A Thesis for the Degree of Master of Science

Identification and Regulatory Characteristics of *gbpA* Encoding a *N*-acetylglucosamine Binding Protein in *Vibrio vulnificus*

N-acetylglucosamine 결합 단백질인 패혈증 비브리오균 *gbpA*의 동정과 조절 특성

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석사학위논문

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ABSTRACT

Attachment of pathogenic bacteria to host cell is crucial for the initial stage of pathogenesis. Previous study showed that VVMO6_03494 encoding putative adhesin is up-regulated by transcriptional regulator IscR which is responsible for the survival and virulence of *V. vulnificus*. VVMO6_03494 is a homologue of *Vibrio cholerae gbpA* encoding *N*-acetylglucosamine binding protein. In this study, the role of *V. vulnificus* GbpA and its regulatory characteristics were investigated. Amino acid sequence analysis revealed that *V. vulnificus* GbpA is predicted to be a secreted chitin binding protein. The *gbpA* mutant showed significantly reduced ability to bind for chitin compared to its parental wild type. The purified GbpA protein was capable of binding to the chitin and *N*-acetylglucosamine, the monomer of chitin, *in vitro*. Also, GbpA was not secreted in the mutant impaired in type II secretion system. These indicated that GbpA is an *N*-acetylglucosamine-sensitive chitin binding protein and secreted via type II secretion system. Furthermore, the disruption of *gbpA* resulted in a substantial decrease in adherence to mucin containing *N*-acetylglucosamine as a component of carbohydrate moiety *in vitro* and the lower adherence was restored to the level of wild type by exogenous addition of purified GbpA. In addition, the *gbpA* mutant was significantly diminished in adherence to HT-29 MTX cells, mucin-secreting human intestinal epithelial cells and exhibited reduced the virulence in the infection of mice. Combined results suggested that GbpA contributes to the pathogenesis of *V. vulnificus* by facilitating the adherence to the mucus layer of human intestinal epithelium. It was observed that the cellular level of *gbpA* transcript*s* is decreased in the growth-dependent manner, implying that the expression level of *gbpA* could

be controlled by quorum sensing. The qRT-PCR analysis and western blot analyses revealed that the expression of *gbpA* is up-regulated by IscR and down-regulated by SmcR, quorum-sensing master regulator, at both the transcription and translation level. The EMSA showed that both IscR and SmcR bind to the promoter region of *gbpA*. In summary, *V. vulnificus* GbpA is a tightly regulated virulence factor responsible for attachment to chitin and mucus layer.

Keywords : *Vibrio vulnificus*, chitin, mucin, GbpA

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I. INTRODUCTION

The marine bacterium *Vibrio vulnificus* is an opportunistic Gram-negative pathogen that contaminates oyster and seafoods (Storm and Parajpye, 2000). Consumption of raw contaminated seafood or exposure of wound to *V. vulnificus* can cause primary septicemia and wound infection, respectively (Gulig *et al*., 2005). The mortality of septicemia caused by *V. vulnificus* is approximately 50% and death can occur within one to two days after the first signs of illness (Linkous and Oliver, 1999; Melissa and Oliver, 2009).

V.vulnificus can attach the chitin in the aquatic environment and enter the host with seafood. In host, *V.vulnficus* moves via gastrointestinal tract and reaches the human intestinal epithelial cells that are covered with mucus layer. The mucus layer protects the intestinal epithelium from various pathogens and toxins. The mucus layer consists of water, salts, lipids and proteins (Allen, 1981). However, the main component of mucus layer is mucins. Mucins are complex and high-molecular-weight glycoproteins with hundreds of oligosaccharide chains. (Rose, 1992). The oligosaccharides of mucin are covalently linked to the theronine and/or serine-rich tandem repeated domain of protein core. The carbohydrates consisting of mucin are primarily *N*-acetylgalactosamine, *N*-acetylglucosamine, fucose, galactose, and sialic acid (*N*-acetylneuraminic acid) and traces of mannose.(Perez-Vilar and Hill. 1999; Bansil and Turner, 2006). Especially, *N*-acetylglucosmine is a monomer of chitin and component of mucin. Previous study showed that glycoprotein moiety of mucin is served as receptor for adhesion in case of *V. mimicus* (Alam *et al*., 1997). Therefore, the microbial adhesin to recognize *N*-

acetylglucosamine can contribute to adhesion to chitin and mucin.

The pathogenic bacteria adhere to intestinal mucus layer in order to survive and cause the disease (Yamamoto and Yokota 1988). The adhesion can help the pathogens colonize on the mucosal surface by binding the mucus layer. The pathogens that attached on the mucus layer can gets a opportunity that invade the mucosal surface and intestinal epithelial cells (Klemm and Schembri, 2000). In addition, abolishment of the ability to bind to mucus layer makes the pathogens less-virulent (Hahn, 1997) Therefore, The microbial adhesin to adhere to mucus layer is a essential virulence factor in the pathogenesis of *V. vulnificus*.

In the present study, *V. vulnificus* GbpA was identified and characterized as a novel adhesin. GbpA is *N*-acetylglucosamine-sensitive chitin binding protein and has the ability to adhere to mucus layer. The mouse survival test revealed that GbpA is important virulence factor of *V. vulnificus*. Also, IscR and SmcR positively and negatively regulate the *gbpA* expression by directly binding its promoter region, respectively..

II. MATERIALS AND METHODS

Strains, plasmids, and culture media The strains and plasmids used in this study are listed in Table 1. *Escherichia coli* were grown in Luria-Bertani(LB) broth or LB broth containing 1.5% (w/v) agar. Unless otherwise noted, *Vibrio vulnificus* were grown in Luria-Bertani (LB) medium supplemented with 2% (w/v) NaCl (LBS). All the media components were purchased from Difco (Detroit, MI), and the chemicals from Sigma (St. Louis, MO)

Amino acid sequence analysis of *V. vulnificus* GbpA The dendrogram showing the amino acid sequence relatedness of *V. vulnificus* GbpA and *V. cholera* GbpA was derived using the CLUSTALW alignment program (http://www.ch.embnet.org/sofware/ClustalW.html) and is based on the amino acid sequence in the Gen-Bank databases (NCBI): *V.vulnificus gbpA* (GenBank accession number VVMO6_03494); *V.cholerae gbpA* (GenBank accession number VCA0811)

Generation of the *V. vulnificus gbpA* mutant *V.vulnificus gbpA* on chromosome was amplified and inactivated *in vitro* by deletion of about four-fifth (1140-bp of 1458-bp) of *gbpA* ORF using the PCR-mediated linker-scanning mutation method as described elsewhere (Lee *et al*.,2007). Pairs of primers GbpA_F1-F and GbpA_F1-R (for amplification of the 5' amplicon) or GbpA_F2-F and GbpA_F2-R (for amplification of the 3' amplicon) were designed and used as listed in Table 1. The 1.7-kb DNA fragment containing Δ*gbpA* was amplified by PCR using the

mixture of both amplicons as the template and GbpA_F1-F and GbpA_F2-R as primers. The resulting Δ*gbpA* were ligated with SphI-SpeI-digested pDM4 (Milton *et al*., 1996) forming pSO1103. E. coli S17-1 *λ pir, tra* (containing pSO1103) was used as a conjugal donor to MO6-24/O. The conjugation and isolation of the transconjugants were conducted using the method previously described (Lim *et al*., 2011)

Chitin beads binding assay with *V. vulnificus* strains The wild type, *gbpA* mutant and complemented strain were grown in LBS. Magnetic chitin beads (New England BioLabs, Ipswich, Massachusetts) were washed five times in modified phosphate-buffered saline containing 3% (w/v) NaCl (NPBS) (Renato Tarsi and Carla Pruzzo, 1999). The 2 mL of cell cultures were centrifuged and removed supernatant to wash media components. The cell pellet were resuspended in 500 μL NPBS and the 100 μL of culture resuspension was mixed with the 400 μL of the washed magnetic chitin beads and the 1.5 mL NBPS added to the mixtures. The mixtures were incubated for 2 hr at room temperature. The supernatant was removed by applying the magnetic rack to the mixtures (Invitrogen, Carlsbad, CA) in order to wash away the unbound bacteria. This wash step was repeated five times. The 1.8 mL of NPBS and 0.2 g of 0.5-mm glass beads were added to the washed magnetic chitin beads and vortexed for 60 s to detach bound bacteria from magnetic chitin beads. The bacterial resuspension was serially diluted and plated. The number of bound bacteria counted from the beads was quantified. The adherence index was calculated by dividing the output by the input numbers (Brooke *et al*., 2009).

Western blot analysis The bacterial cells grown in LBS were harvested, washed and broken by Complete Lysis-B, EDTA-free (Roche, Penzburg, Germany) and separated by centrifugation to generate cell pellet and cell lysates. Supernatant was concentrated by amicon ultra centrifugal filter (Merck millipore, Darmstadt, Germany). For western blot analysis, protein from cell lysate (10 μg) and cell supernatant (14 μL) were resolved by SDS-PAGE and immunoblotting was done according to procedure previously described by Lim *et al*. (Lim *et al*., 2011). The protein concentration was determined by the Bradford method (Bradford, 1976), with bovine serum albumin (BSA) as the standard.

Overexpression and purification of *V. vulnificus* GbpA. The open reading frame of the *gbpA* was amplified by PCR using the primers, GbpA_Exp-F and GbpA_Exp-R (Table 2). The PCR products were ligated into a His_6 tagging expression vector, pET-22b (+) (Novagen, Madison, WI) to result in pSO1201 (Table 1). The His-tagged GbpA proteins were expressed in *E.coli* BL21 (DE3) and purified by affinity chromatography according to the manufacturer's procedure (Qiagen, Valencia, CA). The purified proteins were dialyzed against storage buffer [50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 0.1 mM EDTA, 0.1 mM DTT, 50% (w/w) glycerol] and kept -80◦C until use (Lim *et al*., unpublished).

Chitin beads binding assay of *V. vulnificus* GbpA protein The magnetic chitin beads (New England BioLabs, Ipswich, Massachusetts) were washed five times in

NPBS (Renato Tarsi and Carla Pruzzo, 1999). BSA proteins and purified GbpA proteins were added to the 50 μL of the washed magnetic chitin beads. The mixtures were incubated for 10 min at room temperature. After incubation, the mixtures were applied to the magnet to wash away the unbound proteins. The wash step was repeated ten times. The bound proteins were eluted with 0.1 M DTT and SDS-PAGE gel-loading buffer [50 mM Tris-HCl (pH 6.8), 2% (w/v) Sodium dodecyl sulfate (SDS), 0.1% (w/v) bromopheno blue, 10% (w/v) glycerol] by boiling the mixtures. The samples of each steps were resolved on SDS-PAGE and stained with coomassie blue.

***N*-acetylglucosamine-agarose beads binding assay** *N*-acetylglucosamine-agarose beads (Sigma, St. Louis, Mo) were washed three times in tris buffer [50mM Tris-HCl (pH 8.0), 300mM NaCl]. BSA proteins and Purified proteins were diluted in tris buffer and then 100 μL of washed *N*-acetylglucosamine-agarose beads were added to diluted proteins. The mixtures was gently mixed for 2 hr at 4◦C and transferred to Poly-Prep chromatography column (Bio-Rad, Hercules, CA). After the column was washed in 100 μL of tris buffer, the bound proteins were eluted from column with 100 μL of the tris buffer containing 0.5 M *N*-acetylglucosamine (Tanio and Kohno, 2009). The samples of each steps were resolved on SDS-PAGE and stained with coomassie blue.

Mucin binding assay The pig gastric mucin powder (Sigma, St. Louis, MO) was resuspended in 95% (w/v) ethanol and dried completely at 70◦C. The resuspension

of pig gastric mucin mixed with sterile water. The autoclaved 3% (w/v) agar solution was mixed with the 6% (w/v) mucin solution in the ratio of one to one. The mixture was poured to 12-well microtiter plate (SPL, Kyungki, Korea). As a negative control, the only autoclaved 1.5% (w/v) agar solution was poured to 12-well microtiter plate. The wild type and *gbpA* mutant were grown to early-exponential phase (A_{600} of 0.5). The 2 mL of bacteria cultures of wild type and *gbpA* mutant were washed two times with phosphate-buffered saline (PBS). The cell pellet were resuspended in 2 mL PBS. The 400 μL of culture resuspensions was inoculated in 1.5% agar or 1.5% agar containing 3% mucin and purified GbpA protein (2.5 μM or 5 μM) was added to *gbpA* mutant. These 12-well microtiter plates were incubated for 1 hr at 30◦C room temperature. After incubation, the each well of 12-well microtiter plates was washed with PBS and the 1 mL PBS containing 0.1% (w/v) triton X-100 (Sigma, St. Louis, Mo.) was applied to detach the bound bacteria for 10 min at 30◦C. The bacterial suspension was serially diluted and plated. The number of bound bacteria was counted and quantified. The adherence index was calculated by dividing the output by the input numbers.

Construction of cell line HT-29 MTX Human colon carcinoma epithelial cell lines, HT-29 cells, were purchased from American Type Culture Collection (ATCC, Manassas, VA) and maintained in McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS) (GIBCO-BRL, Gaithersgurg, MD, U.S.A) and antibiotics (1% (w/v) penicillin/streptomycin). HT-29 cells were grown in 75 cm^2 culture flask (SPL, Kyungki, Korea) at 37◦C in 5% CO^2 incubator. For differentiation, HT-29

cells were prepared by seeding 1×10^6 cells in 25 cm^2 culture flask (SPL, Kyungki, Korea) and added the 18 μL of 5×10^{-4} M methotrexte(MTX). The medium was changed daily in all culture conditions. After weekly passages, the HT-29 cells which survived in the presence of 1×10^{-6} M MTX are called HT-29 MTX (Lesuffleur *et al.*, 1990).

Transmission electron microscopy The HT-29 MTX cells were treated with Trypsin-EDTA for 10 min at 37◦C and detached from 75 cm^2 culture flask. The cells were resuspended with modified Karnovsky's fixation solution [2% (w/v) paraformaldehde, 2% (w/v) glutaraldehyde, 0.05 M sodium cacodylate buffer, pH 7.2] and incubated at 4◦C for 2 hr. The samples were washed with 0.05 M sodium cacodylated buffer three times and treated with 1% (w/v) osmium tetroxide in 0.1 M sodium cacodylated buffer at 4◦C. The samples were rinsed with water and incubated with 0.5% (w/v) uranyl acetate for 30 min at 4◦C. The samples were dehydrated and incubated with propylene oxide for 15 min . The propylene oxide and Spurr' resin [10g ERL 4206 (vinyl cyclohexane dioxide), 6g DER (diglycidyl ether of polypropylene alchol), 26g NSA (Nonenyl succinic anhydred), 0.4g DMAE (Dimethylaminoethanol)] were mixed in the ratio one to one. The mixture was mixed with the samples for overnight. The spurr's reisn of samples were replaced with new spurr's resin. The mixtures was incubated for 24 hr at 70◦C and sectioned. The sectioned cells were observed with transmission electron microscopy (JEM1010, JEOL, Japan)

Immunocytochemistry HT-29 MTX cells were grown on the slide glass. The HT-29 MTX cells were fixed with 3.5% (w/v) paraformaldehyde. The fixed HT-29 MTX cells were washed with PBS and incubated with PBS with containing 0.1% (w/v) Triton X-100. The cells were rinsed with PBS and blocked with 1% (w/v) FBS in PBS(FPBS) for overnight at 4◦C. The cells were administrated with the diluted mouse monoclonal antibodies (Merck millipore, Darmstadt, Germany) against human MUC5AC protein in FPBS for 1 hr at room temperature. The cells were rinsed and incubated with rabbit anti-mouse IgG-FITC secondary antibodies (Abcam, Cambridge, UK) in FPBS for 1 hr at room temperature. The secondary antibodies were removed and the cells were incubated with 1 μg/ml DAPI for 1 min. The coverslip were sealed with nail polish to prevent drying and movement under microscope. The confocal laser scanning microscope (Carl Zeiss LSM710, Germany) was used to observe the stained HT-29 MTX cells (Gouyer *et al*., 2001)

Adhesion assay HT-29 MTX cells were seeded at 2×10^5 cells per well in 12-well cell culture plates and infected with *V. vulnificus* strains at an MOI of 10 for 1 h. After incubation with the *V. vulnificus* strains, HT-29 MTX cells were washed two times with pre-warmed PBS to remove nonadhering bacteria. Following final wash, HT-29 MTX cells were incubated in PBS containing 0.1% (w/v) Triton X-100 for 10min to recover adherent bacteria. The percentage of bacterial cells adhered to HT-29 MTX cells was determined by measuring colony-forming units of the recovered bacteria and presented as the percent of the initial inoculum size. HT-29 MTX cells were seeded at 1×10^5 in 60-mm petri dish and infected with *V.vulnificus*

strains at an MOI of 20 for 1h. After incubation with the *V. vulnificus* strains, HT-29 MTX were washed two times with pre-warmed PBS to remove nonadhering bacteria. HT-29 MTX cells were fixed in 100% methanol, stained with 0.4% (w/v) Giemsa solution, and observed using a light microscope (original magnification, ×1,000) after Gimesa staining (Jeong *et al*., 2009) .

Mouse survival test A group of five 6-week-old female mice (ICR specific pathogen-free; Seoul National University) was administered orally with 8.5% (w/v) $NaHCO_3$ followed by immediately bacterial suspension of wild type or *gbpA* mutant at dose of 10^8 CFU and mice were injected intraperitoneally with 12–14 mg/kg zoletile The infected mice were observed for 24 hr (Olivier *et al*., 2009). All manipulations of mice were approved by the Animal Care and Use Committee at Seoul National University

RNA purification and quantative RT-PCR Total cellular RNA from the *V. vulnificus* MO6-24/O strains (wild type, *iscR* mutant and *smcR* mutant) grown to exponential phase were purified using a RNeasy Mini kit (Qiagen, Valencia, CA). In order to synthesize cDNA, iScript^{TM} cDNA Synthesis Kit (Bio-rad, Hercules, CA) was used according to the manufacturer's procedures. Quantative real-time PCR amplification of the cDNA was performed using standard protocols with a pairs of primers. The relative expression level was determined by using the 16S rRNA expression level as internal reference for normalization (Kim *et al*., 2011).

Electrophoretic mobility shift assay(EMSA) The 398-bp upstream region of

gbpA, extending from residues -318 to +79 relative to the first position of the AUG start codon (+1), was amplified by PCR using [γ-^{32}p] ATP-labeled GbpA_EM-R and unlabeled GbpA_EM-F as the primers (Table 2). The radio-labeled DNA fragment (2.5 nM) was incubated with the increasing concentration of purified IscR protein for 20 min at 30◦C in reaction buffer [0.04 M Tris-HCl (pH 7.9), 0.07 M KCl, BSA 100 μg/ml, 1 mM DTT] or purified SmcR for 30 min at 37◦C in reaction buffer [0.05 M Tris-HCl (pH 8.0), 0.1 M KCl, 3 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA]. After incubation, the samples were resolved on 5% polyacrylamide gel. For competition analysis, the same but unlabeled DNA fragment was used as a self-competitor DNA. The labeled DNA probe was incubated with the self-competitor DNA, prior to the addition of 30 nM apo-IscR or 100 nM SmcR (Lim *et al.*, unpublished).

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

Strain or plasmid	Relevant charavteristics[a]	
Bacterial Strains		
V.vulnificus		
MO6-24/O	Clinical isolate; virulent	Laboratory collection
JK	MO6-24/O with *Δ iscR*	Lim et al, unpublished
JK	MO6-24/O with *Δ smcR*	Lim et al, unpublished
SS111	MO6-24/O with *Δ gbpA*	This study
MS023	MO6-24/O with *Δ pilD*	Lim et al, 2011
E.coli		
DH5α	*supE44 ΔlacU169(ϕ80 lacZ ΔM15) hsdR17 recA1 endA1 gyrA96 relA1 relA1 thi-1 relA1;* plasmid replication	Laboratory collection
S17-1λpir	Conjugal donor; host for π-requiring plamid, Sm^r	
BL21(DE3)	*F^-, ompT, hsdS(r_B^-,m_B^-), gal(DE3)*	Laboratory collection
Plasmids		
pGEM-T easy	PCR product cloning vector; Ap^r	Promega
pET-22b(+)	His-tag protein expression vector; Ap^r	Novagen
pDM4	Suicide vector; *ori*R6K; Cm^r	Milton et al., 1996
pJK1113	Complementation vector; *ori*T; Ap^r, Km^r	Lim et al, unpublished

pSO1201	pJK1113 with *gbpA*	This study
pSO1202	pET-22b(+) with *gbpA*	This study

[a] Ap^r, ampicillin resistant; Km^r, kanamaycin resistant; Cm^r, chloramphenicol resistant; Sm^r, streptomycin

Table 2. Oligonucleotides used in this study.

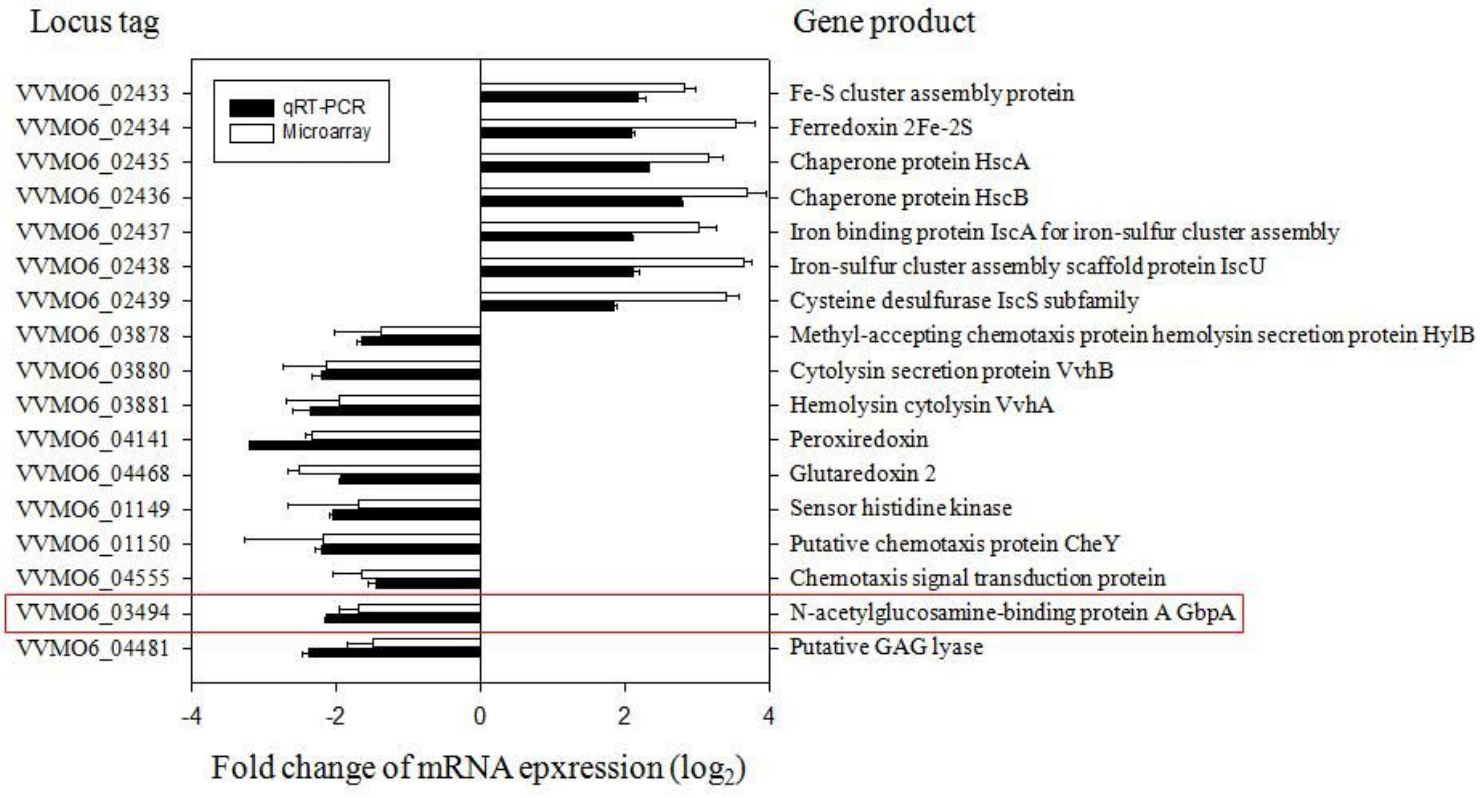
Oligonucleotide	Oligonucleotide sequence, 5' → 3'	Description
GbpA_F1-F	GAGATGCACATCAGCAACGCG	Used for mutant construction
GbpA_F1-R	AAAGGATCCAGCGAACTTACACAGTGT	Used for mutant construction
GbpA_F2-F	GCTGGATCCTTTGTCTTCCCAGATG	Used for mutant construction
GbpA-F2-R	CGCAACAACGGAATCAAACGC	Used for mutant construction
GbpA_Com-F	AAAAAACAACCGCAAAAAACCTTACTG	Used for construction of complementary vector
GbpA_Com-R	TTACAGTTTGTCCCACGCCATTTG	Used for construction of complementary vector
GbpA_Exp-F	TGAAAAAACAACCGCAAAAAACC	Used for construction of expression vector
GbpA_Exp-R	GCAGTTTGTCCCACGCCATT	Used for construction of expression vector
GbpA_qRT-F	CGCCAAGTGATGATTACGA	Used for quantative RT-PCR
GbpA_qRT-R	CGGTTGCTGTCCATTGAA	Used for quantative RT-PCR
GbpA_EM-F	TTTTTCATCACACTTTTTCG	Used for electrophoretic-

		mobility shift assay
GbpA_EM-R	ACCGCAATGAGCCATGGTTATG	Used for electrophoretic-mobility shift assay

II. RESULTS

IscR regulon and sequence analysis of *V.vulnificus* GbpA

IscR is an Fe-S cluster-containing protein that functions as a transcriptional regulator of Fe-S biogenesis and other Fe-S protein-encoding genes (Schwartz *et al*., 2001). Previous study reported that IscR regulates several genes probably involved in survival and pathogenesis of *Vibrio vulnificus* by using a microarray and quantitative RT-PCR analysis(Lim *et al*., unpublished). Among IscR regulon, VVMO6_03494 gene that is up-regulated by IscR was further investigated (Fig. 1). BLASTP analysis showed that VVMO6_03494 is a homologue (75% identical) of *V. cholerae N*-acetylglucosamine binding protein A, GbpA (Fig. 2A). Further analysis of *V.vulnificus* GbpA identified that chitin binding domain is conserved in *V.vulnificus* GbpA (Vaaje-Kolstad *et al*., 2005). In addition, *V. vulnificus* GbpA contains an N-terminal signal peptide with a putative cleavage site located between the 20^{th} amino acid(Thr) and the 21^{th}(Ala), suggesting that GbpA is a secreted protein (Fig. 2B). The results indicated that *V.vulnificus* GbpA is up-regulated by IscR and secreted chitin binding protein.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Gene product
qRT-PCR
Microarray
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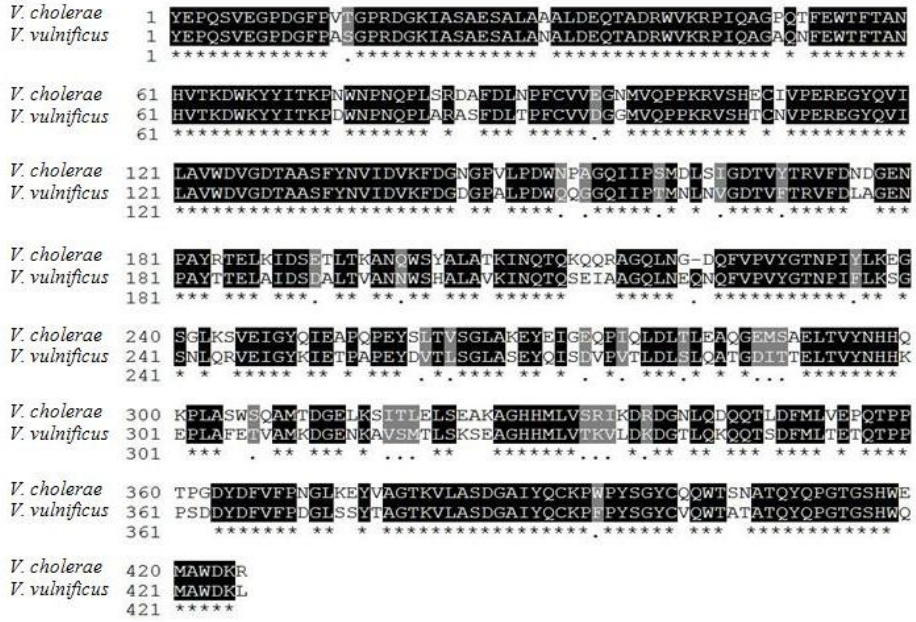
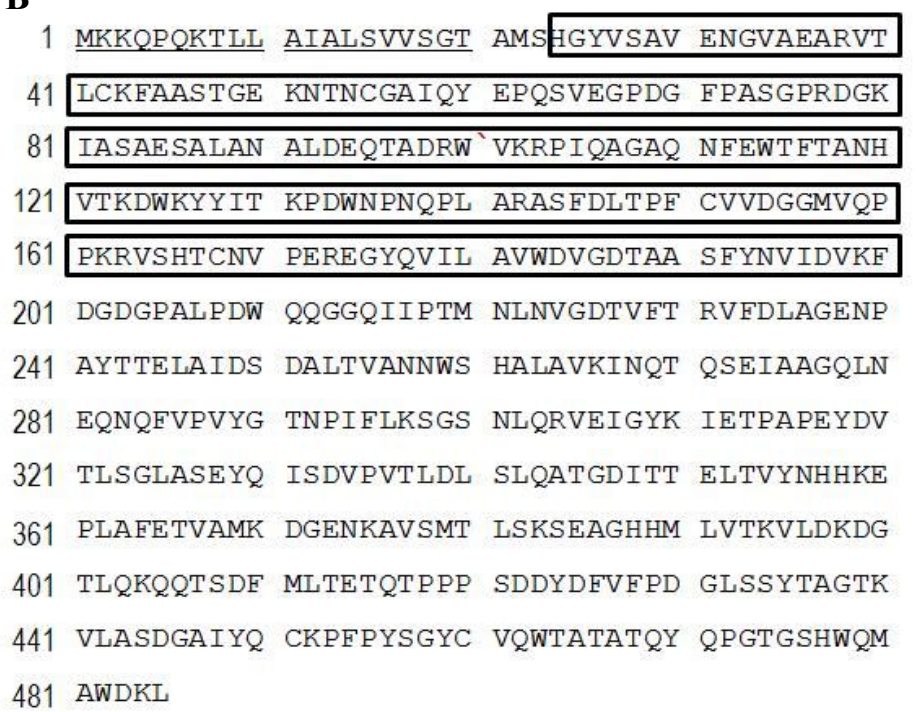
VVMO6_01149
VVMO6_01150
VVMO6_04555
VVMO6_03494
VVMO6_04481
Fe-S cluster assembly protein
Ferredoxin 2Fe-2S
Chaperone protein HscA
Chaperone protein HscB
Iron binding protein IscA for iron-sulfur cluster assembly
Iron-sulfur cluster assembly scaffold protein IscU
Cysteine desulfurase IscS subfamily
Methyl-accepting chemotaxis protein hemolysin secretion protein HylB
Cytolysin secretion protein VvhB
Hemolysin cytolysin VvhA
Peroxiredoxin
Glutaredoxin 2
Sensor histidine kinase
Putative chemotaxis protein CheY
Chemotaxis signal transduction protein
N-acetylglucosamine-binding protein A GbpA
Putative GAG lyase
-4
-2
0
2
4
Fold change of mRNA epxression (log2)

Fig. 1. Verification of the newly predicted genes as part of the IscR regulon. The IscR regulon members newly predicted on the basis of microarray analysis results were analyzed by quantitative real-time PCR. Each column represents the mRNA expression level in the *iscR* mutant relative to that in the wild type. Locus tags are based on the database of the *V. vulnificus* MO6-24/O genome, which was retrieved from GenBank (accession AE016795, AE016796, CP002469.1, and CP002470.1). Error bars represent the standard errors of the means (SEM).

A


V. cholerae
V. vulnificus

B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81 IASAESALAN ALDEQTADRW VKRPIQAGAQ NFEWTFTANH
121 VTKDWKYYIT KPDWNPNQPL ARASFDLTPF CVVDGGMVQP
161 PKRVSHTCNV PEREGYQVIL AVWDVGDTAA SFYNVIDVKF
201 DGDGPALPDW QQGGQIIPTM NLNVGDTVFT RVFDLAGENP
241 AYTTELAIDS DALTVANNWS HALAVKINQT QSEIAAGQLN
281 EQNQFVPVYG TNPIFLKSGS NLQRVEIGYK IETPAPEYDV
321 TLSGLASEYQ ISDVPVTLDL SLQATGDITT ELTVYNHHKE
361 PLAFETVAMK DGENKAVSMT LSKSEAGHHM LVTKVLDKDG
401 TLQKQQTSDF MLTETQTPPP SDDYDFVFPD GLSSYTAGTK
441 VLASDGAIYQ CKPFPYSGYC VQWTATATQY QPGTGSHWQM
481 AWDKL

Fig. 2. Sequence relatedness and amino acid sequence analysis of GbpA proteins. (A) Black boxes and asterisks indicate the identical amino acids and gray boxes and dots represent the similar amino acids. Dashes represent the missing sequences. Alignment was based on the amino acid sequences in the GenBank database and derived by the CLUSTALW alignment program (http://www.ch.embnet.org/sofware/ClustalW.html). The analysis of amino acid sequence of GbpA predicted that GbpA protein contains the signal peptide and chitin binding domain. The putative signal peptide is underlined. The chitin binding domain are indicated by open boxes.

Construction of the *V.vulnificus gbpA* mutant

To study about the role of *V.vulnificus* GbpA, the isogenic *V.vulnificus gbpA* mutant was constructed by allelic exchanges (Fig 3A). A double crossover, in which the wild-type *gbpA* gene on large chromosome was replaced with the *ΔgbpA* allele, was confirmed by PCR using a pair of primers, GbpA_F1-F and GbpA_F2-R (Table 2). Based on PCR analysis, the genomic DNA from MO6-24/O of wild-type with the primers produced a 2.8-kb fragment, but the genomic DNA from isogenic *gbpA* mutant with the primers amplified a 1.7-kb fragment that deleted 1.1-kb fragment in *V.vulnificus gbpA* open reading frame (Fig. 3B) (Lim *et al*., 2011)

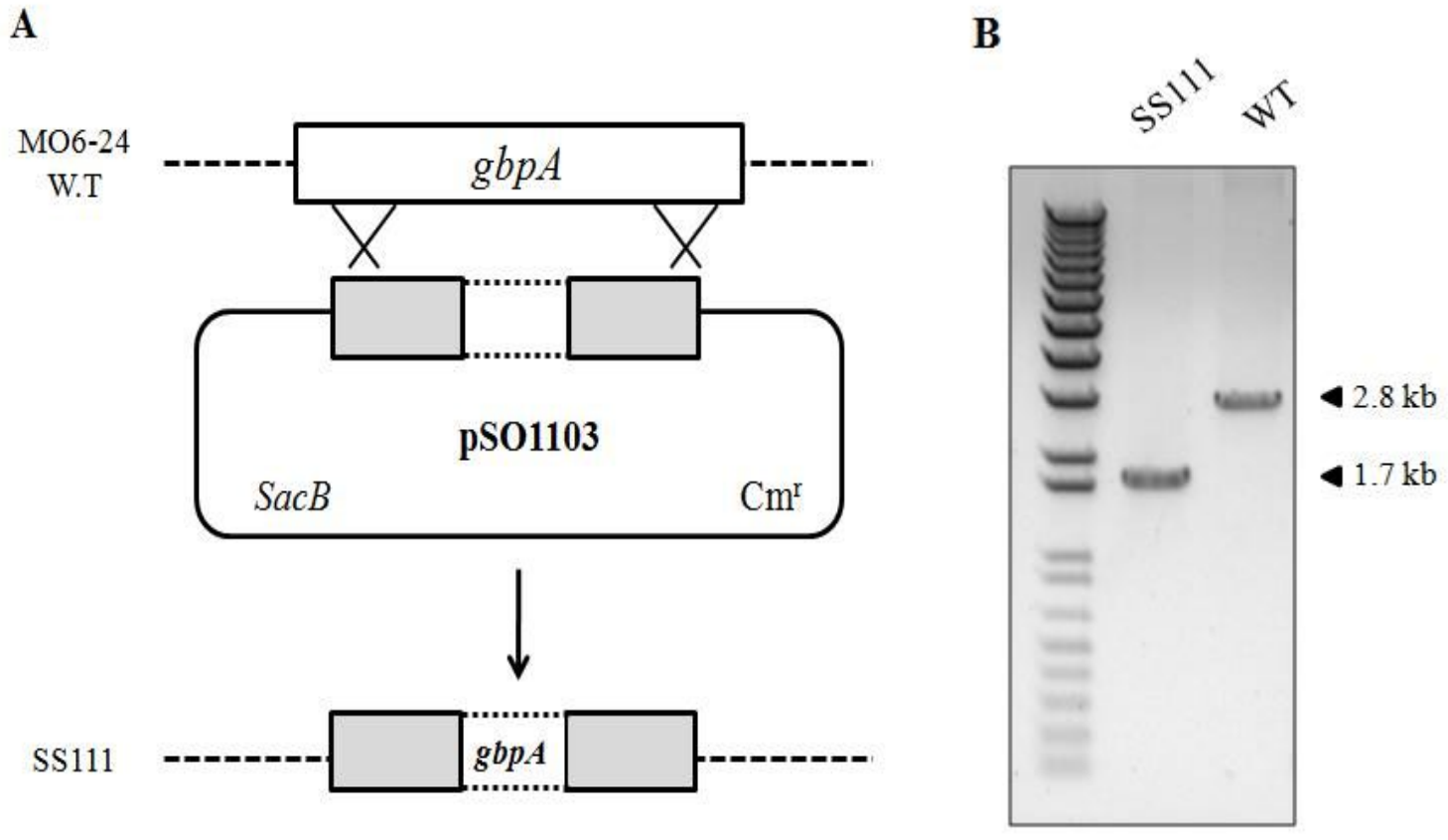

A
MO6-24
W.T
gbpA
pSO1103
SacB
Cmr
SS111
gbpA
B
SS111
WT
2.8 kb
1.7 kb

Fig. 3. Allelic exchange procedure and construction of the *gbpA* mutants. (A) Homologous recombination between the chromosomal *gbpA* gene from wild-type MO6-24/O and pSO1103 led to deletion of the *gbpA* gene and resulted in construction of the *gbpA* mutant. Abbreviations; *sacB*, levansucrase gene; Cm^r, chloramphenicol resistant. (B) PCR analysis of MO6-24/O and the *gbpA* mutant generated by allelic exchange. Molecular size markers (1-kb ladder; Invitrogen) appear on the left of the gels.

Effect of the GbpA mutation on the attachment to chitin *in vitro*

To assess the binding ability of *V. vulnificus* GbpA for chitin, *V. vulnificus* strains were incubated with chitin beads (Brooke *et al*., 2009). As shown in Fig. 4, *gbpA* mutant was exhibited approximately ten times less adherence index than its parental wild type and the ability to adhere to chitin beads was recovered in complemented strain. The result showed that *V.vulnificus* GbpA contributes to adherence to chitin.

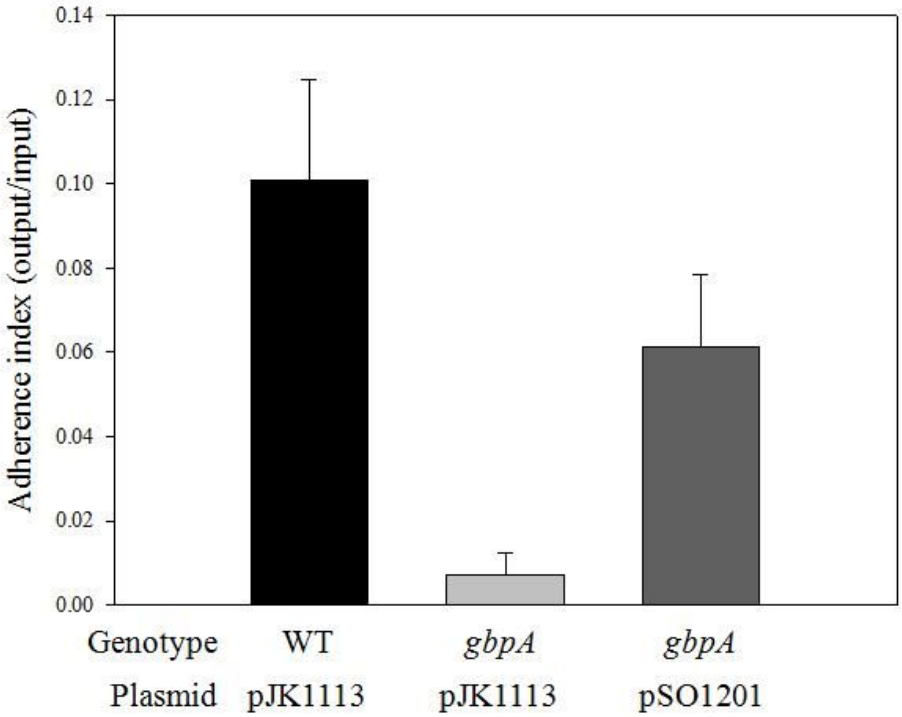

Adherence index (output/input)
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Genotype WT gbpA gbpA
Plasmid pJK1113 pJK1113 pSO1201

Fig. 4. GbpA is involved in the chitin attachment. The cultures of wild type, *gbpA* mutant and complemented strain were resuspended with PBS containing 3% (w/v) NaCl and the magnetic chitin beads were added to the cultures. The mixtures were incubated for 2 hr at room temperature. After unbound bacteria were wash away, the number of bound bacteria recovered from the beads was quantified. The adherence index was calculated by dividing the output by the input numbers. Error bars represent SEM.

Secretion of *V. vulnificus* GbpA

Amino sequence analysis of *V. vulnificus* GbpA revealed that *V. vulnificus* GbpA possesses signal peptide. To investigate that the secretion of *V. vulnificus* GbpA is involved in type II secretion system, western blot analysis was performed using *pilD* mutant that is deficient of a component of type II secretion system (Lim *et al*., 2011). *V. vulnificus* GbpA was not detected in the culture supernatant of *pilD* mutant. In contrast, intracellular level of *V.vulnificus* GbpA increased in *pilD* mutant compared to its parental wild type (Fig. 5). These results indicated that *V. vulnificus* GbpA is secreted by type II secretion system.

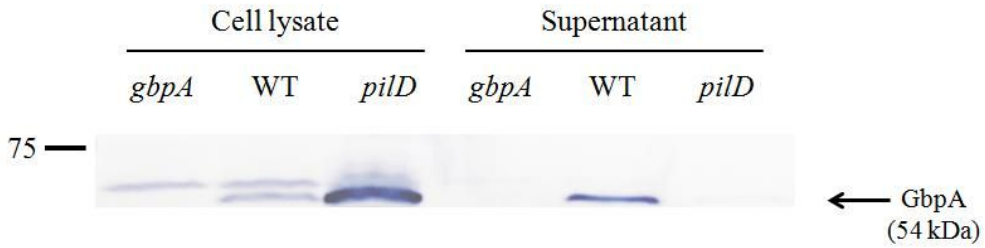
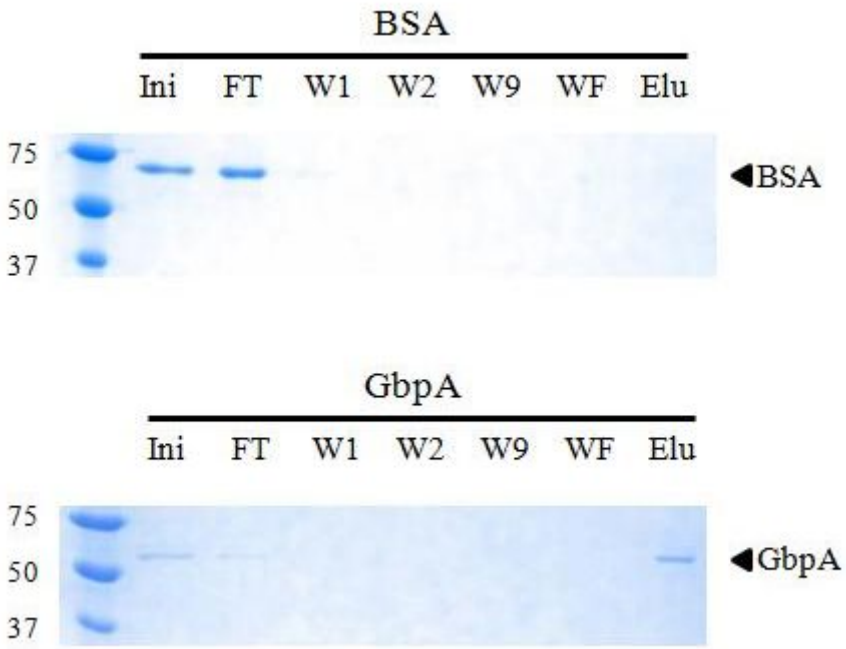

Cell lysate
Supernatant
gbpA
WT
pilD
gbpA
WT
pilD
75
GbpA
(54 kDa)

Fig. 5. Secretion of *V. vulnificus* GbpA mediated by the type II secretion system. The location of *V. vulnificus* GbpA proteins of the wild type, *gbpA* mutant and *pilD* mutant grown in LBS at A_{600} of 0.5 was analyzed by western blot analyses using rabbit anti-GbpA polyclonal antibodies.

GbpA is an important factor for binding to chitin and *N*-acetylglucosamine

In order to examine whether *V. vulnificus* GbpA proteins bind to chitin, the chitin beads binding assay was performed with purified GbpA protein. The chitin binding ability of *V. vulnificus* GbpA was obvious, whereas the chitin binding ability of BSA, as a negative control, was not detectable (Fig 6A). Because chitin is a long-chain polymer of a *N*-acetylglucosamine, *N*-acetylglucosamine binding activity of purified GbpA was assessed in presence of *N*-acetylglucosamine agarose beads (Tanio and Kohno, 2009). As expected, *V. vulnificus* GbpA showed the *N*-acetylglucosamine binding activity. But BSA, as a negative control, did not (Fig 6B). These result revealed that *V. vulnificus* GbpA is an *N*-acetylglucosamine-sensitive chitin binding protein.

A


BSA
Ini
FT
W1
W2
W9
WF
Elu
75
50
37
BSA
GbpA
Ini
FT
W1
W2
W9
WF
Elu
75
50
37
GbpA

B

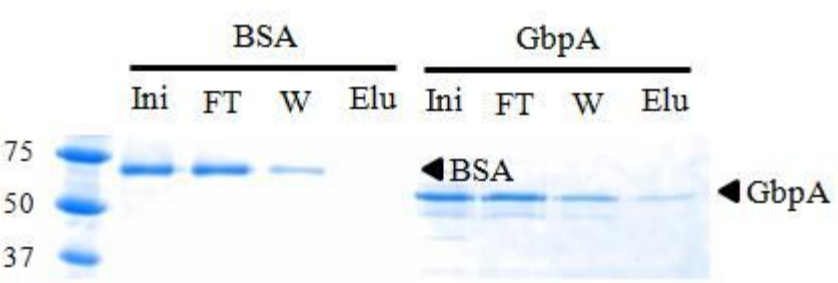

BSA
GbpA
Ini
FT
W
Elu
Ini
FT
W
Elu
75
50
37
BSA
GbpA

Fig. 6. GbpA is a *N*-acetylglucosamine -sensitive chitin-binding protein. (A) BSA proteins and GbpA proteins were added to magnetic chitin beads. The mixtures were incubated for 10 min at room temperature. After unbound proteins were wash away, bound proteins were eluted with 0.1 M DTT. The proteins were resolved on SDS-PAGE. (B) BSA proteins and GbpA proteins were diluted with tris buffer and added to *N*-acetylglucosamine-agarose beads. The mixtures were mixed for 2 hr at 4℃. After unbound protein were wash away, bound proteins were eluted with tris buffer containing 0.5 M *N*-acetylglucosamine. The proteins were resolved on SDS-PAGE.

***GbpA* contributes to adherence to mucin**

Because *N*-acetylglucosamine is a major component of mucin (Bansil and Turner, 2006), *V. vulnificus* GbpA was predicted to adhere to mucin. This prediction was demonstrated by mucin binding assay using the mucin solution and agar. In the experiment using the only agar, all samples showed the similar agar-binding affinity regardless of strains and addition of GbpA proteins, indicating that GbpA proteins are not related to agar-binding. The *gbpA* mutant reduced the ability to bind to mucin compared to its parental wild type. The reduced ability to attach to mucin was recovered by adding the purified GbpA protein to *gbpA* mutant in concentration-dependent manner (Fig 7). These results suggested that the secreted GbpA proteins adhere to mucin and improve the binding affinity of *V. vulnificus* for mucin.

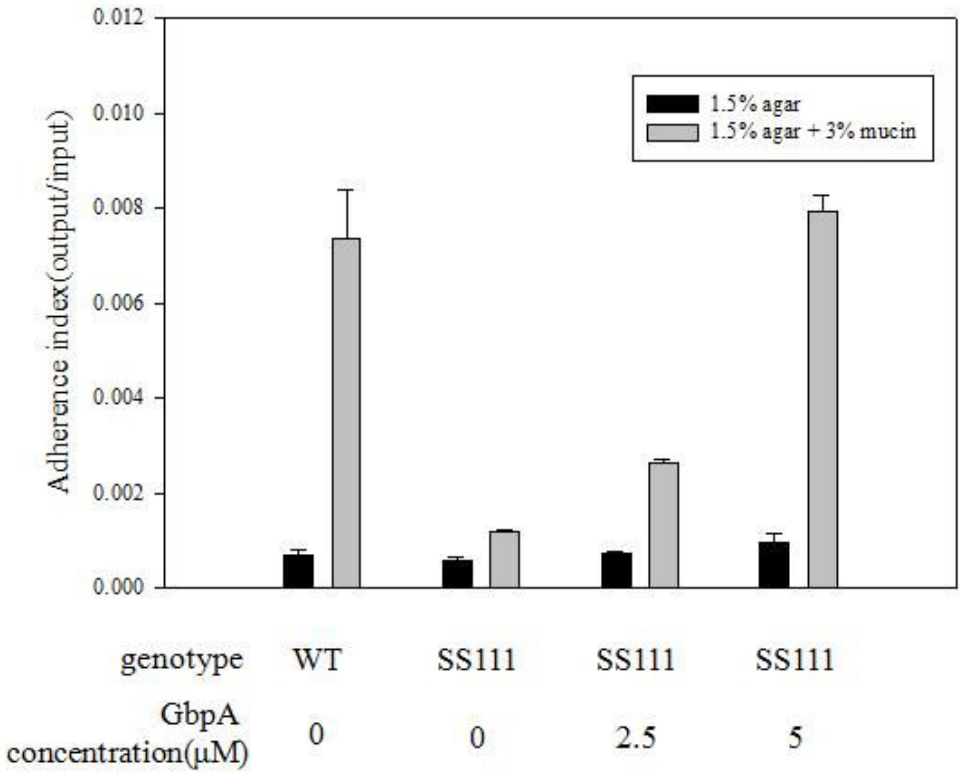
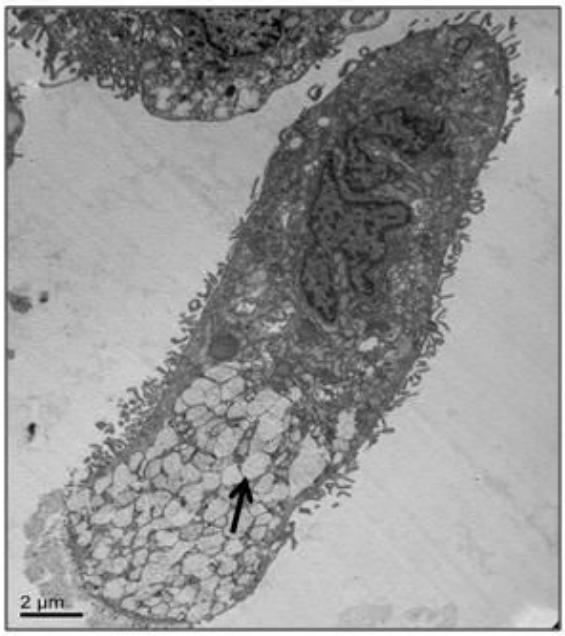

0.012
0.010
0.008
0.006
0.004
0.002
0.000
Adherence index(output/input)
1.5% agar
1.5% agar + 3% mucin
genotype
WT
SS111
SS111
SS111
GbpA concentration(μM)
0
0
2.5
5

Fig. 7. GbpA has ability to bind to mucin. The cell cultures of wild type and *gbpA* mutant were inoculated in 1.5% (w/v) agar or 1.5% (w/v) agar containing 3% (w/v) mucin and GbpA proteins were added. The mixtures were incubated for 1 hr at 30℃. After unbound bacteria were wash away, the number of bound bacteria retrived from the beads was quantified. The adherence index was calculated by dividing the output by the input numbers. Error bars represent SEM.

Construction of the mucus-secreting HT-29 MTX cells

Most HT-29 cells are undifferentiated cells and the only small proportion of HT-29 cells is differentiated mucus-secreting cells (Huet *et al*., 1995). To obtain stably differentiated mucus-secreting cells, HT-29 cells were adapted to 10^{-6} M methotrexte(MTX) (Lesuffleur *et al*., 1990). As shown Fig. 8A, it was confirmed that mucin granules are present inside HT-29 MTX cell through transmission electron microscope (TEM). Also, it was verified that postcofluent HT-29 MTX cell layer is covered with secreted mucin (Fig. 8B). These results suggest that HT-29 MTX cells are overlaid with secreted, gel-forming mucin.

A



B

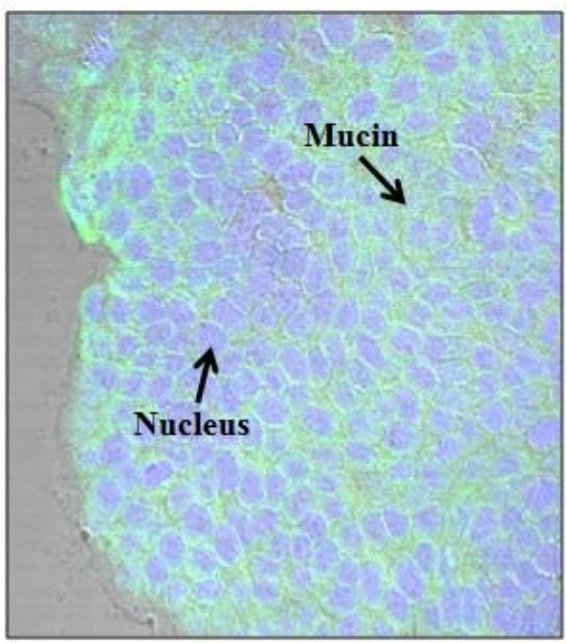
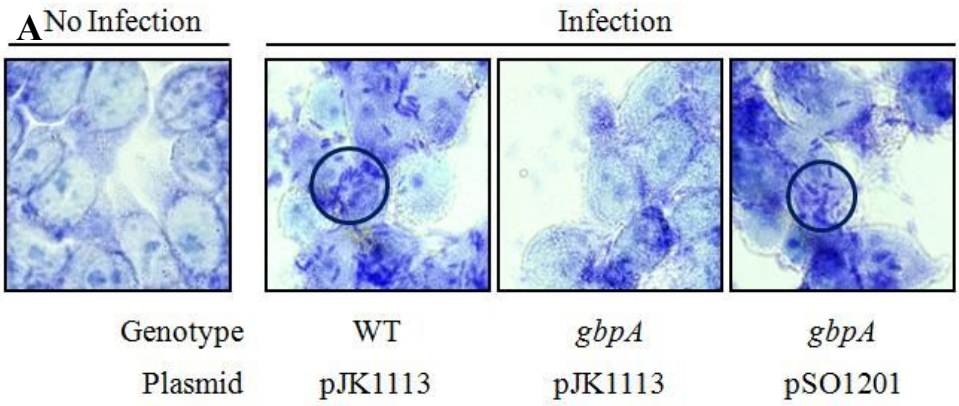


Fig. 8. HT-29 MTX cells produce the secreted gel-forming mucin. (A) HT-29 MTX cells were fixed, dehydrated and sectioned. The pictures of single HT-29 MTX cell were taken by the transmission electron microscopy. The arrow represents the mucin granules (B) HT-29 MTX cells (day 8) were fixed and blocked. HT-29 MTX cell layer was incubated with mouse anti-human MUC5AC primary antibodies and then rabbit anti-mouse IgG–FITC secondary antibodies. After incubation, the cells were stained with DAPI and observed with confocal laser scanning microscope..

GbpA is required for attachment to mucus-secreting epithelial cells *in vitro*.

The adhesion ability of the *V. vulnificus* strains for HT-29 MTX were compared (Jeong *et al*., 2009). The wild type and the complemented strain attached to the surface of HT-29 MTX. In contrast, *gbpA* mutant was impaired in its ability to attach to HT-29 MTX (Fig. 9A).The adherence index of *gbpA* mutant was about twofold lower than the adherence index of the wild type (Fig. 9B). These results revealed that *V. vulnificus* GbpA mediates the adhesion to intestinal mucosal.


A
No Infection
Infection
Genotype
WT
gbpA
gbpA
Plasmid
pJK1113
pJK1113
pSO1201

B

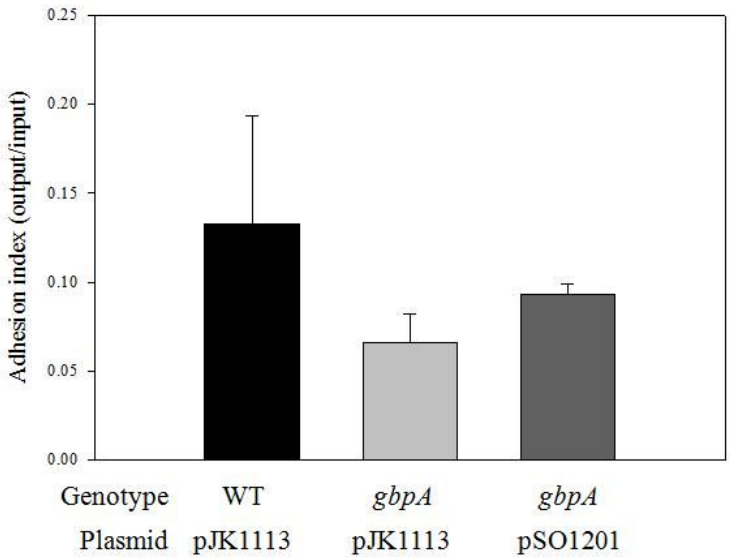

0.25
0.20
0.15
0.10
0.05
0.00
Adhesion index (output/input)
Genotype
WT
gbpA
gbpA
Plasmid
pJK1113
pJK1113
pSO1201

Fig. 9. Cytoadherence of *V. vulnificus* strains. (A) HT-29 MTX cells infected with *V. vulnificus* strains were morphologically observed using a light microscope (original magnification, ×1,200) after Gimesa staining. The adhered *V. vulnificus* cells are indicated by circles. (B) HT-29 MTX cells were infected with the wild type (WT), the *gbpA* mutant, or the complemented strain for 1 h at 37 ℃. After incubation with the bacteria, the HT-29 MTX cells were rinsed to remove any nonadhering bacteria. The bacterial cells adhered to HT-29 MTX cells was counted by measuring colony-forming units of the retrieved bacteria and the adherence index was determined by dividing the output by input. Error bars represent the SEM

Virulence in mice is dependent on *V. vulnificus gbpA*

To investigate the role of the *V. vulnificus gbpA* in virulence, mouse survival test was performed. The none of mice injected with the *gbpA* mutant was dead but the 60% of mice injected with parental wild type were dead (Fig. 10), suggesting that the *gbpA* mutant appeared to be significantly less virulent than its parental wild type. This results indicated that *V.vulnificus* GbpA is important for the virulence of *V. vulnificus* in mice.

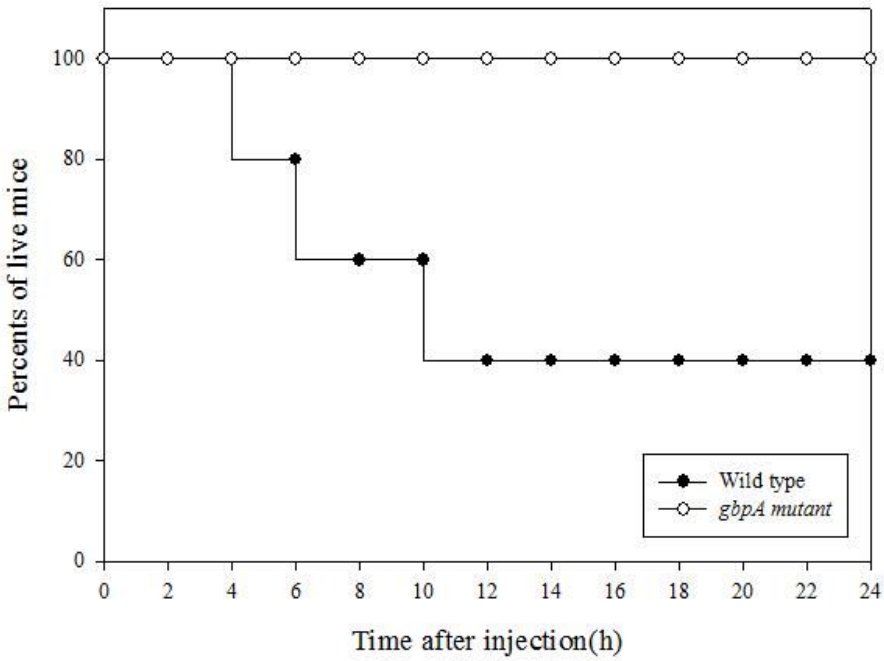

Percents of live mice
100
80
60
40
20
0
0
2
4
6
8
10
12
14
16
18
20
22
24
Time after injection(h)
Wild type
gbpA mutant

Fig. 10. Effect of *gbpA* mutation on virulence of *V. vulnificus* Seven-week-old female ICR mice were administered orally 8.5% (w/v) $NaHCO_3$ and then bacterial suspension of the wild type or *gbpA* mutant. After inoculation, mice were injected intraperitoneally with zoletil. Mouse survival was monitored for 24 h.

Regulation of the cellular level of *V. vulnificus gbpA*

It was observed that the cellular level of the *gbpA* transcript was decreased as cell density increased as shown in Fig. 11. Therefore, we tested whether the expression of *gbpA* is regulated by SmcR, a quorum-sensing master regulator of *V. vulnificus* by using a qRT-PCR. As a result, the level of *gbpA* transcript was increased in the *smcR* mutant compared to that in its parental wild type, suggesting that the expression of *gbpA* is down-regulated by SmcR In addition, it was confirmed that transcription level of *gbpA* is up-regulated by IscR by using a qRT-PCR (Fig. 12A). Furthermore, western blot analysis using *V. vulnificus* strains showed that expression level of GbpA protein was dependent of transcription level of *gbpA* (Fig. 12B). These results implied that IscR and SmcR positively and negatively regulate the *gbpA* expression, respectively.

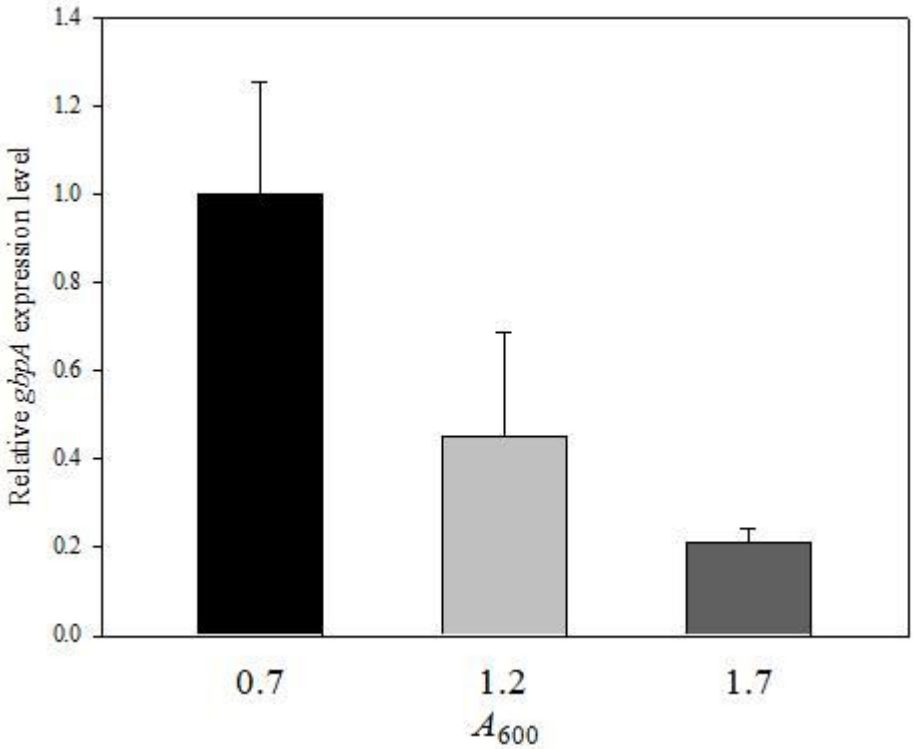
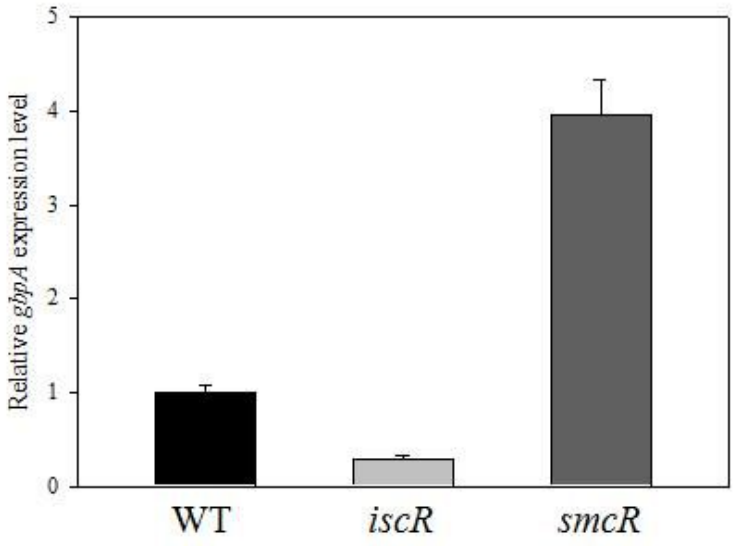

1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Relative gbpA expression level
0.7
1.2
1.7
A600

Fig. 11. Expression of *gbpA* in *V. vulnificus* is dependent of growth-phase. Cultures of the wild type were grown with LBS, and the samples were collected at OD_{600} of 0.7, 1.2 and 1.7. Total cellular RNA was purified from the samples. The relative levels of *gbpA* expression were determined by qRT-PCR analysis and normalized to the 16S rRNA expression level as presented the expression level of the wild type recovered at A_{600} of 0.7 as 1. Error bars represent the SEM.

A


Relative gbpA expression level
5
4
3
2
1
0
WT
iscR
smcR

B

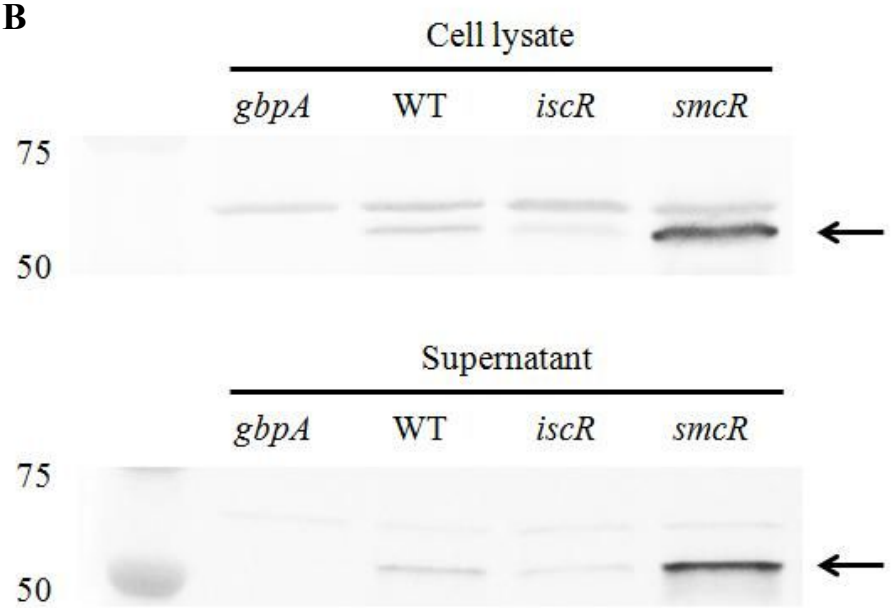
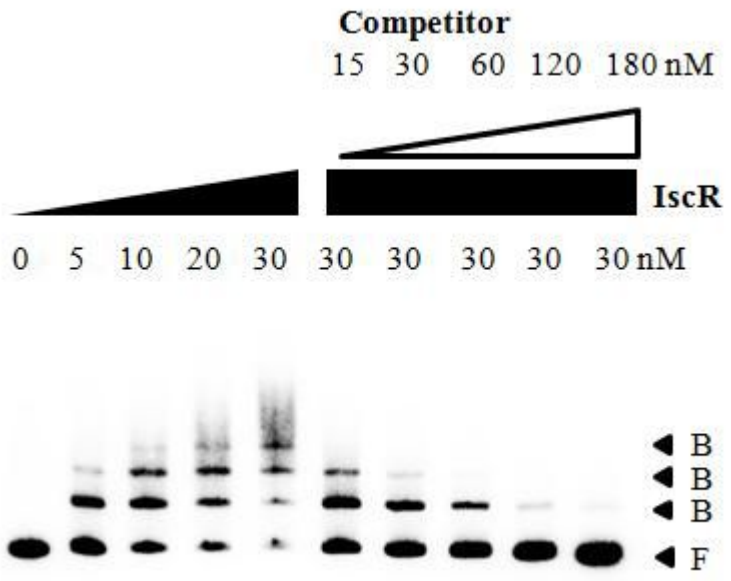

Cell lysate
gbpA
WT
iscR
smcR
75
50
Supernatant
gbpA
WT
iscR
smcR
75
50

Fig. 12. Expression of *gbpA* in *V. vulnificus* with different genetic background. (A) Cultures of the wild type, *iscR* mutant and *smcR* mutants were grown with LBS, and samples removed of OD_{600} of 1.5 were used to isolate total cellular RNA. The relative level of *gbpA* expression was determined by qRT-PCR analysis and normalized to the 16 S rRNA expression level as presented the expression level of the wild typ as 1. Error bars represent the SEM. (B) Culture of the wild type, *iscR* mutant and *smcR* mutant grown to log phase (A_{600} of 0.5) were harvested, broken and separated to cell lysate and supernatant. Total proteins were subjected to western blot analysis using rabbit polyclonal antibodies against *V. vulnificus* GbpA

IscR and SmcR specifically bind to the regulatory region of *gbpA*

The 397-bp labeled DNA fragment encompassing the putative *gbpA* regulatory region was incubated with the increasing amounts of IscR and then subjected to electrophoresis. As shown Fig. 13A, the addition of IscR resulted in concentration-dependent ladder of three retarded bands, indicating that at least three binding sites for IscR are present within the regulatory region of *gbpA*. In competitor assay, the unlabeled DNA fragment was used as a self competitor to confirm the specific binding of IscR to the regulatory region of *gbpA*. The result confirmed that IscR binds specifically to the regulatory region of *gbpA*. In similar DNA-binding assay, SmcR also showed specific binding to the regulatory region of *gbpA* (Fig. 13B). These results showed that the expression of *gbpA* is directly activated by IscR and repressed by SmcR, respectively.

A


Competitor
15 30 60 120 180 nM
IscR
0 5 10 20 30 30 30 30 30 30 nM
B
B
B
F

B

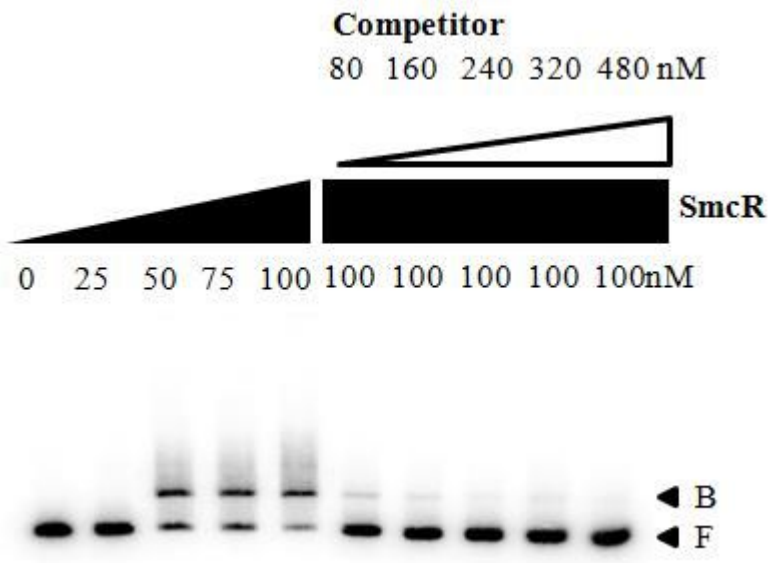

Competitor
80 160 240 320 480 nM
SmcR
0 25 50 75 100 100 100 100 100 100nM
B
F

Fig. 13. Gel mobility shift assay for binding of IscR and SmcR to the *gbpA* regulatory region. (A) 0.4 kb DNA fragment of the *gbpA* regulatory region was radioactively labeled and used as a probe for DNA. The radiolabeled fragments (2.5 nM) were mixed with increasing amounts of IscR (A) or SmcR (B) as indicated and then resolved on a 5% polyacrylamide gel. For competition analysis, the same but unlabeled 0.4 kb DNA fragment was used as a self-competitor DNA. The labeled DNA probe was incubated with the self-competitor DNA, prior to the addition of 30 nM IscR or 100 nM smcR. B, bound DNA; F, free DNA.

IV. DISCUSSION

V. vulnificus is a Gram-negative marine bacterium that cause the primary septicemia and gastroenteritis. Chitin is the most abundant biopolymer in nature. In aquatic environments, *V. vulnificus* attaches the chitin (Montanari, 2006). *V. vulnificus* enters the gastrointestinal track through the contaminated seafood containing the chitin and reach the mucus layer covering the gastrointestinal epithelium (Deplancke and Gaskins, 2001). Attachment and colonization on the surface of intestinal cells is essential steps in the early stage of pathogenesis of *V. vulnificus*. The processes is mediated by microbial adhesin recognizing the receptors that are on the surface of mucus layer (Yuehuei and Friedman, 1997). Therefore, the microbial adhesins that adhere the mucus layer are important virulence factors. In this study, *V. vulnificus* GbpA, a novel microbial adhesin, was identified and characterized. Also, the regulation of the expression of *V. vulnificus* GbpA was studied.

The vibrios has several genes encoding the chitin binding proteins (Montgomery, M. T, and D. L. Kirchman, 1993; Pruzzo *et al*., 1996; Tarsi R. and C. Pruzzo, 1999) *V. cholerae gbpA* encoding *N*-acetylglucosamine binding protein also binds chitin (Kirn *et al*., 2005). The *V. vulnificus* GbpA shows 75% identity in amino sequences with the sequence of the GbpA of *V. cholerae* (Fig. 2A) and contains conserved chitin binding domain (Fig. 2B). As expected, GbpA has binding activity for chitin and *N*-acetylglucosamine . These results revealed that GbpA is chitin binding protein that recognize *N*-acetylglucosamine (Fig. 4; Fig. 6). Because *N*-acetylglucosamine is a abundant sugar present in mucin (Bourlioux *et al*., 2003), it

is reasonable to assume that GbpA attaches to mucin by binding *N*-acetylglucosamine present in mucin. Consistent with this, *gbpA* mutant was impaired adhesion in mucin and mucin-secreting HT-29 MTX cells (Fig. 7 to 9), reflecting that . GbpA contributes to adhesion to mucin *in vitro* and *ex vivo*. Attachment to epithelial cells is essential step for pathogenesis of *V. vulnificus* (Jeong et al., 2009). The *gbpA* mutant significantly diminished virulence in mice as measured by their abilities to cause death (Fig. 10), indicating that GbpA is essential for virulence of *V. vulnificus* in mice.

It is known that chitin binding protein of vibrios is membrane-bound protein Montgomery, M. T, and D. L. Kirchman, 1993; Pruzzo1 *et al*., 1996). However, *V. vulnificus* GbpA include putative signal peptide that is associated with type II secretion system (Fig. 2B) and is trapped in the periplasmic space, rather than supernatant, in the mutant that is inactivated type II secretion system (Fig. 5), indicating that GbpA is secreted via type II secretion system. But, the exact mechanism of GbpA for binnding the chitin and mucus layer has not been solved.

IscR and SmcR directly regulates the expression of gbpA by binding to the regulatory region of *gpbA* (Fig. 11 to 13). At early stage of host infection, *V. vulnificus* commonly is faced with iron-starvation and oxidative stress (Martinez *et al*., 1990; Janssen *et al*., 2003). Under the such conditions, *V. vulnificus* increases the expression of IscR (Zheng, et al., 2001; Outten, *et al*., 2004; .Lim et al, unpublished). IscR positively regulates the expression level of *gbpA* by directly binding the promoter region of *gbpA*. GbpA acts as a adhesin for attachment to the mucus layer of human intestine. In addition, *V. vulnficus* that attach to the mucus layer has the advantage of using the mucin as a nutrient source (Jeong *et al*, 2009).

Some of *V. vulnificus* that adhere on the mucus layer is capable of invading in human intestinal epithelial cells (Chen *et al*., 2002). *V. vulnificus* that enter the intestinal epithelium is not necessary to produce GbpA protein. Furthermore, as cell density increase, the expression level of SmcR increases (Kim *et al*., unpublished). The expression level of GbpA is negatively regulated by SmcR. *V. vulnificus* controls the expression of GbpA through IscR and SmcR to adjust to intestinal environment in host.

The several systematic toxicity tests indicate the dietary *N*-acetylglucosamine is a safe compound even at a high dose. (Lee *et al*., 2004; Takahashi *et al*., 2009). Also, *N*-acetylglucosamine help restore the formation and function of the protective structures of the gastrointestinal tract (*Chen et al., 2010*) Mucus layer, one of the protective structures, protects the intestinal epithelium from pathogenic bacteria (Deplancke and Gaskins, 2001) It was reported that the malfunction of intestinal mucin increases the possibility of infection (McAuley *et al*., 2007). Furthermore, N-acetylglucosamine may reduce the ability of *V. vulnificus* to bind the host intestine. as *V. vulnificus* GbpA binds to the administrated *N*-acetylglucosamine instead of mucin. Therefore, the use of *N*-acetylglucosamine may reduce the infection of *V. vulnificus* by enhancing the structure of the intestinal mucus layer and preventing the attachment to mucin from *V. vulnificus*.

V. REFERNCES

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VI. 국문초록

병원균이 숙주 세포에 결합하는 것은 발병 초기에 중요한 단계이다. VVMO6_03494는 부착소로 추정되며 패혈증 비브리오의 생존과 병원성과 관련된 유전자를 조절하는 전사 조절자인 IscR에 의해 발현이 증가하는 것으로 알려져있다. VVMO6_03494는 콜레라 비브리오의 *N*-acetylglucosamine 결합 단백질을 암호화 하는 *gbpA*와 높은 유사성을 보였다. 이번 연구에서는 패혈증 비브리오균 GbpA의 역할과 조절 특성에 대해 연구하였다. 아미노산 서열 분석을 통해서 GbpA는 키틴에 결합하는 분비 단백질로 예측되었다. *gbpA*가 결여된 균주는 키틴에 결합하는 능력이 크게 감소하였다. 정제된 GbpA 단백질은 키틴과 키틴의 단량체인 *N*-acetylglucosamine에 결합할 수 있었다. 또한, type II 분비 시스템이 망가진 경우 GbpA를 분비하지 못하였다. 이를 통해 GbpA 단백질이 *N*-acetylglucosamine을 인지하여 키틴에 결합하는 단백질이며 type II 분비시스템에 의해 분비되는 분비 단백질임을 알 수 있었다. 뿐만 아니라 *gbpA*가 제대로 작용하지 못하게 되면 N-acetylglucosmaine을 탄수화물 성분으로 가지는 뮤신에 잘 결합하지 못하였으며 이는 외부에서 넣어준 GbpA에 의해 야생종 수준으로 다시 회복되었다. 그리고 gbpA가 결여된 균주는 뮤신을 생성하는 사람의 장 상피세포에 결합하는 능력이 크게 떨어졌다. 이러한 결과는 GbpA가 사람의 장내 뮤신에 결합하는 것을 도움으로써 패혈증 비브리오가 병을 일으키는 데 기여한다는 것을 말해준다.

*gbpA*의 전사체는 패혈증 비브리오의 성장과 밀접한 관련이 있다는 것이 관찰되었으며 이는 패혈증 비브리오의 균 수를 인지하여 *gbpA*의 전사 정도가 조절 받는다는 것을 암시했다. 그리고 qRT-PCR과 western blot의 결과는 전사 수준과 번역 수준 모두에서 IscR에 의해 *gbpA*의 발현이 증가하고 SmcR에 의해 감소한다는 것을 보여주었다. 또한 EMSA는 IscR과 SmcR이 직접적으로 *gbpA* 유전자 위쪽에 결합한다는 것을 알게 하였다. GbpA는 패혈증 비브리오 균이 키틴과 뮤신에 결합하기 위해 필수적이며, 여러 조절자에 의해 세밀하게 조절되는 병원 인자이다.