Bacterial strains

Five clinical strains were used for the present study. Among them, strain ATCC 33149 (isolated in Japan), CECT 5343 (in Spain), E86 (in Spain) and NCIMB 2137 (in Japan)

were from eels, whereas strain 520 (in Taiwan) was from a shrimp. The *vvp/vvpE* genotype of strain CECT 5343 and E86 are type A and type B respectively; however, remaining three strains (strain ATCC33149, NCIMB 2137 and 520) are *vvp/vvpE* negative (Wang et al., 2008). For VvsA purification, strain NCIMB 2137 was used because the culture supernatant from this strain showed the highest proteolytic activity (Wang et al., 2008).

PCR amplification of upstream or downstream genes of *vvp/vvpE*

The bacterium was cultivated at 37 ^{o}C in Luria-Bertani (LB) broth (1 % tryptone, 0.5 % yeast extract, 2.0 % NaCl, pH 7.5) with shaking, and the genomic DNA was extracted at the middle logarithmic growth phase as reported (Senoh et al., 2005). Appropriate PCR primers for amplification of the target genes were designed based on the nucleotide sequences of strain YJ016 (accession number, BA000038).

The bacterial genomic DNA (100 ng in 1 μl), Go Taq Green Master Mix (Promega, Madison, WI, USA) (12.5 μl) and each of the primer set (20 pmol in 2 μl) were mixed, and the total volume of the mixture was adjusted to 25 μl with purified water. Thereafter, the reaction mixture prepared was heat-treated at 94 ^{o}C for 2 min, and PCR amplification was carried out for 25 cycles as following: 30-sec denaturation at 94 ^{o}C, 30-sec annealing at 58 ^{o}C, and 90-sec extension at 72 ^{o}C. After PCR, the products were electrophoresed on a 1.5 % agarose gel and visualized by staining with ethidium bromide.

Purification of VvsA from strain NCIMB 2137

Strain NCIMB 2137 was cultivated in TYE broth (0.5 % tryptone, 0.25 % yeast extract, 2.0 % NaCl, 25 mM K_2HPO_4, pH 7.5) at 26 ^{o}C with shaking. At the early stationary

growth phase (at 20-hr cultivation), the culture supernatant was collected by centrifugation at 7,000 x g for 40 min, and ammonium sulfate was added to 70 % saturation (472 g/l). The resulting precipitate was collected, dissolved in a small volume of distilled water, and dialyzed against 50 mM Tris-HCl buffer (pH 7.5) containing 4 mM $CaCl_2$ for 24 hr. The preparation thus obtained was applied to a HiLoad 26/60 Sephacryl S-300 HR column (GE Healthcare, Buckinghamshire, England) equilibrated with 50 mM Tris-HCl buffer (pH 7.5) containing 4 mM $CaCl_2$ and 0.02 % NaN_3 (buffer A). The fractions showed the high proteolytic activity toward azocasein (Sigma-Aldrich, St Louis, MO, USA) were collected and concentrated by ultra-filtration with a Dioaflo membrane YM30 (Millipore, Billerica, MA, USA). Then, the concentrated preparation was loaded on a Fractogel EMD TMAE 650 5/5 column (Merk, Darmstadt, Germany) equilibrated with buffer A, and the linear gradient elution with 0-0.5 M NaCl in buffer A was carried out. The protein peak with the sufficient proteolytic activity was collected and used as the final VvsA preparation.

Polyacrylamide gel electrophoresis (PAGE) and the N-terminal amino acid sequencing

For SDS-PAGE, the sample was treated with 2 % SDS at 100 ^{o}C for 3 min and was subjected to gel electrophoresis on the PhastSystem using a PhastGel Gradient 10-15 (GE-Healthcare). After electrophoresis, the gel was stained with 0.25 % Coomassie brilliant blue R-250 (CBB) and destained with 25 % ethanol-8 % acetic acid. Native PAGE on the PhastSystem was carried out with a PhastGel Gradient 8-25 (GE Healthcare). After native PAGE, one of the gels was stained with CBB in order to visualize the proteins, whereas another gel was embedded into 2 % skim milk agar (pH 7.5) and incubated at 30 ^{o}C for 3 hr for detection of the proteolytic activity.

For the N-terminal amino acid sequencing, the proteins separated by SDS-PAGE were

transferred to a PVDF membrane and stained with CBB. Thereafter, an appropriate protein band was cut off from the membrane, and its N-terminal amino acid sequence was determined by Edman degradation with an automatic protein sequencer Model 473A (Applied Biosystems, Foster City, CA, USA).

Assay of the protease activity

The proteinase activity was assayed with 1 % azocasein as described previously (Miyoshi et al. 1987) with some modifications. Briefly, the sample was allowed to act on azocasein (1 mg) in 0.3 ml of 50 mM Tris-HCl buffer (pH 8.0) at 30 ^{o}C for 15 min. Thereafter, the reaction was stopped by the addition of 0.7 ml of 5 % trichloroacetic acid, and the reaction mixture was centrifuged at 1,500 x g for 5 min. The supernatant was collected and mixed with an equal volume of 0.5 M NaOH, and then absorbance at 440 nm was measured. One proteinase unit (PU) was defined as the amount of the enzyme hydrolyzing 1 μg of azocasein in 1 min.

The peptidase activity was assayed with peptidyl-4-methylylcoumaryl-7-amide (MCA) substrates developed for human serine proteases. The following substrates, *t*-butyloxycarbonyl (Boc)-Arg-Val-Arg-Arg-MCA (for furin), Boc-Glu-Lys-Lys-MCA (for plasmin), Boc-Gln-Ala-Arg-MCA (for trypsin), succinyl (Suc)-Ala-Ala-Pro-Phe-MCA (for chymotrypsin), and Boc-Gln-Gly-Arg-MCA (for factor XIIa) were used. These substrates were purchased from Peptide Institute (Minoh, Japan) and dissolved in dimethyl sulfoxide at a concentration of 150 mM. For the assay, the sample was allowed to act on the substrate (300 nmol) in 0.3 ml of 50 mM Tris-HCl buffer (pH 8.0) at 30 ^{o}C for 30 min. Thereafter, the reaction was terminated by the addition of 0.7 ml of ice-cold citrate buffer (pH 5.0), and the liberation of 7-amino-4-metylcoumarine from the substrate was

determined by measuring absorbance at 370 nm.

Amplification and sequencing of the *vvsA-vvsB* genes of strain NCIMB2137

In chromosome II of *V. vulnificus* strain YJ016 (accession number, BA000038), *VVA0302* (2025 bp; *vvsA* of strain NCIMB 2137) is separated to *VVA0301* (348 bp; *vvsB* of strain NCIMB 2137) by only 6 bp, suggesting two genes constitute an operon. For PCR amplification of the full-length *vvsA-vvsB* genes, appropriate PCR primers were designed from the nucleotide sequences of *VVA0303* and *VVA0300*. The primers were mixed with the genomic DNA from strain NCIMB 2137 and TaKaRa Ex Taq™ polymerase (Takara-Bio, Otsu, Japan). The reaction mixture thus prepared was heat-treated at 95 °C for 2 min and subjected to the amplification reactions (25 cycles) with the following conditions, 10-sec denaturation at 98 °C, 30-sec annealing at 58 °C and 5-min extension at 72 °C.

After PCR, the nucleotide sequence of the amplicon was determined by using a BigDye™ Terminator Cycle Sequence Kit (Applied Biosystems, Foster City, CA, USA) and ABI PRISM™ 3100 Genetic Analyzer (Applied Biosystems). The nucleotide sequence data of the *vvsA-vvsB* genes of strain NCIMB 2137 was deposited in the DDBJ/EMBL/GenBank database under the accession number AB509375.

Reverse transcription PCR (RT-PCR)

Total RNA was extracted from strain NCIMB 2137 at the early stationary growth phase and treated with DNase I (Invitrogen, Carlsbad, CA, USA) as reported (Kawase et al., 2004). Thereafter, an aliquot (200 ng) was added to the Ready-To-Go RT-PCR Kit (GE

Healthcare) and incubated at 42 °C for 30 min for RT reaction. Then, the reverse transcriptase was inactivated by heat-treatment at 95 °C for 5 min. The PCR amplification using appropriate primers corresponding to the outside or inside of *vvsA*-*vvsB* (Fig. 1) was performed for 30 cycles as follows: 30-sec denaturation at 95 °C, 30-sec annealing at 58 °C and 2-min extension at 72 °C. After PCR, the products were electrophoresed on a 1.5 % agarose gel and visualized by staining with ethidium bromide.

Results and discussion

Deletion of the 80 kb genomic region in the *vvp/vvpE* negative strains

Comparative genomic studies have revealed that the genes around *vvp/vvpE* are commonly conserved in *V. vulnificus* strains from clinical and environmental sources (Quirke et al., 2006; Gulig et al., 2010). In order to clarify whether the *vvp/vvpE* negative strains have lost only the metalloprotease gene or the much larger genomic region, a series of PCR experiments targeting upstream genes of *vvp/vvpE* (*VVA1465*) were carried out (Table 1). Up to *VVA1436*, the amplicons with suitable sizes were produced from all strains tested. However, in the experiment targeting the *VVA1437* gene, no DNA fragment was amplified from the *vvp/vvpE* negative strains (strain ATCC33149, NCIMB2137 and 520). On the other hand, when downstream genes were targeted, amplicons of *VVA1527* or *VVA1526* were ordinarily obtained in all strains; however, the fragment of *VVA1524* was not amplified in the *vvp/vvpE* negative strains (Table 1). The PCR targeting *VVA1525* (156 bp) was not carried out because the suitable primers could not be designed.

In the *vvp/vvpE* negative strains, about 3 kb fragment was commonly produced when

the PCR amplification between *VVA1436* and *VVA1526* was carried out (data not shown). The sequencing of the 3 kb fragment from strain ATCC33149 was performed and the results revealed that the full-size *VVA0256* gene (1338 bp), but not *VVA1525*, was contained in the fragment. This indicates that *VVA0256* has moved and inserted into downstream of *VVA1436*. Although no experimental evidence has been documented, the product of *VVA0256* is a putative transposase or its inactivated derivative (accession number, BA000038). Therefore, this protein might be related to deletion of the genomic elements involving *vvp/vvpE*.

Taken together, as shown in Fig. 2, it may be concluded that the *vvp/vvpE* (*VVA1465*) negative strains has lost the 80 kb genomic region corresponding to the genes from *VVA1437* to *VVA1525* (accession number, BA000038). It should be noted that the G+C nucleotide content of the 80 kb region is 49 %, whereas that of chromosome II is 47 % (Chen et al., 2003). This supports the idea that the 80 kb genomic region might be transferred from another bacterial species. However, in order to verify the idea, the more detailed analysis of G+C variation over different scales is required. Some *Vibrio* species are known to possess orthologues of *vvp/vvpE* (Miyoshi and Shinoda, 1997; Shinoda and Miyoshi, 2011). The comparative analysis of the genes around the orthologues would be useful to clarify whether the 80 kb region is a movable element or not.

Purification and characterization of VvsA produced by strain NCIMB 2137

Wang et al. (2008) reported that LB broth might be not suitable for purification of VvsA, because the protease secreted was found to associate with some proteins contained in the broth. Therefore, we used TYE broth in the present study. As reported by Wang et al. (2008), the culture supernatant was collected at the early stationary growth phase and

fractionated by ammonium sulfate precipitation, gel filtration and ion-exchange column chromatography. However, VvsA showed the apparently different elution profile. Namely, in the final ion-exchange column chromatography, the main protease peak was around 0.2 M NaCl when cultivated in TYE broth (Fig. 3A). In contrast, when cultivated in LB broth, the protease was eluted around 0.3 M NaCl (Wang et al., 2008).

The major protease eluted around 0.2 M NaCl was collected and subjected to SDS-PAGE. As shown in Fig. 3B, a single 45 kDa protein was visualized. The native PAGE analysis showed that the 45 kDa protein had the significant proteolytic activity toward skim milk (data not shown). Additionally, the N-terminal amino acid sequence of the 45 kDa protease was found to be NH_2-SETTPWGYGA, which correspond surely with Ser^{122} to Ala^{131} of VVA0302 (VvsA). Therefore, the final preparation was used as purified VvsA in the next experiments. The typical purification result is summarized in Table 2. The purified protease showed the 1,770-fold specific activity than that of the culture supernatant. However, as small amounts as 80 μg was obtained from 2,400 ml of the culture supernatant.

As reported by Wang et al. (2008), the second peak around 0.3 M NaCl contained the 59 kDa protein, and its N-terminal amino acid sequence was identical with that of 45 kDa VvsA (data not shown). This demonstrates that 45 kDa mature VvsA was derived from the 59 kDa intermediate through removal of the C-terminal 14 kDa polypeptide. The extracellular processing of proteolytic enzymes is not unique in *Vibrio* species. For instance, the C-terminal 10 kDa polypeptide of VVP/VvpE is also digested after secretion into the cultivation broth (Miyoshi and Shinoda, 1997).

In order to determine the optimum pH, VvsA (1.0 μg) was allowed to act on azocasein in 60 mM GTA (3,3-dimethylglutaric acid, Tris, 2-amino-2-methyl-1,3-propandiol) buffer (pH 6.0-10.0). Although the protease showed the significant activity at broad pH range, the

maximal activity was observed at pH 9.0. Next, VvsA (1.0 μg) was allowed to act on azocasein at 20-70 ^{o}C to test the optimum temperature. Up to 50 ^{o}C, the proteolytic activity was increased depending on the incubation temperature; however, the activity was rapidly decreased at higher than 50 ^{o}C.

When purified VvsA was allowed to act on several peptidyl-MCA substrates, only one substrate Suc-Ala-Ala-Pro-Phe-MCA (for chymotrypsin) was steadily hydrolyzed (data not shown). Chymostatin is a well-known competitive inhibitor for both chymotrypsin and chymotrypsin-like bacterial serine protease (Ishihara et al., 2002). To examine the inhibitory potential of chymostatin, VvsA (0.64 μg) was treated with various amounts of the inhibitor at 30 ^{o}C for 10 min, and the residual proteolytic activity was measured with azocasein. Chymostatin could inactivate VvsA in a dose-dependent manner, and 50 % of the activity was abolished by the treatment with 0.12 μg of the inhibitor (data not shown). Taken together, it may be concluded that, likewise VPP1/protese A from *V. parahaemolyticus* (Ishihara et al., 2002), VvsA is a chymotrypsin-like alkaline serine protease.

Genetic analysis of *vvsA* of strain NCIMB 2137

About 3.3 kb DNA fragment of strain NCIMB 2137 containing the full length of the *vvsA*-*vvsB* genes was amplified by PCR, and its nucleotide sequence was determined (accession number, AB509375). It was revealed that both *vvhA* (2025 bp) and *vvhB* (348 bp) of strain NCIMB 2137 were almost identical to the genes of strain YJ016 (*VVA0302* and *VVA0301*). Namely, the identity between *vvhA* and *VVA0302*, and between *vvhB* and *VVA0301* was 97.2 % (1963/2025) and 98.0 % (341/348), respectively (data not shown). additionally, as well as *VVA0302* and *VVA0301*, *vvsA* and *vvsB* were separated by only 6

bp.

The identity of the deduced amino acid sequences is shown in Table 3 and 4. The identity of the VvsA precursor (674 aa) to VVA0302 is 98.8 % (666/674), whereas in the case of the VvsB precursor (115 aa), the identity is as high as 99.1 % (114/115). The homologues of VvsA and VvsB precursors were found in several *Vibrio* species (Table 3 and 4), suggesting widely distribution of the serine protease family in vibrios. The identity was 71.7 % to 77.2 % among the homologues of VvsA precursor; however, the identity was apparently low (36.5 % to 56.5 %) in the case of the VvsB homologues.

VvsA was also similar to an extracellular serine protease, Apa1, produced by *Pseudoalteromonas* sp. strain AS-11. Namely, the identity of the precursors and the mature enzymes were 57.9 % and 68.0 %, respectively. Based on the crystal structure of Apa1 (Dong et al., 2005), the tertiary structure model of VvsA was constructed by using the SWISS-MODEL Workspace (Arnold et al., 2006). The model revealed that three crucial amino acid residues (Asp^{151}, His^{186} and Ser^{490}) mediating the catalytic reaction were located in the cleft of the active center of the enzyme (data not shown).

In order to clarify that the *vvsA* gene constitutes an operon with the downstream gene *vvsB*, we carried out RT-PCR experiments using several sets of the primers, of which locations are the inside or outside of *vvsA*-*vvsB* (Fig. 1). The results showed that, at least in strain NCIMB 2137, the *vvsA* and *vvsB* genes are a single transcriptional unit (Table 5).

V. vulnificus is known to produce an extracellular hemolytic toxin VVH (*V. vulnificus* hemolysin). The *vvhA* gene (1416 bp) encoding the toxin is transcribed together with another gene *vvhB* (507 bp), which are separated by only 1 bp. The recent study apparently indicated that the product of *vvhB* functioned as a chaperone supporting the maturation process of VVH (Senoh et al., 2008). Therefore, the product of *vvsB* may be also a chaperone essential for maturation of VvsA.

In conclusions, the present study demonstrated that the *vvp/vvpE* negative strains had lost the large genomic region and that orthologues of *vvsA* were present in several *Vibrio* species, which suggest that both *vvp/vvpE* and *vvsA* are movable genetic elements.

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Table 1 PCR amplification of the genes locating the downstream or upstream of *vvp/vvpE*

Gene[a]	Strain				
	CECT5343	E86	ATCC33149	NCIMB 2137	520
VVA1435	+	+	+	+	+
VVA1436	+	+	+	+	+
VVA1437	+	+	–[b]	–	–
VVA1465 (*vvp/vvpE*)	+ (Type A)	+ (Type B)	–	–	–
VVA1524	+	+	–	–	–
VVA1526	+	+	+	+	+
VVA1527	+	+	+	+	+

[a] The *VVA1525* gene was not tested because the suitable primers could not be designed

[b] No amplicon was obtained

Table 2 Purification of VvsA

Purification step	Total protein (mg)	Total activity (PU)	Specific activity (PU/mg)	Relative activity	Yield of activity (%)
Culture supernatant	2,400	34,800	15	1.0	100
Ammonium sulfate fractionation	28	24,670	880	60	71.0
Gel filtration	2.4	8,400	3,500	230	24.1
Ion-exchange column chromatography	0.08	2,130	26,600	1,770	6.1

Table 3 Identity of amino acid sequences of the proteins homologous to the VvsA precursor of *V. vulnificus* strain NCIMB 2137

Strain	Protein	Identity (%)
V. vulnificus YJ016	VVA0302 (674 aa)	98.8
V. alginolyticus 12G01	EAS77411 (676 aa)	77.2
V. parahaemolyticus RIMD 2210633	VPA0227 (677 aa)	76.9
Vibrio sp. Ex25	VEA_000775 (677 aa)	76.9
V. orientalis CIP 102891	EEX93955 (673 aa)	72.7
V. brasiliensis LMG 20546	EGAG3986 (676 aa)	71.7

Table 4 Identity of amino acid sequences of the proteins homologous to the VvsB precursor of *V. vulnificus* strain NCIMB 2137

Strain	Protein	Identity (%)
V. vulnificus YJ016	VVA0301 (115 aa)	99.1
V. alginolyticus 12G01	EAS77410 (120 aa)	51.3
V. parahaemolyticus RIMD 2210633	VPA0228 (115 aa)	56.5
Vibrio sp. Ex25	VEA_000774 (116 aa)	52.2
V. orientalis CIP 102891	EEX93956 (115 aa)	44.3
V. brasiliensis LMG 20546	EGAG3985 (110 aa)	36.5

Table 5 RT-PCR to detect *vvsAB* mRNA

Sense primer	Antisense primer	Reverse transcriptase	
		Active	Inactive[a]
F1	R3	–	–
F2	R3	+	–
F3	R2	+	–
F4	R1	–	–

[a] Reverse transcriptase was inactivated by heat treatment, and the PCR amplification was carried out without the RT reaction

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Figure legends

Fig. 1. Locations and nucleotide sequences of primers used in the present study.

Fig. 2. The genes present in the *vvp/vvpE* positive or negative strains. The broken gray line indicates the absence of the genes.

Fig. 3. (A) The elution profile of the ion-exchange column chromatography. The preparation from gel filtration was loaded on a Fractogel EMD TMAE 650 5/5 column, and the linear gradient elution with 0-0.5 M NaCl was carried out at a flow rate of 0.5 ml/min. The protein peak showed the sufficient proteolytic activity was collected. (B) The protein profiles of SDS-PAGE. The final preparation from ion-exchange column chromatography (3.4 μg) was treated with 2 % SDS at 100 ^{o}C for 3 min and subjected to gel electrophoresis on the PhastSystem using a PhastGel Gradient 10-15. After electrophoresis, the gel was stained with 0.25 % Coomassie brilliant blue R-250. Lane M, molecular weight marker proteins (97, 66, 45, 30, 20.1 and 14.1 kDa); and lane S, the final preparation.

Fig. 1

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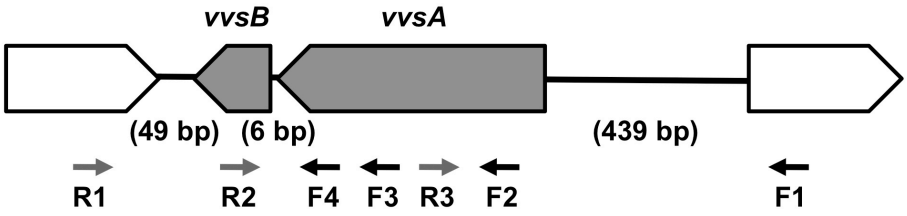

vvs-F1: 5'-AGAATTGCCCGTTCGATACCA-3'
vvs-F2: 5'-TCGACGAAAGTGATCTTCCCA-3'
vvs-F3: 5'-ACAGGCTATGGCTTGATCAATGC-3'
vvs-F4: 5'-CAGAAGAGACACAGGGACGATTA-3'
vvs-R1: 5'-AGTCAAGCGGCCGTAGCAC-3'
vvs-R2: 5'-GTTATTGGCGAAGGAATACCCTGC-3'
vvs-R3: 5'-ATGCTACAAACGGTGCGATTGC-3'

Fig. 2

(Miyoshi et al.)

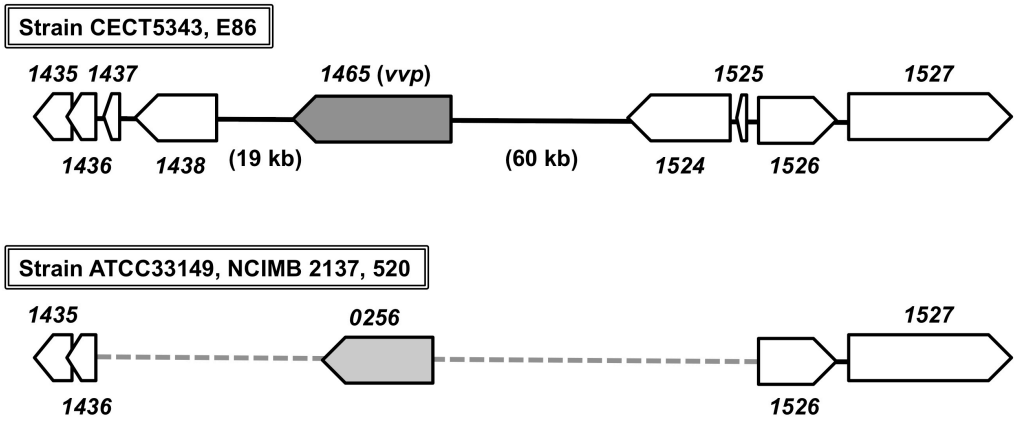

Fig. 3

(Miyoshi et al.)