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온도변화에 의한 패혈증 비브리오균 vulnibactin 수용체 유전자 *vuuA*와 cyclic AMP 수용체 유전자 *crp*의 발현 조절

조선대학교대학원

의 학 과

윤 대 홍

온도변화에 의한 패혈증 비브리오균 **vulnibactin** 수용체 유전자 ***vuuA***와 **cyclic AMP** 수용체 유전자 ***crp***의 발현 조절

Temperature is a Signal for Expression of *vuuA* Encoding Vulnibactin Receptor and *crp* Encoding Cyclic AMP-receptor Protein in *Vibrio vulnificus*

2014 년 2 월 25 일

조선대학교 대학원

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지도교수 김 성 중

이 논문을 의학 박사학위신청 논문으로 제출함

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의 학 과

윤 대 흥

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위원장: 조선대학교 교 수 조 남 수 (인)

위 원: 전남대학교 교 수 민 용 일 (인)

위 원: 조선대학교 교 수 조 수 형 (인)

위 원: 조선대학교 교 수 신 성 희 (인)

위 원: 조선대학교 부교수 김 성 중 (인)

2013 년 12 월

조선대학교 대학원

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윤 대 흥
지도교수: 의학박사 김 성 중
조선대학교 대학원 의학과

패혈증 비브리오균은 호염성(halophilic) 그람음성 간균으로 바닷물에 서식하며 인체에 감염을 일으키기 위해서는 반드시 25°C에서 37°C로의 온도변화를 견뎌내야 한다. 패혈증 비브리오균은 주로 vulnibactin 수용체(VuuA)에 의해 매개되는 철흡수기전(VuuA-mediated iron-uptake system: VuuA-mediated IUS)을 통해 철을 흡수하며 이 철흡수기전의 발현은 분해대사물억제(catabolite repression)에서 통괄조절자(global regulator)로 작용하는 cyclic AMP 수용체(CRP)에 의해 조절된다. 본 연구에서는 철이 결핍된 환경에서 25°C에서 37°C로의 온도변화가 유전자 *vuuA*와 *crp*의 발현에 어떠한 영향을 미치는 가를 관찰하고 VuuA-mediated IUS 와 CRP 사이에 존재하는 상호보완적 관계를 증명하고자 하였다. 철이 풍부한 환경과 비교하여 철이 결핍된 환경에서는 패혈증 비브리오균의 증식이 억제됨과 동시에 *vuuA*의 발현은 증가되고 *crp*의 발현은 감소되었다. 25°C에서 37°C로 온도변화를 주었을 경우 패혈증 비브리오균의 증식이 촉진됨과 동시에 *vuuA*와 *crp*의 발현이 증가되었다. CRP가 결여된 돌연변이 균주에서는 *vuuA*의

발현이 감소되었고, VuuA가 결여된 돌연변이 균주에서는 *crp*의 발현이 감소되었다. 25°C에서 37°C로 온도변화를 주었을 경우 CRP나 VuuA가 결여된 돌연변이 균주에서도 *vuuA* 또는 *crp*의 발현이 증가되었다. 이러한 결과들은 25°C에서 37°C로의 온도변화가 철이 결핍된 환경에서 *vuuA*과 *crp*의 발현을 촉진하는 환경적 신호임을 나타내 주며 VuuA-mediated IUS와 CRP 사이에 최적의 대사(optimal metabolism)를 위한 상호보완적 협력관계(coordinated interaction)가 있음을 나타내 준다.

중심어: 패혈증 비브리오균, 온도, 철, Vulnibactin 수용체, Cyclic AMP 수용체

I. Introduction

Vibrio vulnificus, a gram-negative halophilic bacterium, causes rapidly progressing fatal septicemia and necrotizing wound infections mainly in patients with liver disease and hemochromatosis. Several established and potential virulence factors, such as capsular polysaccharides, iron-uptake systems, flagella, pili, RTX toxin, and exotoxins such as cytolysins (VvhBA) and proteases (VvpE), have been reported to play important roles in the pathogenesis of *V. vulnificus* infection (Jones & Oliver, 2009). The concerted expression of these virulence factors is required for *V. vulnificus* pathogenesis and is under the direct or indirect control of global regulators, such as cyclic AMP-receptor protein (CRP) (Kim *et al*., 2005 & 2013), which is primarily responsible for carbon catabolite repression (Ishizuka *et al*., 1993), and a master regulator SmcR of the quorum sensing system (Kim *et al*., 2003; Lee *et al*., 2007).

Iron is an essential element for survival and proliferation of most bacteria including *V. vulnificus* (Andrews *et al*., 2003; Kim *et al*., 2007). For successful establishment of infection, *V. vulnificus* must be able to acquire iron from its host. The ability to acquire iron is one of the best-established virulence factors in the pathogenesis of *V. vulnificus* infection (Wright *et al*., 1981). *V. vulnificus* has multiple iron uptake systems (IUSs) (Shin, 2013). *V. vulnificus* produces a catechol-type siderophore called vulnibactin and hydroxamate siderophore for acquisition of iron under iron-deficient conditions (Simpson & Oliver,

1983). The ability of *V. vulnificus* to utilize transferrin-bound iron is mainly dependent on the activity of vulnibactin or vulnibactin receptor (VuuA)-mediated IUS (Litwin *et al*., 1996; Webster & Litwin, 2000). *V. vulnificus* also possesses a heme receptor (HupA) for direct acquisition of iron from various heme proteins (Litwin & Byrne, 1998). In addition, *V. vulnificus* can utilize heterologous siderophores, including *Streptomyces* spp. deferoxamine and *Escherichia coli* aerobactin, via DesA- and IutA-mediated IUSs (Aso *et al*., 2002; Tanabe *et al*., 2005; Kim *et al*., 2007). These IUSs are all negatively regulated by iron via ferric uptake regulator (Fur), a transcriptional repressor (Litwin & Calderwood, 1993). The most recent studies have reported that these IUSs are all under the positive control of CRP (Choi *et al*., 2006; Oh *et al*., 2009; Kim *et al*., 2012a; Kim *et al*., 2012b).

V. vulnificus resides in estuarine environments and can infect human body. *V. vulnificus* is capable of sensing and responding to diverse environmental changes between the two environments for survival in the human body or for successful establishment of infection in humans (Kim *et al*., 2003; Lee *et al*., 2007b). Summer estuarine conditions can be simulated at a temperature of 25^{o}C, and 37^{o}C for the human body condition. Entering the human body, *V. vulnificus* must withstand temperature change from 25^{o}C to 37^{o}C (TC). TC modulates the expression of some virulence factors, which is required for survival and for successful infection (McDougald *et al*., 2001; Shao & Hor, 2001; Kawase *et al*., 2004; Kim & Shin, 2011 & 2012).

A recent study reported that *V. vulnificus* can sense and respond to TC by increasing *hupA* expression (Oh *et al*., 2009). However, the effects of TC on the other IUSs and the related mechanisms remain to be clarified. Bacteria undergo metabolic adaptation when they enter a new environment or encounter changes in environmental factors. Bacterial growth is the final result of metabolism. TC stimulates growth of *V. vulnificus* (Kim & Shin, 2011& 2012). Expression of *V. vulnificus* IUSs is positively regulated by CRP for optimal metabolism (Choi *et al*., 2006; Oh *et al*., 2009; Kim *et al*., 2012a; Kim *et al*., 2012b).These findings suggest that TC may modulate expression of VuuA-mediated IUS and/or CRP by changing the requirement of carbohydrates and/or iron for energy production, stimulating growth of *V. vulnificus*. Therefore, in this study we examined the effects of TC on expression of *vuuA* and *crp*, and determined the interdependent relation between VuuA and CRP under iron-limited conditions.

II. Materials and Methods

1. Bacterial strains, plasmids, primers, media, and reagents

The bacterial strains, plasmids and primers used in this study are listed in Table 1, 2 and 3. Heart Infusion (HI; Becton-Dickinson (BD), Franklin Lakes, NJ, USA) broth and agar containing 2.5% NaCl were used in cultivating *V. vulnificus* strains. Luria-Bertani medium (BD) and Thiosulfate-Citrate-Bile-Sucrose medium (TCBS; BD) with or without appropriate antibiotics were used in selection and subculture of recombinant strains. As an iron-limited medium, HI broth containing 200 μM α,α'-dipyridyl (DP-HI) as an iron chelator was used. Antibiotics (BD) were used at the following concentrations: for *Escherichia coli*, ampicillin 50 μg/ml, kanamycin 50 μg/ml, tetracycline 12.5 μg/ml, and chloramphenicol 30 μg/ml; for *V. vulnificus*, ampicillin 20 μg/ml, kanamycin 200 μg/ml, tetracycline 2 μg/ml, and chloramphenicol 2 μg/ml. Unless otherwise stated, all reagents used were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Table 1. Bacterial strains used in this study

Strains, plasmids and primers	Relative characteristics and sequences	References
V. vulnificus		
M06-24/O	Clinical isolate, highly virulent	Reddy *et al*., 1992
CMM710	M06-24/O with a *crp* mutation	Choi *et al*., 2006
CMM714	CMM710 with an *in trans crp* complementation	Choi *et al*., 2006
RC156	M06-24/O with a *vuuA* mutation	This study
RC160	RC156 with an *in trans vuuA* complementation	This study
CMM2101	M06-24/O with an $lacZ_{Vv}$ mutation	Kim *et al*., 2003
RC242	CMM2101 with a merodipliod P_{crp}::$lacZ_{Ec}$ transcriptional reporter	This study
RC130	CMM2101 with a merodiploid P_{vuuA}*::*$lacZ_{Ec}$ transcriptional reporter	This study
Escherichia coli		
SY327 λ*pir*	Host for suicide vector	Miller & Mekalanos, 1988
SM10 λ*pir*	Conjugation donor	Miller & Mekalanos, 1988
DH5α	F- *recA1*; restriction negative	Lab. collection
BL21 (DE3)	F^-, *ompT*, $hsdS_B$ (r_B^-, m_B^-), *gal dcm* (DE3): expression host	Lab. collection

-*Vv* and *Ec* stand for *V. vulnificus* and *E. coli*, respectively.

Table 2. Plasmids used in this study

Plasmids	Relative characteristics	References
pDM4	Suicide vector with R6K origin; Cm^R	McGee *et al*., 1996
pET-30a(+)	Expression vector; Km^R	Novagen
pLAFR3II	pLAFR3 with *bla* inserted at the cos site; Ap^R and Tc^R	Kim *et al*., 2003
pQF52	IncP lacZ transcriptional fusion vector; Ap^R	Farinha & Kropinski, 1990
pRC118	pQF52 with the 856-bp fragment containing the *vuuA* regulatory region	This study
pRC126	pDM4 with the *Bam*HI-*Sca*I fragment containing the P_{vuuA}::*lacZ* fragment	This study
pRC134	2.26-kb *Bgl*II-*Spe*I fragment containing deleted *vuuA*	This study
pRC148	pLAFR3II with the 2.92-kb *Bam*HI-*Hin*dIII fragment containing *vuuA*	This study
pRC182	pQF52 with the 1,057-bp *Bgl*II-*Xba*I fragment containing the *crp* regulatory region	This study
pRC184	pDM4 with the *Bgl*II-*Sca*I fragment containing P_{crp}::$lacZ_{Ec}$ fragment	This study
pRC190	pET-30a(+) with the 1,878-bp *Bam*HI-*Hin*dIII fragment containing *vuuA*	This study
pRC216	pET-30a(+) with a 633-bp *Bam*HI-*Hin*dIII fragment containing the coding region of *crp*	This study
pRK2013	Conjugation helper plasmid	Ditta *et al*., 1980

-Km^R, Tc^R, Cm^R and Amp^R stand for kanamycin-, tetracycline-, chloramphenicol- and ampicillin-resistance, respectively

Table 3. Primers used in this study

Primers	Sequences	References
vuuA-up-1	5'-ga**agatct**gttctcacagagcagcaatcgg-3'	This study
vuuA-up-2	5'-tggtgctcgctcacgcaccgttggcatgctgttacc-3'	This study
vuuA-down-1	5'-ccaacggtgcgtgagcgagcaccaaacataacgg-3'	This study
vuuA-down-2	5'-g**actagt**caggatgcgaaatacgcctcgg-3'	This study
vuuA-rep-F	5'-cg**ggatcc**gttctcacagagcagcaatcgg-3'	This study
vuuA-rep-R	5'-gg**ggtacc**taaagctgccatttgaatttctcc-3'	This study
vuuA-start	5'-atggcagctttacgcccagcg-3'	This study
vuuA-comp-R	5'-ccc**aagctt**gttttactttgggaagggctag-3'	This study
crp-rep-F	5′-ga**agatct**ccaacgatttcccagctctagg-3′ *Bgl*II	This study
crp-rep-R	5′-gc**tctaga**atccttaattagtaccgccacag-3′*Xba*I	This study
His-crp-F	5'-cg**ggatcc**atggttctaggtaaacctcaaac-3' *Bam*HI	This study
His-crp-R-2	5'-ccc**aagctt**ttcatctatttatt**a**acgagtaccgtaaacaacg-3′*Hin*dIII	This study
His-vuuA-F	5'-cg**ggatcc**gaaagaaccatttatgacaccag-3'	This study
His-vuuA-R	5'-ccc**aagctt**ctagaagttcaactgcaatgaag-3'	This study

-Bold letters indicate the restriction enzyme-recognition sequences: **agatct** for *Bgl*I, **tctaga** for *Xba*I, **ggatcc** for *Bam*HI, **actagt** for *Spe*I, **tctaga** for *Kpn*I and **aagctt** for *Hin*dIII.

2. Culture conditions, bacterial growth measurement, and β-galactosidase assay

V. vulnificus strains were preconditioned by culturing in HI broth at 25°C overnight in order to adapt them to a condition mimicking their natural habitat. The adapted strains were inoculated into DP-HI broths at 1 x 10^6 cells/mL and cultured with vigorous shaking (220 rpm) at 25°C, 30°C or 37°C. Culture aliquots were withdrawn at the appropriate times for measurement of bacterial growth and β-galactosidase activity. Bacterial growth was measured by determining the optical densities of culture aliquots at a wavelength of 600 nm (OD_{600}). β-Galactosidase activity in culture aliquots was measured using the Miller method (Miller, 1992).

3. Mutation and complementation

A *crp* deletion mutation in CMM710 and an *in trans crp* complementation in CMM714 were introduced as described previously (Choi *et al*., 2006). A *vuuA* deletion mutation in RC156 was introduced as follows. The 5′ and 3′ fragments of *vuuA* were amplified using the polymerase chain reaction (PCR) primers vuuA-up-1/2 and vuuA-down-1/2. The resulting 1,177-bp and 1,080-bp fragments were used as the DNA template for crossover PCR using the primers, vuuA-up-1 with a *Bgl*II overhang and vuuA-down-2 with a *Spe*I overhang. The resulting 2.26-kb *Bgl*II-*Spe*I fragment with deleted *vuuA* was

cloned into pDM4 (McGee *et al*., 1996). The resulting plasmid pRC148 was transformed into *E. coli* SY327 λ*pir* and SM10 λ*pir* (Miller & Mekalanos, 1988), and subsequently transferred to M06-24/O by conjugation. Transconjugants were selected on TCBS agar containing chloramphenicol to allow for occurrence of the first homogenous recombination, and stable transconjugants were spread onto HI agar containing 10% sucrose to allow for occurrence of the second homologous recombination. Finally, the *vuuA* deletion mutation in RC156 was confirmed by PCR using the vuuA-up-1 and vuuA-down-2 primers.

In order to restore wild-type *vuuA* in RC156, a 2.92-kb *Bam*HI-*Hin*dIII fragment containing the regulatory region and open reading frame of *vuuA* was amplified using the PCR primers, vuuA-rep-F with a *Bam*HI overhang and vuuA-comp-R with a *Hin*dIII overhang, and subsequently cloned into pLAFR3II (Kim *et al*., 2003). The resulting pRC134 was transferred into RC156 via triparental mating using pRK2013 (Ditta *et al*., 1980). A stable transconjugant was selected on TCBS agar plates containing ampicillin and tetracycline and named RC160. The presence of wild-type *vuuA* in RC160 was confirmed by PCR using the vuuA-start and vuuA-comp-R primers.

4. Construction of transcriptional reporters

A merodiploid P_{crp}::$lacZ_{Ec}$ transcriptional fusion was constructed as follows. The 1,057-bp fragment containing the regulatory region of *crp* was amplified using the PCR

primers, crp-rep-F with a *Bgl*II overhang and crp-rep-R with a *Xba*I overhang. The amplified fragment was cloned into pQF52 (Farinha & Kropinski, 1990). From the resulting plasmid pRC182, the *Bgl*II-*Sca*I fragment containing the P_{crp}::$lacZ_{Ec}$ fragment was subsequently cloned into pDM4 (McGee *et al*., 1996). The resulting plasmid pRC184 was transformed into *E. coli* SY327 λ*pir* and SM10 λ*pir* (Miller & Mekalanos, 1988), and transferred to CMM2101 by conjugation. Eventually, a stable transconjugant was selected on TCBS agar containing chloramphenicol and was named RC242. The presence of the P_{crp}::$lacZ_{Ec}$ fusion in RC242 was confirmed by β-galactosidase assay (Miller, 1992).

A chromosomal P_{vuuA}::$lacZ_{Ec}$ transcriptional reporter was constructed as follows. The 856-bp fragment containing the regulatory region of *vuuA* was amplified using the PCR primers; vuuA-rep-F with a *Bam*HI overhang and vuuA-rep-R with a *Kpn*I overhang. The amplified fragment was cloned into pQF52 (Farinha & Kropinski, 1990). From the resulting plasmid pRC118, the *Bam*HI-*Sca*I fragment containing the P_{vuuA}::*lacZ* fragment was subsequently cloned into pDM4 (McGee *et al*., 1996). The resulting plasmid pRC126 was transformed into *E. coli* SY327 λ*pir* and SM10 λ*pir* (Miller & Mekalanos, 1988), and transferred to CMM2101 by conjugation. A stable transconjugant was selected on TCBS agar containing chloramphenicol and was named RC130. The presence of the P_{vvuA}::$lacZ_{Ec}$ fusion was confirmed by β-galactosidase assay (Miller, 1992).

5. Preparation of recombinant proteins and rabbit polyclonal antibodies

For preparation of recombinant CRP, the coding region of *crp* was amplified using the PCR primers His-crp-F with a *Bam*HI overhang and His-crp-R-2nd with a *Hin*dIII overhang. The resulting 633-bp *Bam*HI-*Hin*dIII fragment was then cloned into the pET-30a(+) expression vector (Novagen, Merck, Darmstadt, Germany), and the resulting plasmid pRC216 was transformed into *E. coli* DH5α and BL21 (DE3). The 6x His-tagged CRP fusion protein was over-expressed by treatment of cells with 0.5 mM isopropyl-1-thio-β-$_{D}$-galactopyranoside at 37^{o}C for 4 h. The fusion protein was finally purified using an equilibrated Ni-NTA agarose column and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Recombinant VuuA was prepared using the same methodology. In brief, the coding region of *vuuA* was amplified using the following PCR primers: His-vuuA-F with a *Bam*HI overhang and His-vuuA-R with a *Hin*dIII overhang. The resulting 1,878-bp *Bam*HI-*Hin*dIII fragment was cloned into pET-30a(+) (Novagen). The resulting plasmid pRC190 was transformed into *E. coli* DH5α and BL21 (DE3).

Rabbit polyclonal anti-CRP and -VuuA antibodies were prepared as follows. The CRP and VuuA fusion proteins were mixed with complete or incomplete Freund′s adjuvant, and then injected intradermally into New Zealand White rabbits. After three injections at three-week intervals, blood was collected via cardiac puncture, and antisera were separated

by centrifugation. For removal of cross-reacting antibodies, the antisera were thoroughly adsorbed using boiled cell lysates obtained from the CRP-deficient CMM710 and VuuA-deficient RC156 strain.

6. Western blotting for CRP and VuuA

Intracellular CRP levels were measured using Western blot, as described in our previous studies (Kim *et al*., 2012a; Kim & Shin, 2011 & 2012). In brief, bacterial pellets containing approximately 1 x 10^8 cells were boiled for 10 min, and the resulting cell lysates were electrophoresed on 10% SDS-PAGE gels. Separated proteins were then transferred to nitrocellulose membranes (PROTRAN, Whatman GmbH, Germany). The proprieties of protein loading and transfer among membranes were monitored using a pre-stained protein marker as a control. The membranes were reacted with rabbit polyclonal anti-CRP antibody (1:250) and then with anti-rabbit-IgG antibody conjugated with alkaline phosphatase (1:15,000), and finally visualized using 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium substrate solution. CRP levels on Western blot were quantitatively digitalized using a densitometer program (ImageJ; http://rsb.info.nih.gov/ij). The same methodology as described above was used for measurement of VuuA levels.

III. Results

1. Effect of iron on expression of *vuuA* and *crp*

The effect of iron on *vuuA* transcription was observed by culturing RC130 with the merodiploid P_{vuuA}*::lacZ*$_{Ec}$ fusion fragment and M06-24/O in HI broths containing 0, 100 and 200 μM DP. DP-induced iron limitation inhibited the growth of RC130 (Fig. 1A) and M06-24/O (data not shown). The growth difference among the media could be observed beginning 4 h after culture initiation (mid-exponential growth phase) and showed a continuous increase thereafter. The growth curves of the *V. vulnificus* strains entered into the stationary growth phase 8 h after culture initiation in iron-limited HI broths containing 100 and 200 μM DP. Accordingly, *vuuA* expression was measured 6 h after culture initiation. DP-induced iron limitation resulted in increased *vuuA* transcription levels on a per cell basis in RC130 (Fig. 1B). DP-induced iron limitation also resulted in increased VuuA protein levels on a per cell basis in M06-24/O (Fig. 1C). VuuA was not observed in iron-sufficient HI broth.

The effect of iron on *crp* expression was observed under the same conditions. Growth curves were similar to those described above (Fig. 2A). DP-induced iron limitation resulted in significantly decreased *crp* transcription levels on a per cell basis in RC242 (merodiploid P_{crp}*::lacZ*$_{Ec}$ transcription reporter) (Fig. 2B) and intracellular CRP levels on a per cell basis in M06-24/O (Fig. 2C). CRP was observed at low levels even under more

severe iron-limited conditions (data not shown).

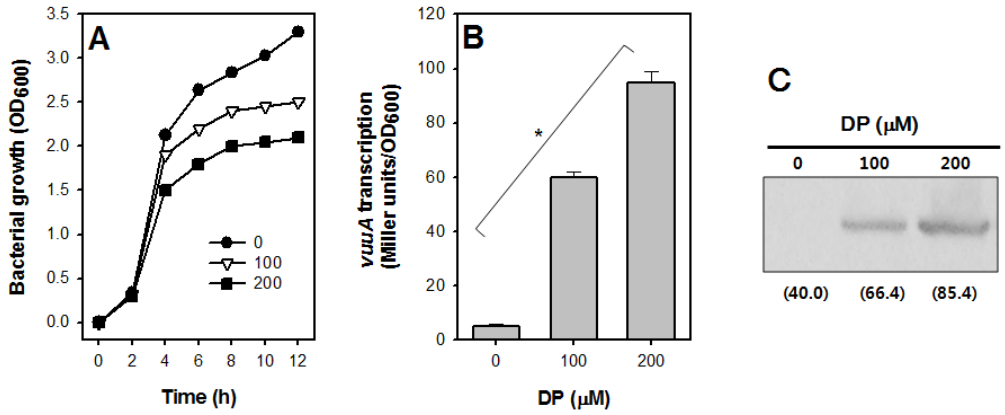

Figure 1. Effect of iron limitation on growth of *Vibrio vulnificus* and expression of *vuuA*. (A and B) *V. vulnificus* RC130 (merodiploid P_{vuuA}*::lacZ* transcription reporter), grown in HI broth overnight, was inoculated into and cultured in HI broth containing dipyridyl (DP) for 12 h at 37°C. Bacterial growth levels were determined by measuring the optical densities of culture aliquots at 600 nm (OD_{600}), and *vuuA* transcription levels were determined by measuring the β-galactosidase activity on a per cell basis (Miller unit) 6 h after culture initiation. The * symbol indicates a significant statistical difference between the media ($p<0.05$, student′s *t* test). (C) *V. vulnificus* M06-24/O was cultured under the same conditions. Culture aliquots were obtained 6 h after culture initiation. Bacterial pellets containing approximately 1 x 10^8 cells were boiled and the resulting bacterial lysates were electrophoresed and transferred to nitrocellulose membranes, and reacted with rabbit polyclonal anti-VuuA antibody. Representative examples of experiments performed in triplicate are shown. The digits in parenthesis indicate the intensities of bands relative to background, which were determined using a computer program (ImageJ; http://rsb.info.nih.gov/ij).

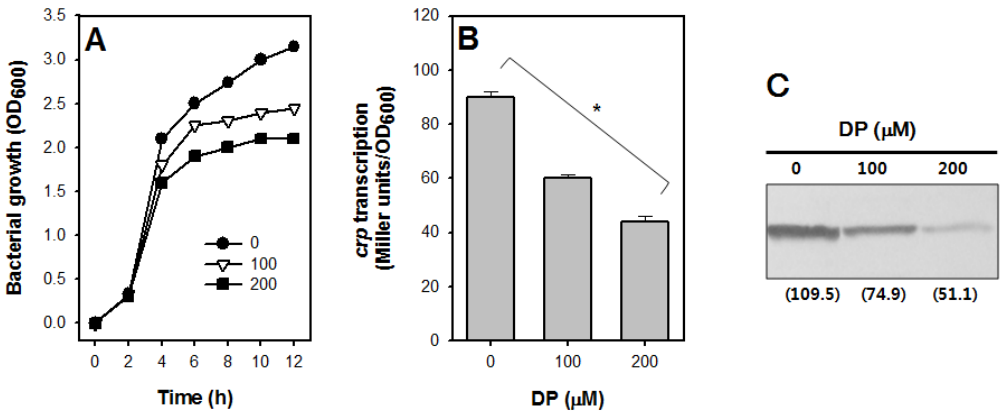

Figure 2. Effect of iron limitation on growth of *Vibrio vulnificus* and expression of *crp*. *V. vulnificus* RC242 (merodiploid P_{crp}*::lacZ* transcription reporter) (A and B) and M06-24/O (C), grown in HI broth overnight, were inoculated into and cultured in HI broth containing dipyridyl (DP) for 12 h at 37^{o}C. Other figure legends are the same as described in Figure 1, except that rabbit polyclonal anti-CRP antibody was used for Western blot.

2. Effect of TC on *vuuA* and *crp* expression

The effect of TC on *vuuA* expression was determined in HI broth containing 100 μM DP. TC stimulated the growth of RC130 (merodiploid P_{vuuA}*::lacZ*$_{Ec}$ transcription reporter) and M06-24/O during the exponential growth phase, but not during the stationary growth phase (Fig. 3A). The resulting difference in growth levels could be observed beginning 2 h after culture initiation, and was greatest 4-6 h after culture initiation, and disappeared 8-10 h after culture initiation. TC induced a significant increase in *vuuA* transcription levels on a per-cell basis, which were measured 6 h after culture initiation in RC130 (Fig. 3B). TC also significantly increased VuuA protein levels on a per-cell basis 6 h after culture initiation in M06-24/O (Fig. 3C). TC from 25°C to 37°C exerted a greater effect on *V. vulnificus* growth and *vuuA* expression than TC from 25°C to 30°C.

Using the same methodology under the same conditions, the effect of TC on *crp* expression was determined using RC242 with the merozygotic P_{crp}*::lacZ*$_{Ec}$ transcriptional fusion fragment and M06-24/O. The growth curves of the two strains were similar to those described above (Fig.4A). TC induced a significant increase in *crp* transcription levels on a per-cell basis at 6 h after culture initiation (Fig. 4B). TC induced a significant increase in intracellular CRP levels on a per-cell basis at 6 h after culture initiation in M06-24/O (Fig. 4C). TC from 25°C to 37°C exerted a greater effect on *V. vulnificus* growth and *crp* expression than TC from 25°C to 30°C.

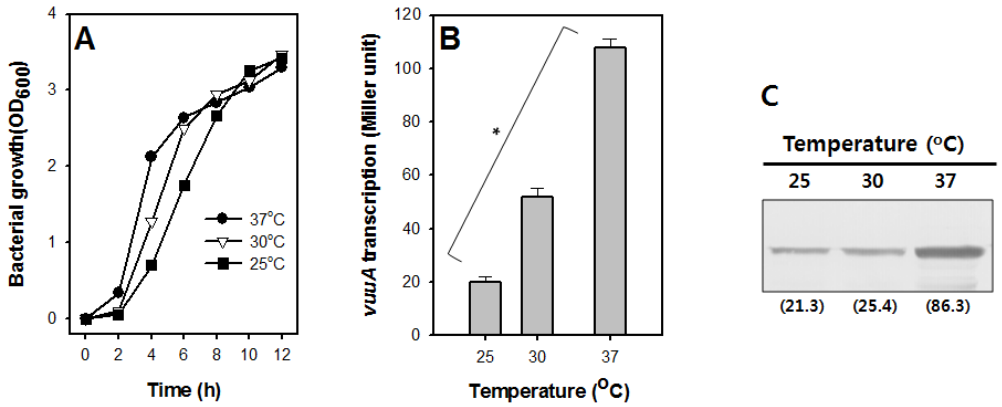

Figure 3. Effect of temperature change on growth of *Vibrio vulnificus* and expression of *vuuA* in an iron-limited condition. *V. vulnificus* RC130 (merodiploid P_{vuuA}*::lacZ* transcription reporter) (A and B) and M06-24/O, grown in HI broth overnight at 25°C, were inoculated into and cultured in HI broth containing 100 μM dipyridyl for 12 h at 25, 30 and 37°C. Other figure legends are the same as described in Figure 1.

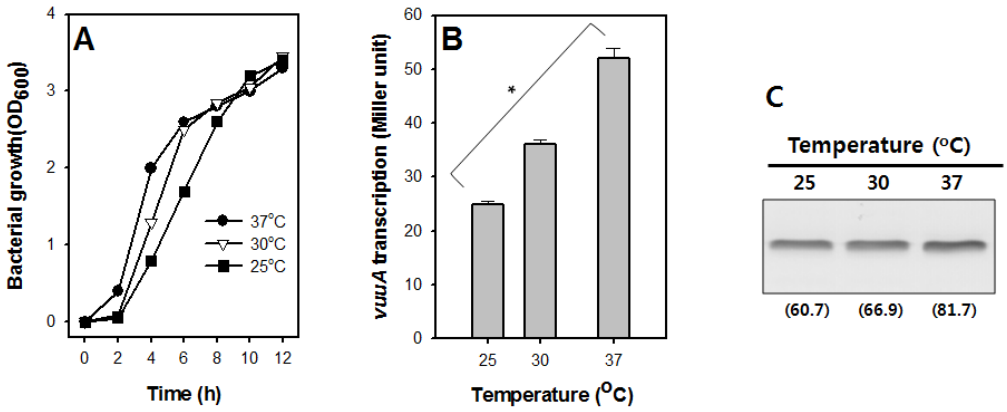

Figure 4. Effect of temperature change on growth of *Vibrio vulnificus* and expression of *crp* in an iron-limited condition. (A and B) *V. vulnificus* RC242 (merodiploid P_{crp}*::lacZ* transcription reporter) and M06-24/O, grown in HI broth overnight at 25°C, were inoculated into and cultured in HI broth containing 100 μM dipyridyl for 12 h at 25, 30 and 37°C. Other figure legends are the same as described in Figure 1, except that rabbit polyclonal anti-CRP antibody was used for Western blot.

3. Effect of a *crp* mutation on the TC-mediated increase of *vuuA* expression

To determine the effect of CRP on *vuuA* expression in HI broth containing 100 μM DP, VuuA protein levels were compared using the three strains, M06-24/O with wild-type *crp*, CMM710 with mutated *crp* and CMM714 with *in trans* complemented *crp*. The growth levels of CMM710 were severely lowered compared with those of M06-24/O and CMM714 (Fig. 5A). VuuA protein levels on a per-cell basis were measured 6 h after culture initiation using Western blot. The VuuA protein levels of CMM710 were also lowered compared with those of M06-24/O and CMM714 (Fig. 5B). The effect of TC on *vuuA* expression in the absence of CRP was determined by comparing VuuA protein levels in CMM710. TC resulted in stimulation of bacterial growth (Fig. 6A) and an increase in VuuA protein levels (Fig. 6B) in CMM710. TC from 25^{o}C to 37^{o}C exerted a greater effect on the growth and *vuuA* expression of CMM710 than TC from 25^{o}C to 30^{o}C.

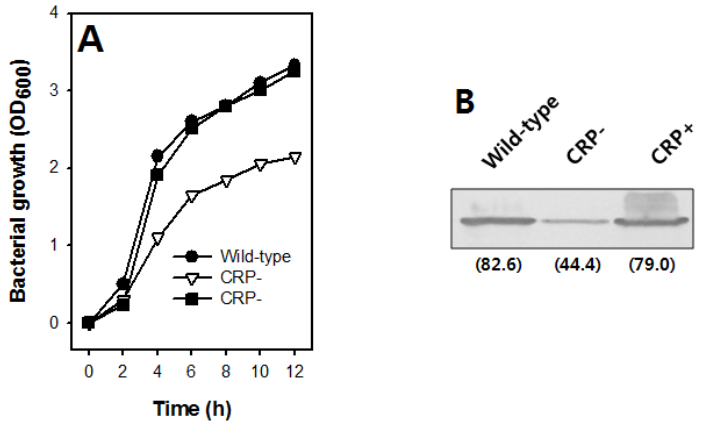

Figure 5. Effect of a *crp* mutation on growth of *Vibrio vulnificus* and expression of *vuuA* in an iron-limited condition. *V. vulnificus* M06-24/O (wild-type), CMM710 (CRP-) and CMM714 (CRP+), grown in HI broth overnight, were inoculated into and cultured in HI broth containing 100 μM dipyridyl for 12 h at 37°C. Other figure legends are the same as described in Figure 1.

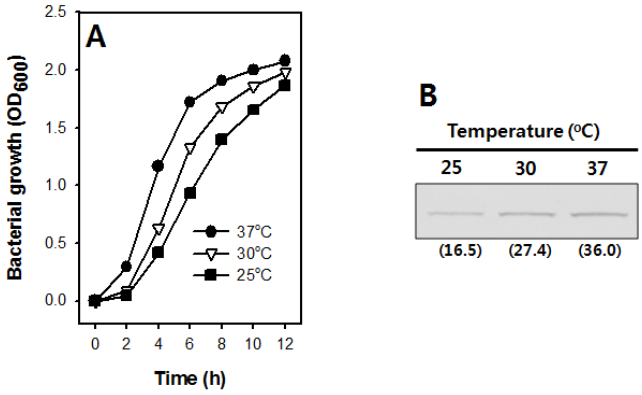

Figure 6. Effect of a *crp* mutation on the TC-mediated increase of *vuuA* expression in an iron-limited condition. *Vibrio vulnificus* CMM710, grown in HI broth overnight at 25°C, was inoculated into and cultured in HI broths containing 100 μM dipyridyl for 12 h at 25, 30 and 37°C. Other figure legends are the same as described in Figure 1.

4. Effect of a *vuuA* mutation in the TC-mediated increase of *crp* expression

To determine the effect of VuuA-mediated IUS on *crp* expression in HI broth containing 100 μM DP, CRP levels were compared using the three strains, M06-24/O with wild-type *vuuA*, RC156 with mutated *vuuA* and RC160 with *in trans* complemented *vuuA*. The growth levels of RC156 were severely lowered compared with those of M06-24/O and RC160 (Fig. 7A). CRP levels on a per-cell basis were measured 6 h after culture initiation using Western blot. The CRP levels of RC156 were lowered compared with those of M06-24/O and RC160 (Fig. 7B). The effect of TC on *crp* expression in the absence of VuuA-mediated IUS was determined by comparison of CRP levels in RC156. TC stimulated the growth of RC156 (Fig. 8A). According to results of Western blot, TC increased intracellular CRP levels in RC156 (Fig. 8B). TC from 25^{o}C to 37^{o}C exerted a greater effect on the growth and *crp* expression of RC156 than TC from 25^{o}C to 30^{o}C.

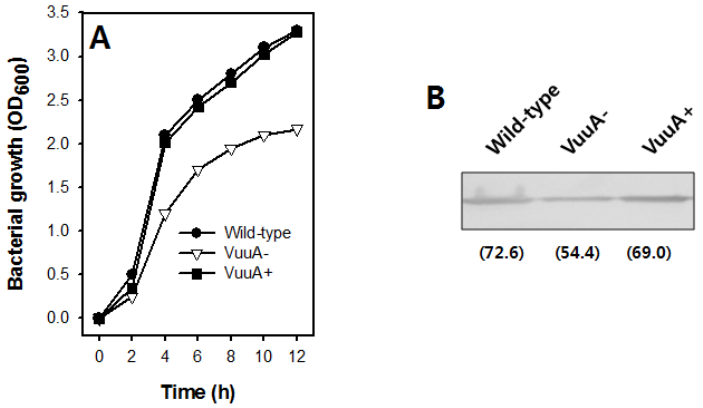

Figure 7. Effect of a *vuuA* mutation on growth of *Vibrio vulnificus* and expression of *crp* in an iron-limited condition. *V. vulnificus* M06-24/O (wild-type), RC156 (VuuA-) and RC160 (VuuA+), grown in HI broth overnight, were inoculated into and cultured in HI broth containing 100 μM dipyridyl for 12 h at 37°C. Other figure legends are the same as described in Figure 1, except that rabbit polyclonal anti-CRP antibody was used for Western blot.

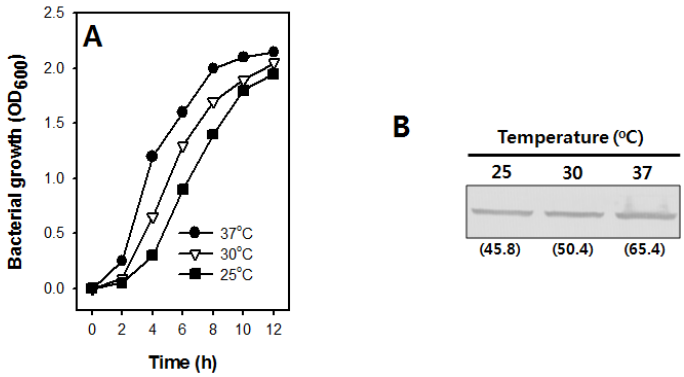

Figure 8. Effect of a *vuuA* mutation on the TC-mediated increase of *crp* expression in an iron-limited condition. *Vibrio vulnificus* RC156, grown in HI broth overnight at 25°C, was inoculated into and cultured in HI broth containing 100 μM dipyridyl for 12 h at 25, 30 and 37°C.Other figure legends are the same as described in Figure 1, except that rabbit polyclonal anti-CRP antibody was used for Western blot.

IV. Discussion

The present study shows that iron limitation led to increased expression of *vuuA* at the transcription level, but inhibited growth of *V. vulnificus*. Iron is an essential element for growth and energy production in most bacteria (Andrews *et al*., 2003). In particular, *V. vulnificus* is a ferrophilic bacterium requiring higher levels of available iron for growth than other pathogenic bacteria (Kim *et al*., 2007). *V. vulnificus* possesses multiple IUSs, however, the activities of the IUSs are relatively low, compared with those of other pathogenic bacteria. Iron limitation is a primary signal for *vuuA* expression and Fur negatively controls *vuuA* expression in proportion to intracellular iron level (Litwin & Calderwood, 1993; Webster & Litwin, 2000; Choi *et al*., 2006).

An interesting finding in the present study is that iron caused an increase in *crp* expression at the transcription level. Glucose availability is a primary signal for *crp* expression (Ishizuka *et al*., 1993). It is likely that iron induces *crp* expression by facilitating metabolism and increasing the requirement of glucose for energy production. Multiple functional interactions are known to exist between CRP and Fur (Zhang *et al*., 2005; Martinez-Antonio *et al*., 2008), and, in addition, CRP directly modulates *fur* expression at the transcription level in *E. coli* (De Lorenzo *et al*., 1998). However, there is no experimental evidence indicating that Fur (or iron) can directly or indirectly affect *crp* expression at the transcription level.

In the present study, TC, particularly from 25°C to 37°C, caused an increase in *vuuA* and *crp* expression at the transcription level, stimulating growth of *V. vulnificus* under iron-limited conditions. The growth stimulation of *V. vulnificus* is a final outcome of increased metabolism and energy production. Both iron and glucose are essential for efficient metabolism and energy production. Therefore, it is likely that the TC-mediated increase of metabolism induces expression of *vuuA* and *crp* by increasing the requirement for iron and glucose. However, the possibility that TC may first increase *vuuA* or *crp* expression in a yet-undefined way, and the resulting increase of intracellular iron or CRP may increase metabolism and facilitate growth of *V. vulnificus* cannot be excluded. TC-mediated changes of gene expressions have been studied extensively in *Shigella* species (Konkel & Tilly, 2000). *Shigella* infection is spread via the fecal-oral route, and is commonly acquired by drinking water contaminated with human fecal material. TC increases the expression of virulence genes in *Shigella* species, including *ipa* (invasion plasmid antigen), *spa* (surface presentation antigens) and *mxi* (membrane expression of invasion) operons. Changes in DNA supercoiling and the transcription repressor H-NS (VirR) are likely to play important roles in TC-mediated changes of virulence gene expression. VirR represses transcription of *virB* and *virF* at 30°C, but de-represses at 37°C. VirF positively regulates *virB* transcription and VirB acts as a positive regulator for transcription of the *ipa*, *mxi*, and *spa* operons. TC also activates the VirF-dependent *virB* promoter by changing DNA supercoiling. Although

there is no experimental evidence, similar mechanisms may also be involved in the TC-mediated change of *crp* or *vuuA* expression in *V. vulnificus*.

The present study shows that a *crp* mutation severely decreased *vuuA* expression. Our previous study using different methods also showed that *vuuA* expression is under the positive control of CRP (Choi *et al*., 2006). In addition, our and other researchers′ studies revealed that expression of *hupA* and *iutA* is also under the positive control of CRP (Oh *et al*., 2009; Kim *et al*., 2012a; Kim *et al*., 2012b). These findings imply that CRP is required for optimal expression of *vuuA*, *hupA* or *iutA* as a positive regulator and that iron metabolism is tightly linked to carbon metabolism. In *E. coli*, CRP is known to be involved in the transcriptional regulation of genes associated with iron uptake (Zhang *et al*., 2005). Under iron-limited conditions, *vuuA* (also *hupA* and *iutA*) is expressed at low levels in the absence of CRP, and induced at high levels in the presence of CRP. Therefore, CRP is likely to enable metabolically coordinated or optimal *vuuA* expression, such that the acquired iron in cells is used for metabolism in the most efficient way. A novel finding in the present study is that TC induced an increase in *vuuA* expression even in the absence of CRP. This finding implies that CRP is not essential for the TC-mediated increase of *vuuA* expression. Accordingly, it is likely that TC increases constitutional expression of *vuuA* (or iron metabolism) which is independent of CRP (or carbon metabolism) under iron-limited conditions.

Conversely, the present study shows that a *vuuA* mutation resulted in a decrease in *crp* expression, however, TC led to increased expression of *crp,* even in the absence of VuuA-mediated IUS. These findings suggest that VuuA-mediated IUS (or the resulting increase of intracellular iron) is required for optimal or coordinated *crp* expression, but it is not essential for the TC-mediated increase of *crp* expression. TC can increase *crp* expression (or carbon metabolism) irrespective of intracellular iron level, but increase the expression of other IUSs besides VuuA-mediated IUS. A previous study reported that higher expression of *hupA* occurs at 37^{o}C than at 30^{o}C (Oh *et al*., 2009). We recently found that TC also increases *iutA* expression (details will be reported elsewhere). Eventually, TC can induce *crp* expression by increasing the levels of intracellular iron via other IUSs, even in the absence of VuuA-mediated IUS.

Virulence expression is a survival strategy of pathogenic bacteria and an intimate functional link exists between metabolic and virulence expression. Metabolic adaptation is a prerequisite for virulence expression when a pathogen encounters a new environment (Rohmer *et al*., 2011). The TC-mediated increase of *crp* and *vuuA* expression can further change the expression of other virulence factors. Indeed, the expression of several virulence factors is under the control of CRP and influenced by intracellular iron levels in *V. vulnificus*. CRP directly regulates the expression of VvhBA, VvpE, and IUSs (Choi *et al*., 2002; Jeong *et al*., 2003; Choi *et al*., 2006; Oh *et al*., 2009; Kim *et al*., 2012a; Kim *et al*.,

2012b), and positively or negatively affects the expression of other virulence factors, including RTX toxin production, capsule formation, and flagella-mediated motility (Kim *et al*., 2005; Kim *et al*., 2013). In addition, iron increases the expression or production of virulence factors including VvhBA and VvpE, stimulating growth of *V. vulnificus* (Kim *et al*., 2006; Kim *et al*., 2009; Kim *et al*., 2012).

In summary, TC, which is encountered upon entering the human body, can increase expression of *vuuA* and *crp*, stimulating growth of *V. vulnificus*, and a coordinated interaction between iron and CRP exists for achievement of optimal metabolism.

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Abstract

Temperature is a Signal for Expression of *vuuA* Encoding Vulnibactin Receptor and *crp* Encoding Cyclic AMP-receptor Protein in *Vibrio vulnificus*

Yoon, Dae-Heung
Advisor: Prof. Kim, Seong-Jung, M.D. & Ph.D.
Department of Medicine
Graduate School of Chosun University

Upon entering the human body, *Vibrio vulnificus*, a gram-negative halophilic marine bacterium, must withstand temperature change from 25°C to 37°C (TC). This bacterium acquires iron mainly via the vulnibactin receptor (VuuA)-mediated iron uptake system (IUS), which is under the positive control of cyclic AMP-receptor protein (CRP), a global regulator responsible for catabolite repression. This study was conducted to examine the effect of TC on expression of *vuuA* and *crp* and to determine the reciprocal relation between VuuA-mediated IUS and CRP under iron-limited conditions. Iron limitation led to increased expression of *vuuA*, but decreased expression of *crp*, inhibiting growth of *V. vulnificus*. TC resulted in increased expression of both *vuuA* and *crp*, stimulating growth of *V. vulnificus*. A *crp* or *vuuA* mutation reciprocally decreased *vuuA* or *crp* expression, and TC increased *vuuA* or *crp* expression even in the *crp*- or *vuuA*-mutated background. These results indicate that TC increases expression of both *vuuA* and *crp* under iron-limited conditions, and that CRP and VuuA-mediated IUS interact coordinately with one another

for achievement of optimal metabolism.

Key words: *Vibrio vulnificus*, Temperature, Iron, Vulnibactin receptor, Cyclic AMP-receptor protein