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A Thesis for the Degree of Master of Science

A Putative U32 Family Collagenase of *Vibrio*
vulnificus Induced upon Exposure to Oyster

굴 접촉에 의해 유도된 패혈증 비브리오균의
잠재적 U32 family Collagenase 규명

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Se Keun Kim

Department of Agricultural Biotechnology
College of Agriculture and Life Sciences
Seoul National University

석사학위논문

A Putative U32 Family Collagenase of *Vibrio
vulnificus* Induced upon Exposure to Oyster

지도교수 최 상 호

이 논문을 석사학위논문으로 제출함

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농생명공학부

김 세 근

김세근의 석사학위논문을 인준함

2016년 2월

위원장 장 판 식 (인)

부위원장 최 상 호 (인)

위원 하 남 출 (인)

Abstract

Vibrio vulnificus is a foodborne pathogen that frequently contaminates seafood, especially oysters. Ingestion of *V. vulnificus*-contaminated seafood can cause foodborne diseases such as gastroenteritis and even life-threatening septicemia (Vugia *et al.*, 2013). In the present study, RNA-sequencing was performed to identify differentially expressed genes of *V. vulnificus* upon exposure to oyster. *V. vulnificus* CMCP6, a clinical isolate from a patient in South Korea, was grown to mid-log phase and incubated in VFMG (*Vibrio fisheri* minimal medium containing glycerol, Kim *et al.*, 2013) with or without oyster tissue for 1hr. Then bacterial cells were harvested and total cellular RNA was isolated. cDNA libraries were generated from the enriched mRNA and sequenced using the Illumina Hi-seq platform. As a result, 2,175 genes (more than 47% of the *V. vulnificus* genome) showing a > 2-fold change in expression with *p*-values of 0.05 or lower were identified, indicating that a variety of cellular processes of *V. vulnificus* were influenced by exposure to oyster. In particular, many genes primarily involved in the transport and metabolism of carbohydrates and amino acids, and iron acquisition were induced, implying that *V. vulnificus* utilizes oyster as a nutrient source. Interestingly, expression of the genes encoding the virulence factors of *V. vulnificus* including RTX toxin (RtxA1) and its transporter (RtxB), thermolabile

hemolysin (Tlh), metalloprotease (VvpE), putative collagenase, and antioxidants were also induced by exposure to oyster. Therefore, the combined results suggest that numerous genes contribute to the *V. vulnificus* pathogenesis in humans as well as in the bacterial's survival in oysters.

The survival and growth of pathogenic bacteria in hosts are dependent on the actions of the bacterial's proteolytic enzymes (Eckhard *et al.*, 2009). Especially, among the proteolytic enzymes, microbial collagenases are strongly related to bacterial survival and pathogenesis (Matsushita & Okabe, 2001). Because collagen is a component of extracellular matrix and is the most abundant protein in animal tissues, destruction of collagen may serve dual roles by providing rich nutrient source, and give an opportunity of infection for pathogenic bacteria (Harrington, 1996). Transcriptomic analysis of *V. vulnificus* genes induced upon exposure to oyster indicated the upregulation of putative collagenase gene. This enzyme belongs to the U32 family of collagenases or peptidases and shares some homology with the *Porphyromonas gingivalis* PrtC one of the representative proteins of the U32 family peptidase (Kato *et al.*, 1992). Considering the potential role of U32 peptidase family in the pathogenicity of *Helicobacter pylori* (gastric colonization, Kavermann *et al.*, 2003), *Streptococcus mutans* (dental root decay, han *et al.*, 2006) and *P. gingivalis* (periodontitis disease, Kato *et al.*, 1992), it is necessary to study how the U32 enzymes contribute to the virulence of pathogenic bacteria. To find

out whether *V. vulnificus* collagenase (VvC) also affects pathogenicity, phenotypic differences between the wild type and the *vvc* mutant were compared *in vitro* and *in vivo*. As a result, VvC may be a player in digesting collagen, especially in the intestinal mucus layer at early infection phase, as well as contributing to nutrient acquirement for bacterial survival. By doing so, *V. vulnificus* enhances its opportunity to infect the host.

Key words: *V. vulnificus*, Transcriptomics, U32 family collagenase, Collagen

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I. INTRODUCTION

Vibrio vulnificus is a Gram-negative, motile, curved rod-shaped bacterium with a single polar flagellum. This bacterium distinguished from other *Vibrio* genus, *V. vulnificus* is a facultative anaerobe having the ability of lactose fermentation (Jones & Oliver, 2009). It is distributed in the estuarine environment and frequently contaminates seafood, especially oysters. *V. vulnificus* causes a severe systemic infection, such as gastroenteritis to life-threatening septicemia. The illness usually contracted through the consumption of raw or undercooked shellfish, as well as wound infections. The mortality from the primary septicemia is very high (> 50%) and death can occur within one to two days after the first signs of illness (Vugia *et al.*, 2013).

Analyzing the transcriptomic changes of *V. vulnificus* induced upon exposure to oyster, provides important insight into the understanding of the strategies for survival and pathogenesis of the *V. vulnificus* in humans as well as the survival in oysters. Recently, advanced RNA-sequencing (RNA-seq) techniques have enabled precise examination of transcriptome at a whole genome level with base-pair resolution in particular physiological conditions. Especially, markedly increased sensitivity of RNA-seq allowed the detection and quantification of RNA expressed at very low level that have been disregarded so far (Pinto *et al.*, 2011).

Collagens are predominant protein constituents of skin, cartilage and blood vessels. Approximately 30% of total proteins in mammalian organisms are various types of collagens. Indeed it is structural components of all connective tissues as fibers embedded in the mucopolysaccharides and extracellular matrix (Ricard-Blum *et al.*, 2011). Collagen is extremely hard to decompose because their stabilized rigid structure, so only a limited proteases can cleave these proteins. Consequently, bacterial collagenolytic proteases are considered as a virulence factors because their potential role: they contribute the spread of the bacteria into host tissues, assist to the acquisition of nutrients for survival and growth, and facilitate host colonization (Harrington *et al.*, 1996). The collagenases include some metalloproteases are suggested to be involved in the digestion of extracellular-matrix components. Unlike mammalian collagenases, bacterial collagenases have broad substrates specificities and can destruction native and denatured collagens (Toyoshima *et al.*, 2001). Especially, the collagenase of *V. vulnificus* was reported to possess potential ability to the bacterial invasion into host tissue through wounds (Smith *et al.*, 1982).

The U32 family peptidase (putative collagenase) contains several collagenolytic proteases, such as *Porphyromonas gingivalis*, *Streptococcus mutans* and *Helicobacter pylori*, and are also suggested to be virulence factors (Kavermann *et al.*, 2003; han *et al.*, 2006; Kato *et al.*, 1992). The peptidase family term “U-“ comes from the MEROPS database which means unknown catalytic mechanism (Rawlings *et al.*, 2013). U32 peptidase contains the consensus sequence: E-x-F-x(2)-G-[SA]-[LIVM]-C-x(4)-G-x-C-x-[LIVM]-S. The most studied U32 protease PrtC from *P. gingivalis* is the first reported.

PrtC is able to degrade type I collagen, synthetic collagen and may require a metal cofactor (Kato *et al.*, 1992). The structure of the U32 protease of the C-terminal domain from *Geobacillus thermoleovorans* has been revealed (Trillo-Muyo *et al.*, 2013). Although a few of U32 proteases are found to collagenolytic activities, the mechanism and roles of these proteases linked in their pathogenicity processes are still unknown.

In this study, I investigated the transcriptomic profiles using a strand-specific RNA-seq method for analysis of differentially expressed genes of *V. vulnificus* in response to exposure to oyster. Based on the RNA-seq data, I selected a target gene may related to pathogenicity of *V. vulnificus* and named the putative U32 family collagenase (VV1_0470) as the *V. vulnificus* collagenase (VvC). This study suggested that VvC will be assist to enhance pathogenicity of *V. vulnificus* by contributed to degradation of collagen, especially embedded in the intestinal mucus layer.

II. MATERIALS AND METHODS

Strain, culture condition and RNA isolation.

The strains and plasmids used in this study are listed in Table 1. *V. vulnificus* CMCP6, a clinical isolate from a patient of South Korea, was grown to mid-log phase (A_{600} of 0.8) and incubated in VFMG (*Vibrio fisheri* minimal medium containing glycerol) medium with the presence or absence of oyster tissue for 1 hr. The culture were filtrated with syringe, sterilized gauze and vacuum filter with Whatman no. 1 filter paper (Whatman International Ltd, Maidstone, England). Subsequently, the culture transferred to 50 ml tubes (SPL, Kyungki, Korea) and centrifuged at 5,000×g and 4°C for 10 minutes. Removing supernatants, pellet was washed with 10 ml of cold-diethylpyrocarbonate (DEPC) treated 0.01M phosphate-buffered saline (PBS, pH 7.4) and transferred to 15 ml tubes (SPL, Kyungki, Korea) and centrifuged at 5,000×g and 4°C for 10 minutes. After centrifugation, pellet was resuspended with using 0.5 ml of cold-DEPC treated PBS and the solutions were quickly mixed with 1 ml of RNAprotect® Bacteria Reagent (QIAGEN, Valencia, CA). Total RNAs were isolated using miRNeasy Mini Kit (QIAGEN) according to the manufacturer's procedure. Contaminated DNAs were digested by TURBO DNase (Ambion, Austin, TX) and then RNAs were cleaned up using RNeasy MinElute Cleanup kit (QIAGEN). The quality of total RNAs was verified using Agilent 2100 Bioanalyzer and Agilent RNA 6000 Nano reagents (Agilent Technologies, Waldbronn, Germany) by Chunlab (Seoul, South Korea).

Strand-specific cDNA library construction and RNA-seq.

The procedures for a strand-specific cDNA library construction and RNA-seq were conducted by Chunlab. mRNA was selectively enriched by depleting ribosomal RNAs by using Ribo-Zero™ rRNA Removal Kits (Epicentre, Madison, WI). Enriched mRNA was subjected to the cDNA library construction using TruSeq Stranded mRNA Sample Prep Kit (Illumina, San Diego, CA) following manufacturer's instruction. The quality of cDNA libraries were evaluated as described above for the quality verification of total RNA, except that Agilent DNA 1000 Reagents (Agilent Technologies) was used. Strand-specific and paired-ended 100 nucleotide reads from each cDNA library were obtained using HiSeq 2500 (Illumina). For biological replication, two libraries were constructed and sequenced from RNAs extracted from two independent filtrated culture supernatants of *V. vulnificus* CMCP6.

Transcriptomic data analyses.

The reads obtained from RNA-seq were mapped to the *V. vulnificus* CMCP6 reference genome (GenBank accession numbers AE016795 and AE016796, www.ncbi.nlm.nih.gov) using CLC Genomics Workbench 5.5.1 (CLC Bio, Aarhus, Denmark). The relative transcript abundance was measured in reads per kilobase of transcript per million mapped sequence reads (RPKM). The fold changes of RPKM values and their significance were assigned and the genes with 2 or greater fold change with P -values ≤ 0.05 were considered to be differentially expressed by exposed to oyster. The graphs describing the results of RNA-seq, principal component analysis (PCA) plot and volcano plot, were created using CLC Genomics Workbench 5.5.1 (CLC Bio).

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
Bacterial strains		
<i>V. vulnificus</i>		
CMCP6	Clinical isolate; virulent	Laboratory collection
SK001	CMCP6 with <i>vvc</i>	This study
SK002	CMCP6 with <i>crp</i>	This study
SK003	CMCP6 with pSK1525	This study
SK004	SK001 with pJH0311	This study
SK005	CMCP6 with pJH0311	This study
<i>E. coli</i>		
DH5 α	supE44 Δ lacU169 (ϕ 80 lacZ Δ M15) hsdR17 recA1 endA1 gyrA 96 thi-1 relA1; plasmid replication	Laboratory collection
S17-1 λ <i>pir</i>	Tc::Mu-Km::Tn7;Tp ^r Sm ^r ; host for π -requiring plasmids; conjugal donor	Simon <i>et al.</i> , 1983
Plasmids		
pGEM-T easy	PCR product cloning vector; Ap ^r	Promega
pDM4	suicide vector; <i>ori</i> R6K; Cm ^r	Milton <i>et al.</i> , 1996
pSK1410	pDM4 with Δ <i>vvc</i> ; Cm ^r	This study
pSK1535	pGEM-T easy with <i>vvc</i> ; Ap ^r	This study
pJH0311	0.3-kb MCS of pUC19 cloned into pCOS5; Ap ^r , Cm ^r	Goo <i>et al.</i> , 2006
pSK1525	pJH0311 with <i>vvc</i> ; Ap ^r , Cm ^r	This study

*Ap^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Km^r, kanamycin resistant; Sm^r, streptomycin resistant.

Table 2. Oligonucleotides used in this study

Oligonucleotide*	Sequence (5' → 3') [†]	Use
For expression of proteins		
vvc_F1_F	CTAGTCAAGGATCACAATTTGGCGTAA	Deletion of <i>vvc</i>
vvc_F1_R_BamH I	<u>ATTGGATCCT</u> CGTCGATACCAATTTTT	
vvc_F2_F_BamH I	<u>CGAGGATCCAAT</u> TATGACTACGGTACTC	Deletion of <i>vvc</i>
vvc_F2_R	TTAGTTACCTGTTGGGTTACGGGTATCC	
vvc_Comp_F	<u>GGTACCAGCGAT</u> TGGAGTTTGTCT	Complementation of <i>vvc</i>
vvc_Comp_R	<u>GAGCTCT</u> TAGTTACCTGTTGGGTTACGGG	
vvc_qRT_F	<u>GAATTC</u> TTATAGGTTTCGCATCCAAAAAC	qRT-PCR
vvc_qRT_R	<u>GGATCC</u> ATGAGTGACAAGATTTTGC	
vvc_PE_F	CACGCTTTGATGCAGACTTTTCTCT	Amplification of <i>vvc</i> upstream region
vvc_PE_R	TTAGGTTCTCGTGATTGAACTCATTGTT	

*The oligonucleotides were designed using the *V. vulnificus* CMCP6 genomic sequence (GenBank accession number AE016795 and AE016796, www.ncbi.nlm.nih.gov).

[†]Regions of oligonucleotides not complementary to the corresponding templates are underlined.

RNA purification and transcript analyses.

Total RNAs from the *V. vulnificus* strains were isolated with an RNeasy[®] Mini Kit (QIAGEN), and cDNA was synthesized using iScript[™] cDNA Synthesis Kit (BIO-RAD). Real-time PCR amplification of the cDNA was performed by using the Chromo 4 real-time PCR detection system (BIO-RAD) with a pair of specific primers listed in Table 2 as described previously (Lim and Choi, 2014). Relative expression levels of the specific transcripts were calculated by using the 16S rRNA expression level as the internal reference for normalization.

To examine the *vvc* mRNA expression in a model of host environment, HT-29 MTX cells were infected with the mid-log phase (A_{600} of 0.8) of *V. vulnificus* CMCP6 at multiplicities of infection (MOI) 1 for 1 hr. Subsequently, the culture harvested to a 15 ml tubes (SPL, Kyungki, Korea) and centrifuged at 250×g for 3 minutes for removing eukaryotic cells. The supernatants were transferred to a new 15ml tubes with double volume of RNAprotect[®] Bacteria Reagent (QIAGEN, Valencia, CA) and total RNAs from the *V. vulnificus* were isolated as described above. The levels of *vvc* mRNA expression were compared between in the basal medium eagle (BME) with or without HT-29 MTX cells.

Bioinformatical Analysis.

The sequenced genomes of *Pseudomonas aeruginosa* PAO1 (Accession, AE004091) and *Helicobacter pylori* 26695 (Accession, AE000511) and *Porphyromonas gingivalis* W83 (Accession, AE015924) were used for the genomic analysis of the U32 peptidase. Alignment analysis was conducted using the CLUSTALW Service at the European Bioinformatics Institute (Thompson *et al.*, 1994).

Generation of the *V. vulnificus* CMCP6 *vvc* mutant.

To inactivate *vvc* *in vitro*, 64% (904-bp of 1401-bp) of the *vvc* open reading frame (ORF) was deleted *in vitro* using the PCR-mediated linker-scanning mutation method as described previously (Lim and Choi, 2014). Briefly, pairs of primers *vvc*_F1_F and *vvc*_F1_R_BamH I for amplification of the 5' amplicon or *vvc*_F2_F_BamH I and *vvc*_F2_R for amplification of the 3' amplicon were designed and used (Table 2). The 904-bp deleted *vvc* (Δvvc) was amplified by PCR using the mixture of both amplicons as the template and *vvc*_F1_F and *vvc*_F2_R as primers. The Δvvc was constructed by ligated with Sph I-Spe I-digested pDM4 to form pSK1410 (Table 1). To generate the *vvc* mutant by using double homologous recombination, *E. coli* S17-1 λ *pir*, *tra* containing pSK1410 was used as a conjugal donor in conjugation with *V. vulnificus* CMCP6 as indicated in Table 1. All of the conjugation and isolation of the transconjugants were conducted using the methods previously described.

Complementation of *vvc* mutant.

Complementation was carried out by introduction of the *vvc* ORF under P_{lac} promoter control of pJH0311. The *vvc* ORF was PCR-amplified from *V. vulnificus* CMCP6 genomic DNA using a pair of primers *vvc*_Comp_F and *vvc*_Comp_R. The amplified *vvc* DNA fragment was cloned into pGEM-T easy vector and digested with Kpn I and Sac I. Subsequently, digested fragment cloned into predigested pJH0311 at Kpn I and Sac I sites. The resultant plasmid, pSK1525 had been transformed to *E. coli* S 17-1 λ *pir*, *tra* and selected by grown on chloramphenicol (20 μ g/ml) treated medium. Selected *E. coli* was used as conjugal donor to Δvvc . Also, *E. coli* S17-1 λ *pir*, *tra* harboring pJH0311 was transformed into wild type *V. vulnificus* CMCP6 for control. Conjugation was conducted and the transconjugants were used for subsequent experiments.

Assay of collagenolytic activity using Azocoll.

Determination of collagenolytic activity was measured using the Azocoll which is an azo-dye impregnated insoluble synthetic collagen. To prepare the substrate, 0.003 g of Azocoll (Sigma, St. Louis, MO) was washed twice using 0.01 M PBS (pH 7.4) as described in Jiang *et al.*, (2007). Culture supernatants used for Azocoll assay were prepared as followed: A supernatant from centrifugation (8000×g at 4°C for 20 min) of 50 ml culture was filtrated with 0.2 µm syringe filter (Puradisc 25 mm, Whatman) and concentrated with 10-kDa-Ultrafree centrifugal ultrafiltration device (Milipore Corporation, Bedford, Mass.) by centrifugation at 8000×g at 4°C up to 2 ml. Briefly, 100 µl of each concentrated supernatants were incubated separately with 400 µl PBS solution which containing of 0.003 g Azocoll. After incubation in a shaking incubator at 37 °C, followed by centrifugation (13,200×g for 3 min), degradation of Azocoll resulted in the purple coloration of the supernatant which was quantified by measuring the absorbance of the azo-dye at 520 nm using Tecan Infinite M 200 reader (Tecan Group Ltd., Mannedorf, Switzerland). For the metal ion chelation challenge, each experiment group were incubated separately with 400 µl PBS solution which containing of 0.003 g Azocoll and 1 mM 1,10-phenanthroline or 10 mM EGTA respectively.

Cytotoxicity and mouse survival test.

Cytotoxicity was evaluated by measuring the cytoplasmic lactate dehydrogenase (LDH) activity that is released from the human cell-lines by damage of plasma membranes. The HT-29 MTX cells were grown in minimal essential medium containing 1% (vol/vol) fetal bovine serum (GIBCO-BRL, Gaithersberg, MD) in 96-well culture dishes (Nunc, Roskilde, Denmark) as described previously (Jeong *et al.*, 2009). Each well with HT-29 MTX cells were infected with the *V. vulnificus* CMCP6 and *vvc* mutant at various MOI for each incubation times. The LDH activity released into the supernatant was determined using a Cytotoxicity Detection Kit (Roche, Mannheim, Germany). Mouse

mortalities of the wild type and *vvc* mutant were compared as described elsewhere (Hwang *et al.*, 2013). Groups of (n=10) 7-week-old ICR female mice (specific pathogen-free, Seoul National University) were starved without food and water for 24 h until infection. Then the mice, without iron-dextran pretreatment, were intragastrically administered with 50 μ l of 8.5% (wt/vol) sodium bicarbonate solution, followed immediately with 100 μ l of the inoculum, representing approximately 5×10^4 cells of either the wild type or *vvc* mutant. Mouse survivals were recorded for 24 hrs. All manipulations of mice were approved by the Animal Care and Use Committee at Seoul National University.

Ex-vivo growth test.

The 3×10^6 HT-29 MTX cells were seeded in cell culture dish (SPL, Kyungki, Korea) and incubated at 37°C in the 5% CO₂ incubator. The McFP medium was changed every 3 days for 9 days, during which time the cells had reached over confluency at 2.5×10^7 cells. Then infected with *V. vulnificus* strains at MOI of 1 using BME medium and incubated at 37°C in the 5% CO₂ incubator. After infection, each time point, infecting *V. vulnificus* were collected as followed: The planktonic cells harvested from supernatant and the attached cells collected by treated 0.1% triton X-100 PBS solution with shaking incubation (700 rpm) for 10 min. Aliquots were withdrawn every hour and serially plated onto Luria-Bertani (LB) medium supplemented with 2.0% (w/v) NaCl (LBS) agar plates. Then incubated in 30°C incubator for 12 hours and colonies were counted.

Primer extension analysis.

Total RNAs from the *V. vulnificus* strains were isolated as described above. A 25-base oligonucleotide primer vvc_PE_R (Table 1) complementary to the coding region of *vvc* was end-labeled with [γ -³²P]ATP and added to the RNA. The primer was then extended with SuperScript II RNase H⁻ reverse transcriptase (Invitrogen). The cDNA products were purified and resolved on a sequencing gel alongside sequencing ladders. The sequencing ladders were generated using the same primer vvc_PE_R from the PCR products, containing 195-bp *vvc* upstream region extending from -195 to + 208 generated using a pair of primers vvc_PE_F and vvc_PE_R (Table 1). The primer extension gels were visualized using a phosphorimage analyzer (BAS1500, Fuji Photo Film Co. Ltd.).

III. RESULTS

Summary statistic for the transcriptomic data analyses.

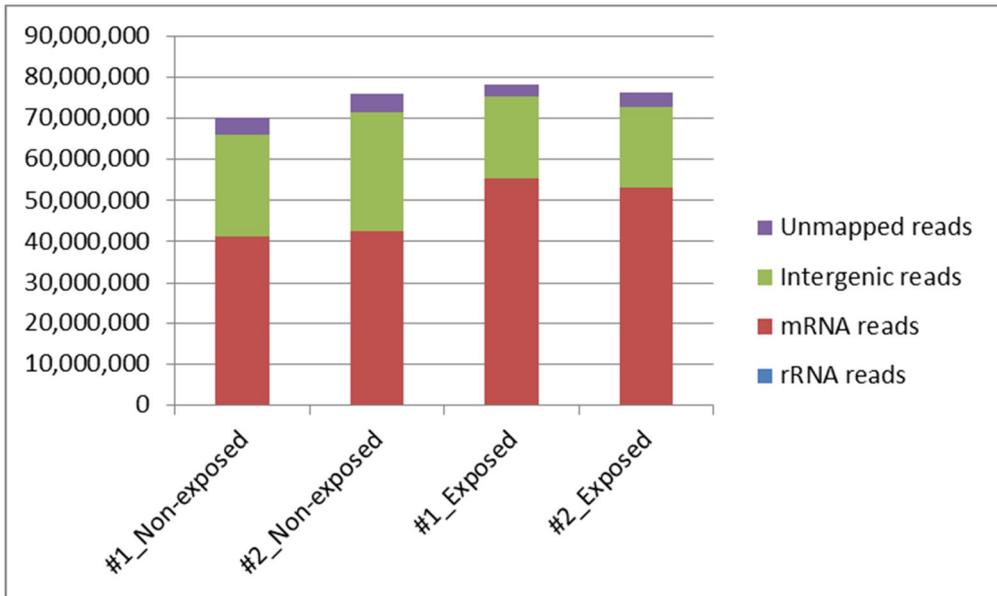
A mean of 81.1 million reads were obtained per each sample, of which ~99.9% were mapped to a single location of *V. vulnificus* CMCP6 reference genome (Table 2). The similarity of the transcriptomes was analyzed by principal component analysis (PCA) (Fig. 1). The PCA plot of the samples exposed and unexposed to oyster were clearly separated, proving the significant differences in their transcriptomic level.

Table 3. Analysis of RNA-seq data mapped to the *V. vulnificus* CMCP6 genome^a

Number of reads	Non-exposed ^b		Exposed ^b	
	#1	#2	#1	#2
rRNA reads	85,584	72,144	4,454	20,888
mRNA reads	41,239,628	42,589,080	55,368,372	53,211,640
Intergenic reads	24,693,102	28,891,120	19,806,352	19,524,058
Unmapped reads	4,171,966	4,388,258	3,119,628	3,598,510
Total reads	75,516,370	82,169,998	84,538,580	82,257,864

^a GenBank accession numbers (AE016795 and AE016796, www.ncbi.nlm.nih.gov).

^b #1 and 2 represent biological duplicates.



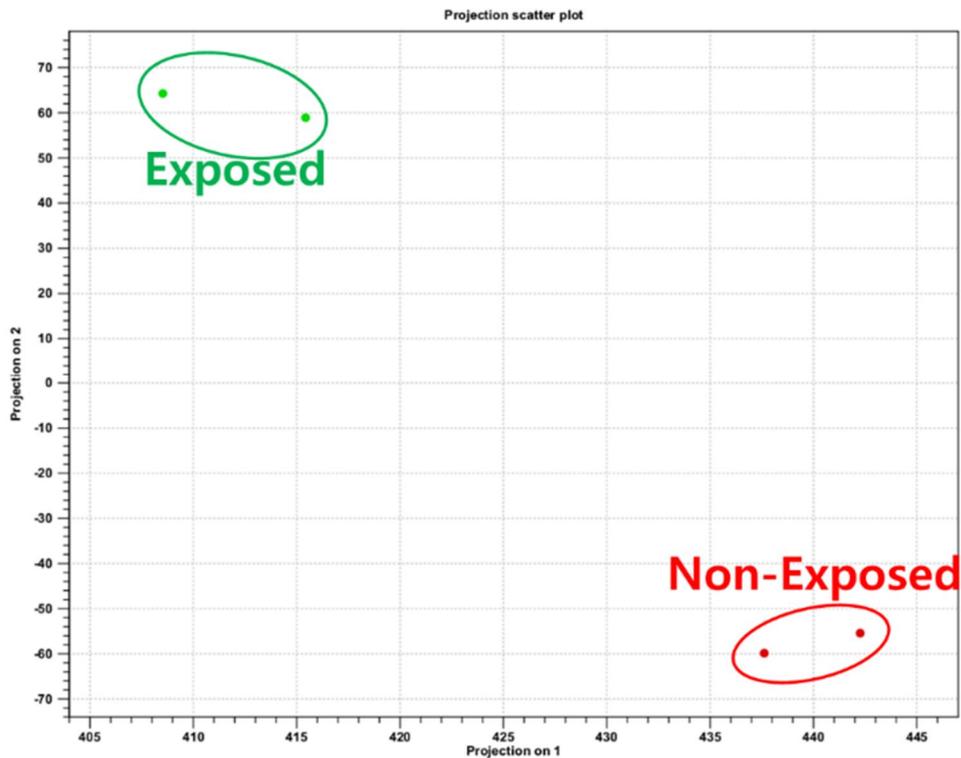


Figure 1. Principal component analysis (PCA) of the RNA-seq samples. PCA analysis was performed for the samples using RPKM values from the RNA-seq analyses. Samples from the cells exposed and non-exposed to oyster were plotted in two dimensional plots across the first two principal components. Samples for each conditions were denoted by a different color. Red dots, RNAs from the control cells unexposed to oyster; green dots, RNAs from the cells exposed to oyster.

Identification of differentially expressed genes under exposed to oyster.

Differentially expressed genes following the exposure to oyster were identified. Average RPKM values from the biological duplicate samples were used to represent the expression level of each genes. The volcano plot showed that a number of genes are differentially expressed with significance (P -value ≤ 0.05 , 2 fold threshold) (Fig. 2). A total of 2,175 genes were identified to be differentially expressed; 1,148 upregulated and 1,027 downregulated. The differentially expressed genes (P -value ≤ 0.05 , 5 fold threshold) were clustered into functionally related groups using the Cluster of Orthologous Groups (COG) database (<http://www.ncbi.nlm.nih.gov/COG/>) for the *V. vulnificus* CMCP6 genome, which showed that the genes with various functions were differentially expressed (Fig. 3).

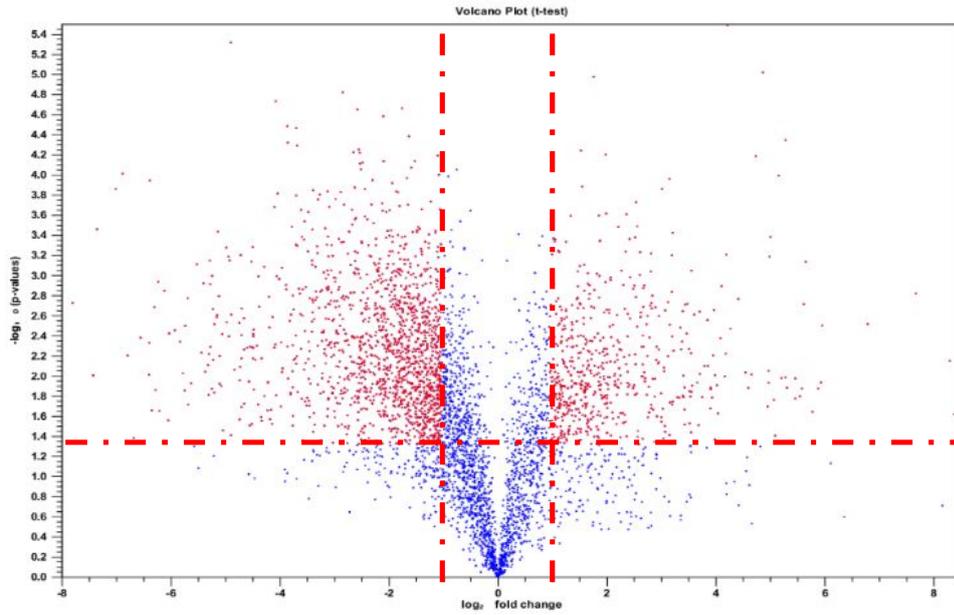


Figure 2. Transcriptome comparisons of the RNA-seq samples. Volcano-plots of genes differentially expressed between the cells exposed and non-exposed to oyster were generated. Numbers on the X- and Y-axis represent the fold change (\log_2) and P -value (\log_{10}). Red dots represent the differentially expressed genes.

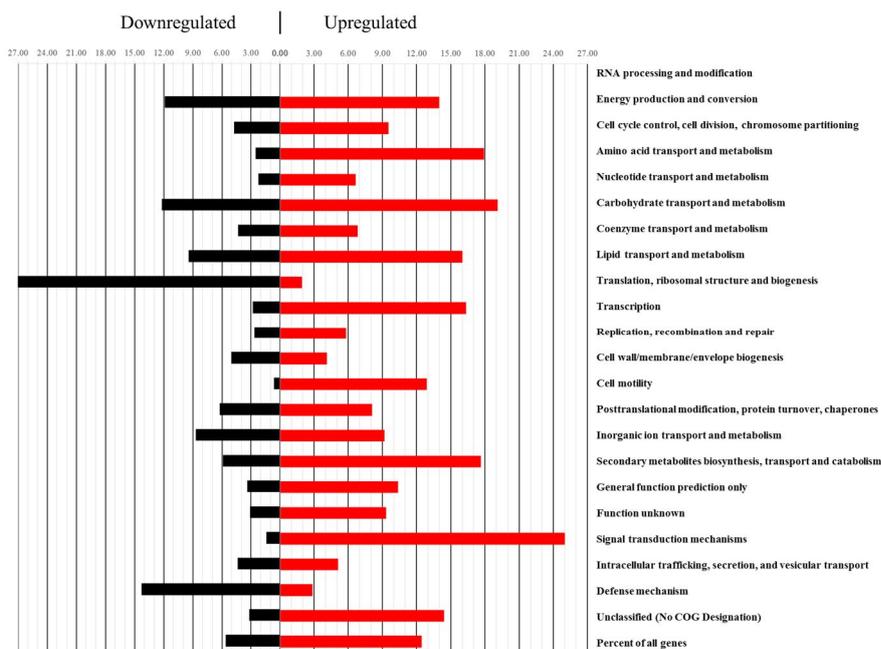


Figure 3. Functional categorization of genes differentially expressed under exposed to oyster. Genes with expression ratios of ≥ 5 on the basis of the RNA sequencing results were considered to be differentially expressed under exposed to oyster. Functional categories (COG) are based on the database for the *V. vulnificus* CMCP6 genome, which was retrieved from GenBank (accession numbers AE016795 and AE016796). Genes upregulated (red) and downregulated (black) under exposed to oyster were represented.

Upregulated genes by exposure to oyster.

Upregulated virulence genes by exposed to oyster were summarized in Table 4.

Table 4. Virulence related genes upregulated by exposed to oyster

Locus tag	Gene product	Fold Change (log ₂)	<i>P</i> -value ^a (< 0.05)
VV1_1634	Quorum-sensing regulator of virulence HapR (SmcR)	2.04975	0
VV1_1907	Zinc metalloprotease	1.2063	0
VV2_0479	autotransporter adhesin	1.46869	0
VV2_0482	RTX toxin transporter	2.20606	0
VV2_0483	RTX toxin transporter	3.14873	0
VV2_0484	RTX toxin transporter	1.02742	0.00037
VV1_0470	Putative peptidase (collagenase)	2.20028	0
VV1_0439	Iron-sulfur cluster assembly transcription factor IscR	3.55429	0

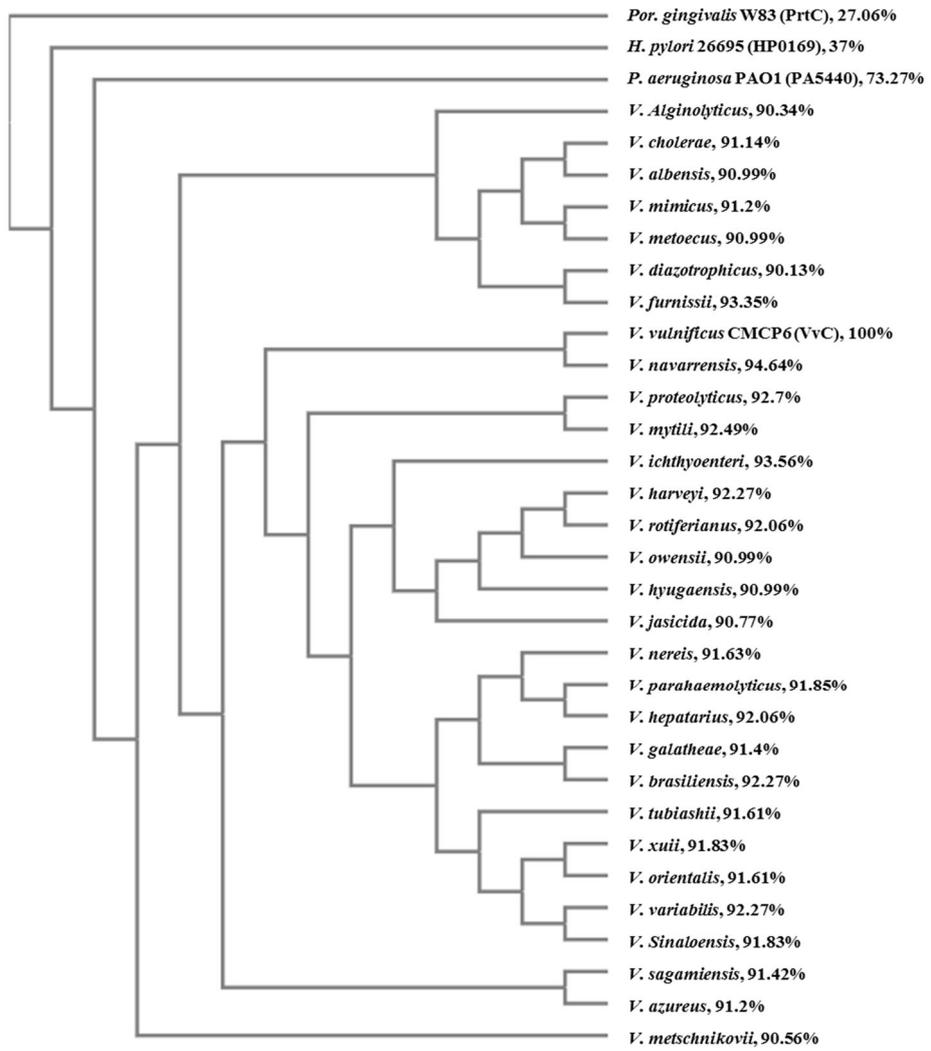
^aThe *P*-values less than six decimal places were denoted as zero.

Bioinformatical sequence analysis of U32 peptidase.

A 1,402 bp *vvc* gene was identified in the genome of *V. vulnificus* CMCP6 (GenBank accession numbers AE016795 and AE016796) as one of the upregulated gene under exposure to oyster (Table 4). The *vvc* gene encodes a protein product of 466 amino acid residues with a calculated molecular mass of 52,585 Da and an isoelectric point (pI) of 5.4 (http://www.alphalyse.com/gpmaw_lite.html). The protein belongs to U32 peptidase family which was so far poorly characterized except that *P. gingivalis* PrtC contributed to the periodontal tissue destruction (Kato *et al.*, 1992).

BLAST, multiple amino acid sequence alignment, and phylogenetic analyses revealed that protein homologues of VvC are conserved in *Vibrio* species with the amino acid sequence identity above 90% (Fig. 4A and B). In bacteria other than *Vibrio* species, several strains in *Pseudomonas*, *Helicobacter*, and *Porphyromonas* species also have proteins homologous to VvC with the amino acid sequence identity. In addition, they have the conserved signature of U32 peptidase family (E-x-F-x(2)-G-[SA]-[LIVM]-C-x(4)-G-x-C-x-[LIVM]-S, Rawlings *et al.*, 1993, Fig. 4B).

(A)



(B)

Por. gingivalis W83 (PrnC) LSTQLNISNAEALRFYSRFA-DVVVLARELNMDQVRTIHETIVRDNICGPKGHFVRIEMF
H. pylori 26695 (HP0169) LSTQANVNLNLLDAQVFDLGVKRVICARELSLNDIAIEIKKAL-----PNLELEIF
P. aeruginosa PAO1 (PA5440) LSVQANAVNWAAYQVFRWQOGLTRVILSRELSLEEIEEIRQQV-----PDMELEVF
V. metschnikovii LSVQANAVNWATVKFWSTQGVVERVILSRELSLEEIEEIRQQC-----PETELEIF
V. alginolyticus LSVQANAVNWATVKFWASQGVVERVILSRELSLEEIEEIRQQC-----PDTELEIF
V. albensis LSVQANAVNWATVKFWASQGVVERVILSRELSLEEIEEIREKC-----PNTEIEVF
V. cholerae LSVQANAVNWATVKFWASQGVVERVILSRELSLE---EIREKC-----PNTEIEVF
V. mimicus LSVQANAVNWATVKFWASQGVVERVILSRELSLEEIEEIREKC-----PNTEIEVF
V. metoecus LSVQANAVNWATVKFWASQGVVERVILSRELSLEEIEEIREKC-----PNTEIEVF
V. diazotrophicus LSVQANAVNWATVKFWSTQGVVERVILSRELSLEEIEEIRQQC-----PEMEIEIF
V. navarrensis LSVQANAVNWATVKFWAAYGVVERVILSRELSLEEIEEIRQQC-----PETELEVF
V. vulnificus CMCp6 (VvC) LSVQANAVNWATVKFWAAYGVVERVILSRELSLEEIEEIRQQC-----PETELEVF
V. sagamiensis LSVQANAVNWATVKFWAANGIERVIVSRELSLEEIEEIREHC-----PETELEVF
V. azureus LSVQANAVNWATVKFWAANGVERVIVSRELSLEEIEEIREHC-----PETELEVF
V. furnissii LSVQANAVNWATVKFWSANGVERVIVSRELSLEEIEEIREHC-----PETELEVF
V. proteolyticus LSVQANAVNWATVKFWAANGVERVIVSRELSLEEIEEIREHC-----PETELEVF
V. mytili LSVQANAVNWATVKFWAANGVERVIVSRELSLEEIEEIREHC-----PETELEVF
V. ichtyoventer LSVQANAVNWATVKFWAAYGVVERVILSRELSLEEIEEIREHC-----PETELEVF
V. harveyi LSVQANAVNWATVKFWAANGVERVIVSRELSLEEIEEIREHC-----PETELEVF
V. rotiferianus LSVQANAVNWATVKFWAANGVERVIVSRELSLEEIEEIREHC-----PETELEVF
V. hyugaensis LSVQANAVNWATVKFWAANGVERVIVSRELSLEEIEEIREHC-----PETELEVF
V. jastida LSVQANAVNWATVKFWAANGVERVIVSRELSLEEIEEIREHC-----PETELEVF
V. owensii LSVQANAVNWATVKFWAANGVERVIVSRELSLEEIEEIREHC-----PETELEVF
V. nereis LSVQANAVNWATVKFWAANGVERVIVSRELSLEEIEEIREHC-----PETELEVF
V. parahaemolyticus LSVQANAVNWATVKFWAANGVERVIVSRELSLEEIEEIREHC-----PETELEVF
V. hepatarius LSVQANAVNWATVKFWAANGVERVIVSRELSLEEIEEIREHC-----PETELEVF
V. galathea LSVQANAVNWATVKFWAANGVERVIVSRELSLEEIEEIREHC-----PETELEVF
V. tubiashii LSVQANAVNWATVKFWAANGVERVIVSRELSLEEIEEIREHC-----PETELEVF
V. brasiliensis LSVQANAVNWATVKFWAANGVERVIVSRELSLEEIEEIREHC-----PETELEVF
V. xuii LSVQANAVNWATVKFWAANGVERVIVSRELSLEEIEEIREHC-----PETELEVF
V. orientalis LSVQANAVNWATVKFWAANGVERVIVSRELSLEEIEEIREHC-----PETELEVF
V. sinaloensis LSVQANAVNWATVKFWAANGVERVIVSRELSLEEIEEIREHC-----PETELEVF
V. variabilis LSVQANAVNWATVKFWAANGVERVIVSRELSLEEIEEIREHC-----PETELEVF
* * * * * . . . : : : * * * : : * * : : * * *

Por. gingivalis W83 (PrnC) AHGALCMAYSGRCYLSLHEHNSANRGAQAICRRGYIV---KDKDSGLELDIE-----
H. pylori 26695 (HP0169) VHGSMLCFAFSGRCLISALQKGRVFNRSRGCANDCRFDYEEYV-KNFDNVMRRLIVE-----
P. aeruginosa PAO1 (PA5440) VHGALCMAYSGRCLLSGYINRRDPNQGCTNACRWEYKAVEGREDELGNVVAR-----
V. metschnikovii VHGALCMAYSGRCLLSGYINRRDPNQGCTNACRWEYKVEKQEDALGQIVEQFDPNAAQ
V. alginolyticus VHGALCMAYSGRCLLSGYINKRDPNQGCTNACRWEYKVEAKADEAGQIVEQFDPNAAQ
V. albensis VHGALCMAYSGRCLLSGYINKRDPNQGCTNACRWEYKVEAAKEDAGQIVEQFDPNAAQ
V. cholerae VHGALCMAYSGRCLLSGYINKRDPNQGCTNACRWEYKVEAAKEDAGQIVEQFDPNAAQ
V. mimicus VHGALCMAYSGRCLLSGYINKRDPNQGCTNACRWEYKVEAAKEDAGQIVEQFDPNAAQ
V. metoecus VHGALCMAYSGRCLLSGYINKRDPNQGCTNACRWEYKVEAAKEDAGQIVEQFDPNAAQ
V. diazotrophicus VHGALCMAYSGRCLLSGYINKRDPNQGCTNACRWEYKVEKGEDEAGQIVEQFDPNAAQ
V. navarrensis VHGALCMAYSGRCLLSGYINKRDPNQGCTNACRWEYKVEQKEDAGQIVEQFDPNAAQ
V. vulnificus CMCp6 (VvC) VHGALCMAYSGRCLLSGYINKRDPNQGCTNACRWEYKVEQKEDAGQIVEQFDPNAAQ
V. sagamiensis VHGALCMAYSGRCLLSGYINRRDPNQGCTNACRWEYKVEKGEDEAGQIVEQFDPKQAA
V. azureus VHGALCMAYSGRCLLSGYINRRDPNQGCTNACRWEYKVEKGEDEAGQIVEQFDPKQAA
V. furnissii VHGALCMAYSGRCLLSGYINKRDPNQGCTNACRWEYKVEKGEDEAGQIVEQFDPNAAQ
V. proteolyticus VHGALCMAYSGRCLLSGYINKRDPNQGCTNACRWEYKVEKGEDEAGQIVEQFDPNAAQ
V. mytili VHGALCMAYSGRCLLSGYINKRDPNQGCTNACRWEYKVEKGEDEAGQIVEQFDPNAAQ
V. ichtyoventer VHGALCMAYSGRCLLSGYINKRDPNQGCTNACRWEYKVEEGKENDAGQIVEQFDPNAAQ
V. harveyi VHGALCMAYSGRCLLSGYINKRDPNQGCTNACRWEYKVEEGKENDAGQIVEQFDPNAAQ
V. rotiferianus VHGALCMAYSGRCLLSGYINKRDPNQGCTNACRWEYKVEEGKENDAGQIVEQFDPNAAQ
V. hyugaensis VHGALCMAYSGRCLLSGYINKRDPNQGCTNACRWEYKVEEGKENDAGQIVEQFDPNAAQ
V. jastida VHGALCMAYSGRCLLSGYINKRDPNQGCTNACRWEYKVEEGKENDAGQIVEQFDPNAAQ
V. owensii VHGALCMAYSGRCLLSGYINKRDPNQGCTNACRWEYKVEEGKENDAGQIVEQFDPNAAQ
V. nereis VHGALCMAYSGRCLLSGYINKRDPNQGCTNACRWEYKVEEGKENDAGQIVEQFDPNAAQ
V. parahaemolyticus VHGALCMAYSGRCLLSGYINKRDPNQGCTNACRWEYKVEEGKENDAGQIVEQFDPNAAQ
V. hepatarius VHGALCMAYSGRCLLSGYINKRDPNQGCTNACRWEYKVEEGKENDAGQIVEQFDPNAAQ
V. galathea VHGALCMAYSGRCLLSGYINKRDPNQGCTNACRWEYKVEEGKENDAGQIVEQFDPNAAQ
V. tubiashii VHGALCMAYSGRCLLSGYINKRDPNQGCTNACRWEYKVEEGKENDAGQIVEQFDPNAAQ
V. brasiliensis VHGALCMAYSGRCLLSGYINKRDPNQGCTNACRWEYKVEEGKENDAGQIVEQFDPNAAQ
V. xuii VHGALCMAYSGRCLLSGYINKRDPNQGCTNACRWEYKVEEGKENDAGQIVEQFDPNAAQ
V. orientalis VHGALCMAYSGRCLLSGYINKRDPNQGCTNACRWEYKVEEGKENDAGQIVEQFDPNAAQ
V. sinaloensis VHGALCMAYSGRCLLSGYINKRDPNQGCTNACRWEYKVEEGKENDAGQIVEQFDPNAAQ
V. variabilis VHGALCMAYSGRCLLSGYINKRDPNQGCTNACRWEYKVEEGKENDAGQIVEQFDPNAAQ
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Figure 4. Phylogenetic tree and CLUSTALW alignment of the amino acid sequences of the peptidases U32 family and VvC. (A) Phylogenetic tree showing proteins highly related to VvC. 33 proteins closely related to VvC were selected from the BLASTP analysis (<http://blast.ncbi.nlm.nih.gov/>), and phylogenetic analysis was conducted using the European Molecular Biology Laboratory (EMBL) database (http://www.ebi.ac.uk/Tools/phylogeny/clustalw2_phylogeny/). The amino acid sequence identity of each protein with VvC from *V. vulnificus* CMCP6 are indicated in %. (B) Sequences from NCBI database. PA5440, HP0169, and PrtC are the U32 peptidase proteins of the putative collagenase precursor from *Pseudomonas aeruginosa* PAO1, *Helicobacter pylori* 26695, and *Porphyromonas gingivalis* W83 respectively. ‘’, Column of the alignment contains identical amino acid residues in all sequences. ‘:’, Column of alignment contains conserved substitutions of amino acids. ‘.’, Column of alignment contains semi-conserved substitutions. ‘-’, Gaps in the amino acid sequence. Blank, column of the alignment contains dissimilar amino acids. *Gray box*, the signature sequence for the peptidase U32 family (E-x-F-x(2)-G-[SA]-[LIVM]-C-x(4)-G-x-C-x-[LIVM]-S).

Increased expression of *vvc* under exposed to HT-29 MTX which human intestinal cells.

The expression of *vvc* was significantly induced by exposure to confluent grown HT-29 MTX cells, mucin-secreted, as upregulated by exposed to oyster (Table 4).

When examined the *vvc* mRNA expression by qRT-PCR, it was increased about 2.3-fold upon exposed to HT-29 MTX cells (Fig. 5). The results indicated that the expression of *vvc* is induced not only by exposed to oyster but also by exposed to human intestinal cell-line (Lesuffleur *et al.*, 1990).

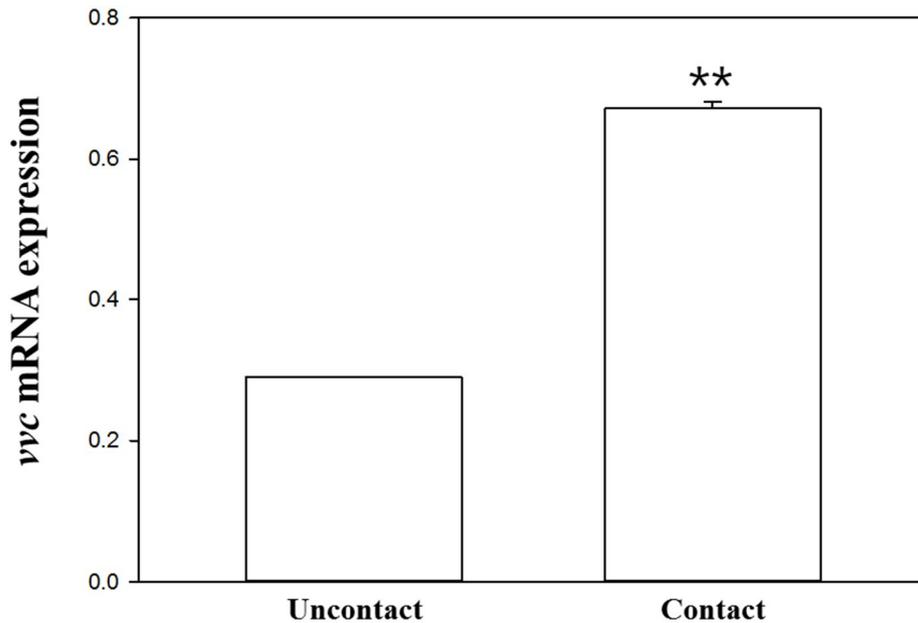


Figure 5. *vvc* mRNA expression under exposure to HT-29 MTX cells. *V. vulnificus* wild type was grown to A_{600} of 0.5 in LBS, and diluted to minimal medium (BME), and exposed to HT-29 MTX cells for 1 hr. Relative levels of the *vvc* transcript in the total RNA was determined by qRT-PCR. Error bars represent the SEM from two independent experiments. Uncontact, exposed to none; **, $P < 0.005$ relative to the uncontact cells.

Construction and confirmation of *vvc* mutant.

To obtain insight into the role of VvC, *V. vulnificus* *vvc* mutant was constructed by allelic exchange (Fig. 5A). A double crossover, in which wild type *vvc* gene was replaced with Δvvc allele, was confirmed by PCR using a pair of primers, *vvc_F1_F* and *vvc_F2_R* (Table 2). The PCR analysis of the genomic DNA from *V. vulnificus* CMCP6 with the primers produced a 1.4-kb fragment, but the genomic DNA from Δvvc mutant resulted in an amplified DNA fragment approximately 0.5-kb in length (Fig. 5B).

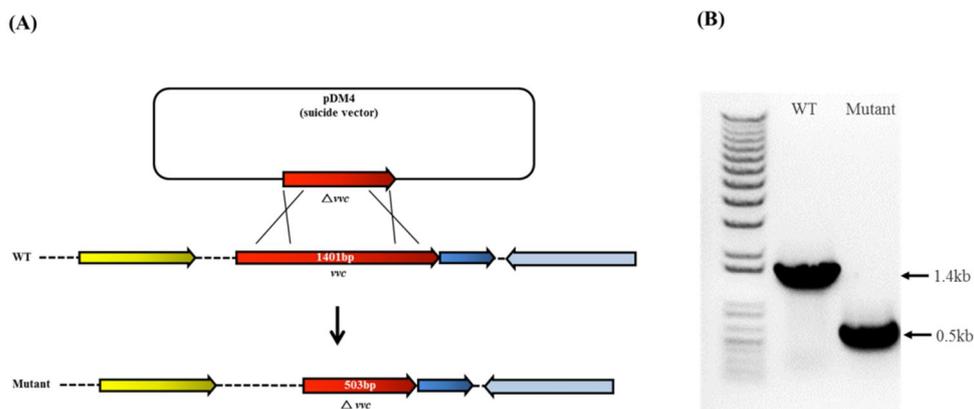


Figure 6. Schematic diagram of the procedure used to create the deleted *vvc* gene fragment. (A) Homologous recombination between chromosomal *vvc* gene from *V. vulnificus* CMCP6. Dashed lines, chromosomal DNA; solid line, plasmid DNA; red arrows, the target *vvc* gene; green boxes, the BamHI I restriction site; X, crossover. (B) PCR analysis of *V. vulnificus* CMCP6 and the *vvc* mutant generated by allelic exchange.

Enzyme assays.

Collagenolytic activity was determined by an Azocoll, synthetic insoluble collagen, as described. Reaction was performed at 37°C for 8 hr. In this assay, collagenolytic activity is measured by quantitating the production of soluble azo-dye, which are readily separated from digested Azocoll substrate. The purified PrtC, represent of the U32 peptidase, is slightly stimulated in the presence of Ca²⁺ and inhibited by EDTA (Kato *et al.*, 1992). To determine the inhibitory effects of metal ion chelation, two common chelators were used as described earlier. The wild type supernatants have approximately 145% more total collagenolytic activity than the *vvc* mutant supernatants. Complementation of the *vvc* gene in *vvc* mutant with a functional *vvc* gene (pSK1525) restored the collagenolytic activity to levels comparable to that of the wild type (Fig. 7). Collagenolytic activity of each samples were inhibited approximately 90% by the presence of 1 mM 1,10-phenanthroline and 10 mM EGTA, respectively (Fig. 7). This result suggested that the VvC activity may be needed the presence of metal ion, especially Ca²⁺.

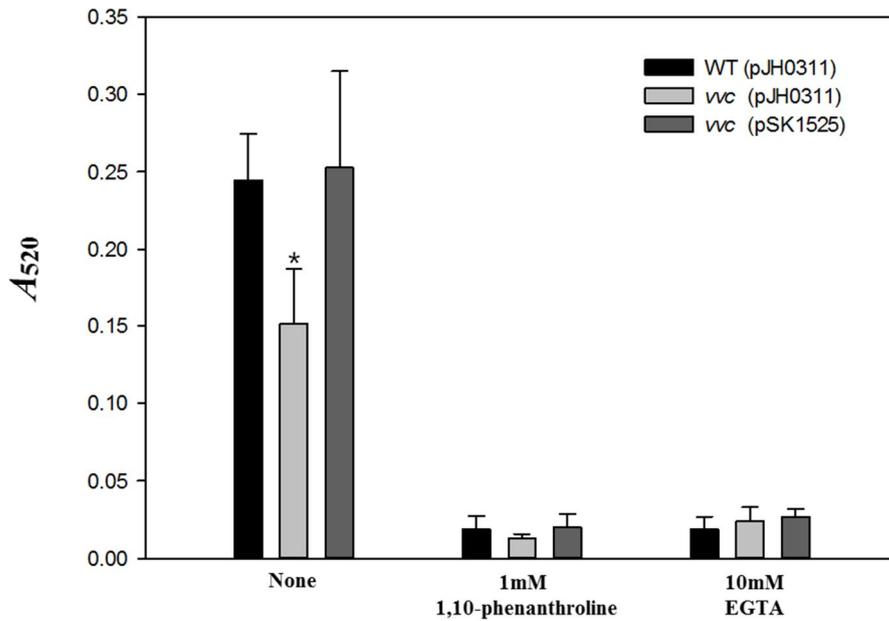


Figure 7. Effects of *vvc* mutation and metal ion on the collagenolytic activity.

An aliquot of the culture supernatants of *V. vulnificus* was mixed with an equal volume of Azocoll (0.003 g) and then incubated at 37°C for 8 hr. Two common protease inhibitors were used to characterize the *vvc* (1,10-phenanthroline, EGTA). The degradation of Azocoll was determined, and results are presented by measured at 520 nm absorbance of the solution supernatant. Error bars represent the SEM from biological duplicate experiments. *, $P < 0.05$ relative to the absorbance of wild type.

VvC is important for virulence and growth of *V. vulnificus*.

To measure the effect of VvC, compared the virulence and growth of the wild type and *vvc* mutant toward cell culture system. For examine the virulence in human epithelial cell damage, the post-confluent cells of HT-29 MTX were infected with the wild type and *vvc* mutant. Subsequently, the activities of LDH released from the HT-29 MTX cells were compared at different MOI as shown in Fig. 7A. The *vvc* mutant exhibited significantly lower LDH-releasing activities than the wild type, indicating that VvC is important for *V. vulnificus* to injure host cells. Complementation of the *vvc* gene using pSK1525 restored the LDH-releasing activities to levels comparable to those of the wild type (Fig. 7A).

Since the expression of *vvc* was induced specifically by exposure to oyster (Table 4) and the bacterial collagenolytic protease well known for important to fix nitrogen into their biological process (Zhang *et al.*, 2015), the growth rate compared between the wild type and the *vvc* mutant under exposed to HT-29 MTX cells. The result indicated that the growth rate of the *vvc* mutant is lower than that of the wild type (Fig. 7B). It suggests that the VvC is required for the growth of *V. vulnificus* by contributes to digest of extracellular components or cellular matrices. However, it is not a sole factor for the growth of *V. vulnificus*, as seen from the reduced growth rate of the *vvc* mutant (Fig. 7B)

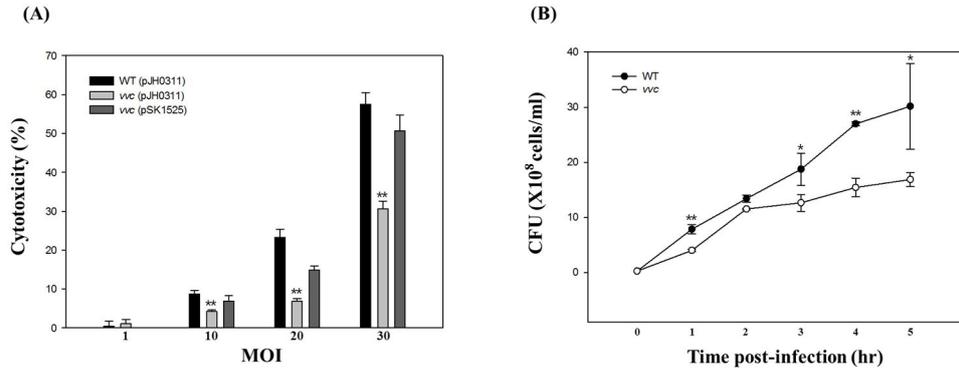


Figure 8. Effects of *vvc* mutation on the virulence and growth of *V. vulnificus* towards HT-29 MTX cells. (A) HT-29 MTX cells were infected with the wild type and the *vvc* mutant at various MOIs for 1 hr 30 min, respectively. The cytotoxicity was determined by an LDH release assay. (B) In order to examine the effects of the *V. vulnificus* growth on the HT-29 MTX, the wild type and the *vvc* mutant were infected in HT-29 MTX cells at the MOI of 1 for various incubation times. Then bacterial cells were withdrawn each hour and serially plated onto LBS agar plates. After incubated in 30 °C for 12 hours, colonies were counted. WT, wild type type; *vvc*, *vvc* mutant. Error bars represent the SEM. *, $P < 0.05$; **, $P < 0.005$ relative to groups of infected with the wild type at each MOI or times.

Mouse survival test.

To further understand the role of VvC in *V. vulnificus* pathogenesis, mice were infected intragastrically with the wild type and *vvc* mutant and then numbers of dead mice were counted for 24 hrs. As shown in Fig. 9, the deaths of mice infected with the *vvc* mutant were significantly delayed ($P = 0.0228$, Log rank test, Lim *et al.*, 2014), compared to those of mice infected with the wild type. These result suggested that VvC is essential for pathogenesis of *V. vulnificus*.

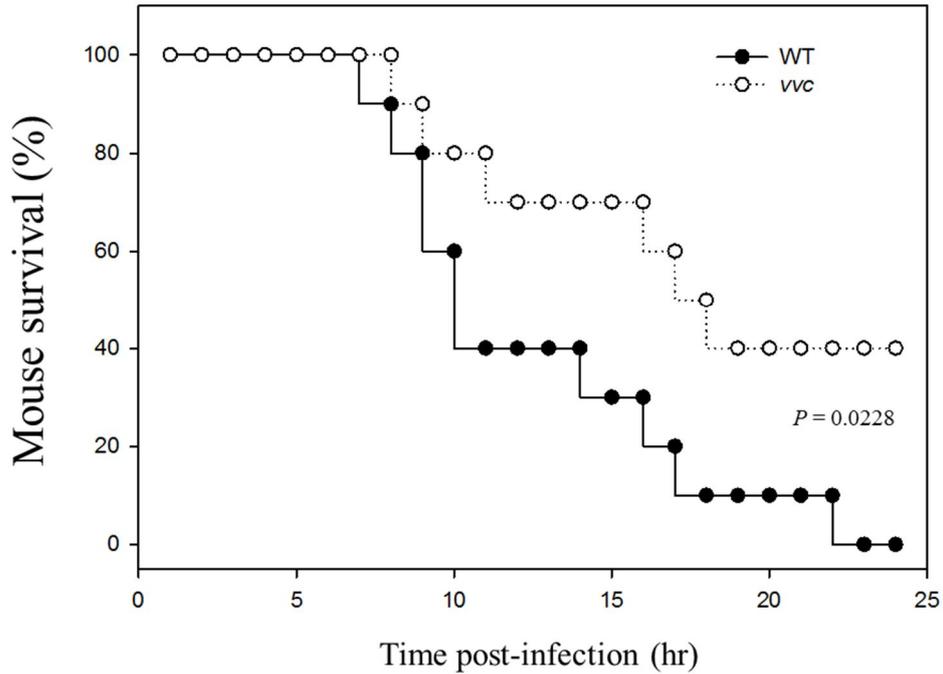


Figure 9. Mouse mortality of the *V. vulnificus* strains. Seven-week-old specific-pathogen-free female ICR mice ($n = 10$), without iron-dextran pretreatment, were intragastrically administered with 50 μ l of 8.5% (wt/vol) sodium bicarbonate solution, followed immediately with 100 μ l of the inoculum, representing approximately 5×10^4 CFU of either the wild type or *vvc* mutant. Mouse survival was monitored for 24 hrs. The data were analyzed by using a log rank test. WT, wild type type; *vvc*, *vvc* mutant.

Effects of the growth and *crp* mutation on the *vvc* mRNA expression.

In order to examine the effect of the growth on the expression of *vvc*, the levels of *vvc* mRNA in the wild type were determined in lag to stationary growth phase with qRT-PCR (Fig. 10A). The level of *vvc* mRNA in lag-phase cells (A_{600} 0.1) was more than 30-fold higher when compared to that in the stationary-phase cells (A_{600} 1.4) as shown in Fig. 10B.

The cyclic AMP receptor protein (CRP) has been reported that it modulates quorum sensing, motility and multiple genes that affect intestinal colonization in *Vibrio cholera* (Liang *et al.*, 2007). To tested whether *V. vulnificus* CRP controls the expression of *vvc*, the levels of *vvc* mRNA in the stationary phase of wild type and *crp* mutant cells determined by qRT-PCR. The levels of *vvc* mRNA in *crp* mutant cells was more than 13-fold higher when compared to that in the wild type cells (Fig. 10C). This result suggests that CRP may act as a repressor when increase the cyclic AMP concentration, and play a role in the growth phase-dependent variation of *vvc* expression.

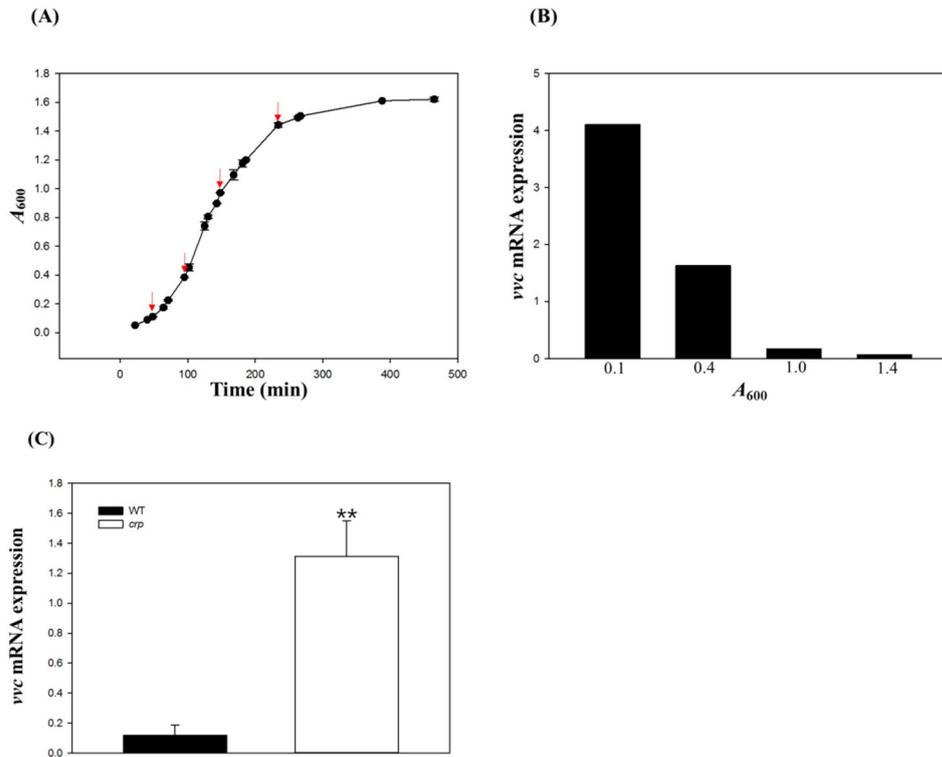


Figure 10. Effects of the growth and *crp* mutation on the *vvc* mRNA expression.

(A to B) Cultures of the wild type grown with LBS were harvested at various growth phases and used to isolate total cellular RNA. The *vvc* mRNA levels were determined by qRT-PCR analyses and normalized to the 16 S rRNA expressions.

(C) Cultures of the wild type and the *crp* mutant grown with LBS were harvested at A_{600} of 2.0 and the *vvc* mRNA expressions were determined by qRT-PCR. Error bars represent the SEM. **, $P < 0.005$ relative to the wild type. WT, wild type; *crp*, *crp* mutant SK002.

Analysis of P_{vvc} in *V. vulnificus* and sequence analysis of the *vvc* regulatory region.

The transcription start site of *vvc* was determined by primer extension of the RNA derived from the wild type. Total RNA was prepared from the stationary-phase cells (A_{600} 2.0). Lanes *C*, *T*, *A*, and *G* represent the nucleotide sequencing ladders of pSK1535 (Table 1). An *asterisk* indicated the transcription start site (TSS) of P_{vvc}.

TSS of P_{vvc} is indicated by *bent arrow*, and the position of putative -10 and -35 regions are *underlined*. The CRP-binding sequences are predicted and represented as a *gray box*. The ATG translation initiation codon and the putative ribosome-binding site (AGAA) are indicated in *boldface*. The consensus sequences for the binding of CRP from *V. vulnificus* are shown below the DNA sequence (Choi *et al.*, 2002).

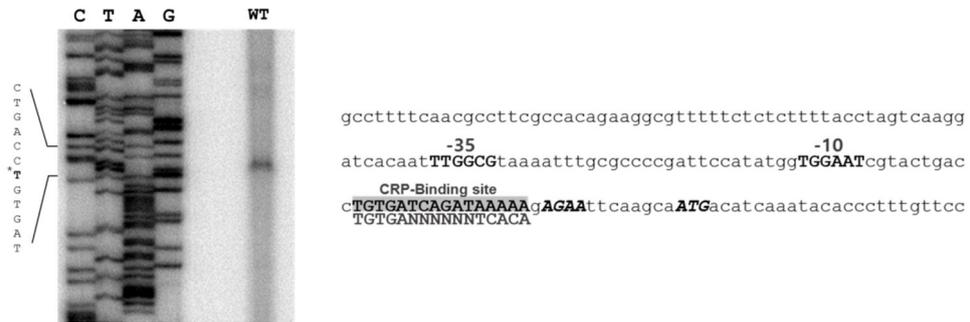


Figure 11. Determination of the *vvc* promoter region. Transcription start site of P_{vvc} indicated by *asterisks*, was determined by primer extension of the RNA derived from the wild type grown to A_{600} of 0.5. Lanes *C*, *T*, *A*, and *G* represent the nucleotide sequencing ladders of P_{vvc} . The positions of the putative -10 and -35 regions are *underlined*. The ATG translation initiation codon and putative ribosome binding site (AGAA) are indicated by *italic type*. The CRP-binding sequences predicted in this study are represented as a *gray box*. N, any base.

IV. DISCUSSION

Collagens are predominant protein constituents of marine organisms (Mizuta *et al.*, 2005), including oyster tissues, and *V. vulnificus* has been known to cause foodborne illnesses by residing in the gills and digestive tract of bivalves (Vugia *et al.*, 2013; Jones *et al.*, 2009). In addition, collagens are essential molecules of the extracellular matrices and the most abundant proteins in tissues and organs in animals (Ricard-Blum *et al.*, 2011). Due to the complex and rigid structure of collagens, and it being insoluble in water, only a few proteases have the ability to degrade collagens. In summary, the degradation of collagen layers is related to the pathological process (Zhang *et al.*, 2015).

In the marine environment, digestion of insoluble components, such as collagens, might serve a critical role in nitrogen recycling (Park *et al.*, 2015). The family of the genus *Vibrio* which includes *V. vulnificus* is well known for its collagenolytic activity (Smith *et al.*, 1982). However, the predicted collagenolytic activities that may contribute to the degradation of collagens by *V. vulnificus* remain largely unknown.

The *V. vulnificus* collagenase (VvC), the subject of this studies, belongs to the U32 peptidase family and contain the conserved E-x-F-x(2)-G-[SA]-[LIVM]-C-x(4)-G-x-C-x-[LIVM]-S signature (Fig. 4B, Rawlings *et al.*, 2013). Based on the BLASTP

analysis (<http://blast.ncbi.nlm.nih.gov/>), VvC is conserved in the *Vibrio* species, and the similarity of amino acid sequence is above 90% (Fig. 4B). Alignment of the VvC with the PrtC, one of the well-characterized members of the U32 peptidase from *P. gingivalis* (Kato *et al.*, 1992), showed only 27% homology of amino acid sequences (Fig. 4A). This result suggested that the VvC may have somewhat different characteristics compared to those of the PrtC.

The present study showed that VvC will contribute to the growth and pathogenesis of *V. vulnificus*. As shown in Fig. 5, the VvC is induced upon exposure to HT-29 MTX cells which is a human intestinal cell line, and disrupting the functional *vvc* gene by double homologous recombination (Fig. 6) resulted in reduced virulence toward the host cells (Fig. 8A) and in mice (Fig. 9). In addition, the *vvc* mutants, when compared with the wild type, showed reduced collagenolytic activity (Fig. 7), and decreased growth rate when exposed to the human cells with minimal medium (Fig. 8B). Therefore, it is likely that the deficiency of VvC may be a reason, not the sole factor, for the reduced growth and virulence by decreasing the collagenolytic activity. Furthermore, VvC seemed to be inhibited by metal ion chelation (Fig. 7), and it suggests a requirement of Ca^{2+} for enzyme activity as already studied in PrtC (Kato *et al.*, 1992). But in this study, the VvC collagenolytic activity was only measured from the culture supernatant, so further work using purified VvC is needed to confirm this idea. Actually, his-tagged VvC protein was successfully

expressed in a soluble form from *Escherichia coli* BL21 (DE3) by using pHisparallel, the protein overexpression plasmid, and purified by affinity chromatography according to the manufacturer's procedure. However, his-tagged VvC protein didn't show collagenolytic activities even after the his-tag was removed (data not shown). I hypothesized that the VvC is activated from its inactive proenzyme via the functioning of some other protease(s) or secretion systems that are present in *V. vulnificus*, but not in *E. coli* (Fig. 12). In *V. cholerae*, the type II secretion system (T2SS)-dependent secretome analyses revealed that there are various extracellular proteins which rely on across the outer membrane via T2SS, such as lipase, sialidase, cytolysin, serine proteases and metalloproteases. From the several lines of evidence presented here, it is suggested that the VvC also will be secreted via the T2SS, like any other metalloprotease(s).

It is noteworthy that the *vvc* expression is downregulated by CRP at stationary-phase, a state in which the cell density is high enough (Fig. 10C). I hypothesized that during the stage of early infection, when the cell density is low, the VvC contributes to the degradation of collagen, leading to the proliferation and colonization of *V. vulnificus*. However, at the high cell density (HCD) stage, the HCD regulator CRP is activated by high concentration of cAMP, leading to the decreased level of VvC. After digesting the collagen layer, release of additional protease is unnecessary in terms of energy conservation. For example, when INT-

407 cells, which do not secrete mucin and are non-polarized were infected with either the wild type and *vvc* mutant at various MOIs for several incubation times, the cytotoxicity displays insignificant differences and a saturated tendency (data not shown). The mechanism for the negative regulation of CRP has already been studied in *V. vulnificus rpoS* (Lee *et al.*, 2008). Primer extension revealed that the transcription start site is located at the first nucleotide of the predicted CRP-binding consensus sequence (Choi *et al.*, 2002). Therefore, *V. vulnificus* VvC might be repressed by directly binding to the transcription start region of CRP (Fig. 11).

In conclusion, VvC is a novel enzyme that seems to share some characteristics with the *P. gingivalis* PrtC. The presented data suggests that the VvC contributes to the pathogenesis of *V. vulnificus* by collagenolytic activity. Without a doubt, further work is needed to fully understand the mechanism of VvC in *Vibrio* pathogenesis.

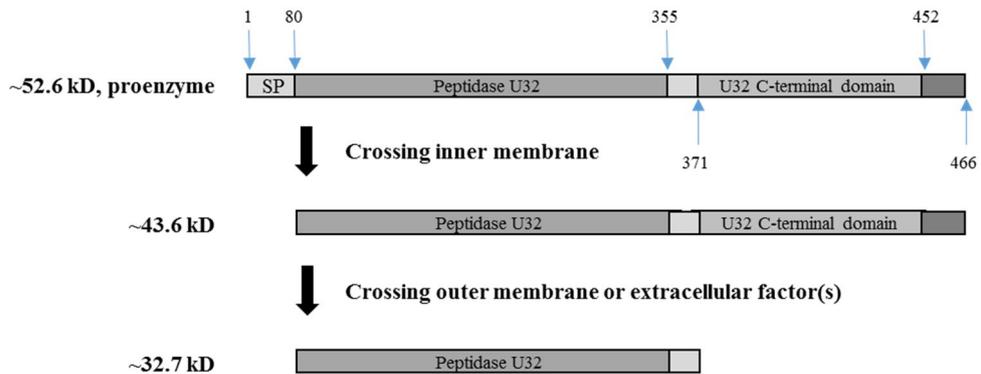


Figure 12. A presumed scenario of VvC transformation. Signal peptide (SP) is cleaved when translocation of the VvC to the periplasm. Subsequently, the secretion of VvC into the extracellular space, the C-terminal tale domain is cleaved from the protein with the help of another secreted factor(s) or secretion systems in *V. vulnificus*.

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VI. 국문초록

패혈증 비브리오균은 대표적인 식중독 원인균으로 해산물을 날것으로 섭취하거나 상처 부위가 바닷물에 노출되었을 경우 감염될 수 있다. 특히 생굴 섭취로 인한 식중독 사고가 빈번하게 일어나고 있으며 감염 시 심각한 위장염이나 패혈증을 유발하여 치사율이 50%가 넘는다. 본 연구는 패혈증 비브리오균이 식품 내 생존하여 인간에게 식중독을 일으키는 전략을 이해하기 위해 RNA-sequencing 기법을 통해 생굴과 접촉 시 특이적으로 발현되는 유전자의 발현 양상을 분석하였다. 발현이 변화하는 유전자를 기능적으로 분류하여 보면 영양물질의 생합성과 관련한 유전자 발현은 줄어든 반면 영양물질 대사와 관련한 유전자의 발현이 증가하였다. 또한, 패혈증 비브리오균의 독성을 나타내는 데 중요한 독성인자들과 그 조절자의 발현이 증가한 것이 관찰되었다. 따라서 패혈증 비브리오균은 생굴을 영양분으로 인식하여 대사에 활용 할 수 있으며 증가한 독성인자를 바탕으로 이를 섭취한 사람에서 식중독을 유발할 수 있음을 추측해 볼 수 있다.

생굴과 접촉하여 발현이 증가한 유전자 중 잠재적 collagenase로 명명된 *Vibrio vulnificus* collagenase (vvc)의 특성을 규명하였다. 미생물 collagenases는 동물성 세포의 세포외기질 혹은 세포 간 접합을 구성하는 collagen을 기질로 분해하는 능력을 가진 효소로 다양한 생물 종에서 중요한 독성인자로 작용할 수 있다고 알려져 있다. 하지만 현재까지 이

러한 효소들의 과학적 정의와 분류가 부족한 상태이다. 특히 VvC 단백질 서열 분석을 통해 밝혀낸 U32 peptidases는 *Vibrio* 종 내에 약 90% 이상의 상동성을 가지며 잘 보존되어 있으나 현재까지 연구된 사례가 없는 것이 확인되었으며, 일부 계통분류학적으로 거리가 먼 균에서 독성과 관련이 있음이 밝혀져있다.

해당 연구는 *vvc* 유전자 돌연변이와 야생형간 표현형의 차이를 분석하여 생장의 차이는 나타나지 않으나 돌연변이에서 합성 collagen (Azocoll)의 분해능력이 크게 감소하고 인간 세포에 대한 세포 독성, 인간 세포를 단일 영양소로 제공하였을 때 패혈증 비브리오균의 성장속도가 감소하는 것을 관찰하였다. 또한 실험을 통해 *vvc* 유전자는 패혈증 비브리오균의 성장 곡선상 성장 유도기와 초기 대수증식기에서 그 발현이 높다가 이후에는 발현이 감소하고 이는 CRP에 의한 repression일 가능성이 있음을 확인하였다. 추가적으로 primer extension 기법을 통해 *vvc* 유전자의 전사 시작점과 조절자의 부착 위치를 예측할 수 있었다. 결과를 종합하면 VvC는 해양 환경에서 접하게 되는 굴과 같은 유기물이나 혹은 인간에게 감염되었을 때 체내에 존재하는 collagen 성분을 분해하는 데 작용하여 패혈증 비브리오균의 성장과 숙주에 대한 독성을 나타내는 데 기여할 것으로 판단된다.

주요어 : 패혈증 비브리오균, 전사체 분석, U32 family collagenase, collagen