A Thesis for the Degree of Master of Science

Functional Analysis of HlyU, a Transcriptional Regulator of *Vibrio vulnificus*

패혈증 비브리오균의 전사조절자 HlyU 의 기능 분석

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석사학위논문

Functional Analysis of HlyU, a Transcriptional Regulator of *Vibrio vulnificus*

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이 논문을 석사학위논문으로 제출함

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Abstract

Vibrio vulnificus is an opportunistic pathogen capable of causing severe food-borne diseases to susceptible individuals with hematological disorders or immunocompromised states. This bacterium possesses several virulence factors including capsular polysaccharide, lipopolysaccharide, toxins, and proteins involved in iron acquisition, motility, attachment and adhesion. RtxA toxin is one of the major virulence factors that plays an important role in the pathogenesis of *V. vulnificus* by triggering cellular damages after host cell contact, which results in necrotic and apoptotic cell death. Expression of *rtxA* is up-regulated by HlyU, a transcriptional regulator that directly binds to the promoter region of the *rtxHCA* operon. To further examine the function of HlyU, the *hlyU* mutant was constructed and its virulence was evaluated. Compared with the wild type, the *hlyU* mutant showed significantly reduced virulence in mice and tissue culture, indicating HlyU is essential for the virulence of *V. vulnificus*. In a growth phase-dependent manner, HlyU appeared to up-regulate the expression of *rtxA*. To further identify the genes regulated by HlyU, transcriptomic profiles of the wild type and *hlyU* mutant were analyzed by RNA-sequencing. In addition to *rtxA*, genes encoding virulence factors such as hemolysin and phospholipase were down-regulated in the *hlyU* mutant, suggesting that HlyU contributes to the *V. vulnificus* pathogenesis by regulating various virulence genes. This study also revealed that the induction of *rtxA* was mediated by HlyU upon exposure to host cells or under anaerobic conditions, and it might be attributed to

altered activity rather than amounts of HlyU. A mutagenesis study proposed that Cysteine 30 is a critical residue of HlyU for *rtxA* activation. Taken together, HlyU might act as a regulator for the various virulence genes and induce *rtxA* under host environment by altering its activity.

Key words: *Vibrio vulnificus*, RtxA toxin, Transcriptional regulator, HlyU

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I. INTRODUCTION

Vibrio vulnificus, a Gram negative, motile, and curved-rod shaped bacteria, is the causative agent of food-borne disease such as gastroenteritis and life-threatening septicemia (Jones and Oliver, 2009). Infection with *V. vulnificus* may result from consumption of contaminated seafood or from exposure of wound skin to contaminated salt or brackish water (Horseman and Surani, 2011). Individuals with chronic liver disease, immunodeficiency, iron storage disorders, or diabetes mellitus are at high risk of infection. *V. vulnificus* infection can cause fever, chills, nausea, or the formation of skin lesions. With an average mortality rate exceeding 50%, this primary septicemia happens as a result of production of virulence factors, including capsular polysaccharide, lipopolysaccharide, toxins such as cytotoxin (RtxA), cytolytic hemolysin (VvhA), and metalloprotease (VvpE), and proteins involved in iron acquisition, motility, attachment, and adhesion (Jones and Oliver, 2009; Horseman and Surani, 2011).

The ability of *V. vulnificus* infecting a host is strongly linked to a production of RTX toxin and VvhA, which promotes rapid growth and dissemination of this pathogen to the bloodstream (Jeong and Satchell, 2012). RtxA, a member of the RTX (repeats in toxin) family of toxins, is produced after the contact host cells and triggers excessive production of reactive oxygen species (ROS) by the host and cellular changes including rearrangement of cytoskeletal structure, bleb formation, and aggregation of actin, thereby resulting in necrotic cell death and apoptosis (Lee *et al*., 2007; Kim *et*

al., 2008). By synergistically functioning with VvhA, RtxA plays multiple roles during infection including promotion of rapid growth *in vivo*, destruction of epithelial cells, and probably causing death of patients through tissue destruction in peripheral organs (Jeong and Satchell, 2012).

HlyU, a homolog of *V. cholerae* HlyU that up-regulates expression of the hemolysin (HlyA), belongs to the transcriptional regulators of SmtB/ArsR family (Williams *et al*., 1993). Known as a positive regulator of *rtxA* and *vvhA, V. vulnificus* HlyU was first identified as one of the genes induced *in vivo*, and mutation of *hlyU* exhibited the loss of cytotoxicity and an increased LD_{50} (Shao *et al*., 2011; Liu *et al*., 2007; Kim *et al*., 2003). HlyU activates the expression of *rtxHCA* operon at the transcriptional level by direct binding to the *rtxHCA* promoter region (Liu *et al*., 2009). The molecular mechanism of activation by HlyU is considered as de-repression of bound H-NS, a global transcriptional repressor in Gram-negative bacteria, from the region extending from upstream and downstream of the *rtxHCA* promoter with higher DNA binding affinity (Stoebel *et al*., 2008; Liu *et al*., 2009). However, the detailed mechanisms of *rtxA* regulation by HlyU is still unknown. Moreover, studies about how *V. vulnificus* expresses virulence factors in response to environmental cues, such as host signals, are very limited.

In this study, the functional characteristics of HlyU were investigated using genetic and biochemical analyses. The role of HlyU in the pathogenesis of *V. vulnificus*, especially in *rtxA* induction, was explored. Transcriptomic profiles were analyzed by RNA-sequencing to identify the genes differentially expressed between the wild type and the *hlyU* mutant. In addition, mediation by HlyU in *rtxA* induction was tested

under conditions which is similar to host environment. Furthermore, *hlyU* mutants with a single point mutation were constructed to elucidate the mechanism of HlyU, and an essential residue in function of HlyU was identified.

II. MATERIALS AND METHODS

Strains, plasmids, and culture conditions. The strains and plasmids used in this study are listed in Table 1. Unless noted otherwise, the *V. vulnificus* strains were grown aerobically in Luria-Bertani (LB) medium supplemented with 2.0% (*w*/*v*) NaCl (LBS) at 30℃ with shaking. When required, M9 (Sambrook and Russell, 2001) supplemented with glucose (0.4%, *w*/*v*) was used as a minimal medium. Anaerobic conditions were obtained by using an anaerobic chamber with an atmosphere of 90% N_2, 5% CO_2, and 5% H_2 (Coy Laboratory Products, Grass Lake, MI).

Generation and complementation of *hlyU* mutant. The *hlyU* gene was inactivated *in vitro* by deletion of the *hlyU* ORF (249 of 297 bp) using the PCR-mediated linker-scanning mutation method as described previously (Lim *et al.*, 2014). Pairs of primers HLYUF1-F and -R (for amplification of the 5′ amplicon) or HLYUF2-F and -R (for amplification of the 3′ amplicon) were designed (Table 2). The 249-bp deleted *hlyU* was amplified by PCR using the mixture of both amplicons as the template and HLYUF1-F and HLYUF2-R as primers. The resulting DNA fragment containing the deleted *hlyU* was ligated with *Spe*I-*Sph*I-digested pDM4 (Milton *et al*., 1996) to generate pZW1401 (Table 1). The *E. coli* S17-1 λ *pir, tra* strain (Simon *et al*., 1983) containing pZW1401 was used as a conjugal donor to *V. vulnificus* MO6-24/O to generate the *hlyU* mutant, ZW141 (Table 1). The conjugation and isolation of the transconjugants were conducted as described previously (Lim *et al*., 2014).

For complementation, a pair of primers HLYUC-F and –R was designed and used to amplify the *hlyU* ORF including its own promoter region (Table 2). The amplified *hlyU* ORF containing its own promoter region was cloned into a broad host-range vector pJH0311 (Goo *et al*., 2006) in the opposite direction of the Lac promoter, which is supposed to be regulated by its own promoter, and the resulting plasmid was named pZW1510 (Table 1). Then, pZW1510 was transferred into ZW141 by conjugation as described above.

Mouse mortality test. Mortalities of mice infected with the wild type and *hlyU* mutant strains were compared as described elsewhere (Lim and Choi, 2014). Groups of (n = 15) 7-week-old ICR female mice (specific-pathogen-free; Seoul National University) were starved without food and water for 24 hours until infection. The mice were injected intraperitoneally (i.p.) with 25 μg of iron dextran (Sigma)/g of body weight prior to inoculation of bacteria. Then, the mice were intragastrically (i.g.) administered with 50 μl of 8.5% (*w*/*v*) sodium bicarbonate solution, followed immediately with 100 μl of inoculum, representing approximately 10^7 cells of either the wild type or *hlyU* mutant strain suspended in PBS. Mouse mortalities were recorded for 24 hours. All manipulations of mice were approved by the Animal Care and Use Committee of Seoul National University.

Cytotoxicity assay. Cytotoxicity was evaluated by measuring cytoplasmic lactate dehydrogenase (LDH) activity that is released from the mucus-secreting human intestinal epithelial cells, HT-29 MTX (Lesuffleur *et al*., 1990, Jang, K. K., 2015

unpublished data). The HT-29 MTX cells were grown at 37 ℃ in 5% CO_2 in a 6McCoy's 5A medium supplemented with 10% (*v*/*v*) fetal bovine serum and antibiotics (10 units/ml penicillin G and 10 μg/ml streptomycin (Gibco-BRL, Gaithersburg, MD) in 96-well culture dishes (SPL) as described previously. Each well with 1×10^{6} cells was infected with the *V. vulnificus* strains at a various multiplicity of infection (MOI) for various incubation times. The LDH activity released into the supernatant was determined using a cytotoxicity detection kit (Roche, Mannheim, Germany).

RNA purification and transcript Analysis. Total cellular RNAs from the *V. vulnificus* strains were isolated using 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 *et al*., 2014). Relative expression levels of the specific transcripts were calculated by using the 16S rRNA expression level as the internal reference for normalization.

RNA sequencing and data analysis. Total RNAs from the wild type and *hlyU* mutant strains grown to exponential phase (A_{600} of 0.5) 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).

The procedures for a strand-specific cDNA library construction and RNA-sequencing were conducted by Chunlab. Briefly, mRNA was selectively enriched by depleting ribosomal RNAs by using Ribo-Zero™ rRNA Removal kit (Epicentre, Madison, WI). Enriched mRNA was subjected to the cDNA library construction using TruSeq Stranded mRNA Sample Preparation kit (Illumina, San Diego, CA) following manufacturer's instruction. The quality of cDNA libraries was evaluated as described above for the quality verification of total RNA, except that Agilent DNA 1000 Reagents (Agilent Technologies) was used. Strand-specific 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 isolated from two independent exponential phase cultures of *V. vulnificus*.

The reads obtained from RNA-sequencing were mapped to the *V. vulnificus* MO6-24/O reference genome (GenBank™ accession numbers CP002469 and CP002470, www.ncbi.nlm.nih.gov) using CLC Genomics Workbench 5.5.1 (CLC Bio, Aarhus, Denmark). The relative transcript abundance was measured by reads per kilobase of transcript per million mapped sequence reads (RPKM) (Mortazavi *et al.*, 2008). 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 in samples.

Western blot analysis. The purified His-tagged HlyU were used to raise rabbit polyclonal antibodies against HlyU (AB Frontier, Seoul, South Korea). *V. vulnificus* strains grown aerobically and anaerobically in M9 minimal media supplemented with glucose (0.4%, *w*/*v*) at 30 ℃ with shaking. The cells grown to A_{600} of 0.3 were harvested and lysed using complete lysis B buffer (Roche) for 5 min. Residual cell debris was removed by centrifugation (Kim *et al*., 2012) to isolate total proteins. Protein samples from the cell lysates, equivalent to 160 μg of total protein, were resolved by using SDS-PAGE. The resolved proteins were then transferred to a nitrocellulose membrane (Bio-rad) and probed with a 1:400 dilution of the anti-HlyU polyclonal antibodies. The bound antibodies were detected using goat anti-rabbit IgG conjugated with alkaline phosphatase (Sigma) and visualized by incubation with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Sigma) (Lim and Choi, 2014).

Site-directed mutagenesis of HlyU. Cysteine 30 (C30) or Cysteine 96 (C96) of HlyU was replaced with serine by using a QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies) (Kim *et al*., 2010). Methionine 87 (M87) of HlyU was replaced with leucine in the same way as above. The complementary mutagenic primers were listed in Table 2 and used in conjunction with pZW1516 (as a template DNA) to create pZW1516-C30S, pZW1516-M87L, and pZW1516-C96S (Table 1). The C30S, M87L, and C96S mutant *hlyU* genes were then subcloned into the *Apa*I and *Sac*I sites of the pDM4 to yield pZW1520 (for HlyU-C30S), pZW1521 (for

HlyU-M87L), and pZW1522 (for HlyU-C96S), respectively (Table 1). The mutations were confirmed by DNA sequencing.

Construction of *hlyU* mutants with a single point mutation. To construct the *hlyU* mutants with a single point mutation, pZW1519 was generated using the PCR-mediated linker-scanning mutation method as described above. Pairs of primers hlyU_chromo-F1 and –R1 (for amplification of the 5′ amplicon) or hlyU_chromo-F2 and –R2 (for amplification of the 3′ amplicon) were designed (Table 2). Both amplicons contain a *Bam*HI restriction sites. The 822-bp deleted *hlyU* was amplified by PCR using the mixture of both amplicons as the template and hlyU_chromo-F1 and hlyU_chromo-R2 as primers. The 0.8-kb PCR product was cloned into pGEM-T easy vector and 1.2-kb *nptI* DNA conferring resistance to kanamycin (Oka *et al*., 1981) was inserted into a unique *Bam*HI site present within the deleted *hlyU* ORF to result in Δ*hlyU::nptI*. The 2-kb Δ*hlyU::nptI* cartridge was then ligated with *Spe*I-*Sph*I digested pDM4 to form pZW1519 (Table 1).

To generate the Δ*hlyU::nptI* mutant, ZW151, by homologous recombination, *E. coli* S17-1 λ *pir*, *tra* (containing pZW1519) was used as a conjugal donor to *V. vulnificus* MO6-24/O. The conjugation and isolation of the transconjugants were achieved as described above. Subsequently, *E. coli* S17-1 λ *pir*, *tra* harboring pZW1520 was mated with ZW151 to generate *hlyU* C30S mutant, ZW152. Similarly, *E. coli* S17-1 λ *pir*, *tra* containing pZW1521 and pZW1522, respectively, was used as a conjugal donor in conjunction with ZW151 to generate the *hlyU* M87L mutant, ZW153 and *hlyU* C96S mutant, ZW154 (Table 1).

Data analyses and statistics. Mean values and standard deviations (SD) of results were calculated from at least three independent experiments. Mouse mortality was evaluated using a log rank test program (http://bioinf.wehi.edu.au/software/russell/logrank/). All other data were analyzed by Student's *t* tests with the SAS program (SAS software, SAS Institute, Inc.). Significance of differences between experimental groups was accepted at a *P*-value of 0.05.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics [a]	Reference or source
Bacterial strains		
V. vulnificus		
MO6-24/O	wild type *V. vulnificus*, virulent	Laboratory collection
ZW141	MO6-24/O with Δ*hlyU*	This study
ZW151	MO6-24/O with Δ*hlyU*::*nptI*; Km^r	This study
ZW152	MO6-24/O with *hlyU* C30S[b]	This study
ZW153	MO6-24/O with *hlyU* M87L[b]	This study
ZW154	MO6-24/O with *hlyU* C96S[b]	This study
E. coli		
DH5α	*supE44 DlacU169 (f80 lacZ D*M15*) hsdR17 recA1 endA1 gyrA96 relA1 relA1 thi-1 relA1*; plasmid replication	Laboratory collection
S17-1 λ *pir*	Tc::Mu-Km::Tn7;Tp^r Sm^r; host for π-requiring plasmids; conjugal donor	Laboratory collection
Plasmids		
pGEM-T easy	PCR product cloning vector; Ap^r	Promega
pDM4	suicide vector; *ori* R6K; Cm^r	Milton *et al.*, 1996
pZW1401	pDM4 with Δ*hlyU*; Cm^r	This study
pJH0311	0.3-kb MCS of pUC19 cloned into pCOS5; Ap^r, Cm^r	Goo *et al.*, 2006
pZW1510	pJH0311 with *hlyU*, Ap^r, Cm^r	This study
pZW1516	pGEM-T easy with *hlyU* gene ORF; Ap^r	This study
pZW1516-C30S	pGEM-T easy with *hlyU* C30S; Ap^r	This study
pZW1516-M87L	pGEM-T easy with *hlyU* M87L; Ap^r	This study
pZW1516-C96S	pGEM-T easy with *hlyU* C96S; Ap^r	This study
pZW1519	pDM4 with *hlyU*::*nptI*; Cm^r, Km^r	This study
pZW1520	pDM4 with *hlyU* C30S; Cm^r	This study
pZW1521	pDM4 with *hlyU* M87L; Cm^r	This study
pZW1522	pDM4 with *hlyU* C96S; Cm^r	This study

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

[b] First letter is an amino acid to be substituted, number is the position of mutation and the last letter is new generated amino acid.

Table 2. Oligonucleotides used in this study

Name	Oligonucleotide sequences (5′→ 3′)[a]	Use
For mutant construction		
HLYUF1-F	CTTGTTTTGGTCGCGATGG	Construction of *hlyU* deletion mutant
HLYUF1-R	CAGAATTCTTTATTGCGAAGAATAATGC	
HLYUF2-F	AAAGAATTCTGCTCCATATCTTTTAAGTTC	
HLYUF2-R	CGACACAAAGTCCCGGCC	
hlyU_chromo_F1	CAAATAACAAATCTGCTAAGAAGCGCG	Construction of *hlyU* mutants with a single point mutation
hlyU_chromo_R1	AGAGGATCCGTGCCGTGTAGAAAAG	
hlyU_chromo_F2	CACGGATCCTCTAGCTACAACTCACATG	
hlyU_chromo_R2	GCAGAAAAAACTGTTGATTCCTGGGAG	
For amplification of gene ORF		
HLYUC-F	GGTACCTCTCAAAGAAGAGTATTACGTG	Amplification of *hlyU* ORF
HLYUC-R	GAGCTCTTACACCGATCCTAGACC	
For site-directed mutagenesis		
HlyUC30S-F	GACGCCTGCAAATCTTATCCATGCTACACAATCAAGAG	Construction of *hlyU* C30S mutant
HlyUC30S-R	CTCTTGATTGTGTAGCATGGATAAGATTTGCAGGCGTC	
HlyUM87L-F	GTGAAGAAGTAAAAGCATTGATTAAACTGCTGCACAGTC	Construction of *hlyU* M87L mutant
HlyUM87L-R	GACTGTGCAGCAGTTTAATCAATGCTTTTACTTCTTCAC	
HlyUC96S-F	GCACAGTCTTTATTCCGAAGAATAATGCTTTTGCGTGCC	Construction of *hlyU* C96S mutant
HlyUC96S-R	GGCACGCAAAAGCATTATTCTTCGGAATAAAGACTGTGC	

For qRT-PCR		
HLYU-qRT-F	CATGGCCAATGAAAGACGCC	qRT-PCR of *hlyU*
HLYU-qRT-R	ACCATGCCAGATGCTGAGAC	
RTXA-qRT-F	TAGCGGCGACAATGAAACCT	qRT-PCR of *rtxA*
RTXA-qRT-R	CCCATCACCGCAAGGGTATT	

[a] The oligonucleotides were designed using the *V. vulnificus* MO6-24/O genomic sequence (GenBank™ accession number CP002469 and CP002470).

III. RESULTS

HlyU is essential for virulence of *V. vulnificus*.

To examine the role of HlyU in the pathogenesis of *V. vulnificus*, iron dextran-treated mice were infected intragastrically with the wild type and *hlyU* mutant strains, and the numbers of dead mice were counted. Because *V. vulnificus* is an opportunistic pathogen capable of causing severe to life-threatening infection in susceptible individuals, especially with elevated iron levels, iron dextran-treated mice is considered as a useful model for studying systemic disease caused by *V. vulnificus* (Starks *et al.*, 2000; Horseman and surani, 2011). As shown in Fig. 1, the death of mice infected with the *hlyU* mutant was significantly delayed and inhibited, while more than 50 % of mice infected with the parental wild-type strain were dead by this time point ($P < 0.05$, log rank test).

In order to investigate the cytotoxicity of the *hlyU* mutant when cultured with the human intestinal epithelial cells where the infection of *V. vulnificus* mainly occurs, LDH release assay was performed using the mucus-secreting human intestinal epithelial cells, HT-29 MTX (Lesuffleur *et* al., 1990; Lee *et al.*, 2008). HT-29 MTX cells were infected with the wild type, the *hlyU* mutant, and the complemented strains, and the activities of LDH released from the HT-29 MTX cells were compared at various MOIs for 2 hours and at various incubation times at an MOI of 50 (Fig. 2). The *hlyU* mutant exhibited significantly lower LDH-releasing activity than the wild type. Also, complementation of the *hlyU* gene in the *hlyU* mutant with a functional

hlyU gene (pZW1510) restored the LDH-releasing activity to levels comparable to the wild-type level (Fig. 2). The combined results suggested that HlyU is essential in the pathogenesis of *V. vulnificus* in mice and tissue culture.

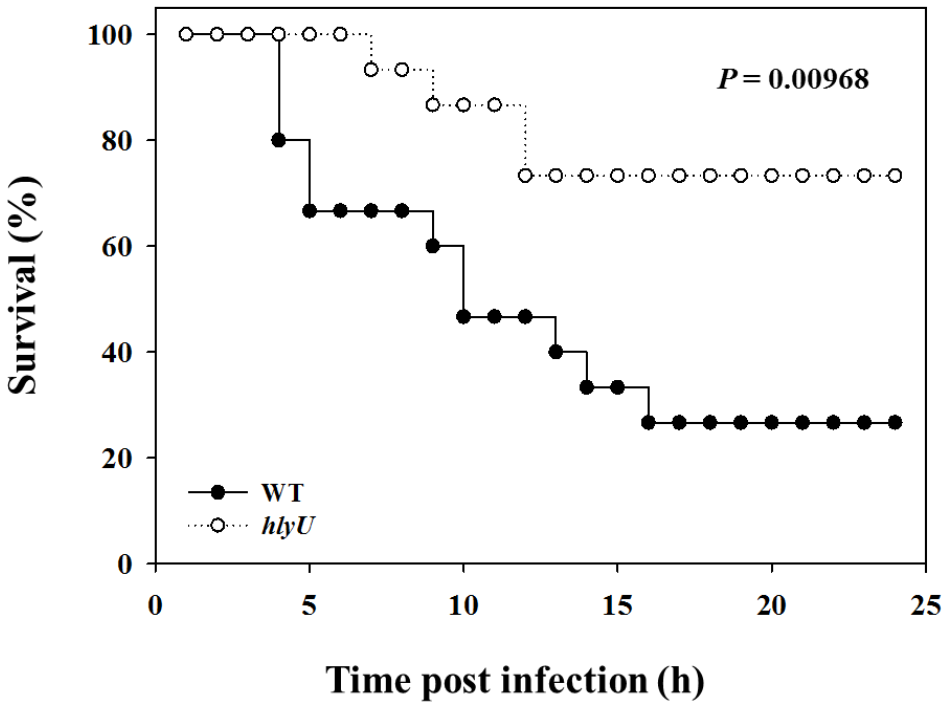


Fig. 1. Mortality of the *V. vulnificus* strains for mice. Seven-week-old specific-pathogen-free female ICR mice (n = 15) were intraperitoneally injected with 25 μg of iron dextran/g body weight and intragastrically infected with the wild type or the *hlyU* mutant at doses of 10^7 CFU. Mouse survival was monitored for 24 h. The data were analyzed by using a log rank test. WT, wild type; *hlyU*, *hlyU* mutant.

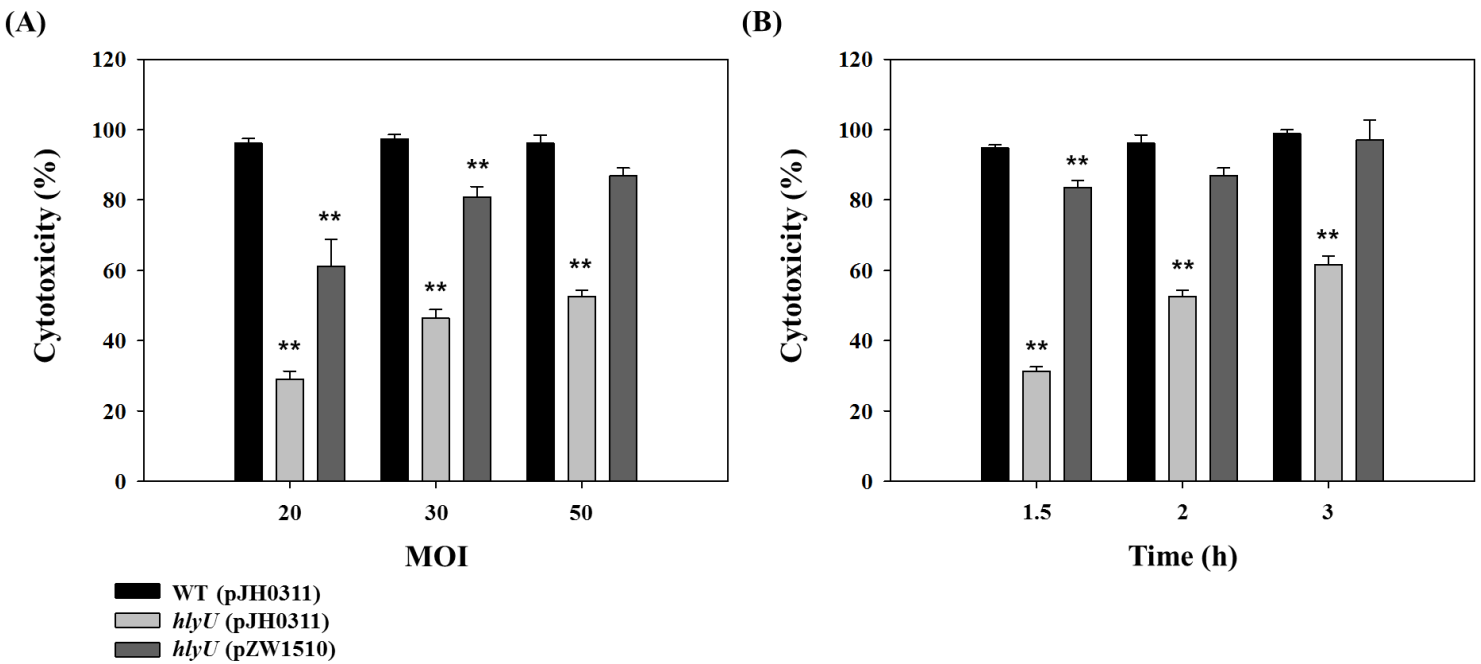


Fig. 2. Effect of *hlyU* mutation on virulence of *V. vulnificus* toward HT-29 MTX cells. HT-29 MTX cells were infected with the wild type or the *hlyU* mutant at various MOIs for 2 h (A) and at an MOI of 50 for various incubation times (B). The cell cytotoxicity was determined by an LDH release assay. Error bars represent the SD. ** $P < 0.005$ relative to groups infected with the wild type at each condition. WT (pJH0311), wild type; *hlyU* (pJH0311), *hlyU* mutant; *hlyU* (pZW1510), complemented strain.

Growth phase-dependent expression of *hlyU*.

It was previously described that the activity of *rtxHCA* is maximum in mid-exponential phase, and decreased in stationary phase (Park *et al*., 2012). To examine the regulation by HlyU on the expression of *rtxA*, the relative revels of *hlyU* expression were analyzed at various growth stages (Figs. 3A and B). The *hlyU* transcript gradually increased as the cell grows, reached a maximum in mid-exponential phase, and then decreased in stationary phase. These results is consistent with the previous results, suggesting that HlyU might act as a positive regulator for *rtxA* expression in a growth phase-dependent manner.

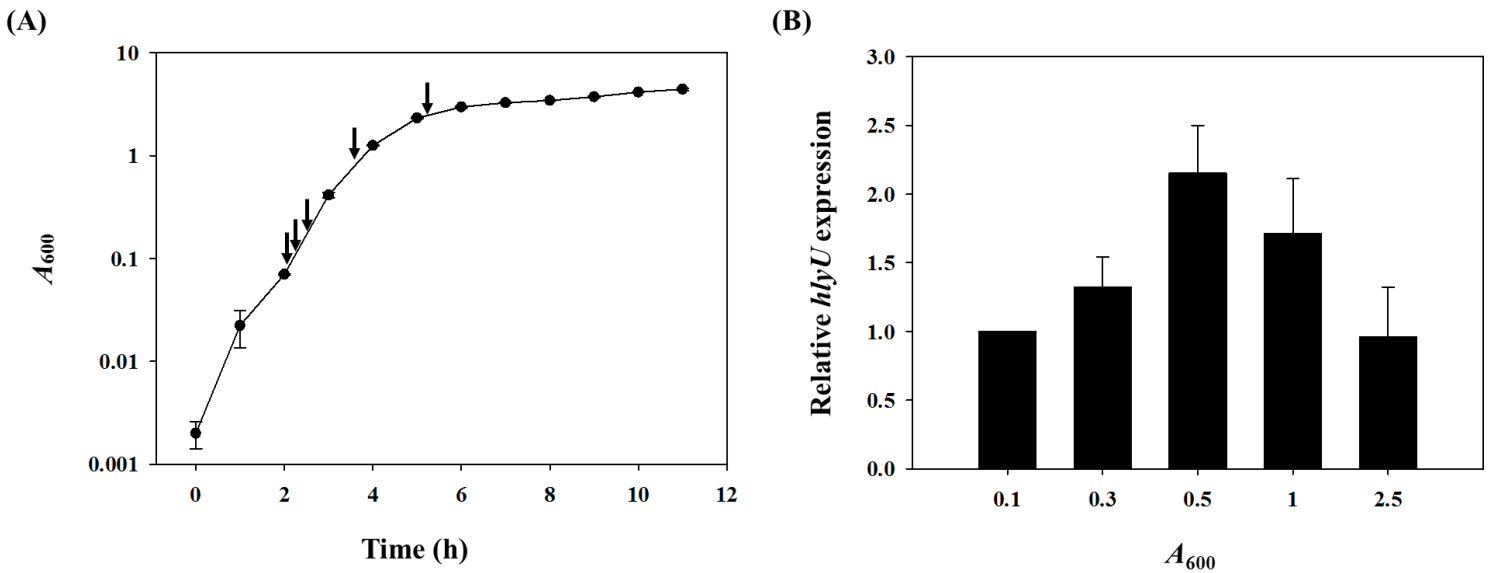


Fig. 3. Growth kinetics of *V. vulnificus* and growth-phase dependent expression of *hlyU*. (A) Growth of the wild type culture in LBS was monitored spectrophotometrically at 600 nm (A_{600}) and total RNAs were isolated from cells harvested at different growth phases (from left, at A_{600} of 0.1, 0.3, 0.5, 1.0, and 2.5) as indicated by arrows. (B) The *hlyU* mRNA levels were determined by qRT-PCR analyses, and the expression levels of *hlyU* at A_{600} of 0.1 was set as 1. Error bars represent the SD.

Identification of differentially expressed genes in the *hlyU* mutant.

Mouse mortality and cytotoxicity tests revealed that HlyU is essential for the virulence of *V. vulnificus*. To further identify the genes regulated by HlyU, transcriptomic profiles of the wild type and *hlyU* mutant were analyzed by RNA-sequencing, and differentially expressed genes in the *hlyU* mutant were identified. Average RPKM values from the biological duplicate samples were used to represent the expression level of each gene. The Volcano plot showed that genes are differentially expressed with significance (P-value<0.05, 2 fold threshold) (Fig. 4). A total of 30 genes were identified to be differentially expressed in the *hlyU* mutant; 13 up-regulated and 17 down-regulated. The differentially expressed genes were clustered into functionally related groups using the Cluster of Orthologous Groups (COG) database for the *V. vulnificus* MO6-24/O genome (http://www.ncbi.nlm.nih.gov/COG/), which showed that the genes with various functions were differentially expressed and listed in Tables 3 and 4. In addition to *rtxA* and *vvhA*, which encode RtxA toxin and cytolysin, respectively, genes encoding virulence factors such as thermolabile hemolysin and protease-related protein were down-regulated in the *hlyU* mutant, implying that HlyU contributes to the *V. vulnificus* pathogenesis by regulating various virulence genes (Kim *et al*., 2003; Kim *et al*., 2008; Liu *et al*., 2012).

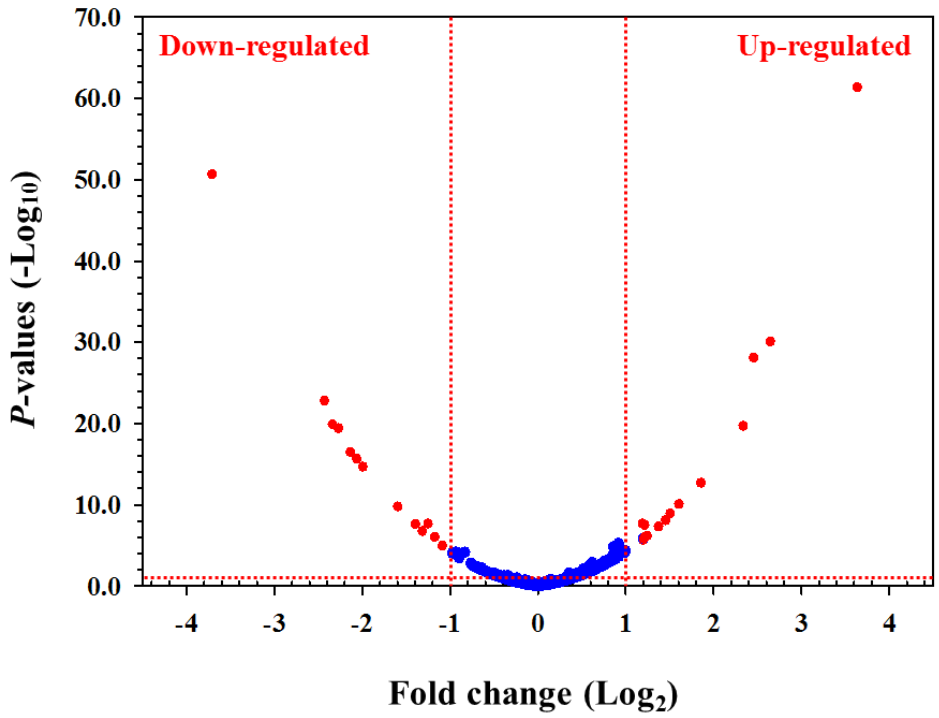


Fig. 4. Transcriptome comparisons of the wild type and *hlyU* mutant. Volcano-plot of genes differentially expressed between the wild type and *hlyU* mutant were generated. Numbers on the X- and Y-axis represent the fold change (Log_2) and *P*-value ($-Log_{10}$). Red dots represent the differentially expressed genes.

Table 3. List of genes up-regulated by HlyU

Locus tag [a]	Gene product	Fold change (Log_2)	*P*-value [b] (<0.05)
Transcription			
VVMO6_02507	transcriptional activator HlyU	**-3.70762**	**0**
Virulence			
VVMO6_03257	thermolabile hemolysin	**-1.08688**	**0.00001**
VVMO6_03300	protease-related protein	**-1.25019**	**0**
VVMO6_03880	cytolysin secretion protein VvhB	**-1.39441**	**0**
VVMO6_03881	cytolysin VvhA	**-1.31537**	**0**
VVMO6_03947	autotransporter adhesion RtxA	**-2.4292**	**0**
VVMO6_03948	cytolysin-activating lysine-acyltransferase RtxC	**-2.33751**	**0**
VVMO6_03949	hypothetical protein	**-2.1349**	**0**
VVMO6_03950	RTX toxin transporter	**-2.06135**	**0**
VVMO6_03951	RTX toxin transporter	**-2.27012**	**0**
VVMO6_03952	RTX toxin transporter	**-1.99169**	**0**
Coenzyme metabolism			
VVMO6_02720	sulfate adenylyltransferase subunit 2	**-1.17357**	**0**
Function unknown			
VVMO6_04436	hypothetical protein	**-1.59459**	**0**

[a] Locus tag numbers, functional categories, and annotation of gene products are based on the database for the *V. vulnificus* MO6-24/O genome, which was retrieved from GenBank™ (Accession number CP002469 and CP002470). Functional categories in boldface are shown above the first gene in each category.

[b] The *P*-values less than six decimal places were denoted as zero.

Table 4. List of genes down-regulated by HlyU

Locus tag [a]	Gene product	Fold change (Log_2)	*P*-value [b] (<0.05)
Defense			
VVMO6_00539	multidrug ABC transporter ATPase	**1.19479**	**0**
VVMO6_00540	multidrug ABC transporter permease	**1.21836**	**0**
VVMO6_03818	acriflavin resistance protein	**2.46004**	**0**
Adherence			
VVMO6_01209	pilus assembly protein CpaE-like protein	**1.20244**	**0**
VVMO6_01210	flp pilus assembly protein TadA	**1.22918**	**0**
VVMO6_01211	Flp pilus assembly protein TadB	**1.37507**	**0**
VVMO6_01212	flp pilus assembly protein TadC	**1.24781**	**0**
VVMO6_01213	flp pilus assembly protein TadD, contains TPR repeat	**1.20487**	**0**
Cell wall/membrane/envelope biogenesis			
VVMO6_03819	membrane-fusion protein	**2.64761**	**0**
Transcription			
VVMO6_03822	ArsR family transcripitonal regulator	**1.50756**	**0**
Inorganic ion transport and metabolism			
VVMO6_03384	pyridoxamine 5'-phosphate oxidase-related heme iron utilization protein	**1.20005**	**0**
Others			
VVMO6_03820	transporter component	**1.85977**	**0**
VVMO6_03823	rhodanese-related sulfurtransferase	**1.60751**	**0**

VVMO6_03824	coA-disulfide reductase	**3.63729**	**0**
Function unknown			
VVMO6_01214	hypothetical protein	**1.45776**	**0**
VVMO6_01216	hypothetical protein	**1.19746**	**0**
VVMO6_03821	hypothetical protein	**2.3385**	**0**

[a] Locus tag numbers, functional categories, and annotation of gene products are based on the database for the *V. vulnificus* MO6-24/O genome, which was retrieved from GenBank™ (Accession number CP002469 and CP002470). Functional categories in boldface are shown above the first gene in each category.

[b] The *P*-values less than six decimal places were denoted as zero.

HlyU mediates the induction of *rtxA* upon exposure to host cells.

Previous studies reported that the contact of *V. vulnificus* with host cells is a prerequisite to the cytotoxicity of the bacteria, and increases the expression of *rtxA* in a time-dependent manner (Kim *et al*., 2008). To further elucidate the effect of HlyU on the host cell-induction of *rtxA*, the wild type and the *hlyU* mutant were exposed to the INT-407 human epithelial cells, and the levels of *rtxA* expression were compared (Fig. 5A). The exposure to the INT-407 cells induced the *rtxA* expression in the wild type, but not in the *hlyU* mutant, indicating that the induction of *rtxA* by host cells is mediated by HlyU. Interestingly, the levels of *hlyU* transcript does not exhibit a significant change after exposure to host cells, suggesting that the induction of *rtxA* might be attributed to an increase of transcriptional activity of HlyU, rather than an increase of the *hlyU* transcript (Fig. 5B).

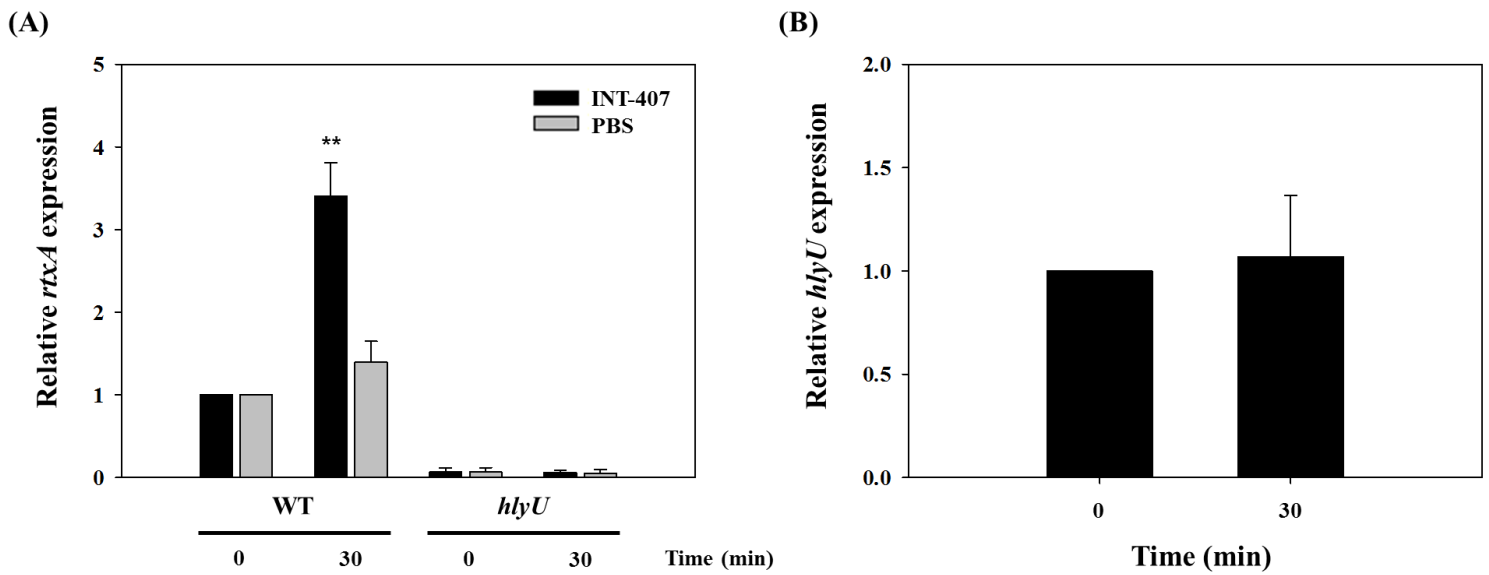
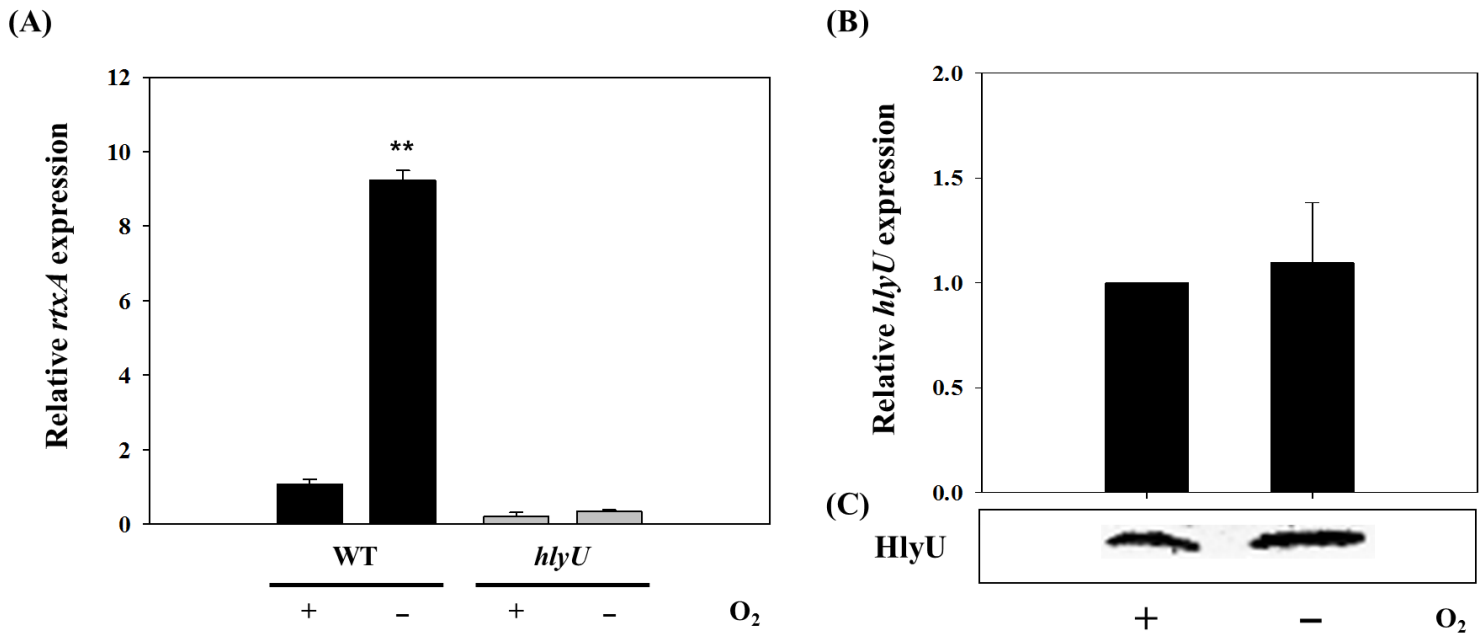


Fig. 5. Induction of *rtxA* upon exposure to INT-407 host cells is mediated by HlyU. Wild type and *hlyU* mutant were exposed to the INT-407 human epithelial cells for 30 min. The relative levels of *rtxA* (A) and *hlyU* (B) expression were determined by qRT-PCR analyses, and the expression levels of *hlyU* and *rtxA* in the wild type at 0 min was set as 1. Error bars represent the SD. **, $P < 0.005$ relative to the cells of each strain unexposed to host cells. WT, wild type; *hlyU*, *hlyU* mutant.

Anaerobiosis-induced *rtxA* expression is mediated by HlyU.

HlyU-mediated *rtxA* expression is induced when *V. vulnificus* is exposed to host cells (Fig. 5A). Considering that *V. vulnificus* infection and most severe tissue necrosis occurs in the intestine, which is thought be primarily anaerobic, it was hypothesized that *rtxA* expression is highly induced in oxygen-limiting conditions (Chen *et al.*, 2002; Lee *et al.*, 2008; Eckburg *et al.*, 2005). To test this hypothesis, levels of the *rtxA* mRNA of the wild type and the *hlyU* mutant grown under aerobic and anaerobic conditions were measured by qRT-PCR analyses. The level of *rtxA* in the wild type grown under anaerobic conditions increased and was almost 9-fold greater compared with that reached by the bacteria grown aerobically (Fig. 6A). In contrast, this increase of the *rtxA* transcript under anaerobic conditions was not apparent in the *hlyU* mutant, indicating that *rtxA* expression is induced by HlyU under anaerobic conditions. Surprisingly, the *hlyU* transcript and cellular level of HlyU determined by Western blot analysis were not significantly different between aerobic and anaerobic conditions (Fig. 6B and C). The combined results proposed that the activation of *rtxA* might be due to the increased transcriptional activity of HlyU, rather than increased amounts of HlyU.

Fig. 6. Expression of *rtxA* and *hlyU* under aerobic and anaerobic conditions. Total RNAs and proteins were isolated from the wild type and the *hlyU* mutant grown to A_{600} of 0.3 in M9 minimal medium under aerobic and anaerobic conditions, respectively. The relative levels of *rtxA* (A) and *hlyU* (B) expression were determined by qRT-PCR analyses, and the expression level of the wild type under aerobic condition was set as 1. (C) Protein samples were resolved by SDS-PAGE, and HlyU was detected by Western blot analysis using a rabbit anti-HlyU antiserum. Error bars represent the SD. **, $P < 0.005$ relative to the strains grown aerobically. WT, wild type; *hlyU*, *hlyU* mutant.


(A)
Relative rtxA expression
12
10
8
6
4
2
0
**
WT
hlyU
+
–
+
–
O2
(B)
Relative hlyU expression
2.0
1.5
1.0
0.5
0.0
(C)
HlyU
+
–
O2

Construction of *hlyU* mutants with a single point mutation.

It was found that HlyU up-regulates the expression of *rtxA* when exposed to host cells and under anaerobic conditions, respectively. One possible hypothesis to explain these results is that the transcriptional activity of HlyU may change in response to host cells or oxygen-limiting conditions. To further elucidate the mechanism of HlyU in *rtxA* regulation, loss-of-function mutations were introduced to the residues of HlyU considered to affect the transcriptional activity of HlyU.

Because cysteine, methionine, and histidine are redox-sensitive amino acid side chains in proteins, Cysteine 30 and Cysteine 96 of HlyU were replaced with serine, respectively, by site-directed mutagenesis (Kumsta and Jakob, 2009). Conservation of Cys residues of HlyU in *Vibrio* species also suggests the possibility of Cys residues as a redox switch (Mukherjee *et al*., 2015). Considering that the dimerization of HlyU is required for DNA binding, mutation of Met87 residue located at the dimeric surface of HlyU may alter the transcriptional activity of HlyU by inhibiting HlyU dimerization (Mukherjee *et al*., 2015, Nishi *et al*., 2010). In the same way, Methionine 87 was replaced with leucine.

As shown in Fig. 8A, the *hlyU* mutant carrying *nptI* encoding kanamycin resistant gene, ZW151, was constructed by allelic exchange first. The PCR analysis of the genomic DNA from *V. vulnificus* MO6-24/O with the primers produced a 1.1-kb fragment, but the genomic DNA from ZW151 resulted in an amplified DNA fragment approximately 2-kb in length (Fig. 8B). Sequentially, *hlyU* mutants with a single point mutation were constructed in the same way and selected by kanamycin resistance, and the mutations were confirmed by DNA sequencing (Fig. 8C).

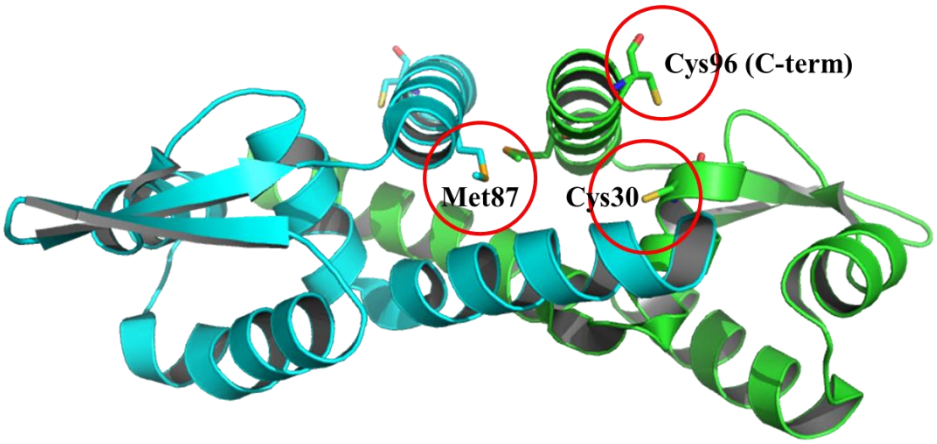


Fig. 7. Structure of HlyU dimer and position of the target residues for mutation in HlyU. Cys30, Cysteine 30; Met87, Methionine 87; Cys96; Cysteine 96.

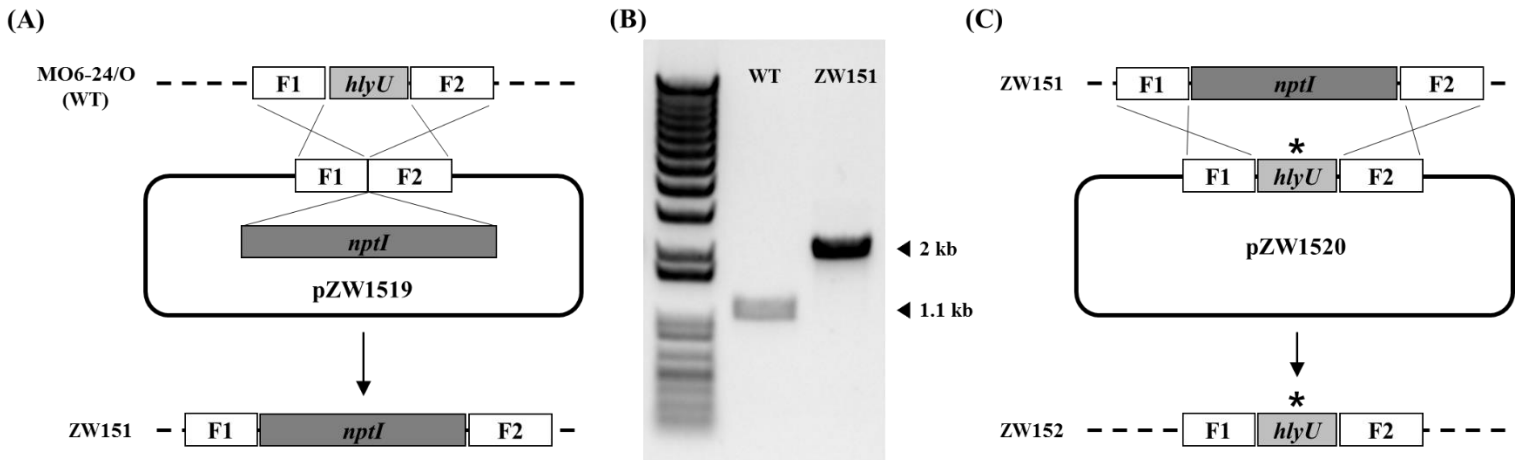


Fig. 8. Construction of *hlyU* mutants with a single point mutation. (A) Homologous recombination between the flanking regions of *hlyU* gene from wild type MO6-24/O and pZW1519 resulted in generation of *hlyU*::*nptI* mutant, ZW151. (B) The PCR analysis of the genomic DNA from *V. vulnificus* MO6-24/O and ZW151 produced a 1.1-kb and 2-kb fragments, respectively. (C) Homologous recombination between ZW151 and pZW1520 resulted in construction of HlyU C30S mutant, ZW152. *Dashed lines*, chromosomal DNA; *solid line*, plasmid DNA; *open boxes*, the flanking regions of *hlyU; gray boxes*, the target *hlyU* gene; *dark gray boxes*, the *nptI* gene; *, point mutation; ×, crossover.

Cysteine 30 is a critical residue for *rtxA* activation.

In order to investigate the effect of point mutations in HlyU, the levels of *rtxA* in the *V. vulnificus* strains grown under aerobic and anaerobic conditions were measured by qRT-PCR analyses. The C30S mutant almost abolished the transcription of *rtxA*, while the M87L and C96S mutants still showed partial expression of *rtxA* (Fig. 9). To further verify that the decreased *rtxA* level affects virulence of *V. vulnificus*, LDH release assay was performed using the HT-29 MTX cells. The host cells were infected with the wild type, *hlyU* mutant, and the C30S mutant, and the activities of LDH released from the HT-29 MTX cells were compared at various MOIs for 2 hours and at various incubation times at an MOI of 50 (Fig. 10). Consistently, LDH-releasing activity of the C30S mutant was significantly lower than the wild type when the multiplicity of infection (MOI) was up to 50, indicating that C30S mutation resulted in lower *rtxA* activation in tissue culture. Taken together, the results suggested that Cys30 is a critical residue of HlyU for the transcriptional activation of *rtxA*.

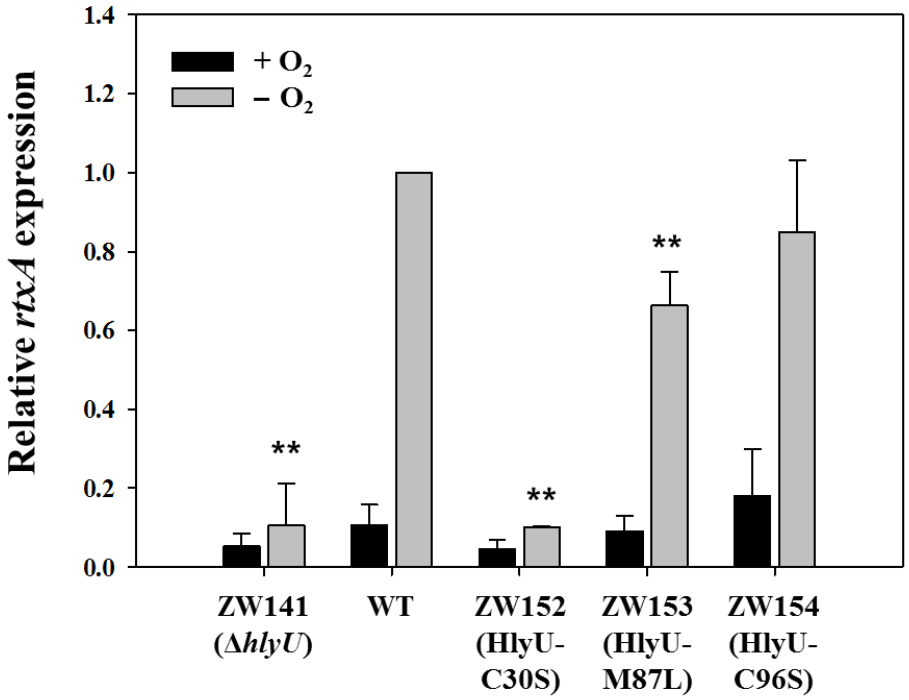


Fig. 9. Effects of HlyU point mutation on *rtxA* expression under aerobic and anaerobic conditions. Total RNAs were isolated from the *V. vulnificus* strains grown to A_{600} of 0.3 in M9 minimal medium under aerobic and anaerobic conditions, respectively. The relative levels of *rtxA* expression were determined by qRT-PCR analyses, and the expression level of the wild type under anaerobic condition was set as 1. Error bars represent the SD. **, $P < 0.005$ relative to the wild-type cells grown in each condition. ZW141, *hlyU* mutant; WT, wild type; ZW152, HlyU C30S mutant; ZW153, HlyU M87L mutant; ZW154, HlyU C96S mutant.

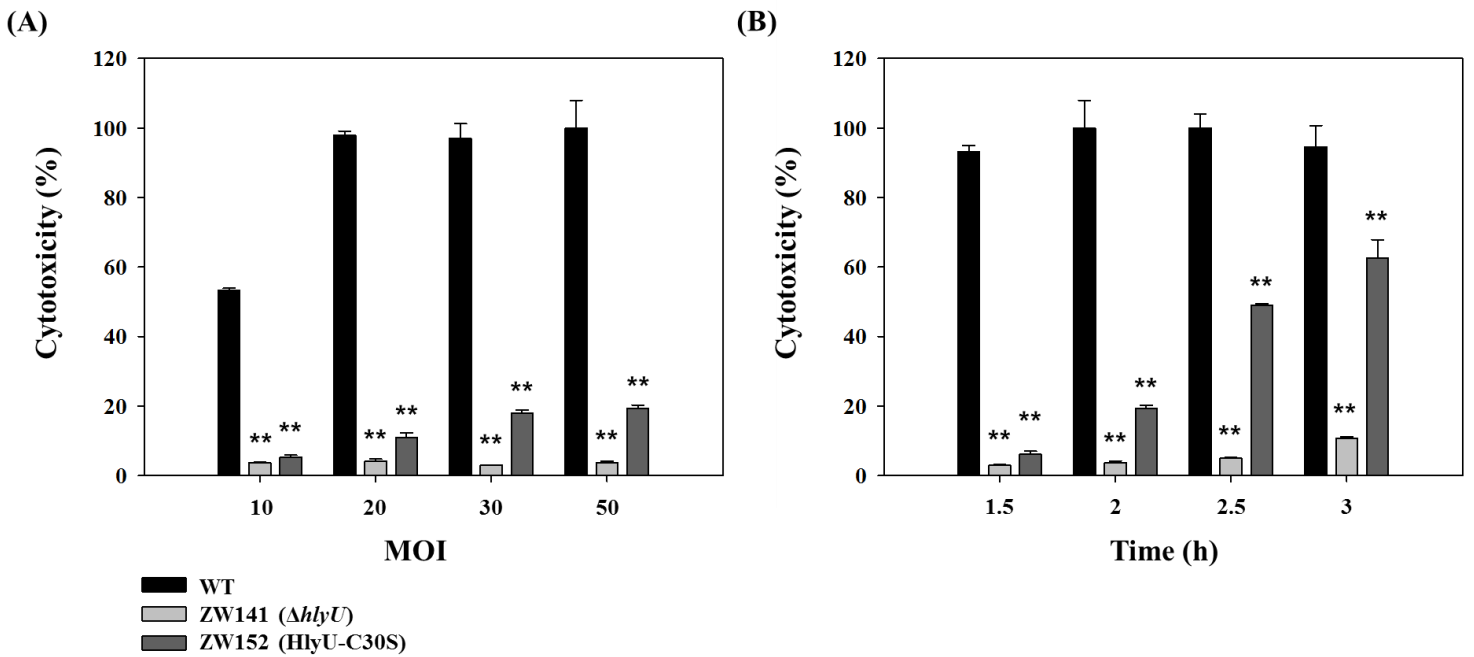


Fig. 10. Effects of HlyU C30S mutation on virulence of *V. vulnificus* toward HT-29 MTX cells. HT-29 MTX cells were infected with the *V. vulnificus* strains at various MOIs for 2 h (A) and at an MOI of 50 for various incubation times (B). The cell cytotoxicity was determined by an LDH release assay. Error bars represent the SD. **, $P < 0.005$ relative to groups infected with the wild type at each condition. WT, wild type; ZW141, *hlyU* mutant; ZW152, HlyU C30S mutant.

IV. Discussion

Pathogenic bacteria has evolved sophisticated regulatory systems to cooperatively control their virulence gene expression, as a result of co-evolution between bacteria and their hosts (Cotter and DiRita, 2000). To successfully infect a host, they must alter their phenotypic properties to survive and multiply in the host environment (Liu *et al.*, 2011). Expression of virulence factors is coordinately regulated by global regulatory proteins in response to environmental changes, contributing to the pathogenesis of bacteria during infection (Cotter and DiRita, 2000; Matson *et al*., 2007).

HlyU is a transcriptional regulator known to activate the expression of two cytotoxins, VvhA and RtxA, in the human pathogen *V. vulnificus* (Shao *et al*., 2011; Liu *et al*., 2007). RtxA is one of the major virulence factors that contributes to the pathogenesis of *V. vulnificus* and triggers cellular damages after host cell contact, which leads to necrotic and apoptotic cell death. As shown in Fig. 3, HlyU appears to activate *rtxA* expression in a growth phase-dependent manner, however, studies about the mechanisms of *rtxA* regulation by HlyU are very limited. Although the contact with host cells was found as a prerequisite to induce *rtxA* expression in *V. vulnificus*, the correlation of the host-cell exposure with *rtxA* regulation by HlyU is still unknown (Kim *et al*., 2008).

In this study, it was demonstrated that the induction of *rtxA* upon exposure to host cells was mediated by HlyU (Fig. 5A). Similarly, HlyU mediates the increase of *rtxA*

level under anaerobic conditions, a similar condition to the human intestines, compared to that under aerobic conditions (Fig. 6A) (Eckburg *et al*., 2005). It is worth mentioning that the *hlyU* transcript did not change in response to such conditions (Figs. 5B and 6B). The cellular level of HlyU also exhibited no difference under both aerobic and anaerobic conditions (Fig. 6C). These results proposed a novel mechanism of *rtxA* regulation, which is that the expression of *rtxA*, when *V. vulnificus* was exposed to host cells or under anaerobic conditions, might be induced by altered activity, rather than amounts of HlyU. In addition, mutation of *hlyU* significantly reduced the virulence of *V. vulnificus* in mice and tissue culture (Figs. 1 and 2). Taken together, HlyU might act as an important regulator involved in virulence gene expression with recognition of the host environment, contributing to virulence of *V. vulnificus* during infection.

In pathogenic bacteria, virulence genes are usually expressed conditionally in certain conditions, such as in response to specific stimuli. For example, *V. cholerae* virulence genes are expressed under oxygen-limiting conditions, an environment similar to the human intestines, using a thiol-based switch mechanism (Liu *et al*., 2011). In this study, mutations on specific residues of HlyU revealed that Cys 30 is a critical residue for the expression of *rtxA* (Fig. 9). *V. vulnificus* expressing HlyU-C30S showed lower LDH-releasing activity in the HT-29 MTX cells than the wild type (Fig. 10). Based on the conservation of Cys residues in HlyU in *Vibrio* species, the probable role of Cys residues was proposed in terms of that HlyU may serve as a redox switch (Mukherjee *et al*., 2015). Thus, one possible explanation for these results is that the reduced level of *rtxA* and decreased cytotoxicity might be attributed to such redox

role of Cys30, probably resulting from a decreased transcriptional activity of HlyU. Induction of *rtxA* by HlyU upon exposure to host cells or anaerobic conditions supported the possibility of HlyU as a redox switch.

Furthermore, this study demonstrated that *V. vulnificus* HlyU might be a key regulator associated with transcription of several virulence factors (Fig. 4 and Table 3). As expected, *vvhA* and *rtxA*, encoding hemolysin and cytotoxin, respectively, were down-regulated in the *hlyU* mutant. Thermolabile hemolysin (Tlh), a homolog of *V. parahaemolyticus* Tlh which is a major pathogenic factor phospholipase A2, was also down-regulated in the *hlyU* mutant (Wang *et al*., 2012; Gutierrez *et al*., 2013). In addition to these virulence genes, the transcriptome analysis revealed that genes involved in metabolism, transcription, and adherence were also regulated by HlyU (Tables 3 and 4). Therefore, this coordinate regulation of genes by HlyU contributes to the pathogenesis of *V. vulnificus.* The combined results indicate that HlyU might act as a regulator for the various virulence genes and induce the expression of *rtxA* under host environment by altering its activity. Further studies are required to elucidate the detailed mechanisms of *rtxA* regulation by HlyU, and this would expand our understandings of the role of HlyU in *V. vulnificus* pathogenesis and provide knowledge to successfully control the pathogen.

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VI. 국문초록

패혈증 비브리오균은 만성 간 질환, 혈액학적 장애나 면역 체계가 약화된 상태의 환자에게 치명적인 식중독을 일으킬 수 있는 기회 감염성 병원균이다. 패혈증 비브리오균의 발병은 협막다당(capsular polysaccharide), 리포다당(lipopolysaccharide), 독소, 철 획득, 이동성, 부착 등에 관련된 단백질을 포함한 여러 독성 인자에 발현에 기인한다. RtxA 독소는 주요한 독성 인자 중 하나로, 패혈증 비브리오균에 의한 질병에 기여하며, 숙주 세포와의 접촉 후 세포 손상을 유발함으로써 괴저성 세포 사멸을 일으킨다. 이러한 *rtxA* 의 발현은 전사 조절자 HlyU 가 *rtxHCA* 오페론(operon)의 프로모터(promoter)에 직접 결합함으로써 활성화된다. 정상 균주와 비교하였을 때, *hlyU* 돌연변이 균주는 쥐를 이용한 생체 조건과 숙주의 장 점막 세포를 이용한 비 생체 조건 모두에서 현저히 감소한 독성을 나타내었다. 이는 HlyU 가 패혈증 비브리오균이 일으키는 질병에 필수적인 역할을 하고 있음을 의미한다. HlyU 는 생장 단계에 의존하여 *rtxA* 의 발현을 상향 조절하는 것으로 보인다. HlyU 에 의해 조절되는 유전자들을 동정하기 위하여, RNA-sequencing 을 이용하여 정상 균주와 *hlyU* 돌연변이 균주의 전사체 프로파일을 분석하였다. *hlyU* 돌연변이 균주에서 다르게 발현되는 유전자들을 동정하였고, 이로부터 HlyU 가 *rtxA* 를 포함한 여러 독성인자의 전사에 관여함으로써 패혈증

비브리오균의 독성에 기여하는 중요한 전사 조절자임을 확인하였다. 또한, 숙주 세포에 노출되었을 때 또는 무산소 환경에서의 *rtxA* 발현이 HlyU 에 의해 매개됨을 관찰하였으며, 이러한 *rtxA* 발현이 HlyU 의 양적인 변화보다는 전사 활성화 능력의 변화에 기인하여 이루어짐을 관찰하였다. 이어 HlyU 의 돌연변이 생성 연구를 통해 Cysteine 30 잔기가 *rtxA* 발현과 그로 인한 패혈증 비브리오균의 세포 독성에 있어 중요한 잔기임을 확인하였다. 많은 단백질에서 Cysteine 의 산화-환원 상태 변화가 단백질의 구조 변화를 매개한다는 점에 미루어, HlyU 의 Cysteine 30 잔기 또한 HlyU 가 산화-환원 스위치로써 작용하는 데 구조 변화를 매개할 가능성을 제안하였다. 종합하면, HlyU 는 숙주와 흡사한 환경에서 *rtxA* 의 발현을 유도하며, 여러 독성인자 유전자의 발현에 관여하는 전사조절자로서 작용한다. 이러한 HlyU 의 기능 연구를 통해 패혈증 비브리오균이 숙주에 감염하여 질병을 일으키는 기전을 설명할 수 있다. 나아가 패혈증 비브리오균의 여러 독성인자 발현을 조절하는 HlyU 가 패혈증 비브리오균의 제어 타겟이 될 수 있음을 제시하고자 한다.

주요어: 패혈증 비브리오균, RtxA 독소, 전사 조절자, HlyU

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