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2009 년 2 월
박사학위논문

# 패혈증 비브리오균에 있어 cyclic AMP 수용체 단백에 의한 vulnibactin 수용체 단백의 발현 조절

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**Regulation of the Vulnibactin Receptor Expression by Cyclic AMP-receptor Protein in *Vibrio vulnificus***

2009 년 2 월 일


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# 초 록

## 패혈증 비브리오균에 있어 cyclic AMP 수용체 단백에 의한 vulnibactin 수용체 단백의 발현 조절

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패혈증 비브리오균은 여러 가지 다양한 철흡수체계를 가지고 있다. 이러한 체계 중, 패혈증 비브리오균이 생산하는 시데로포아 중 하나인 vulnibactin과 세포 외막에 존재하는 수용체 단백인 VuuA에 의해 매개되는 철흡수체계는 철농도가 매우 낮거나 철이 트렌스페린에 결합된 형태로 존재하는 환경에서 패혈증 비브리오균이 철을 이용하여 증식하고 실험동물의 체내에서 독력을 발현하는데 매우 중요한 역할을 담당하고 있다. 그럼에도 불구하고 철이 높은 환경에서 전사억제자인 ferric uptake regulator (Fur)와 철이 결합하여 Fe-Fur 복합체를 이루어 이 철흡수체계의 발현을 억제한다는 사실 외에 다른 조절기전에 대해서는 알려져 있지 않다. Cyclic AMP (cAMP)와 그 수용체 단백(cAMP-receptor protein: CRP)의 복합체는 일차적으로 에너지원인 탄수화물의 획득가능성(availability)에 반응하는 포괄적인 전사조절자(global regulator)로서 다른 여러 가지 균독력인자의 발현도 조절하는 것으로 알려지고 있다. 최근 한 연구에서 VuuA에 의해 매개되는 철흡수체계의 발현이 전사조절자 CRP를 암호화하는 *crp* 유전자의 돌연변이에 의해 심각하게 억제됨을 보고하였다. 이는 탄수화물 대사와 철 대사과정이 서로 연관되어 있음을 시사하며 cAMP-CRP 복합체에 의해 많은

철흡수 관련 유전자들의 발현이 조절될 가능성을 시사한다. 따라서 본 연구에서는 cAMP-CRP와 Fur에 의해서 조절되는 철흡수체계 사이에 긴밀한 상호작용이 있음을 보다 명백하게 규명하고자 하였다. 먼저 두 전사조절자 사이에 상호작용이 있는지를 알아보기 위해 *crp* 유전자의 돌연변이가 *fur* 유전자의 발현에 미치는 영향을 관찰하였고, 이어서 *crp* 또는 *fur* 유전자의 돌연변이가 *vuuA* 유전자의 발현에 미치는 영향을 관찰하였다. 유전자 *lacZ*가 결합된 전사리포터를 이용하여 *crp*, *fur* 또는 *vuuA* 유전자의 발현을 전사수준에서 관찰하였고, 토끼에서 얻은 다클론 VuuA 항체를 이용하여 *vuuA* 유전자의 발현을 단백수준에서 관찰하였으며, 6 M 요소를 함유한 겔 전기영동법을 이용하여 VuuA에 의해 매개되는 철흡수체계의 활성을 기능수준에서 관찰하였다.

연구결과를 요약하면, *crp* 유전자의 발현은 철농도에 영향을 받지 않았다. 야생형(wild-type) *crp* 유전자를 가진 경우, *fur* 유전자는 철농도에 비례하여 상당한 수준으로 발현되었다. 그러나 *crp* 유전자에 돌연변이를 유발한 경우 *fur* 유전자의 발현은 철에 반응하지 않으면서 현저히 감소되었다. 이와 같은 *fur* 유전자의 발현이 플라스미드로 *crp* 유전자를 보완한 경우 다시 회복되었다. 야생형 *crp* 유전자를 가진 경우 *vuuA* 유전자는 철농도에 반비례하여 상당한 수준으로 발현되었다. 그러나 *crp* 유전자에 돌연변이를 유발한 경우 *vuuA* 유전자의 발현은 철이 결핍된 조건에서도 현저히 억제되었고 이러한 *vuuA* 유전자의 발현은 플라스미드로 *crp* 유전자를 보완한 경우 다시 회복되었다. 야생형 *crp* 유전자를 가진 경우, VuuA 단백은 철농도에 반비례하여 상당한 수준으로 생산되었다. 그러나 *crp* 유전자에 돌연변이를 유발한 경우 VuuA 단백의 생산은 현저히 감소하였고

플라스미드로 *crp* 유전자를 보완한 경우 다시 회복되었다. 야생형 *crp* 유전자를 가진 경우 패혈증 비브리오균은 트렌스페린에 결합된 철을 이용하여 비교적 잘 자랐다. 그러나 *crp* 유전자에 돌연변이를 유발한 경우 패혈증 비브리오균의 증식은 현저히 억제되었고 플라스미드로 *crp* 유전자를 보완한 경우 다시 회복되었다. 야생형 *crp* 유전자를 가진 경우, 패혈증 비브리오균은 트렌스페린에 결합된 철을 상당 수준 탈취할 수 있었다. 그러나 *crp* 유전자에 돌연변이를 유발한 경우 트렌스페린에 결합된 철을 탈취하는 능력은 현저히 약화되었고 플라스미드로 *crp* 유전자를 보완한 경우 다시 회복되었다. 이에 비해, *fur* 유전자에 돌연변이를 유발한 경우 *vuuA* 유전자의 발현과 트렌스페린에 결합된 철을 이용하여 증식하는 능력은 특히 철농도가 높은 환경에서 탈억제(de-repression)되어 증가되었고 플라스미드로 *fur* 유전자를 보완한 경우 다시 회복되었다.

본 연구결과를 종합하여 보면, 첫째로 cAMP-CRP복합체가 *fur* 유전자의 발현을 양성적으로(positively) 조절하고 있어 두 전사조절자인 cAMP-CRP복합체와 Fe-Fur 복합체 사이에 밀접한 상호작용이 있음을 알 수 있다. 둘째로, cAMP-CRP 복합체는 철이 결핍된 환경에서 VuuA에 의해 매개된 철흡수체계의 활성을 양성적으로 조절하여 활성화한다. 셋째로 cAMP-CRP 복합체는 철이 풍부한 환경에서 Fe-Fur 복합체를 통해 VuuA에 의해 매개된 철흡수체계의 활성을 음성적으로(negatively) 조절하여 활성을 약화시킬 수 있다.

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핵심용어: 패혈증 비브리오균, 철, Vulnibactin, cAMP 수용체 단백, Fur

# INTRODUCTION

## 1. Overview

### 1) Iron availability

Iron is an essential element for all living things including microorganisms. As iron is rapidly oxidized from $Fe^{2+}$ to $Fe^{3+}$ and exists as insoluble oxyhydroxide (FeOOH) polymers in oxygen-containing environments at neutral pH, the level of freely available iron is severely low (approximately $10^{-18}$ M). This low iron availability affects significantly many biological systems, including survival and proliferation of bacterial pathogens in natural environments [1, 2]. Similarly, iron availability is also very low within the human body despite large amounts of iron being present. Most iron is sequestered within cells, especially red blood cells. Only a very small portion of iron exists in extracellular body fluids such as plasma and mucosal secretions. Moreover, most of the extracellular iron is tightly bound to the high-affinity iron-binding glycoproteins such as transferrin and lactoferrin. Accordingly, despite iron being an essential element for the survival and proliferation of microbial pathogens, the level of freely available iron is too low to sustain bacterial growth [3-5]. However, most microbial pathogens have successfully adapted to these iron-limited environments by developing their specific iron uptake systems (IUS). To satisfy their needs for iron, microbial pathogens can respond to the environmental cue of limited iron-availability by de-repressing their high-affinity IUS.

### 2) Iron and *V. vulnificus* infections

*Vibrio vulnificus* is a gram-negative halophilic estuarine bacterium that opportunistically causes necrotizing wound infection and septicemia in suscepti-

ble patients. These *V. vulnificus* infections are closely associated with the consumption of raw seafood or the exposure to seawater contaminated with the bacterium in patients with underlying hepatic diseases, heavy alcohol-drinking habits, or other immunocompromised conditions. Particularly, *V. vulnificus* septicemia progresses very rapidly and exhibits a high mortality rate of over 50%. Most of patients with *V. vulnificus* septicemia die of multi-organ failure within 1 to 2 days after hospitalization despite of aggressive supportive shock and antibiotic therapies [6-8].

Several putative virulence factors, such as lipopolysaccharide, capsular polysaccharide, hemolysin/cytolysin, metalloprotease, RTX (repeats in toxin) toxin and several IUS, have been implicated to be associated with the pathogenesis of *V. vulnificus* infections [7, 8]. Of these factors, IUS has been confirmed to be an authentic virulence factor, in accordance with the molecular version of Koch's postulates [9].

Iron availability is increased under some pathologic conditions [3]. Such increased iron-availability plays important roles in the pathogenesis of *V. vulnificus* infections. Several experimental data clearly show that increased iron-availability is directly correlated with increased host susceptibility to *V. vulnificus* [10-15]. Injection of iron lowers the $LD_{50}$ of mouse to *V. vulnificus* and the increased mouse lethality directly correlated with the serum iron levels elevated following liver damage by injection of $CCl_4$ [10]. Virtually, iron-overloaded mice have been used as the most susceptible animal model to *V. vulnificus* [10, 13].

Like other microbial pathogens, *V. vulnificus* also possesses multiple IUS (Figure 1). Nevertheless, *V. vulnificus* preferentially infects patients with elevated serum iron levels because it is a ferrophilic bacterium that requires more iron for growth and survival than other microbial pathogens [15]. Interestingly,

this ferrophilic characteristic implies that IUS may be an effective candidate for the development of vaccine and new chemotherapeutic agents in several pathogenic microorganisms including *V. vulnificus* [16, 17 ].

### 3) *V. vulnificus* IUS

#### (1) Vulnibactin- or VuuA-mediated IUS

*V. vulnificus* produces the two types of siderophores: catechol (phenolate)- and hydroxamate siderophores in low-iron media [18]. Catechol-siderophore (also called vulnibactin) is a dihydroxybenzoic acid-containing compound and is structurally related to vibriobactin produced by *V. cholerae* [19]. Generally, virulent isolates of *V. vulnificus* produce vulnibactin and can efficiently utilize transferrin-bound iron, whereas avirulent isolates neither produce vulnibactin nor utilize transferrin-bound iron [20]. Vulnibactin plays more important role in the growth stimulation of *V. vulnificus* under iron-deficient conditions than hydroxamate siderophore [21]. The *ven* operon consists of the genes essentially required for vulnibactin synthesis [22]. The *ven* operon shows a significant homology with the *Escherichia coli ent* operon which consists of the genes for the synthesis of *E. coli* catechol siderophore (called enterobactin). A mutation of *venB* abolishes the ability to produce vulnibactin and to utilize transferrin-bound iron, and significantly attenuates the virulence of *V. vulnificus* in an infant mouse model [22]. In addition, a mutation of *vis* encoding isochorismate synthase, which is another enzyme required for the synthesis of vulnibactin, also abolishes the ability of *V. vulnificus* to produce vulnibactin and to utilize transferrin-bound iron [23]. Overall, the vulnibactin- or VuuA-mediated IUS plays a crucial important role in the transferrin-bound iron utilization and the virulence of *V. vulnificus*. The *vuuA* gene encoding vulnibactin receptor protein was first

cloned and characterized by Webster and Litwin [24]. VuuA is an iron-regulated outer membrane protein with 72 kDa and is highly expressed in a deletion mutant of *fur* encoding a transcriptional repressor Fur (ferric uptake regulator) [25], and a mutation of *vuuA* abolishes the ability of *V. vulnificus* to use vulnibactin.

A few researchers reported that the activity of the *V. vulnificus* metalloprotease (VvpE) was required for efficient utilization of transferrin- and lactoferrin-bound iron, making the bound iron more accessible to the vulnibactin-mediated IUS via proteolytic cleavage of these proteins [26]. More recently, however, VvpE is demonstrated to have no direct effect on iron uptake from human transferrin via the vulnibactin-mediated IUS [23, 27].

**(2) Hydroxamate siderophore-mediated IUS**

It is known that avirulent *V. vulnificus* strain A1402 produces only hydroxamate siderophore in iron-deficient condition [18]. The addition of hydroxamate siderophore enhances the growth of *V. vulnificus* in iron-deficient media and also stimulates the growth of a hydroxamate auxotroph. *V. vulnificus* biotype 2, a primary pathogen for eels and an opportunistic pathogen for human, also produces and uses hydroxamate siderophore for iron acquisition from transferrin [28]. However, neither genes related to the hydroxamate siderophore-mediated IUS have been known, nor the structure of hydroxamate siderophore have been known until now. Accordingly, the actual function of hydroxamate siderophore in the virulence of *V. vulnificus* remains totally unknown.

**(3) Versatile DesA- or IutA-mediated IUS**

*V. vulnificus* is able to use desferrioxamine (also called deferoxamine) as an exogenous siderophore to reverse iron limitation [10], via DesA (desferriox-

amine receptor) which is an iron-repressible outer membrane protein with 78-kDa and is induced in iron-starved *V. vulnificus* cells only in the presence of exogenous desferrioxamine [16, 29]. In addition to the DesA-mediated IUS, *V. vulnificus* can also utilize exogenously-supplemented aerobactin under iron-deficient conditions, along with enhanced production of the 76 kDa iron-repressible ferric-aerobactin receptor (named IutA). The expression of *iutA* is also induced by the exogenous addition of *Escherichia coli* aerobactin into iron-deficient conditions [30].

Noticeable is that *V. vulnificus* can utilize desferrioxamine by actively inducing DesA. Desferrioxamine was originally a hydroxamate siderophore derived from *Streptomyces* spp. This siderophore is currently being used as the iron-chelating therapeutic agent to reduce iron-overload. *V. vulnificus* preferentially infects such patients with iron-overload and is able to actively utilize desferrioxamine; that is to say, the desferrioxamine therapy can seriously exacerbate the infections by *V. vulnificus*. Other microbial pathogens including *Staphylococcus aureus* and *Yersinia enterocolitica* are also known to be capable of utilizing desferrioxamine for iron uptake [31, 32]. Accordingly, this adverse effect of desferrioxamine warrants that it is necessary to develop new iron-chelating agents which cannot be utilized by bacteria.

**(4) HupA-mediated IUS**

A mutation of *fur* constitutively expresses an iron-regulated outer membrane protein with 77 kDa, which is encoded by *hupA*. A HupA-deficient mutant failed to utilize haemin or hemoglobin as an iron source, but the role of HupA in virulence has not been determined yet [33]. HupR is known to act as a positive regulator for *hupA* in the presence of haemin under low-iron conditions [34].

## 2. The aim of the present study

As in other microbial pathogens, the expression of virulence factors is also under the control of several global regulators in *V. vulnificus* [35]. Of these global regulators, the complex between cyclic AMP (cAMP) and its receptor protein (CRP), which is primarily responsive to carbon availability and regulates the expressions of a variety of genes, including genes related to carbon and iron metabolism in *Escherichia coli* and *Salmonella typhimurium* [36-39]. The expression of microbial IUS is primarily regulated by Fur, which is responsive to iron availability [38, 39]. Genes associated with iron uptake are generally constitutively expressed but repressed by the Fe-Fur complex under iron-sufficient conditions because they are repressible genes. In *V. vulnificus*, the cAMP-CRP complex also regulates the expression of a variety of virulence factors [40-42]. The expression of *V. vulnificus* IUS is also regulated by Fur [23, 24, 33]. The interaction between the cAMP-CRP complex and Fur-regulated IUS has been recently suggested but not directly demonstrated yet in accordance with the molecular version of Koch's postulates in *Escherichia coli* [38, 39]. More recently, the interaction between the cAMP-CRP complex and the VuuA-mediated IUS was first demonstrated in *V. vulnificus* [43]. Vulnibactin production is decreased and the ability of *V. vulnificus* to utilize transferrin-bound iron is attenuated by *crp* mutation. In addition, the expression of *vis* or *vuuA* is down-regulated by *crp* mutation. These findings suggest that there is a link between carbon and iron metabolic processes and the cAMP-CRP complex can also regulate expression of several genes associated with iron-uptake.

The present study attempted to unequivocally demonstrate the interaction between the two systems, cAMP-CRP and Fur-regulated IUS, as shown in Figure 2. Accordingly, the effect of *crp* mutation on *fur* expression was first ob-

served to demonstrate the interaction between the two transcriptional regulators CRP and Fur, and then the effect of *crp* or *fur* mutation on the expression of the VuuA-mediated IUS was observed. The *lacZ*-fused transcriptional reporters were used to measure *crp*, *fur* or *vuuA* expression at the transcription level, the rabbit polyclonal anti-VuuA-body was used to measure *vuuA* expression at the protein level, and 6 M urea-gel electrophoresis was used to measure the activity of the VuuA-mediated IUS at the functional level.

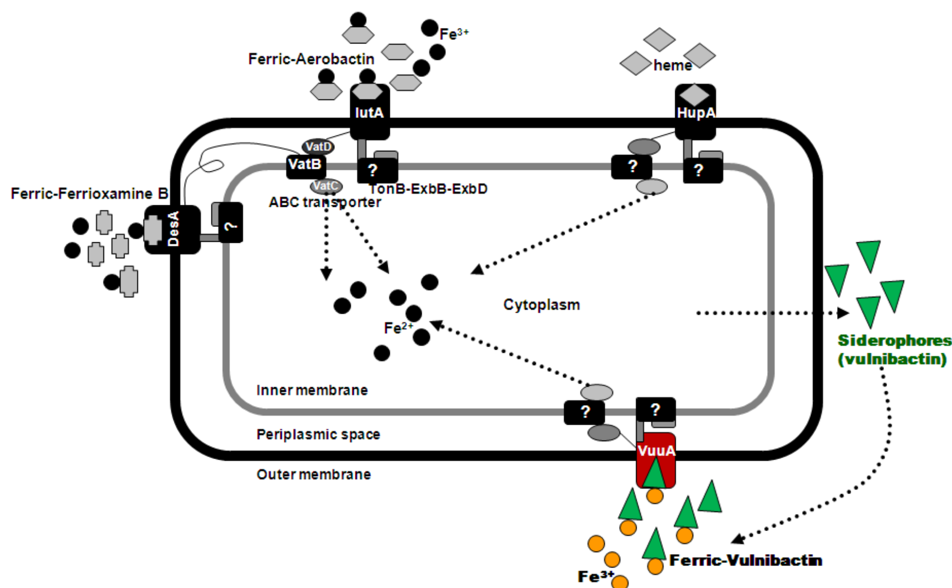


**Figure 1. Hypothesized iron-uptake systems of *Vibrio vulnificus*.** The four kinds of iron-repressible receptors on the outer membrane of *V. vulnificus* have been identified. HupA is a receptor that can directly bind heam proteins. IutA is a receptor that can bind aerobactin, which is a hydroxamate siderophore produced by *Escherichia coli*. DesA is a receptor that can bind desferrioxamine which is produced by *Streptomyces* and is being used as a standard iron chelating therapeutic agent. *V. vulnificus* siderophores cognate to IutA and DesA has not been identified yet. VuuA is a receptor that can bind vulnibactin produced by *V. vulnificus*. The VuuA- or vulnibactin-mediated iron-uptake system is known to play a crucial role in the utilization of transferrin-bound iron and pathogenesis of *V. vulnificus* infections.

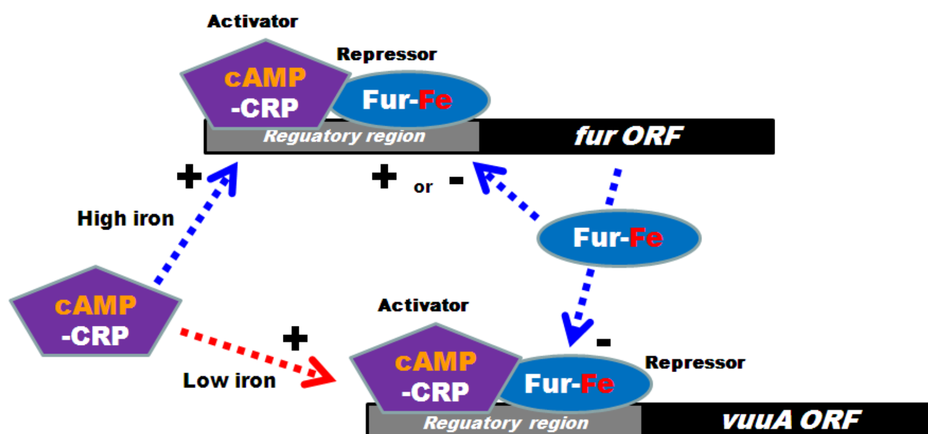


**Figure 2. Hypothetical regulation of the expression of *fur* encoding ferric uptake regulator (Fur) and *vuuA* encoding vulnibactin receptor protein (VuuA).** It is well-documented that the Fur-Fe complex negatively (-) regulates *vuuA* expression. Fur can positively or negatively auto-regulate *fur* expression. This study newly proposes that the cAMP-CRP complex can positively (+) regulate *fur* expression as well as *vuuA* expression: the complex can directly activate *vuuA* expression especially under iron-deficient conditions (red arrow), whereas the complex can indirectly repress *vuuA* expression by directly activating *fur* expression (blue arrows).

# MATERIALS and METHODS

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

The bacterial strains, plasmids and primers used in this study are listed in Tables 1, 2 and 3, respectively. Heart Infusion (HI; BD, Flanklin Lakes, NJ, USA) medium containing 2.5% NaCl was used as a basal medium for the cultivation of *V. vulnificus* strains. Iron-limited HI broth was prepared by adding 200 μM dipyridyl into HI broth. In addition, deferrated (DF) HI broth was prepared by the method described by Leong and Neilands [51] to a residual iron concentration of less than 1 μM. When necessary, ferric chloride (FC), partially iron-saturated human transferrin (PT; 300~600 μg iron per 1 g protein) or holotransferrin (HT; 1,200~1,600 μg iron per 1 g protein) was added to DF-HI broth as an iron-source. HI agar and Thiosulfate-Citrate-Bile-Sucrose (TCBS; BD) agar containing appropriate antibiotics were used to select and maintain *V. vulnificus* strains. Luria-Bertani (BD) media containing appropriate antibiotics were used for the cultivation and maintenance of *Escherichia coli* strains. Unless noted otherwise, all reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Table 1. Bacterial strains used in this study.

| Bacterial strains | Relative characteristics | Sources |
|---|---|---|
| *Vibrio vulnificus* | | |
| M06-24/O | Wild type strain | Lab stock |
| CMM2101 | M06-24/O with *lacZ* deletion mutation | 44 |
| CMM710 | M06-24/O with *crp* deletional mutation | 43 |
| CMM714 | CMM710 with *in trans crp* complementation | 43 |
| CMM2303 | M06-24/O with *fur* deletional mutation | 23 |
| RC144 | CMM2303 with *in trans fur* complementation | This study |
| CMM2304 | M06-24/O with *fur* and *lacZ* deletional mutation | # |
| RC100 | M06-24/O with *crp* and *lacZ* deletion mutation | This study |
| RC130 | CMM2101 with $P_{vuuA}$::*lacZ* transcriptional fusion | This study |
| RC132 | RC100 with $P_{vuuA}$::*lacZ* transcriptional fusion | This study |
| RC260 | RC132 with *in trans crp* complementation | This study |
| RC134 | CMM2304 with $P_{vuuA}$::*lacZ* transcriptional fusion | This study |
| RC156 | M06-24/O with *vuuA* deletional mutation | This study |
| RC160 | RC156 with *in trans vuuA* complementation | This study |
| RC190 | CMM2101 with $P_{fur}$::*lacZ* transcriptional reporter | This study |
| RC194 | RC100 with $P_{fur}$::*lacZ* transcriptional reporter | This study |
| RC242 | CMM2101 with $P_{crp}$::*lacZ* chromosomal reporter | This study |
| RC300 | RC194 with *in trans crp* complementation | This study |
| *Escherichia coli* | | |
| SY327 λ*pir* | Δ(*lac pro*) *argE*(Am) *rif nalA recA56* λ*pir* lysogen; Host for suicide vector | 45 |
| SM10 λ*pir* | *thi thr leu tonA lacY supE recA*::RP4-2-$Tc^r$::Mu $Km^r$ λ*pir* lysogen; Conjugation donor | 45 |
| BL21 (DE3) | $F^-$, *ompT*, *hsdS*$_B$ ($r_B^-$, $m_B^-$), *gal dcm* (DE3) | Lab stock |

#: In manuscript submission

Table 2. Plasmids used in this study.

| Plasmids | Characteristics | Sources |
|---|---|---|
| pKAS32 | Suicide vector with *ori*R6K, *rpsL* and $Amp^R$ | 46 |
| pLAFR3 | IncP cosmid vector; $Tc^R$ | 47 |
| pLAFR3II | pLAFR3 with *bla* inserted at the cos site; $Ap^R$ and $Tc^R$ | 16 |
| pDM4 | Suicide vector with R6K origin; $Cm^R$ | 48 |
| pQF52 | IncP lacZ transcriptional fusion vector; $Amp^R$ | 49 |
| pCR®2.1-TOPO® | PCR cloning vector | Lab. stock |
| pRC118 | 856-bp *Bam*HI-*Kpn*I fragment containing the *vuuA* promoter region cloned into pQF52 | This study |
| pRC126 | *Bam*HI-*Sca*I fragment containing $P_{vuuA}$::*lacZ* fragment from pRC118 cloned into *Bgl*II-*Sma*I cut pDM4 | This study |
| pRC132 | 861-bp *Bam*HI-*Hin*dIII fragment containing the *fur* gene cloned into pLAFR3II | This study |
| pRC134 | 2.26-kb *Bgl*II-*Spe*I fragment containing an in-frame deletion of the *V. vulnificus vuuA* gene cloned into pDM4 | This study |
| pRC148 | 2.92-kb *Bam*HI-*Hin*dIII fragment containing the *vuuA* gene cloned into pLAFR3II | This study |
| pRC160 | 882-bp *Kpn*I-*Hin*dIII fragment containing the *fur* promoter region cloned into pQF52 | This study |
| pRC166 | *Sph*I-*Sca*I fragment containing $P_{fur}$::*lacZ* fragment from pRC160 cloned into *Sph*I-*Sma*I cut pDM4 | This study |
| pRC182 | 1,057-bp *Bgl*II-*Xba*I fragment containing the *crp* promoter region cloned into pQF52 | This study |
| pRC184 | *Bgl*II-*Sca*I fragment containing $P_{crp}$::*lacZ* fragment from pRC182 cloned into *Bgl*II-*Sma*I cut pDM4 | This study |
| pCMM236 | 1.8-kb fragment containing an in-frame deletion of *V. vulnificus fur* gene cloned into pCR®2.1-TOPO® | |
| pCMM237 | *Sac*I-*Xba*I fragment of pCMM236 cloned into pDM4 | |
| pCMM712 | pLAFR3 cloned with 6 kb *Hin*dIII fragment containing *V. vulnificus crp* | 43 |
| pCMM714 | pUC19 cloned with 1,721bp *Hin*dIII-*Sal*I fragment containing *V. vulnificus crp* | 43 |
| pCMM715 | pCMM714 deleted in 513 bp DNA fragment encompassing a majority of *crp* ORF by blunt ligation after *Pst*I-*Sph*I cut | 43 |
| pCMM716 | pKAS32 cloned with the *Eco*RI and *Eco*RV fragment from pCMM715 | 43 |
| pRK2013 | IncP, $Km^r$, Tra $Rk2^+$ *repRK2 repE1* | 50 |
| pET-30a(+) | Inducible expression vector carrying N-terminal | Lab stock |

| | | |
|---|---|---|
| | His·Tag/thrombin/S·Tag/enterokinase configuration plus an optional C-terminal His·Tag sequence; $Km^r$ | |
| pRC190 | pET-30a(+) with 1,878-bp *Bam*HI-*Hin*dIII fragment containing *vuuA* gene | This study |

Table 3. Primers used in this study.

| Primers | Characteristics | Sources |
|---|---|---|
| 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-comp-R | 5'-ccc**aagctt**gttttactttgggaagggctag-3' | This study |
| vuuA-start | 5'-atggcagctttacgcccagcg-3' | This study |
| vuuA-1 | 5'-gcctatgctcaaaccgagag-3' | This study |
| vuuA-3 | 5'-gcagcggtttgtgaatagtttcg-3' | This study |
| fur-rep-F | 5'-gg**ggtacc**atgagttcgaagcatcaaaagc-3' | This study |
| fur-rep-R | 5'-ccc**aagctt**tactttcccgttgatgatctgc-3' | This study |
| CRP1 | 5'-tacctactggcgatgatcgatg-3' | This study |
| CRP7 | 5'-cggaatctgagagggtttagt-3' | This study |
| crp-rep-F | 5'-ga**agatct**ccaacgatttcccagctctagg-3' | This study |
| crp-rep-R | 5'-gc**tctaga**atccttaattagtaccgccacag-3' | This study |
| fur-comp-F | 5'-cg**ggatc**cccgttaaagagaaaatactgcc-3' | This study |
| fur-comp-R | 5'-ccc**aagctt**tatcgagcgtcgatattagttc-3' | This study |
| His-vuuA-F | 5'-cg**ggatcc**gaaagaaccatttatgacaccag-3' | This study |
| His-vuuA-R | 5'-ccc**aagctt**ctagaagttcaactgcaatgaag-3' | This study |
| 16s rRNA-F | 5'-aacgagcgcaacccttatcc-3' | This study |
| 16s rRNA-R | 5'-actccaatggactacgacgcac-3' | This study |

*Bam*HI: ggatcc, *Hin*dIII: aagctt, *Bgl*II: agatct, *Spe*I: actagt, *Xba*I:tctaga, *Kpn*I:ggtacc.

**2. Preparation of rabbit polyclonal anti-VuuA-body**

**1) Production of His-tagged VuuA recombinant protein**

The coding region of *vuuA* was amplified using the PCR primers; His-vuuA-F with *Bam*HI overhang and His-vuuA-R with *Hin*dIII overhang. The resulting 1,878-bp *Bam*HI-*Hin*dIII fragment was cloned into the pET-30a(+) expression vector (Novagen, Korea). The resulting plasmid pRC190 was transformed into *Escherichia coli* DH5α and BL21 (DE3). In *Escherichia coli* BL21 (DE3), 6x His-tagged VuuA protein was over-expressed at 37$^{o}$C for 3 h by 0.5 mM isopropyl-1-thio-β-$_{D}$-galactopyranoside. The bacterial pellet was suspended in phosphate-buffered saline (PBS, pH 7.2) and was sonicated on an ice bath at amplitude of 30% with a Vibra Cell$^{TM}$ microtip sonicator (Model VCX500, Sonics & Materials, CT, USA). The cell-free supernatant was removed by centrifugation at 10,000 rpm for 30 min, and the remaining pellet was washed with 0.5% Triton X-100 and phosphate-buffered saline (PBS), and was solubilized with 8 M urea solution, which contains 10 mM Tris (pH 8.0), 0.5 M NaCl and 5 mM imidazole. After incubation for 1 h at room temperature, the cell debris was removed by centrifugation at 11,000 rpm for 30 min. The recovered supernatant was loaded to the equilibrated Ni-NTA agarose column, and the resulting matrix was fully washed with the 8 M urea solution. The 6x His-tagged VuuA protein was eluted with the 8 M urea solution without 50 or 250 mM imidazole, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

**2) Rabbit polyclonal anti-VuuA-body**

The 6x His-tagged VuuA protein was mixed with the complete or incomplete Freund's adjuvant, and injected intradermally to a New Zealand White

rabbit. After three times-repeated injections at 3-week intervals, blood was collected via cardiac puncture, and serum was separated after centrifugation at 4°C. To remove cross-reacting antibodies, the antiserum was thoroughly adsorbed using cell lysates obtained from RC156 and immobilized onto nitrocellulose membrane.

**3. Deletional mutation and *in trans* complementation of *vuuA***

A *vuuA* deletion mutation was constructed using crossover PCR and the suicide vector pDM4 with R6K origin [48], as shown in Figure 3. The 5' and 3' fragments of *vuuA* were amplified using the 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. The resulting plasmid pRC148 was transformed into *Escherichia coli* SY327 λ*pir* and SM10 λ*pir* [45], and subsequently transferred to M06-24/O by conjugation. Transconjugants were selected on TCBS agar containing chloramphenicol to allow the first homogenous recombination to occur, and stable transconjugants were spread onto 2.5% NaCl HI agar containing 10% sucrose to allow the second homologous recombination to occur. Finally, the *vuuA* deletion mutation in RC156 was confirmed by PCR using the vuuA-up-1 and vuuA-down-2 primers.

To restore wild-type *vuuA* in the *vuuA*-deletion mutant 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 was subsequently subcloned into the broad host range vector pLAFR3II (named pRC134), which

was prepared by subcloning a 1,453-bp *Bam*HI-*Bgl*II fragment containing *bla* of pUTKm1 into a *Bgl*II site of pLAFR3 [16, 47]. The pRC134 was transferred into RC156 via triparental mating using the conjugative helper plasmid pRK2013 [50]. A stable transconjugant (named RC160) was selected on TCBS agar plates containing ampicillin and tetracycline. The presence of wild-type *vuuA* in RC160 was confirmed by PCR using the vuuA-start and vuuA-comp-R primers.

**4. Deletional mutation and *in trans* complementation of *crp***

A *crp* deletion mutation was constructed in M06-24/O using the suicide vector pKAS32 [43, 46]. The *Hind*III-*Sal*I fragment containing *crp* was cloned into pUC19 (pCMM714). The DNA fragment encompassing the majority of the open reading frame of *crp* was deleted from pCMM714 by digestion with *Pst*I and *Sph*I and blunt-end ligation (pCMM715). The *Eco*RI-*Eco*RV fragment from pCMM715 was cloned into pKAS32 (pCMM716), and then transformed into SY327 λ*pir* and *E. coli* SM10 λ*pir* [45], and subsequently transferred to CMM2100 [43] by conjugation. Transconjugants were selected on TCBS agars containing ampicillin or streptomycin. The deletion mutation of *crp* in CMM710 was confirmed by PCR using the CRP1 and CRP7 primers.

To restore wild-type *crp*, the plasmid pCMM712 was transferred into CMM710 by triparental mating using the conjugative helper plasmid pRK2013 [50]. Transconjugants were selected on TCBS agars containing tetracycline and confirmed by PCR using the CRP1 and CRP7 primers. The resulting *crp*-complemented strain was designated CMM714.

**5. Deletional mutation and *in trans* complementation of *fur***

A *fur*-deletion mutant was constructed in M06-24/O by crossover PCR, as

previous described [23]. Two pairs of the PCR primers (Fur-1/Fur-2 and Fur-3/Fur-4) were used for the PCR amplification of *fur*. The two PCR products were used as a template for crossover PCR using the Fur-1 and Fur-4 primers. The resulting PCR product was cloned into pCR®2.1-TOPO® vector (Invitrogen, Korea) to generate pCMM236. The *Sac*I and *Xba*I fragment was excised from pCMM236 and cloned into the suicide vector pDM4 [48]. The resulting plasmid pCMM237 was transformed into *E. coli* SY327 λ*pir* and SM10 λ*pir* [45], and subsequently transferred to M06-24/O by conjugation. Transconjugants were selected on TCBS agar containing chloramphenicol and on HI agar containing 10% sucrose. The resulting deletional mutation of *fur* was confirmed by PCR using the Fur-1 and 4 primers and the strain was designated CMM2303.

To restore wild-type *fur* in CMM2303, the 861-bp *Bam*HI-*Hin*dIII fragment containing the regulatory region and open reading frame of *fur* was amplified using the PCR primers; fur-comp-F with a *Bam*HI overhang and fur-comp-R with a *Hin*dIII overhang, and was subsequently subcloned into the broad host range vector pLAFR3II (named pRC132). The plasmid pRC132 was transferred into CMM2303 via triparental mating using the conjugative helper plasmid pRK2013 [50]. A stable transconjugant was selected on TCBS agar plates containing ampicillin and tetracycline and was named RC144. The presence of wild-type *fur* on plasmid in RC144 was confirmed by PCR using the fur-comp-F/R primers.

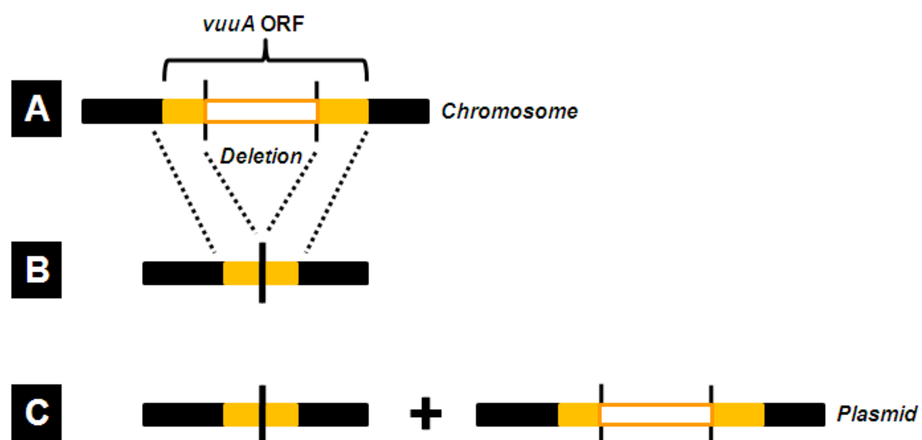


**Figure 3. An example of the construction of deletion mutation and *in trans* complementation.** (A) The wild-type *vuuA* gene. (B) A *vuuA* deletion mutation was constructed by deleting the internal portion of *vuuA* open reading frame (ORF) via various means such as cross-over PCR. (C) The *vuuA* deletion mutation was *in trans* complemented by introducing a plasmid harboring the regulatory region and whole *vuuA* ORF, which is originated from the wild-type strain.

### 6. $P_{vuuA}$::*lacZ* transcriptional fusion

A chromosomal $P_{vuuA}$::*lacZ* transcriptional reporter was constructed using the R6K origin suicide vector pDM4 in *V. vulnificus* CMM2101, as shown in Figure 4. First, the $P_{vuuA}$::*lacZ* transcriptional fusion was constructed using pQF52 containing the promoterless *lacZ* [49]. The 856-bp fragment from -814 to +12 bp from the start codon of *vuuA* was amplified using PCR primers; vuuA-rep-F with *Bam*HI overhang and vuuA-rep-R with *Kpn*I overhang. The amplified fragment was subcloned into pQF52 (named pRC118). The *Bam*HI-*Sca*I fragment containing the $P_{vuuA}$::*lacZ* fragment from pRC118 was subsequently subcloned into pDM4 cut with *Bgl*II and *Sma*I (named pRC126). The resulting plasmid pRC126 was transformed into *E. coli* SY327 λ*pir* and SM10 λ*pir* [45], and transferred to CMM2101 by conjugation. A stable transconjugant were selected on TCBS agar containing chloramphenicol and was named RC130. The presence of the $P_{vvuA}$::*lacZ* fusion was confirmed by β-galactosidase assay [52].

The *crp*-deleted $P_{vuuA}$::*lacZ* transcriptional reporter RC132 was constructed by incorporating the $P_{vuuA}$::*lacZ* fusion on pRC126 into the chromosome of RC100 by conjugation, as described above. RC100 was constructed by introducing a *lacZ* deletion mutation into CMM710 as described previously with only one exception that pDM4 was used instead of pKAS32 [43, 44].

To restore wild-type *crp* in the *crp*-deleted $P_{vuuA}$::*lacZ* transcriptional reporter RC132, the plasmid pCMM712 containing wild-type *crp* was transferred into RC132 via triparental mating using the conjugative helper plasmid pRK2013 as described previously [43, 50]. A stable transconjugant was selected on TCBS agar plates containing chloramphenicol, ampicillin and tetracycline, and was named RC260. The presence of wild-type *crp* on plasmid in

RC260 was confirmed by PCR using the CRP1 and CRP7 primers.

The *fur*-deleted $P_{vuuA}$::*lacZ* transcriptional reporter RC134 was constructed by introducing a *fur*-deletion mutation in RC130, as described above.

### 7. Construction of a chromosomal $P_{crp}$::*lacZ* transcriptional reporter

A chromosomal $P_{crp}$::*lacZ* transcriptional reporter was constructed using the R6K origin suicide vector pDM4 in CMM2101. The $P_{crp}$::*lacZ* transcriptional fusion was constructed using pQF52 containing the promoterless *lacZ* [49]. 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 subcloned into pQF52 (named pRC182). The *Bgl*II-*Sca*I fragment containing the $P_{crp}$::*lacZ* fragment from pRC182 was subsequently subcloned into pDM4 cut with *Bgl*II and *Sma*I (named pRC184). The resulting plasmid pRC184 was transformed into *E. coli* SY327 λ*pir* and SM10 λ*pir* [45], and was transferred to CMM2101 by conjugation. A stable transconjugant was selected on TCBS agar containing chloramphenicol and was named RC242. The presence of the $P_{crp}$::*lacZ* transcriptional fusion was confirmed by β-galactosidase assay [52].

### 8. Construction of chromosomal $P_{fur}$::*lacZ* transcriptional reporters

A chromosomal $P_{fur}$::*lacZ* transcriptional reporter was constructed using the R6K origin suicide vector pDM4 in CMM2101. The $P_{fur}$::*lacZ* transcriptional fusion was constructed using pQF52 containing a promoterless *lacZ* [49]. The 882-bp fragment containing the regulatory region of *fur* was amplified using the PCR primers; fur-rep-F with a *Kpn*I overhang and fur-rep-R with a *Hin*dIII overhang. The amplified fragment was subcloned into pQF52 (named pRC160).

The *Sph*I-*Sca*I fragment containing the $P_{fur}$::*lacZ* fragment from pRC160 was subsequently subcloned into pDM4 cut with *Sph*I and *Sma*I (named pRC166). The resulting plasmid pRC166 was transformed into *E. coli* SY327 λ*pir* and SM10 λ*pir* [45], and was transferred to CMM2101 by conjugation. A stable transconjugant was selected on TCBS agar containing chloramphenicol and was named RC190. The presence of $P_{fur}$::*lacZ* transcriptional fusion in RC190 was confirmed by β-galactosidase assay [52].

A *crp*-deleted $P_{fur}$::*lacZ* transcriptional reporter RC194 was constructed by introducing the $P_{fur}$::*lacZ* transcriptional fusion into RC100 by conjugation, as described above. To restore wild-type *crp* in RC194, the plasmid pCMM712 was transferred into RC194 by triparental mating, as described above. The resulting *crp*-complemented $P_{fur}$::*lacZ* transcriptional reporter strain was designated RC300.

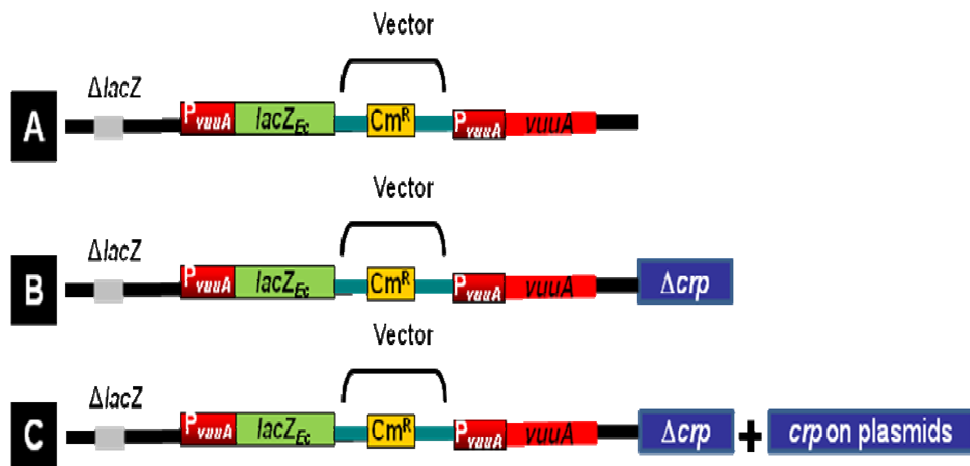


**Figure 4. An example of the construction of the *lacZ-fused* merozygotic transcriptional reporters.** (A) First of all, *V. vulnificus lacZ* must be mutated (*ΔlacZ*). The promoter region of *vuuA* ($P_{vuuA}$) was fused with a new *lacZ*, which is originated from *Escherichia coli* (*Ec*) and displaces the open-reading frame of *vuuA.* The simultaneous presence of the recombinant reporter portion $P_{vuuA}$::$lacZ_{Ec}$ and the orginal wild-type *vuuA* gene $P_{vuuA}$::*vuuA,* is called merozygote. (B and C) This *vuuA* transcription reporter was also constructed into CMM710 with crp-deletion (*Δcrp*) and into CMM714 with *crp-in trans* complementation (*crp* on plasmids) to observe the effect of *crp* mutation on *vuuA* expression.

**9. Growth condition and measurement**

In order to adapt bacteria to iron-limited conditions and to reduce intracellular iron storage, bacteria were cultured in HI broth containing 200 μM dipyridyl for 12 h. These preconditioned bacteria in the late exponential growth phase were inoculated into DF-HI broth or DF-HI broths containing 0.5 mg/ml PT or HT, and cultured with vigorous shaking (220 rpm) at 37°C for 24 h. In addition, to further limit free iron in PT-containing DF-HI, dipyridyl was additionally added to the media. The initial bacterial inoculum was about 1 x $10^6$ cells/ml. Bacterial growth was spectrophotometrically measured by the optical density at 600 nm ($OD_{600}$) of the aliquots.

**10. Measurement of β-galactosidase activity**

During culture, aliquots was harvested at indicated times and the $OD_{600}$) value of the aliquots was measured. A portion (0.1 ml) of the aliquots was mixed with 0.9 ml of Z buffer (0.06 M $Na_2HPO_4$, 0.04 M $NaH_2PO_4$, 0.01 M KCl, 0.001 M $MgSO_4$, 0.05 M β-mercaptoethanol). The mixture was solubilized with chloroform and 0.1% SDS. The solubilized aliquots was mixed with 0.2 ml of 4 mg/ml *o*-nitrophenylgalactoside, and allowed to react at 37°C. After the reaction was terminated with 0.25 ml of 2 M $Na_2CO3$, the optical density at 420 nm ($OD_{420}$) and 550 nm ($OD_{550}$) was measured. Miller units are derived using the formula: 1000 x ($OD_{420}$-(1.75 x $OD_{550}$)/(time in min x 0.1 x $OD_{600}$), as previously described [52].

**11. 6 M urea-gel electrophoresis**

To observe the ability of *V. vulnificus* strains to remove iron from transferrin

molecules during culture, culture supernatants were obtained from DF-HI broths containing 0.5 mg/ml PT or HT at appropriate times by centrifugation. Equal volumes (20 μl) of culture supernatants were electrophoresed on 6 M urea-PAGE gels, as previously described [53], and were stained with Coomassie blue.

**12. Western blot**

Western blot was conducted using the adsorbed rabbit polyclonal anti-VuuA-body described above. The bacterial pellets containing approximately 1 x $10^8$ cells of *V. vulnificus* strains were boiled for 10 min, and the supernatants were used as cell lysates. The cell lysates were electrophoresed on 10% SDS-PAGE gels. Thereafter, separated proteins were transferred to nitrocellulose transfer membrane (PROTRAN, Whatman GmbH, Germany). The membranes were incubated with blocking solution (0.2% Tween 20 and 5% Skim milk in PBS) at $4^oC$ overnight, allowed to react with the adsorbed rabbit polyclonal anti-VuuA-body (1:250 diluted in washing buffer consisting of 0.1% Tween 20 and 1% Skim milk in PBS) and with anti-rabbit-IgG-body conjugated with alkaline phosphatase (Sigma; 1:15,000 diluted in washing buffer), and finally visualized with 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium substrate solution (Sigma).

# RESULTS

## 1. Specificity of rabbit polyclonal anti-VuuA-body

First of all, to confirm the specificity of the absorbed polyclonal anti-VuuA-body prepared in this study, the three *V. vulnificus* strains, M06-24/O with wild-type *vuuA*, RC156 with deleted *vuuA* and RC160 with *in trans* complementation of wild-type *vuuA,* were cultured in DF-HI containing 5 or 25 μM FC, the bacterial densities of the three strains were then adjusted to approximately 1 x $10^8$ cells/ml to obtain the cell lysates. After electrophoresis of the cell lysates, Western blot was conducted using the absorbed anti-VuuA-body as primary antibody (Figure 5). The antibody could bind only VuuA of *ca.* 72 kDa although a variety of proteins were observed on SDS-PAGE. At 5 μM FC, VuuA was profoundly expressed in M06-24/O but not in RC156, and was recovered to the M06-24/O level in RC160. In contrast, at 25 μM FC, *vuuA* was not or expressed or expressed at a very low level in all the three strains. These results clearly indicate that the absorbed polyclonal anti-VuuA-body is a useful antibody that can specifically detect only VuuA, as well as that VuuA is iron-repressible protein. Accordingly, this antibody was used for further experiments employed in this study.

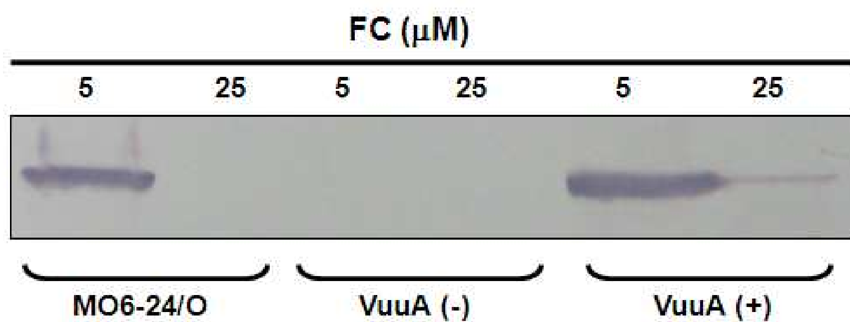


**Figure 5. Specificity of the absorbed polyclonal anti-VuuA-body produced in this study.** The three *V. vulnificus* strains, M06-24/O with wild-type *vuuA*, RC156 with deleted *vuuA* and RC160 with *in trans* complementation of wild-type *vuuA,* were cultured in DF-HI containing 5 or 25 μM ferric chloride (FC) for 12 h. The final bacterial densities were then adjusted to approximately 1 x $10^8$ cells/ml, and the cell lysates were obtained from the bacterial pellets. After electrophoresis of the cell lysates, Western blot was conducted using the absorbed anti-VuuA-body as primary antibody, as described in the Materials and Methods section.

### 2. Effect of iron concentration on *V. vulnificus* growth and *vuuA* expression

To observe the effect of iron concentration on *vuuA* expression and to determine appropriate iron concentrations which is suitable for the measurement of bacterial growth as well as *vuuA* expression levels, the *V. vulnificus* M06-24/O strain was cultured in DF-HI containing various FC concentrations (0 to 30 μM). Bacterial growth was measured 6 and 12 h after culture initiation and the bacterial densities at those times were then adjusted to approximately 1 x $10^8$ cells/ml, and the cell lysates were obtained from the bacterial pellets. After electrophoresis of the cell lysates, Western blot was conducted using the rabbit polyclonal anti-VuuA-body as primary antibody. Iron stimulated the growth of M06-24/O in a dose-dependent manner at FC concentrations of less than 10 μM, but not at FC concentrations of more than 10 μM (Figure 6A). In contrast, VuuA production was inhibited by iron in a dose-dependent manner and completely inhibited at the FC concentration of 30 μM (Figure 6B). These results indicate that excessive iron no longer stimulates *V. vulnificus* growth, and that VuuA production is tightly controlled by iron concentrations regardless of *V. vulnificus* growth levels, and that the Western blot method using the anti-VuuA-body produced in this study can detect VuuA production very sensitively. Furthermore, the FC concentration of 10 μM was determined to be critical for further experiments: namely, FC concentrations of more than 10 μM was regarded to be relatively iron-sufficient conditions, while FC concentrations of less than 10 μM was regarded to be relatively iron-deficient conditions.

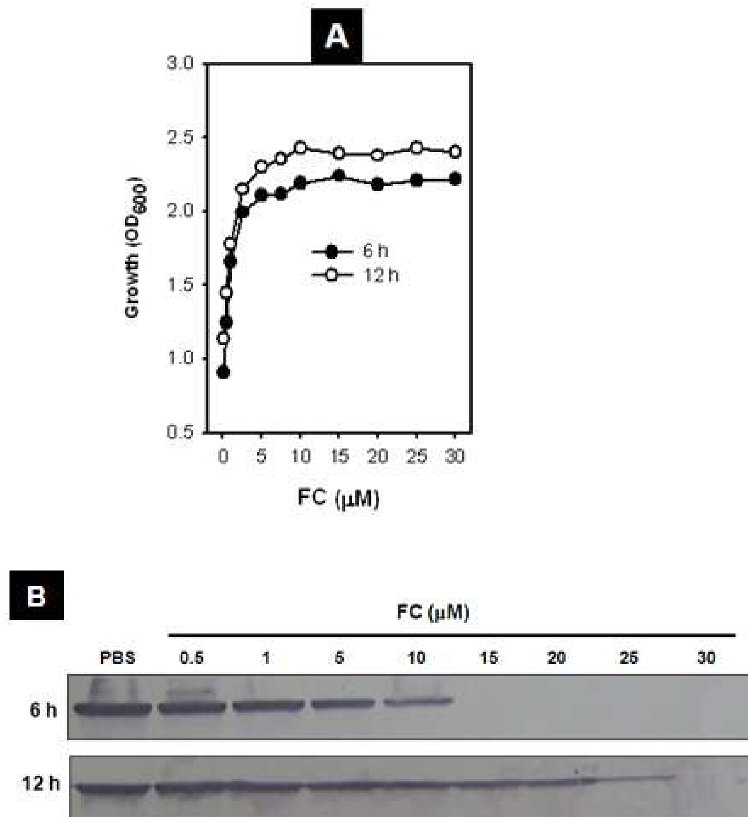


**Figure 6. The effect of iron concentration on *V. vulnificus* growth and *vuuA* expression.** The *V. vulnificus* M06-24/O strain were cultured in DF-HI containing various ferric chloride (FC) concentrations for 6 or 12 h. (A) Bacterial growth was measured by $OD_{600}$. (B) The final bacterial densities were then adjusted to approximately 1 x $10^8$ cells/ml, and the cell lysates were obtained from the bacterial pellets. After electrophoresis of the cell lysates, Western blot was conducted using the absorbed anti-VuuA-body as primary antibody, as described in the Materials and Methods section.

**3. Effect of *vuuA* mutation on the ability of *V. vulnificus* to utilize and grow on transferrin-bound iron**

To determine whether the ability of *V. vulnificus* to utilize transferrin-bound iron is dependent on the activity of the VuuA-mediated IUS, the three strains, M06-24/O with wild-type *vuuA*, RC156 with deleted *vuuA* and RC160 with *in trans* complementation of wild-type *vuuA,* were cultured in DF-HI with 0.5 mg/ml PT or HT. During culture, aliquots were removed to measure bacterial growth. The growth of M06-24/O was stimulated by the addition of transferrin-bound iron and was in proportion to the amount of iron contained in PT and HT (Figure 7). In contrast, the growth of RC156 was severely retarded, but not completely, despite addition of transferrin-bound iron, when compared with that of M06-24/O. This growth retardation in RC156 was completely recovered to the M06-24/O levels in RC160. Accordingly, these results clearly indicate that the ability of *V. vulnificus* to utilize and grow on transferrin-bound iron is mostly dependent on the activity of the VuuA-mediated IUS.

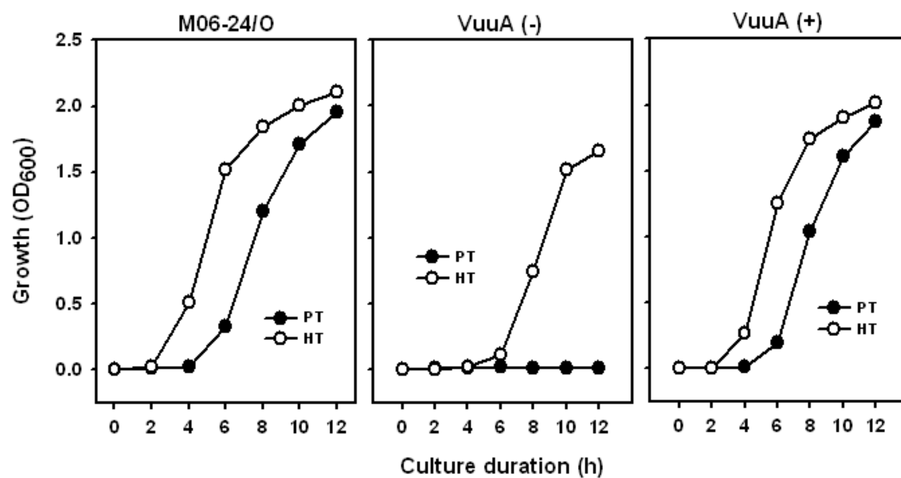


**Figure 7. Effect of *vuuA* mutation on the ability of *V. vulnificus* to grow on transferrin-bound iron**. The three strains, M06-24/O with wild-type *vuuA*, RC156 with deleted *vuuA* and RC160 with *in trans* complementation of wild-type *vuuA,* were inoculated at about 1 x $10^6$ cells/ml in DF-HI with 0.5 mg/ml partially iron-saturated transferrin (PT) or holotransferrin (HT), and cultured with vigorous shaking (220 rpm) at 37°C for 12 h. During culture, aliquots were removed to monitor bacterial growth, which was measured by $OD_{600}$.

### 4. The presence of putative CRP- and Fur-binding sites on the regulatory region of *fur* and *vuuA*

On analyzing sequences based on reported data [38], putative CRP-binding sites showing a similarity with *Escherichia coli* CRP-binding consensus sequences (5'-aattgtgatctagatcacattt-3' or 5'-aattgtgattcgtttcacattt-3') were found in the regulatory regions of both *fur* and *vuuA.* The putative CRP-binding sites in *fur* and *vuuA* were partially overlapped or juxtaposed with a putative Fur-binding sequence (5'-gataatgataaatcattatc-3') (Figure 8). Moreover, the putative CRP- or Fur-binding sequence was also founded in the regulatory region of *fur* or *vuuA* from other *V. vulnificus* strains such as CMCP6 and YJ016 and showed no difference among the strains. Accordingly, these results primarily suggest the two possibilities: (1) the global transcriptional regulator CRP can positively regulate the expression of *vuuA* encoding the vulnibactin receptor protein in *V. vulnificus,* and (2) CRP can also negatively regulate the expression of *vuuA* by controlling the expression of *fur* encoding Fur, which is well-known as a transcriptional repressor on *vuuA* [24, 25].

**Figure 8. Putative binding sites for cAMP-receptor protein (CRP) and ferric uptake regulator (Fur) in the regulatory region of *fur* (A: accession no. AY072036) and *crp* (B: accession no. AF156496) in *Vibrio vulnificus*.** Sequences were analyzed using the program DNAssist (ver.2.2; http://www.dnassist.org). The putative CRP- or Fur-binding consensus of *Escherichia coli* is as in the text.

### 5. Effect of iron concentration on *crp* expression

To determine the effect of iron concentration on *crp* expression at the transcription level, the merozygotic $P_{crp}$::*lacZ* transcription reporter RC242 strain was constructed. The merozygotic transcription reporter of a gene allows measuring its expression level without its mutation and is suitable for the measurement of the expression level of a transcription factor. The strain was cultured in DF-HI with 1.0, 5.0 and 10.0 μM FC. During culture, aliquots were removed to measure bacterial growth and β-galactosidase activity. The growth of RC242 was stimulated by iron in a dose-dependent manner, but the *crp* expression level was not increased by iron (Figure 9). Nevertheless, this result suggests that *crp* expression may remain slightly repressed by iron-Fur complex, rather than that it may not be affected at all by iron.

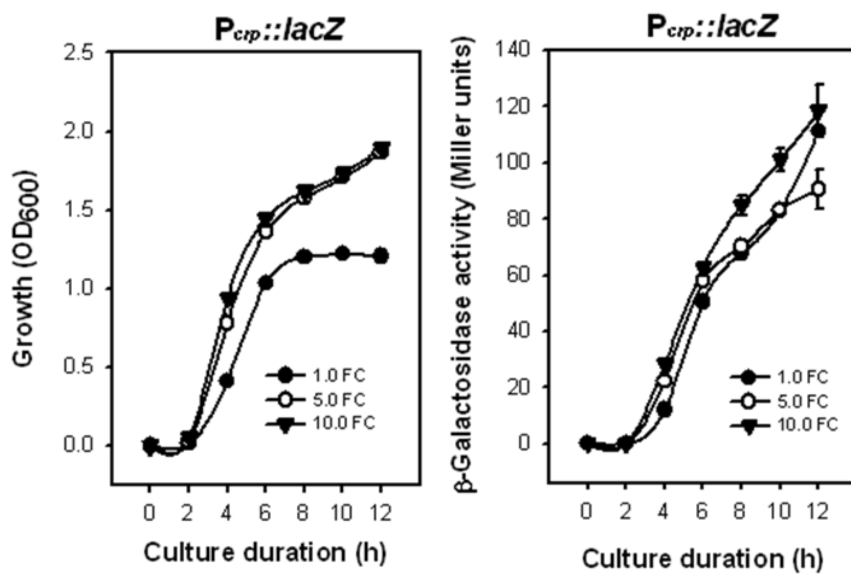


**Figure 9. The effect of iron concentration on *crp* expression.** The merozygotic $P_{crp}$*::lacZ* transcription reporter RC242 was inoculated at about 1 x $10^6$ cells/ml in DF-HI with 1.0, 5.0 and 10.0 μM FC, and cultured with vigorous shaking (220 rpm) at 37°C for 12 h. During culture, aliquots were removed to monitor bacterial growth and *crp* expression. Bacterial growth was measured by $OD_{600}$, and the level of *crp* expression was determined by measuring β-galactosidase activity with the Miller method [52].

**6. Effect of *crp* mutation on *fur* expression**

To determine the effect of CRP on *fur* expression at the transcription level, the three $P_{fur}$::*lacZ* transcription reporter strains, RC190 with wild-type *crp*, RC194 with deleted *crp* and RC300 with *in trans* complementation of wild-type *crp,* were cultured in DF-HI with 1.0, 5.0 or 10.0 μM FC. During culture, aliquots were removed to measure bacterial growth and β-galactosidase activity. The growth of RC190 was stimulated by iron in a dose-dependent manner and the *fur* expression level was also increased by iron in a dose-dependent manner (Figure 10). Expression of *fur* as well as bacterial growth was repressed in RC194; moreover, the *fur* expression in RC194 appeared not to be affected by iron concentration. Both the repressed *fur* expression and bacterial growth were recovered to the RC190 levels in RC300. Accordingly, these results clearly indicate that *fur* expression is positively regulated by iron and CRP. In addition, these results suggest that *fur* expression may be positively auto-regulated by the Fe-Fur complex.

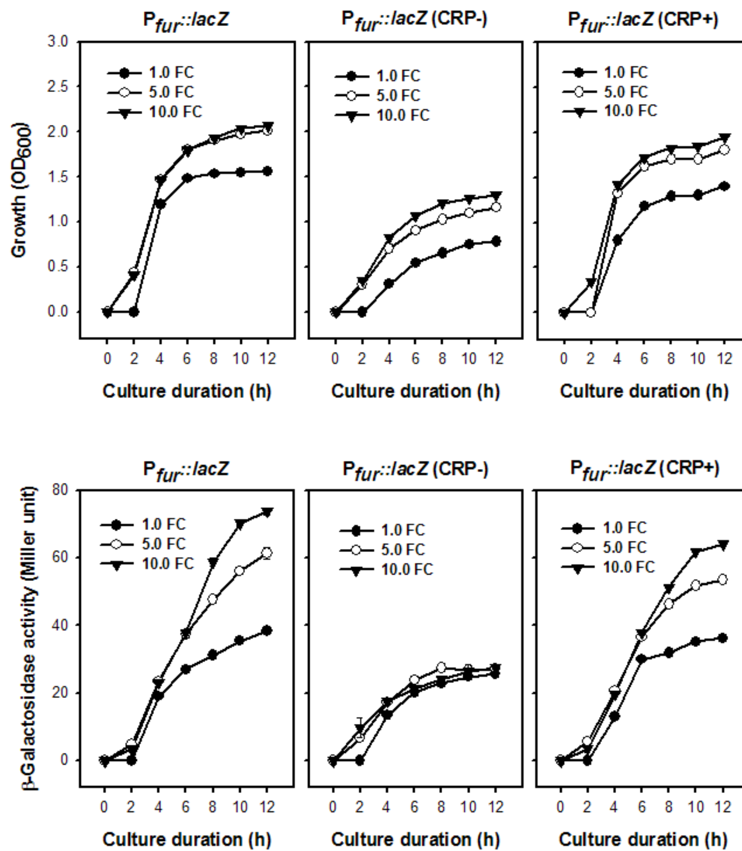


**Figure 10. The effect of *crp* mutation on *fur* expression.** The three $P_{fur}$::*lacZ* transcription reporters, RC190 with wild-type *crp*, RC194 with deleted *crp* (CRP-) and RC300 with *in trans* complementation of wild-type *crp* (CRP+), were inoculated in DF-HI containing 1.0, 5.0 or 10.0 μM FC and cultured with vigorous shaking (220 rpm) at 37°C for 12 h. During culture, aliquots were removed to measure bacterial growth (Upper row) and *fur* expression (Lower row). Bacterial growth was measured by $OD_{600}$, and the level of *fur* expression was determined by measuring β-galactosidase activity with the Miller method [52].

### 7. Effect of *crp* mutation on *vuuA* expression at the transcription level

To determine the effect of CRP on *vuuA* expression at the transcription level, the three $P_{vuuA}$*::lacZ* transcription reporter strains, RC130 with wild-type *crp*, RC132 with deleted *crp* and RC260 with *in trans* complementation of wild-type *crp,* were cultured in DF-HI with FC concentrations of 5.0 or 25.0 μM. During culture, aliquots were removed to measure bacterial growth and β-galactosidase activity. The growth of RC130 was stimulated by iron in a dose-dependent manner (data not shown). However, *vuuA* expression in RC130 was repressed by iron in a dose-dependent manner (Figure 11AB). Expression of *vuuA* as well as bacterial growth was almost completely repressed in RC132. Both the repressed *vuuA* expression and bacterial growth were recovered to the levels comparable to RC130 in RC260. Noticeably, *vuuA* expression was almost completely repressed in DF-HI with 25.0 μM FC regardless of the presence or absence of CRP. Accordingly, these results clearly indicate that the iron-repressible *vuuA* expression is positively regulated by CRP especially under iron-deficient conditions, whereas, especially under iron-sufficient conditions, *vuuA* expression is negatively regulated by the transcriptional repressor iron-Fur complex regardless of the presence or absence of CRP acting as a transcriptional activator.

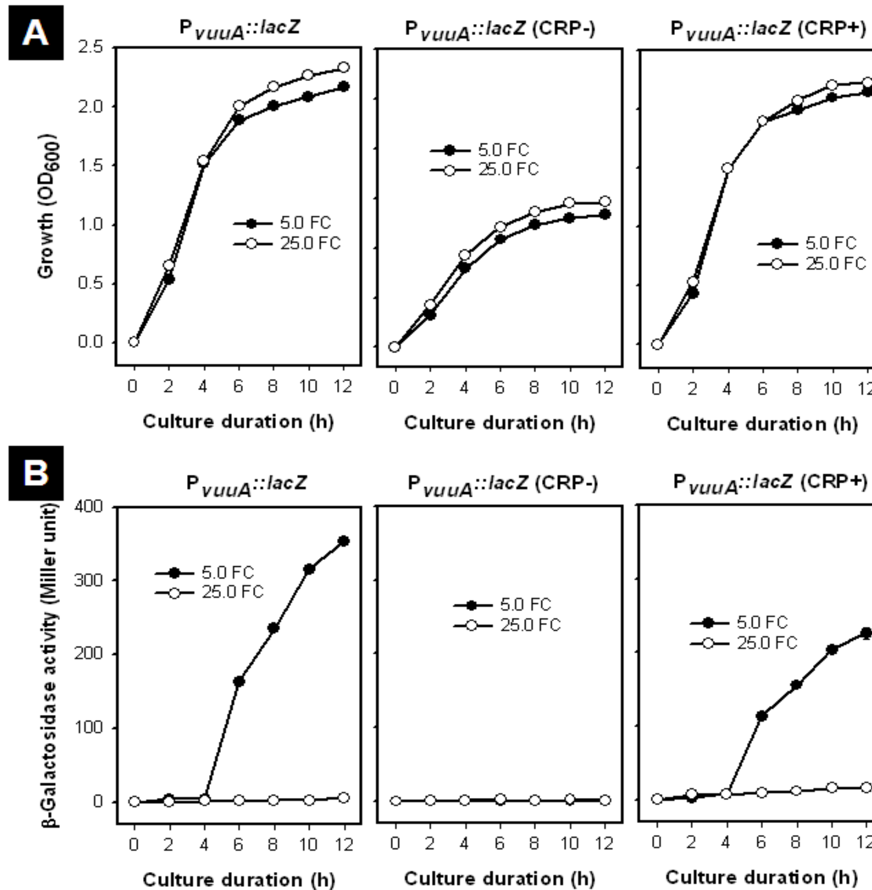


**Figure 11. The effect of *crp* mutation on *vuuA* expression at the transcription level.** The three $P_{vuuA}$*::lacZ* transcription reporters, RC130 with wild-type *crp*, RC132 with deleted *crp* (CRP-)and RC260 with *in trans* complementation of wild-type *crp* (CRP+), were inoculated at about 1 x $10^6$ cells/ml in DF-HI containing 5.0 or 25.0 μM FC and cultured with vigorous shaking (220 rpm) at 37°C for 12 h. During culture, aliquots were removed to measure bacterial growth (A) and *vuuA* expression (B). Bacterial growth was measured by $OD_{600}$ and the level of *vuuA* expression was determined by measuring β-galactosidase activity with the Miller method [52].

### 8. Effect of *crp* mutation on *vuuA* expression at the protein level

To determine the effect of CRP on *vuuA* expression at the protein level, the three strains, MO6-24/O with wild-type *crp*, CMM710 with deleted *crp* and CMM714 with *in trans* complementation of wild-type *crp,* were cultured in DF-HI with 5.0 or 25.0 μM FC. The final bacterial densities were then adjusted to approximately 1 x $10^8$ cells/ml, and the cell lysates were obtained from the bacterial pellets. After electrophoresis of the cell lysates, Western blot was conducted using the absorbed anti-VuuA-body as primary antibody. The growth of the three strains was stimulated by iron in a dose-dependent manner (data not shown). However, VuuA production in M06-24/O was decreased by iron in a dose-dependent manner (Figure 12). VuuA production was severely, but not completely, decreased in CMM710. Unlike at the *vuuA* transcription level, VuuA production was observed albeit at a very low level even in CMM710. Both the decreased VuuA production was recovered to 70~80% of the M06-24/O levels in CMM714. Noticeably, VuuA production was almost completely repressed in DF-HI with 25.0 μM FC regardless of the presence or absence of CRP. Accordingly, these results clearly indicate that the iron-repressible VuuA production is positively regulated by CRP especially under iron-deficient conditions, whereas, especially under iron-sufficient conditions, VuuA production is negatively regulated by the transcriptional repressor iron-Fur complex regardless of the presence or absence of CRP acting as a transcriptional activator.

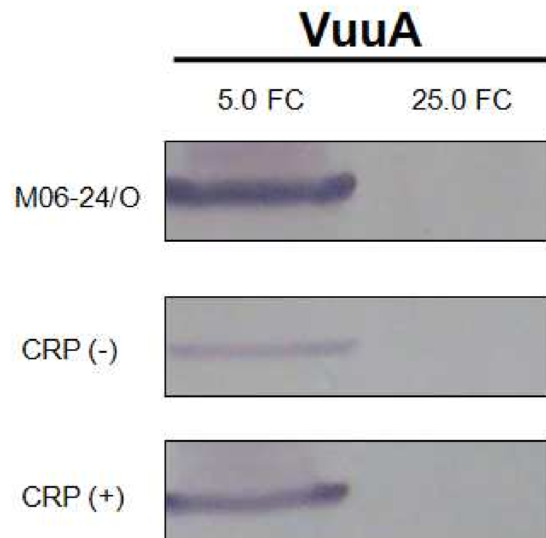


**Figure 12. The effect of *crp* mutation on *vuuA* expression at the protein level.** The three strains, M06-24/O with wild-type *crp*, CMM710 with deleted *crp* (CRP-) and CMM714 with *in trans* complementation of wild-type *crp* (CRP+), were inoculated at about 1 x $10^6$ cells/ml in DF-HI containing 5.0 or 25.0 μM FC, and cultured with vigorous shaking (220 rpm) at 37°C for 12 h. The final bacterial densities were then adjusted to approximately 1 x $10^8$ cells/ml, and the cell lysates were obtained from the bacterial pellets. After electrophoresis of the cell lysates, Western blot was conducted using the ab-sorbed anti-VuuA-body as primary antibody, as described in the Materials and Methods section.

**9. Effect of *crp* mutation on the ability of *V. vulnificus* to grow on and to utilize transferrin-bound iron**

To determine the effect of CRP on the ability of *V. vulnificus* to grow on and to utilize transferrin-bound iron, the three strains, M06-24/O with wild-type *crp*, CMM710 with deleted *crp* and CMM714 with *in trans* complementation of wild-type *crp,* were cultured in DF-HI with PBS or 0.5 mg/ml PT. During culture, aliquots were removed to measure bacterial growth and to obtain culture supernatants to measure the ability of the three strains to utilize transferrin-bound iron on 6 M urea-gels. The growth of the three strains was stimulated by the addition of transferrin-bound iron (Figure 13A, B), but the growth of CMM710 was less stimulated by addition of transferrin-bound iron than that of M06-24/O. This growth defect in CMM710 was completely recovered to the M06-24/O levels in CMM714.

To directly observe iron-removal from transferrin molecules, 6 M urea-gel electrophoresis was conducted (Figure 13C). Transferrin molecules in PT were separated into four forms on 6 M urea-gel, according to their iron-saturation levels; diferric (DF)-, N-terminal monoferric (MN)-, C-terminal monoferric (MC)-, and apoferric (AF)-forms. Because *V. vulnificus* continued to remove and utilize iron during culture, transferrin molecules in PT were increasingly shifted up from DF-form to MN- or MC-forms to AP-form with the culture duration. M06-24/O could efficiently acquire iron from PT, shifting-up transferrin molecules especially from DF-form to MN- or MC-form. In contrast, CMM710 did neither acquire iron from PT, nor shift up transferrin molecules. This defect of CMM710 was completely recovered to the M06-24/O levels in CMM714. Accordingly, these results clearly indicate that the ability of *V. vulnificus* to grow on and to utilize transferrin-bound iron is under the positive control of CRP.

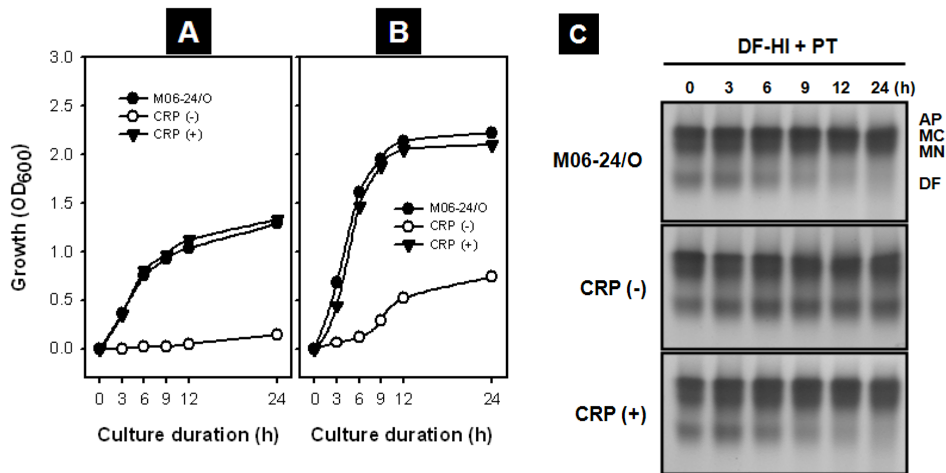


**Figure 13. Effect of *crp* mutation on the ability of *V. vulnificus* to grow on and to utilize transferrin-bound iron.** The three strains, M06-24/O with wild-type *crp*, CMM710 with deleted *crp* (CRP-) and CMM714 with *in trans* complementation of wild-type *crp* (CRP+), were inoculated at about 1 x $10^6$ cells/ml in DF-HI with phosphate-buffered saline (A) or 0.5 mg/ml partially iron-saturated transferrin (PT; B), and cultured with vigorous shaking (220 rpm) at 37°C for 24 h. During culture, aliquots were removed to monitor bacterial growth (A and B) and the ability to remove iron from PT (C). Bacterial growth was measured by $OD_{600}$. The ability to remove iron from PT was determined on 6 M urea-gel electrophoresis, as described in the Materials and Methods section. Transferrin molecules in PT are separated into four forms on 6 M urea-gel, according to their iron-saturation levels; diferric (DF)-, N-terminal monoferric (MN)-, C-terminal monoferric (MC)-, and apoferric (AF)-forms.

### 10. Effect of *fur* mutation on *vuuA* expression

To determine the effect of Fur on *vuuA* expression at the transcription level, the two $P_{vuuA}$*::lacZ* transcription reporter strains, RC130 with wild-type *fur* and RC134 with deleted *fur,* were cultured in DF-HI with 1.0, 5.0 or 25.0 μM FC. During culture, aliquots were removed to measure bacterial growth and β-galactosidase activity. The growth of RC130 was stimulated by iron in a dose-dependent manner (Figure 14). However, *vuuA* expression in RC130 was repressed by iron in a dose-dependent manner. Bacterial growth was slightly stimulated in RC134. Nevertheless, *vuuA* expression was de-repressed in RC134. The extent of *vuuA* de-repression was increased in proportion to FC concentration; namely, the effect of Fur on *vuuA* expression was increased in proportion to FC concentration. In addition, *vuuA* expression was almost completely repressed in DF-HI with 10.0 μM FC regardless of the presence of CRP. Accordingly, these results clearly indicate that the iron-repressible *vuuA* expression is negatively regulated by the iron-Fur complex especially under iron-sufficient conditions, whereas, especially under iron-deficient conditions, the iron-Fur complex acting as a transcriptional repressor has a lesser effect on *vuuA* expression than CRP acting as a transcriptional activator.

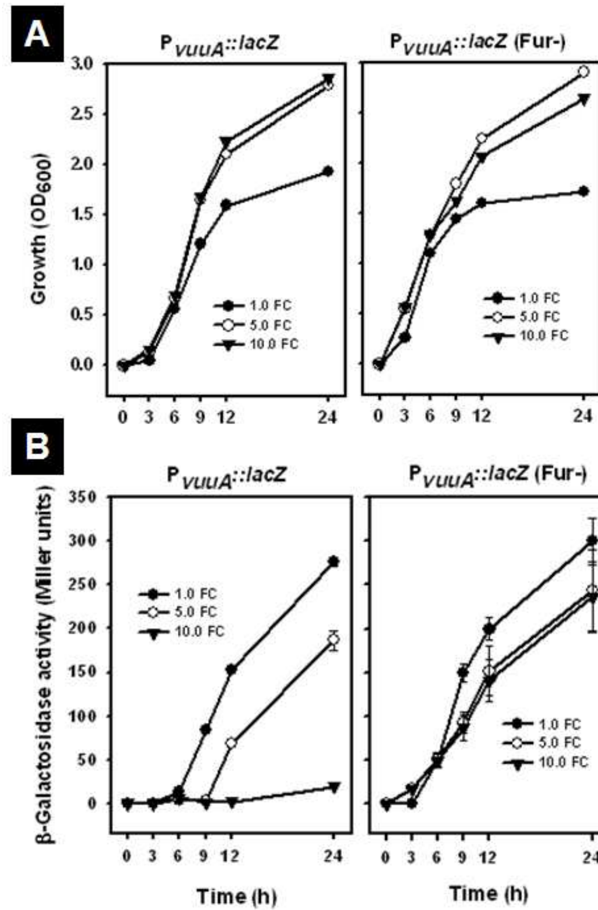


**Figure 14. Effect of *fur* mutation on *vuuA* expression.** The two $P_{vuuA}$::*lacZ* transcription reporter strains, RC130 with wild-type *fur* and RC134 with deleted *fur,* were inoculated at about 1 x $10^6$ cells/ml in DF-HI containing 1.0, 5.0 or 10.0 μM FC, and cultured with vigorous shaking (220 rpm) at 37°C for 24 h. During culture, aliquots were removed to measure bacterial growth (A) and *vuuA* expression (B). Bacterial growth was measured by $OD_{600}$ and the level of *vuuA* expression was determined by measuring β-galactosidase activity with the Miller method [52].

### 11. Effect of *fur* mutation on the ability of *V. vulnificus* to grow on and to utilize transferrin-bound iron

To determine the effect of Fur on the ability of *V. vulnificus* to grow on and to utilize transferrin-bound iron, the three strains, M06-24/O with wild-type *fur*, CMM2303 with deleted *fur* and RC144 with *in trans* complementation of wild-type *fur,* were cultured in DF-HI with 0.5 mg/ml PT or HT. During culture, aliquots were removed to measure bacterial growth and to obtain culture supernatants to measure the ability of the three strains to utilize transferrin-bound iron on 6 M urea-gels. The growth of the three strains was stimulated by the addition of transferrin-bound iron and was in proportion to the iron level contained in PT and HT (Figure 15A). The growth of CMM2303 was more stimulated by addition of transferrin-bound iron than that of M06-24/O. This growth stimulation in CMM2303 was completely recovered to the M06-24/O levels in RC144.

To directly observe iron-removal from transferrin molecules, 6 M urea-gel electrophoresis was conducted (Figure 15B). M06-24/O could efficiently acquire iron from PT or HT and shifting-up of transferrin molecules especially from DF-form to MN- or MC- to AP-form was clearly observed. In contrast, CMM2303 acquired iron from PT or HT and shifted up transferrin molecules more efficiently than M06-24/O. This increase in CMM2303 was completely recovered to the M06-24/O levels in RC144. Accordingly, these results clearly indicate that the ability of *V. vulnificus* to grow on and to utilize transferrin-bound iron is under the negative control of the iron-Fur complex.

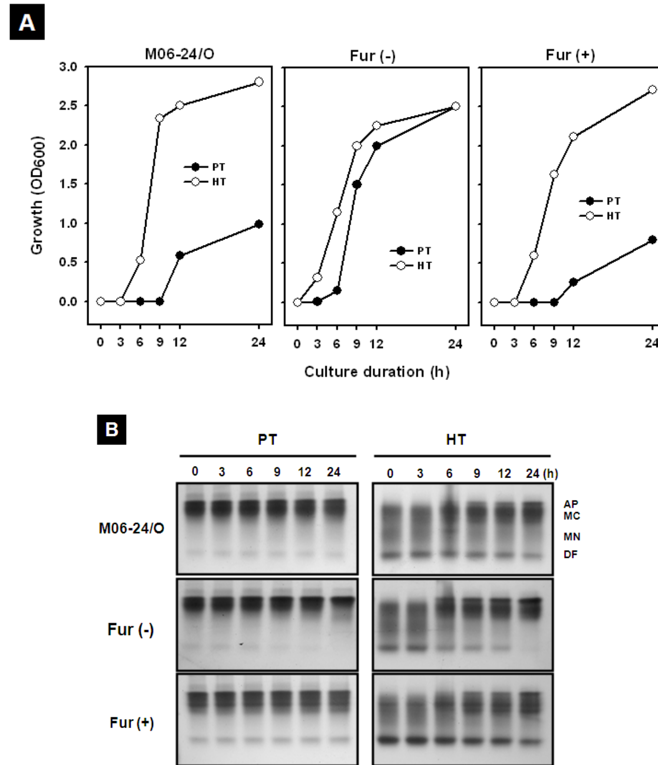


**Figure 15. Effect of *fur* mutation on the ability of *V. vulnificus* to utilize transferrin-bound iron.** The three strains, M06-24/O with wild-type *fur*, CMM2303 with deleted *fur* and RC144 with *in trans* complementation of wild-type *fur,* were inoculated at about 1 x $10^6$ cells/ml in DF-HI with 0.5 mg/ml partially iron-saturated transferrin (PT) and holotransferrin (HT), and cultured with vigorous shaking (220 rpm) at 37°C for 24 h. During culture, aliquots were removed to monitor bacterial growth (A) and the ability to remove iron from PT or HT (B). Bacterial growth was measured by $OD_{600}$. The ability to remove iron from PT was determined on 6 M urea-gel electrophoresis, as described in the Materials and Methods section. DF, MN, MC and AF indicate diferric, N-terminal monoferric, C-terminal monoferric and apoferric.

# DISCUSSION

It is well documented in accordance with the molecular version of Koch's postulates that *V. vulnificus* IUS, especially the VuuA-mediated *IUS*, plays a crucial role in the pathogenesis of *V. vulnificus* infections as one of the authentic virulence factors [9, 18-27]. However, there has been little understanding with regard to regulation of the VuuA-mediated IUS, except that it is negatively regulated by the Fe-Fur complex [24, 25]. A previous study suggested that *crp* mutation down-regulates the expression of the VuuA-mediated IUS [43]. Subsequently, the present study shows the unequivocal evidence for the previous results and the presence of a new sophisticated regulatory mechanism for expression of the VuuA-mediated IUS. In summary, (1) there is a close link between the cAMP-CRP complex which is responsive to carbon, especially glucose, availability as an energy source and the iron-Fur complex which is responsive to iron availability, (2) that the cAMP-CRP complex positively regulates *fur* expression, as well as *vuuA* expression, (3) that the cAMP-CRP complex positively regulates *vuuA* expression especially under iron-deficient conditions, and (4) that the cAMP-CRP complex can negatively regulate *vuuA* expression via the iron-Fur complex especially under iron-sufficient conditions.

Based on published reports, the Western blot method using anti-VuuA-body, as well as 6 M urea-gel electrophoresis which is a functional assay, employed in this study was first used to measure and compare the VuuA-mediated IUS activity. The rabbit polyclonal anti-VuuA-body used in this study was specific enough to discriminate only VuuA in cell lysates and could detect VuuA on Western blot more sensitively than the *lacZ*-fused transcription reporter assay (Figures 5, 6, 11 and 12). Empirically, it is sometimes inaccurate to measure and compare the expression levels of *vuuA* only at the transcriptional level and

the activity of the VuuA-mediated IUS only with functional assays such as 6 M urea gel electrophoresis, especially under the condition that bacterial growth is suppressed. Because it still has the ability to produce vulnibactin, even a VuuA-negative mutant can capture iron from transferrin molecules but not take up the Fe-vulnbactin complex into cells. As a result, no difference in the ability to remove iron from transferrin molecules was observed on 6 M urea-gel electrophoresis between a VuuA-negative mutant and its wild-type strain. Accordingly, the functional assays such as 6 M urea-gel electrophoresis should be combined with the more sensitive and specific Western blot method to accurately measure and compare the VuuA-mediated IUS activity, as in this study.

Iron stimulated *V. vulnificus* growth in a dose-dependent manner at less than 10 μM FC, but not at more than 10 μM FC (Figure 6A), which indicates that excessive iron is rather harmful to *V. vulnificus* growth. Because excess intracellular iron is toxic due to its tendency to catalyze free radical generation, iron-uptake should be tightly controlled in respond to intracellular iron level. [1-5]. However, iron levels required for optimal growth or the ability to acquire iron are extremely variable among bacteria [3, 17, 32, 54]. Recently, *V. vulnificus* is found to be a ferrophilic bacterium that requires relatively more iron for growth and survival than other microbial pathogens [15]. Ferrophilic bacteria generally possess impaired IUS and cannot efficiently acquire iron. *V. vulnificus* produces only a very small amount of siderophores, thus shows ferrophilic characteristics despite the presence of several IUS. Although *V. vulnificus* shows ferrophilic characteristics, FC of more than 10 μM appears to be toxic for *V. vulnificus*. Accordingly, the FC concentrations of more than 10 μM represent relatively iron-sufficient conditions, while the FC concentration of less than 10 μM represent relatively iron-deficient conditions in this study. Unlike *V. vulnifi-*

*cus* growth, VuuA production was iron-repressible in a dose-dependent manner and completely repressed at the 30 μM FC (Figure 6B). These results indicate that VuuA production is tightly controlled in respond to intracellular iron level regardless of growth levels.

The growth of M06-24/O with wild-type *vuuA* or of RC160 with *in trans*-complementation of *vuuA* was stimulated by the addition of transferrin-bound iron, but the growth of RC156 with deleted *vuuA* remained severely retarded, but not completely, despite addition of transferrin-bound iron (Figure 7), which clearly indicates that the ability of *V. vulnificus* to utilize and grow on transferrin-bound iron is dependent mostly on the activity of the VuuA-mediated IUS and partly on the activity of the other IUS. *V. vulnificus* produces the two types of siderophores: vulnibactin and hydroxamate siderophores [18]. Hydroxamate-siderophore can also stimulate *V. vulnificus* growth even though it shows lower affinity for iron than vulnibactin [18-23]. However, neither the genes related to the hydroxamate siderophore-mediated IUS have been known, nor the structure of hydroxamate-siderophore has been determined until now. Accordingly, the actual function of hydroxamate siderophore in the virulence of *V. vulnificus* remains totally unknown. Moreover, *V. vulnificus* has the four different IUS, as described above. Further studies are required for determine which IUS is involved in the utilization of transferrin-bound iron together with the VuuA-mediated IUS. More recently, the author and colleagues constructed a deletion mutant of *iutA*, which encodes an outer membrane receptor for heterologous *E. coli* aerobactin (Figure 1) [30], and found that the mutant has the impaired ability to utilize transferrin-bound iron. This finding provides a clue that the IutA-mediated IUS may partially be involved in the utilization of transferrin-bound iron (details will be reported elsewhere).

Putative CRP-binding sites showing a similarity with *Escherichia coli* CRP-binding consensus sequences [38] were found in the regulatory regions of both *fur* and *vuuA.* The sites in *fur* and *vuuA* were partially overlapped or juxtaposed with a putative Fur-binding sequence (Figure 8). Moreover, the similar putative CRP- or Fur-binding sequence was also founded in other *V. vulnificus* strains such as CMCP6 and YJ016 (data not shown). These findings need to be confirmed using protein-DNA binding assays such as electrophoretic mobility shift assay called EMSA or foot-printing [41, 42], but suggest the two possibilities: (1) the global transcriptional regulator cAMP-CRP complex can positively regulate *vuuA* expression, and (2) the complex can also negatively regulate *vuuA* expression by controlling *fur* expression [24, 25]. Moreover, these findings primarily provide a clue that carbon metabolic processes may be closed linked to iron metabolic processes and thus the metabolic processes may be mutually regulated in responsive to carbon or iron-availability. Iron is an essential element required for carbon metabolic processes, particularly the aerobic Krebs cycles and electron transport system. Accordingly, the expression of IUS should be directly or indirectly affected by the cAMP-CRP system responsive to carbon-availability. The interaction between the two systems has been documented in *Escherichia coli* [38, 39]. The mutation of *Escherichia coli crp* down-regulates the expression of the enterobactin-mediated IUS, and putative CRP-binding sites are found in the regulatory regions of the *entC* encoding enterobactin-specific isochorismate synthase and of the *fepA* gene encoding enterobactin receptor, moreover, the sites are overlapped with Fur-binding sites. The present study also offers a novel information that the cAMP-CRP complex can directly regulate the expression of *fur* in *V. vulnificus*. There has been only a report regarding the direct interaction between CRP and Fur in *Escherichia coli*

[39].

First of all, the effect of iron concentration on *crp* expression at the transcription level needs to be determined in order to verify the interaction between carbon and iron metabolic processes. However, *crp* expression appeared not to be influenced by iron concentrations (Figure 9). This finding can be simply interpreted: *crp* expression may not be affected by iron. However, if there is a close link between carbon and iron metabolic processes, the finding can be interpreted differently: *crp* expression may remain repressed by the Fe-Fur complex. In addition, CRP required cAMP to act as a transcription activator and the synthesis of cofactor cAMP is controlled by adenylate cyclase, which is encoded by *cya* [40]. Iron or Fur may affect the synthesis of cAMP by changing *cya* expression instead of *crp* expression. To solve this puzzle, *crp* or *cya* expression needs to be determined in the *fur*-mutated background through further studies.

To determine the presence or absence of the interaction between CRP and Fur, *fur* expression was compared in the background with wild-type *crp* or mutated *crp* (Figure 10). Iron-inducible *fur* expression was decreased by *crp* mutation, indicating that *fur* expression is positively regulated by the cAMP-CRP complex. The effect of *crp* mutation on *fur* expression in *V. vulnificus* was first observed in this study, and the effect of iron on *fur* expression was also observed by another research group [54, 55]. Interestingly, they reported that *fur* expression was repressed by iron. As the result of literature review, only the two different things was noted between the two research groups: (1) as *fur* transcription reporters, they used the $P_{fur}$::*luxAB* fusion, and the $P_{fur}$::*lacZ* fusion was used in this study, and (2) they added dipyridyl as an iron chelator into media, and iron was extracted from media by using hydroxyquinoline in

this study. This difference needs to be verified through further fine studies. In addition, *fur* expression was not affected by iron concentration in the *crp*-mutated background, suggesting that *fur* expression may be autoregulated by Fur. Recent studies have demonstrated that Fur can regulate its expression by direct binding [55, 56].

In the background with wild-type *crp*, both *vuuA* expression and VuuA production were iron-repressible (Figures 11 and 12). The *vuuA* expression at the transcription level was almost completely repressed by *crp* mutation, VuuA production was also severely decreased by *crp* mutation. The bacterial growth on transferrin-bound iron was severely retarded by *crp* mutation, and the ability to utilize transferrin-bound iron was also severely lowered by *crp* mutation (Figure 13). All these findings were observed even under iron-deficient conditions. Accordingly, these results clearly indicate that *vuuA* expression and VuuA production are positively regulated by the cAMP-CRP complex especially under iron-deficient conditions. These findings were first observed in a recent study [43] and more definitively evidenced in this study.

The role of CRP as a transcription activator is well known in the *lac* operon of *Escherichia coli* [57]. If glucose is not available, cAMP levels increase, cAMP binds and activates CRP, and the cAMP-CRP complex binds the CRP-binding site near the *lac* promoter and facilitates binding of RNA polymerase. Under this condition, transcription of *lac* operon occurs at maximal levels. If glucose is available, cAMP is not increased and CRP remains inactive. Under this condition, RNA polymerase cannot bind the promoter efficiently and transcription levels of *lac* operon remain low.

A noticeable thing is that, unlike at the *vuuA* transcription level, VuuA production was observed albeit at a very low level (Figure 12) and bacterial growth

was slightly stimulated by the addition of PT (Figure 13) even in the *crp*-mutated background, suggesting that *vuuA* expression constitutively occurs albeit at very low levels. Based on these findings, the *lacZ*-fused transcription reporter assay is thought to be less sensitive than the Western blot method especially under conditions that bacterial growth is severely retarded.

Another noticeable thing is that both *vuuA* expression and VuuA production was almost completely repressed under iron-sufficient conditions regardless of the presence or absence of CRP (Figures 11 and 12). These results clearly indicate that, especially under iron-sufficient conditions, *vuuA* expression and VuuA production is negatively regulated by the transcriptional repressor iron-Fur complex regardless of the presence or absence of CRP acting as a transcriptional activator.

In the *fur*-mutated background, bacterial growth was slightly stimulated and *vuuA* expression was de-repressed (Figure 14). The effect of Fur on *vuuA* expression was increased in proportion to iron concentration. In addition, *vuuA* expression was almost completely repressed under iron-sufficient conditions regardless of the presence or absence of CRP. The ability of *V. vulnificus* to grow on transferrin-bound iron and to utilize transferrin-bound iron was also increased in the *fur*-mutated background (Figure 15 AB). These results clearly indicate that the Fe-Fur complex negatively regulates *vuuA* expression especially under iron-sufficient conditions. The effect of Fur on *vuuA* expression is well documented in previous studies [24, 25]. Fur is primarily produced as an inactive apo-form. If a bacterium is under the condition with high iron-availability, intracellular iron levels are increased. Moreover, according to the results in this study, Fur is produced more under the condition with high iron-availability. Increased iron binds and activates increased Fur. The Fe-Fur com-

plex binds the Fur-binding site near the promoter of iron-repressible genes and facilitates binding of RNA polymerase. Under this condition, transcription of such genes is repressed. If a bacterium is under the condition with low iron-availability, intracellular iron levels are low, iron cannot bind Fur sufficiently and most Fur remains inactive. Inactive apo-Fur cannot bind the promoter of iron-repressible genes and interfere with binding of RNA polymerase. Under this condition, RNA polymerase can bind the promoter efficiently and transcription levels of such genes remain maximal.

A null mutation of the *V. vulnificus cya* or *crp* gene results in a pleiotropic change in virulence phenotype [40, 58]. The production of capsule, protease, and hemolysin and swarming motility, cytotoxicity, and mouse or *Caenorhabditis elegans* lethality were all reduced by this *cya* or *crp* mutation. These results indicate that the cAMP-CRP system plays an essential role in the global regulation of *V. vulnificus* virulence, as in other bacteria [59, 60]. In addition, pathogenic potential of a bacterium is primarily determined by many factors that influence its ability to survive and multiply within the host. As shown in this study, *in vitro or ex vivo V. vulnificus* growth especially under iron-deficient conditions is severely suppressed by *cya* or *crp* mutation, and this grow defect would primarily attenuates virulence. A *cya* or *crp* mutant in *Salmonella typhimurium* has proven to be useful for the preparation of live oral vaccine derivatives because of their avirulence [60]. Accordingly, a *cya* or *crp* mutant in *V. vulnificus* would have a similar utility for vaccine development because of their attenuated virulence.

# Conclusion

The present study shows the presence of a new sophisticated regulatory mechanism for expression of the VuuA-mediated IUS in accordance with the molecular version of Koch's postulates [9]. (1) There is a close link between the cAMP-CRP complex which is responsive to carbon availability especially glucose as an energy source and the iron-Fur complex which is responsive to iron availability. (2) The cAMP-CRP complex positively regulates Fur, as well as the activity of the VuuA-mediated IUS. (3) The cAMP-CRP complex positively regulates the activity of the VuuA-mediated IUS especially under iron-deficient conditions. (4) The cAMP-CRP complex can negatively regulate the activity of the VuuA-mediated IUS via the iron-Fur complex especially under iron-replete condition.

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# Abstract

## Regulation of the Vulnibactin Receptor Expression by Cyclic AMP-receptor Protein in *Vibrio vulnificus*

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*Vibrio vulnificus* possesses several iron-uptake systems (IUS). Of these IUS, the vulnibactin receptor (VuuA)-mediated IUS is known to play an essential role in the utilization of transferrin-bound iron and in the expression of *V. vulnificus* virulence in animal models. Nevertheless, there is little understanding about the regulatory mechanisms of the VuuA-mediated IUS expression, except that Fur, a transcription repressor, negatively regulates the IUS expression via the Fe-Fur complex under iron-sufficient conditions. The cyclic AMP (cAMP) and cAMP receptor protein (CRP) complex is known as a global regulator that primarily responds to carbon availability and can also regulate the expression of several virulence factors. A previous study first reported that *crp* mutation severely inhibited the expression of the VuuA-mediated IUS, which suggests that there is a link between carbon and iron metabolic processes and the cAMP-CRP complex can regulate expression of several genes associated with iron-uptake. The present study attempted to unequivocally demonstrate the interaction between the two systems, cAMP-CRP and Fur-regulated IUS. Accordingly, the effect of *crp* mutation on *fur* expression was first observed to demonstrate the interaction between the two transcriptional regulators CRP and Fur, and then the effect of *crp* or *fur* mutation on the expression of the

VuuA-mediated IUS was observed. The *lacZ*-fused transcriptional reporters were used to measure *crp*, *fur* or *vuuA* expression at the transcription level, the rabbit polyclonal anti-VuuA-body was used to measure *vuuA* expression at the protein level, and 6 M urea-gel electrophoresis was used to measure the activity of the VuuA-mediated IUS at the functional level. First, *crp* expression appeared not to be influenced by iron concentration. In the background with wild-type *crp*, *fur* was expressed at high levels and the *fur* expression was iron-inducible. However, the *fur* expression was unresponsive to iron and down-regulated by *crp* mutation, and the down-regulated or unresponsive *fur* expression was recovered to wild-type levels by *in trans crp* complementation. In the background with wild-type *crp*, *vuuA* was expressed at considerable levels and the *vuuA* expression was iron-repressible. However, the *vuuA* expression was almost completely suppressed by *crp* mutation even under the iron-deficient conditions, and the suppressed *crp* expression was recovered to wild-type levels by *in trans crp* complementation. In the background with wild-type *crp*, VuuA was produced at considerable levels in a inverse proportion to iron concentration. However, the VuuA production was severely inhibited by *crp* mutation, and the inhibited VuuA production was recovered to wild-type levels by *in trans crp* complementation. In the background with wild-type *crp*, *V. vulnificus* grew relatively well on transferrin-bound iron. However, the *V. vulnificus* growth on transferrin-bound iron was severely inhibited by *crp* mutation, and the inhibited growth was recovered to wild-type levels by *in trans crp* complementation. In the background with wild-type *crp*, *V. vulnificus* could utilize transferrin-bound iron at considerable levels. However, the ability of *V. vulnificus* to utilize transferrin-bound iron was severely attenuated by *crp* mutation, and the attenuation was recovered to wild-type levels by *in trans crp* complementation. In

contrast, *vuuA* expression and the ability of *V. vulnificus* to utilize and grow on transferrin-bound iron were all de-repressed by *fur* mutation especially under iron-sufficient conditions, and these de-repressions were all recovered to wild-type levels by *in trans fur* complementation. Taken together, these results indicate (1) that there is a close link between the cAMP-CRP complex and the iron-Fur complex; the cAMP-CRP complex positively regulates Fur, (2) that the cAMP-CRP complex positively regulates the activity of the VuuA-mediated IUS especially under iron-deficient conditions, and (3) that the cAMP-CRP complex can negatively regulate the activity of the VuuA-mediated IUS via the iron-Fur complex especially under iron-sufficient conditions.

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Key words: *Vibrio vulnificus*, Iron, Siderophore, Vulnibactin, cAMP-receptor protein

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# 저작물 이용 허락서

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| 연락처 | E-mail: **channa6041229@hanmail.net** | | | | |
| 논문 제목 | 한글: **패혈증 비브리오균에 있어 cyclic AMP 수용체 단백에 의한 vulnibactin 수용체 발현 조절** | | | | |
| | 영문: **Regulation of the Vulnibactin Receptor Expression by Cyclic AMP-receptor Protein in *Vibrio vulnificus*** | | | | |

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건 아래 -조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

\- 다 음 -

1. 저작물의 DB 구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함.
2. 위의 목적을 위하여 필요한 범위 내에서의 편집과 형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포・전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5 년으로 하고, 기간종료 3 개월 이내에 별도의 의사 표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 출판을 허락을 하였을 경우에는 1 개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물 이용의 허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음.
7. 소속 대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송・출력을 허락함.

**동의여부 : 동의( 0 ) 반대( )**

2009 년 2 월

저작자: 김 안 나 (인)

# 조선대학교 총장 귀하