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Regulatory Mechanism of the *hupA* Gene Encoding Heme Receptor Protein in *Vibrio vulnificus*

조선대학교대학원

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패혈증 비브리오균에서 햄수용체 단백 유전자의 조절기전

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이 강 욱

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패혈증 비브리오균은 감수성이 있는 환자, 특히 혈청 철 농도가 높아진 환자에서 매우 빠르게 진행하며 치명적인 감염증을 초래한다. 높아진 철 농도를 선호하는 패혈증 비브리오균은 외막에 존재하는 햄수용체 단백인 HupA 를 통해 헤모글로빈과 같은 햄단백으로부터 직접 철을 획득할 수 있다. 햄수용체 단백을 암호화하는 *hupA* 유전자의 발현은 철 농도에 반응하여 유전자발현을 억제하는 철흡수조절인자(Ferric uptake regulator: Fur)에 의해 음성적으로 조절되며, 최근에는 포도당 고갈에 반응하여 유전자발현을 활성화시키는 cyclic AMP 수용체 단백(cAMP−receptor protein: Crp)에 의해 양성적으로 조절된다고 알려졌다. 본 연구에서는 일차적으로 패혈증 비브리오균이 헤모글로빈을 철 공급원으로 직접 이용하는데 있어 HupA 의 역할을 검증하고, 패혈증 비브리오균 임상 그리고 환경 분리주에서 HupA 의 분포를 조사하며, *hupA* 유전자의 발현 조절을 위해 Fur 와 Crp 가 어떻게 협조적으로 작용하는가를 규명하고자 하였다. 유전자결손 방법에 의해 *hupA* 유전자의 돌연변이를 유발하였을 경우 패혈증 비브리오균의 헤모글로빈

이용능력은 소실되었고 이러한 결함은 플라스미드를 통해 온전한 *hupA* 유전자를 보충하였을 때 다시 회복되었다. 패혈증 비브리오균 임상 분리주 10 주 중 2 주, 그리고 환경 분리주 10 주 중 1 주만이 *hupA* 유전자를 가지고 있지 않았고 HupA 단백을 발현하지도 않았다. 유전자결손 방법에 의해 *crp* 유전자에 돌연변이를 유발하였을 때 *hupA* 유전자의 발현은 현저히 억제되었고 이러한 결함은 플라스미드를 통해 온전한 *crp* 유전자를 보충하였을 때 다시 회복되었다. 더불어, cyclic AMP 합성에 필요한 효소인 adenylate cyclase 를 암호화하는 *cyaA* 에 유전자결손 돌연변이를 유발하였을 경우에도 *hupA* 유전자의 발현은 현저히 억제되었고, 이러한 결함은 플라스미드를 통해 온전한 *cyaA* 유전자를 보충하였을 때와 외부에서 cyclic AMP 을 첨가하여 주었을 때 다시 회복되었다. 유전자 결손 방법에 의해 *fur* 유전자에 돌연변이를 유발하였을 때 *hupA* 유전자의 발현이 철에 의해 억제되지 않았고, 이러한 변화는 플라스미드를 통해 온전한 *fur* 유전자를 보충하였을 때 다시 회복되었다. 철을 더욱 결핍시킨 환경에서 *hupA* 유전자는 Crp 가 없을 때에도 발현되었으나 Crp 가 없을 때 보다는 Crp 가 존재할 때 더 높은 수준으로 발현되었다. 포도당은 Fur 가 존재할 때에는 *hupA* 유전자의 발현을 용량의존적으로 그리고 완전하게 억제할 수 있었으나 Fur 가 존재하지 않을 때에는 용량의존적으로 억제하지 못하였다. 이러한 결과를 종합하면, (1) HupA 는 철공급원인 햄단백을 직접 이용하는데 관여하며, (2) HupA 는 패혈증 비브리오균 임상분리주뿐 아니라 환경분리주에도 널리 분포되어 있으며, (3)

철이 결핍된 환경에서 포도당 고갈은 *hupA* 유전자의 발현을 위해 상승적으로 작용하고, (4) Fur 는 철 농도에 반응하여 *hupA* 유전자의 발현을 억제하기 위해 필수적이며, (5) Crp 는 필수적으로 필요하지는 않을지라도 포도당 농도에 반응하여 *hupA* 유전자의 발현을 최적화하는 데 필요한 전사활성인자임을 알 수 있다.

중심어: 패혈증 비브리오균, 철, 포도당, 햄수용체, cyclic AMP 수용체 단백, 철흡수조절인자

Introduction

Backgrounds

Vibrio vulnificus is a gram-negative halophilic bacterium capable of causing gastroenteritis, life-threatening septicemia, and necrotizing wound infections that can progress rapidly and have high mortality rates. Human infections can be acquired either by consuming raw seafood or by contact between contaminated sea water and open wounds. Furthermore, *V. vulnificus* is an opportunistic pathogen in certain susceptible individuals, such as, in patients with liver cirrhosis, hemochromatosis, or β-thalassemia [Strom and Paranjpye, 2000; Gulig et al., 2005; Jones and Oliver, 2009]. Several established and potential virulence factors have reported to play important roles in the pathogenesis of *V. vulnificus* infections, such as, RTX (repeats in toxin) toxin [Lee et al., 2007; Kim et al., 2008], capsular polysaccharides [Wright et al., 1990], lipopolysaccharides [McPherson et al., 1991], iron-assimilation systems [Stelma et al., 1992], flagella or motility [Kim and Rhee, 2003; Lee et al., 2004], pili [Paranjpye et al., 1998], and exotoxins, such as, cytolysin/hemolysin [Grey and Kreger, 1987] and proteases [Kothary and Kreger, 1987]. The concerted expression of these factors is required for pathogenesis, and appears to be under the control of global regulators, such as, cyclic AMP (cAMP) and cAMP receptor protein (Crp) complex [Kim et al., 2005], the quorum sensing master regulator SmcR [Lee et al, 2007], LysR-like AphB [Jeong and Choi, 2008], the small regulatory protein HlyU

[Liu et al., 2007], and the alternative sigma factor of RNA polymerase RpoS [Hulsmann et al., 2003].

Elevated serum or tissue iron is one of the well-known predisposing host factors. For example, iron treatment in mice reduces the intraperitoneal 50% lethal dose (LD_{50}) from 10^6 cells to only 1 cell [Wright et al., 1981], and increases the mortality rate to 100% [Stelma et al., 1992]. *V. vulnificus* grows better in the blood of patients with hemochromatosis, if transferrin saturation by iron is increased, or if hematin is added [Bullen et al., 1991]. Iron is an essential element for the survival and proliferation of most bacteria. In some bacteria, called ferrophilic or iron-sensitive bacteria, iron acquisition is impaired, and thus, these bacteria cause diseases primarily in iron-overloaded hosts [Weinberg, 2000]. *V. vulnificus* is a ferrophilic bacterium that requires higher levels of readily available iron for growth initiation than other pathogens [Kim et al., 2007]. Precisely how elevated serum iron confers an advantage to *V. vulnificus* is unclear. Nevertheless, three theories have been proposed. First, elevated serum iron levels enhance the growth of *V. vulnificus* [Starks et al., 2000; Bogard and Oliver, 2007]. Second, elevated serum iron levels inhibit neutrophil activity [Hor et al., 2000], and third, they facilitate the productions of some virulence factors in *V. vulnificus* [Kim et al., 2006; Kim et al., 2009]. In addition, Ashrafian [2003] proposed that hepcidin, an antimicrobial protein expressed in liver and involved in iron regulation in the host, could be one of the important factors

determining host susceptibility against *V. vulnificus* in patients with liver diseases. The existence of these different theories suggests that elevated serum iron plays multiple roles in susceptibility to *V. vulnificus* infections, and highlights the need for further research in this area.

To establish infection successfully, bacteria must be able to acquire iron from their hosts. Most bacteria have evolved specific iron uptake systems (IUSs). The ability to acquire iron is a well-established virulence factor in the pathogenesis of *V. vulnificus* infections. In fact, *V. vulnificus* possesses multiple IUSs. For examples, the vulnibactin- or vulnibactin receptor (VuuA)-mediated IUS plays a crucial role in the utilization from various sources, especially transferrin-bound iron [Choi et al., 2006; Kim et al., 2006; Litwin et al., 1996; Webster and Litwin, 2000], and the heme-receptor (HupA)-mediated IUS plays a role in the direct utilization of heme iron from heme-containing proteins, such as, hemoglobin and hemin [Litwin and Byrne, 1998; Litwin and Quackenbush, 2001; Oh et al., 2009]. In addition, *V. vulnificus* can utilize heterologous siderophores; *Streptomyces pilosus* deferoxamine via the cognate receptor DesA [Aso et al., 2002; Takata et al., 2005; Kim et al., 2007] and *Escherichia coli* aerobactin via the cognate receptor IutA [Tanabe et al., 2005; Kim et al., 2012]. Of these IUSs, the HupA-mediated IUS may play an important role in iron acquisition *in vivo* [Helms et al., 1984], because about 99% of iron within the human body is present as hemoglobin within red blood cells [Weinberg, 1978], and *V.*

vulnificus produces several cytotoxins, such as, cytolysin/hemolysin (VvhA) [Grey and Kreger, 1987] and RTX toxin [Lee et al., 2007; Kim et al., 2008], which can destroy various cells including red blood cells, and thus release intracellular iron in the form of hemoglobin, hemin, ferritin and hemosiderin.

The expression of the *hupA* gene is regulated at the transcription level by Fur (ferric uptake regulation), which acts as a repressor, employing iron as a cofactor to bind the operator region of iron-regulated genes [Litwin and Byrne, 1998; Litwin and Calderwood, 1993]. Recently, Crp, a global regulator primarily responsible for catabolite repression [Deutscher, 2008], was found to act as an transcriptional activator of *hupA* expression [Oh et al., 2009]. However, the detailed roles of Fur and Crp in regulating *hupA* expression remains to be determined. In addition, the presence of heme (a co-inducer) under iron limited conditions was observed to synergistically activate *hupA* expression in a LysR-like activator HupR-mediated manner [Litwin and Quackenbush, 2001]. However, the role of HupR in the regulation of *hupA* expression appears to be less important than the roles of Fur or Crp because the presence of iron-containing heme increases iron availability and represses the expressions of *vuuA* and *hupA* by itself [Litwin and Quackenbush, 2001]. The potential and established binding sites of Fur, Crp and HupR are shown in Fig. 1.

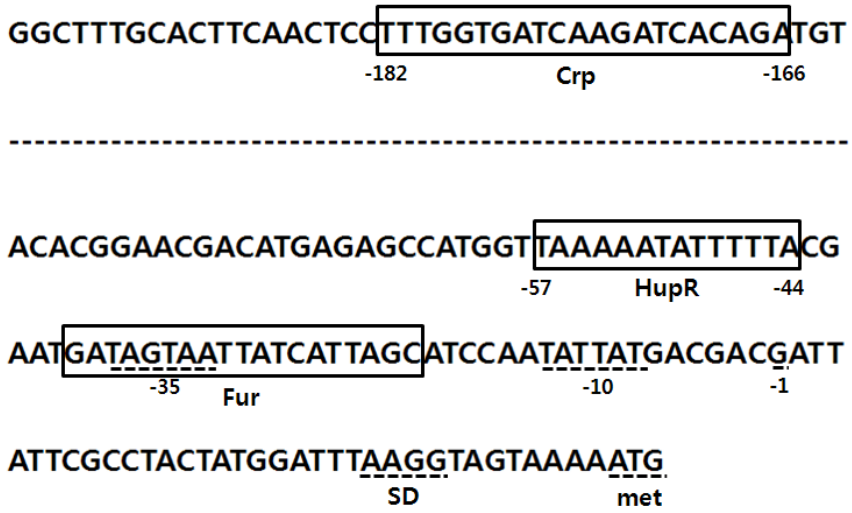

Figure 1. The established or putative binding sites of Crp, HupR, and Fur in the regulatory region of *hupA*. The Crp-binding site was confirmed by DNA foot-printing [Oh et al., 2009], and the putative HupR [Litwin and Quackenbush, 2001] and Fur binding sites [Litwin and Byrne, 1998] were identified by sequence analysis. SD: Shine-Dalgano, met: methione or start codon.

The aim of this study

The aim of the present study is to detail the roles of Fur and Crp in regulating *hupA* expression and further to determine the presence of a direct connection or hierarchy between iron and carbon metabolisms in *V. vulnificus*. In the present study, the author confirmed that HupA is involved in the direct utilization of hemoglobin, examined the prevalence of HupA-mediated IUS in *V. vulnificus* clinical and environmental isolates, and determined the detailed roles of Fur and Crp in the regulation of *hupA* expression under conditions containing various concentrations of iron and glucose. For these, a *hupA*-inactivated mutant strain, a *fur*-inactivated mutant strain, a *crp*-inactivated mutant strain, and a *cyaA*-inactivated strain, were constructed. In addition, the *lacZ*-fused *hupA* transcriptional reporter strains were constructed in the wild-type *fur* and *crp*, *fur*-inactivated, and *crp*-inactivated backgrounds. Finally, polyclonal anti-HupA antibody was prepared and Western blot was conducted to measure *hupA* expression at the protein level.

Materials and Methods

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. Heart Infusion (HI; BD, Franklin Lakes, NJ, USA) agar or broth containing additional 2.0% NaCl, Thiosulfate-Citrate-Bile Salt-Sucrose (TCBS, BD) agar, and Synbase agar [Simpson and Oliver, 1987] were used to cultivate *V. vulnificus* strains. Luria Bertani (LB; BD) medium was used to cultivate *Escherichia coli* strains. Antibiotics (BD) were used at the following concentrations (μg/mL): for *E. coli*, ampicillin 50, kanamycin 50, tetracycline 12.5, and chloramphenicol 30; and for *V. vulnificus*, ampicillin 20, kanamycin 200, tetracycline 2, and chloramphenicol 2. Unless otherwise stated, all other 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, highly virulent clinical isolate	Reddy et al., 1992
RC110	M06-24/O with *hupA* mutation	This study
RC114	RC110 with *in trans hupA* complementation	This study
CMM710	M06-24/O with *crp* mutation	Choi et al., 2006
CMM714	CMM710 with *in trans crp* complementation	Choi et al., 2006
CMM2303	M06-24/O with *fur* mutation	Kim et al., 2006
RC144	CMM2303 with *in trans fur* complementation	This study
RC386	M06-24/O with *cyaA* mutation	Kim et al., 2012
RC390	RC386 with *in trans cyaA* complementation	Kim et al., 2012
CMM2101	M06-24/O with *lacZ* mutation	Kim et al., 2003
CMM2304	CMM2101 with *fur* mutation	Kim et al., 2009
RC100	CMM2101 with *crp* mutation	This study
RC120	CMM2101 with merodiploid P_{hupA}*::lacZ* transcriptional fusion	Kim et al., 2011
RC122	RC100 with P_{hupA}*::lacZ* transcriptional fusion	This study
RC258	RC122 with *in trans crp* complementation	This study
RC124	CMM2304 with P_{hupA}*::lacZ* transcriptional fusion	This study
RC312	RC124 with *in trans fur* complementation	This study
others	9 clinical isolates and 10 environmental isolates; randomly selected	Laboratory stock
Escherichia coli		
SY327 λ*pir*	Host for suicide vector	Miller and Mekalanos, 1988
SM10 λ*pir*	Conjugation donor	Miller and Mekalanos, 1988
DH5a	F- *recA1*; restriction negative	Laboratory stock
BL21	F-, *ompT*, *hsdS* (r_B-, m_B-), *gal*	Laboratory stock

Table 2. Plasmids used in this study

Plasmids	Relative characteristics[a]	Sources
pET-30a(+)	Inducible expression vector carrying N-terminal His·Tag/thrombin/S·Tag/enterokinase configuration plus an optional C-terminal His·Tag sequence; Km^R	Novagen
pDM4	Suicide vector with R6K origin; Cm^R	McGee et al., 1996
pQF52	IncP lacZ transcriptional fusion vector; Amp^R	Farinha and Kropinski, 1990
pRK2013	IncP, Km^R, Tra $Rk2^+$ *repRK2 repE1*	Ditta et al., 1980
pLAFR3	IncP cosmid vector; Tc^R	Staskawicz et al., 1987
pLAFR3II	pLAFR3 with *bla* inserted at the cos site; Amp^R and Tc^R	Kim et al., 2003
pRC110	pDM4 with a 1.82-kb *Sma*I-*Spe*I fragment containing an in-frame deletion of *hupA*	This study
pRC114	pLAFR3II with a 2.75-kb *Eco*RI-*Bam*HI fragment containing *hupA*	This study
pRC132	pLAFR3II with a 861-bp *Bam*HI-*Hin*dIII fragment containing *fur*	This study
pRC192	pET-30a(+) with a 1,626-bp *Bam*HI-*Eco*RI fragment containing *hupA*	This study
pRC316	pDM4 with 1.63-kb *Xba*I-*Sma*I fragment containing *in-frame* deleted *cyaA*	Kim et al., 2012
pRC318	pLAFR3II with 3.16-kb *Bam*HI-*Eco*RI fragment containing wild-type *cyaA*	Kim et al., 2012

[a]: Km^R (kanamycin-resistant), Cm^R (chloramphenicol-resistant), Amp^R (ampicillin-resistant), Tc^R (tetracycline-resistant).

Table 3. Primers used in this study

Primers	Sequences	Sources
hupA-1	5’-aaattgcaatctcagctgagcg-3’	This study
hupA-2	5’-ctaccttgtaagtggcacctag-3’	This study
His-hupA-F	5’-cg**ggatcc**tcatactcatccgaagataaatcg-3’	This study
His-hupA-R	5’-**ggaatt**cttagaactcgtattttacactgatg-3’	This study
hupA-up-1	5’-tcc**cccggg**tctgactctggttttactcacg-3’	This study
hupA-up-2	5’-gtagttggttacattcccctaatattgatactttg-3’	This study
hupA-down-1	5’-attaggggaatgtaaccaactacaaaaacaaaagc-3’	This study
hupA-down-2	5’-g**actagt**caatgttcgccaaagtgagccg-3’	This study
hup-comp-for	5’-g**gaattc**tctgactctggttttactcacg-3’	This study
hup-comp-2	5’-cg**ggatcc**tggttagaactcgtattttacactg-3’	This study
CRP1	5'-tacctactggcgatgatcgatg-3'	Choi et al., 2006
CRP7	5'-cggaatctgagagggtttagt-3'	Choi et al., 2006
fur-comp-F	5'-cg**ggatcc**ccgttaaagagaaaatactgcc-3'	This study
fur-comp-R	5'-ccc**aagctt**tatcgagcgtcgatattagttc-3'	This study
cyaA-up-1	5’-gc**tctaga**agccagcggccgcgagaatgatc-3’	Kim et al., 2012
cyaA-up-2	5’-cgctttggacatcttctgactttgcaaatccataagcgccag-3’	Kim et al., 2012
cyaA-down-1	5’-gatttgcaaagtcagaagatgtccaaagcggtcaacgtatag-3’	Kim et al., 2012
cyaA-down-2	5’-tcc**cccggg**tgcctactgtgattgctcagattgttg-3’	Kim et al., 2012
cyaA-comp-1	5’-cg**ggatcc**tgcacgcccttccagcattgc-3’	Kim et al., 2012
cyaA-comp-2	5′-g**gaattc**gcgtagctatcgttaagccattaag-3′	Kim et al., 2012

*Bam*HI: ggatcc, *Eco*RI: gaattc, *Bgl*II: agatct, *Sma*I: cccggg, *Spe*I: actagt, *Hin*dIII: aagctt, *Xba*I: tctaga.

DNA manipulations

General procedures for the isolation of plasmid DNA and genomic DNA and transformation were carried out as described by Sambrook and Russell [2001]. Restriction and DNA-modifying enzymes were used as recommended by the manufacturer (Takara, Japan). DNA fragments were purified from agarose gels using the QIA quick® Gel Extraction kit (Qiagen, Germany). Plasmid DNA was extracted using the Wizard Plus SV Minipreps DNA Purification System (Promega corporation, Madison, WI, USA). Genomic DNA was isolated from *V. vulnificus* M06-24/O using the G-spin™ for Bacteria Genomic DNA extraction kit (iNtRON, Korea). Oligonucleotides were synthesized at Bioneer (Korea). PCR reactions were carried out as specified by the manufacturer (Mastercycler gradient, Eppendorf, Germany), and using standard protocols.

Limitation and supplementation of iron and glucose

Three types of iron-limited media were used in this study. First, HI broth containing 200 μM α,α'-dipyridyl as an iron chelator was used for the adaption of *V. vulnificus* strains to iron limited conditions. Second, HI broth was deferrated using 8-hydroxyquinoline, as previously described [Leong and Neilands, 1982], and third, the chemically defined Synbase broth was deferrated in the same manner. Residual iron concentrations in deferrated (DF) HI and Synbase broths were less than 1 μg/dL, which was measured by the

method devised by Stookey [1970]. To observe the effect of iron on growth and gene expression, various concentrations of ferric chloride (FC) as an iron source were added to DF-HI broths. In addition, DF-HI was used as a glucose-poor medium because it contains only an undefined low level of glucose. To observe the effect of glucose, various concentrations of glucose were added to TES-DF-HI broths containing 100 mM N-trismethyl-2-aminoethanesulfonic acid (TES) as a buffering agent, as previously described [Kim et al., 2003].

Plate bioassay

Human hemoglobin was used as a heme iron source. To assess the utilization of hemoglobin by *V. vulnificus* strains, a plate bioassay was used. In brief, about 10^4 cells, preconditioned and grown in HI broth containing 200 μM dipyridyl overnight, were inoculated into 2 ml of molten DF-Synbase agar [top agar containing 0.5% Bacto agar (BD)], which was then poured onto and allowed to solidify on the surface of DF-Synbase agar (bottom agar containing 1.5% Bacto agar). Paper discs containing various amounts of hemoglobin were placed onto the surface of agar. After agar plates were incubated at 37^{o}C for 24 h, the diameters of growth-enhanced area around discs were measured.

Culture conditions and the measurements of bacterial growth and β-

galactosidase activity

V. vulnificus strains were preconditioned by culturing in HI broth containing 200 μM dipyridyl at 37°C overnight to adapt to iron-limited conditions. Preconditioned strains were inoculated into test broths at a bacterial density of 5 x 10^6 cells/mL, and then cultured with vigorous shaking at 37°C for 12 h. Culture aliquots were withdrawn at appropriate times to determine bacterial growth and gene transcription levels. Bacterial growth levels were determined by measuring the optical densities of culture aliquots at 600 nm (OD_{600}), and gene transcription levels were determined by measuring β-galactosidase activities on a per cell basis in culture aliquots, as previous described [Miller, 1992].

In frame deletion mutation and in trans complementation of genes

RC110 containing deleted *hupA* and RC114 containing *in trans* complemented *hupA* were constructed as shown in Figs. 2 and 3. Two pairs of the PCR primers hupA-up-1/hup-up-2 and hupA-down-1/hupA-down-2 were used for the amplification of the 5' and 3' fragments of *hupA*, respectively. The resulting two PCR products were used as templates for second PCR amplification using the PCR primers hupA-up-1 with a *Sma*I overhang and hupA-down-2 with a *Spe*I overhang. The *Sma*I-*Spe*I fragment with deleted *hupA* so obtained was then cloned into pDM4 [McGee et al., 1996]. The resulting plasmid pRC110 was transformed into *E. coli* SY327 λ*pir* and SM10 λ*pir* [Miller and Mekalanos, 1988],

and subsequently transferred to *V. vulnificus* M06-24/O by conjugation. A stable transconjugant (RC110) was selected on TCBS agar containing chloramphenicol, and subsequently on HI agar containing 10% sucrose. The *hupA* deletion in RC110 was confirmed by PCR using the primers hupA-up-1 and hupA-down-2 (data not shown).

To restore wild-type *hupA* in the *hupA*-deleted RC110 strain, a 2.75-kb *Eco*RI-*Bam*HI fragment containing intact wild-type *hupA* was amplified using the PCR primers hupA-comp-for with a *Eco*RI overhang and hupA-comp-2 with an *Bam*HI overhang. The resulting PCR product was then subcloned into pLAFR3II [Kim et al., 2003]. The resulting plasmid pRC114 was transferred into RC110 by triparental mating using pRK2013 [Ditta et al., 1980]. A stable transconjugant (RC114) was then selected on TCBS agar containing ampicillin and tetracycline. The presence of wild-type *hupA* in RC114 was confirmed by PCR using the primers hupA-comp-for and hupA-comp-2 (data not shown).

The *crp*-deleted CMM710 strain and the *crp*-complemented CMM714 strains were constructed as described previously [Choi et al., 2006]. The *fur*-deleted CMM2303 strain was constructed as described previously [Kim et al., 2006]. The *fur*-complemented RC144 strain was constructed in this study. In brief, an 861-bp *Bam*HI-*Hin*dIII fragment containing intact wild-type *fur* was amplified using the PCR primers, fur-comp-F with a *Bam*HI overhang and fur-comp-R with a *Hin*dIII overhang. The resulting plasmid pRC132 was transferred to CMM2303 as described above. The *cyaA*-deleted RC386 strain and the

cyaA-complemented RC390 strain were constructed as described in our recent study [Kim et al., 2012]. In briefly, two pairs of PCR primers cyaA-up-1/cyaA-up-2 and cyaA-down-1/cyaA-down-2 were used for the PCR amplification of the 5' and 3' ends of *cyaA*, respectively. The resulting PCR products were used as templates for the second PCR amplification using the PCR primers, cyaA-up-1 with an *Xba*I overhang and cyaA-down-2 with a *Sma*I overhang. The *Xba*I-*Sma*I fragment containing deleted *cyaA* was cloned into pDM4 [McGee *et al*., 1996]. The resulting plasmid (pRC316) was transformed into *E. coli* SY327 λ*pir* and SM10 λ*pir* [Miller & Mekalanos, 1988], and subsequently transferred to M06-24/O by conjugation. To restore wild-type *cyaA* in RC386, a 3.16-kb *Bam*HI-*Eco*RI fragment encompassing wild-type *cyaA* was amplified using the PCR primers, cyaA-comp-1 with a *Bam*HI overhang and cyaA-comp-2 with an *Eco*RI overhang. The resulting PCR product was subcloned into pLAFR3II [Kim *et al*., 2003], and the resulting plasmid pRC318 was transferred into RC386 by triparental mating using pRK2013 [Ditta *et al*., 1980]. The resulting *cyaA*-complemented strain was named RC390.

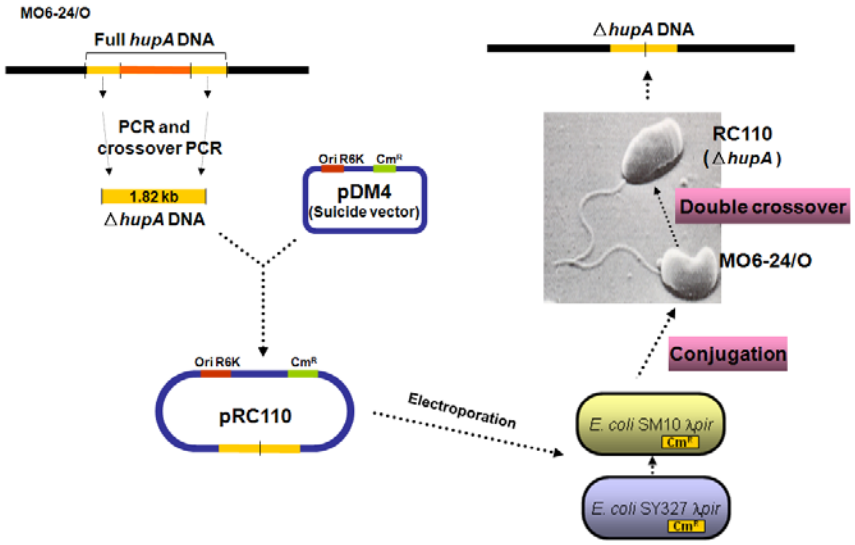

Figure 2. The construction of the *Vibrio vulnificus* RC110 strain containing *in frame* deleted *hupA.* Details are described in the text.PCR (polymerase chain reaction), Cm^R: chloramphenicol-resistant cassette, Δ*lacZ* (deleted *lacZ*).

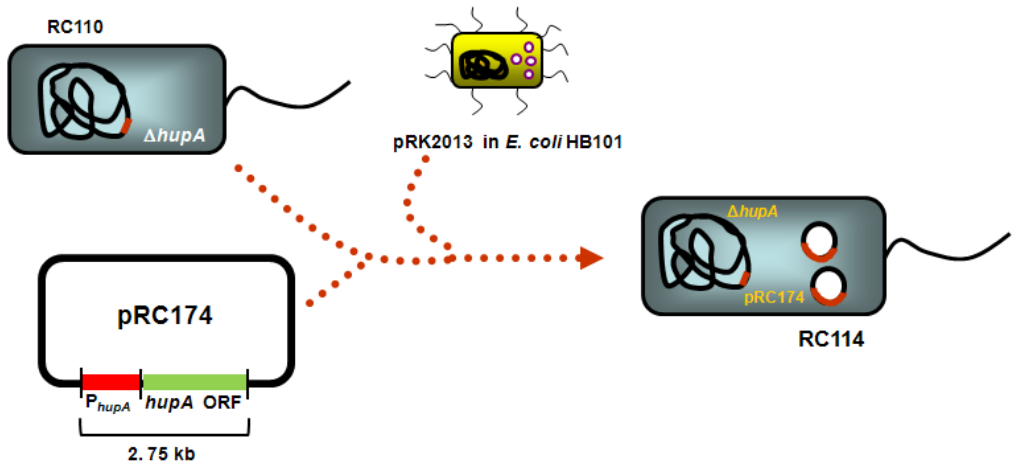

Figure 3. The construction of the *Vibrio vulnificus* RC114 strain containing *in trans*-complemented *hupA.* Details are described in the text. P_{hupA}: the promoter region of *hupA*, ORF (open reading frame), Δ (deletion mutation).

Construction of *lacZ*-fused *hupA* transcription reporters

RC120 strain containing the *lacZ*-fused *hupA* transcription reporter was constructed by introducing the merodiploid P_{hupA}::*lacZ* construct into CMM2101 with wild-type *crp* and *fur* as shown in Fig. 4 and described previously [Kim et al., 2011]. In brief, a 916-bp fragment from -881 to +19 bp from the *hupA* start codon was amplified using the PCR primers, hupA-rep-F with *Bam*HI overhang and hupA-rep-R with *Kpn*I overhang. The amplified fragment was subcloned into pQF52 containing promoterless *lacZ* [Farinha and Kropinski, 1990]. From the resulting plasmid pRC116, a *Bam*HI-*Sca*I fragment containing the P_{hupA}::*lacZ* construct was then subcloned into pDM4 [McGee et al., 1996], yielding pRC124. The plasmid pRC124 containing the P_{hupA}::*lacZ* construct was transformed into *E. coli* SY327 *λpir* and SM10 *λpir* [Miller and Mekalanos, 1988], and introduced into CMM2101 by conjugation. Finally, a transconjugant (RC120) was selected on TCBS agar containing chloramphenicol. The presence of the P_{hupA}::*lacZ* construct in RC120 was confirmed by β-galactosidase assay [Miller, 1992]. Using the same methodology, the merodiploid P_{hupA}::*lacZ* construct was introduced into the *crp*-deleted RC100 strain and the *fur*-deleted CMM2304 strain. Finally, the *crp*-deleted P_{hupA}::*lacZ* transcription reporter strain and the *fur*-deleted P_{hupA}::*lacZ* transcription reporter strain were named RC122 and RC124, respectively.

To restore wild-type *crp* in RC122, the plasmid pCMM712 containing wild-type *crp*

was introduced into RC122, as previously described [Choi et al., 2006], and the resulting *crp*-complemented strain was named RC258. The presence of wild-type *crp* in RC256 was confirmed by PCR using the CRP-1 and CRP-7 primers (data not shown). Using the same methodology, wild-type *fur* on the plasmid pRC132 was introduced into RC124, and the resulting *fur*-complemented strain was named RC312. The presence of wild-type *fur* in RC312 was confirmed by PCR using the fur-comp-F and fur-comp-R primers (data not shown).

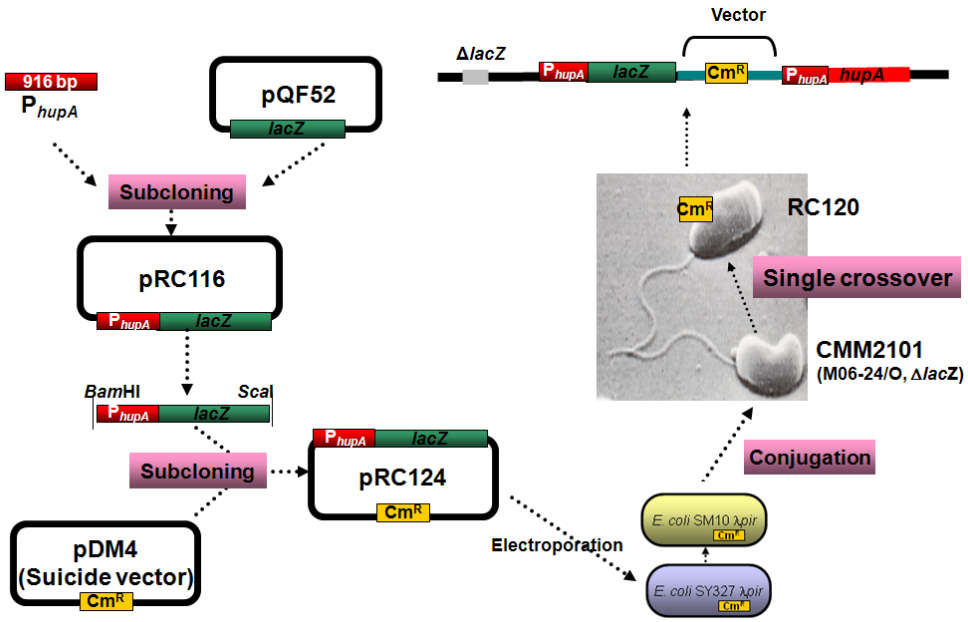

Figure 4. The construction of the *Vibrio vulnificus* RC120 strain containing the *lacZ*-fused *hupA* transcription reporter. Details are described in the text. P_{hupA}: the promoter region of *hupA*, Cm^R: chloramphenicol-resistant cassette, Δ*lacZ* (deleted *lacZ*).

Preparation of His-tagged HupA protein and polyclonal anti-HupA antibody and Western blotting

Recombinant HupA protein and rabbit polyclonal anti-HupA antibody were prepared and Western blot for HupA was conducted as described previously [Kim et al., 2011]. In brief, the coding region of *hupA* was amplified using the PCR primers His-hupA-F with *Bam*HI overhang and His-hupA-R with *Eco*RI overhang. The resulting 1,626-bp *Bam*HI-*Eco*RI fragment was cloned into the pET-30a(+) expression vector (Novagen, Madison, WI, USA). The resulting plasmid pRC192 was transformed into *E. coli* DH5α and BL21 (DE3). His-tagged HupA fusion protein was over-expressed by treating *E. coli* BL21 (DE3) with 0.5 mM isopropyl-1-thio-β-$_{D}$-galactopyranoside for 3 h at 37^{o}C. The over-expressed HupA fusion protein was purified using Ni-NTA agarose columns (Qiagen).

His-tagged HupA fusion protein was mixed with complete or incomplete Freund's adjuvant, and then injected into a New Zealand White rabbit. After three repeated injections at 3-week intervals, blood was collected via cardiac puncture, and serum was separated by centrifugation at 4^{o}C and filtration. To remove cross-reacting antibodies, the serum was thoroughly adsorbed using cell lysates obtained from the HupA-deficient RC110 strain. The specificity of the antibody was confirmed using the M06-24/O, RC110, and RC114 strains, as shown in Fig. 5.

V. vulnificus strains were preconditioned and cultured as described above. Bacterial

pellets containing approximately 1 x 10^8 cells were boiled for 10 min to obtain cell lysates, which were electrophoresed on 10% SDS-PAGE gels. Separated proteins were transferred to nitrocellulose transfer membranes (PROTRAN, Whatman GmbH, Germany). The membranes were incubated with blocking solution at 4^{o}C overnight, allowed to react with rabbit polyclonal anti-HupA antibody (1:250) as the primary antibody, then with the secondary antibody anti-rabbit IgG conjugated with alkaline phosphatase (1:15,000), and finally visualized with 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium substrate solution.

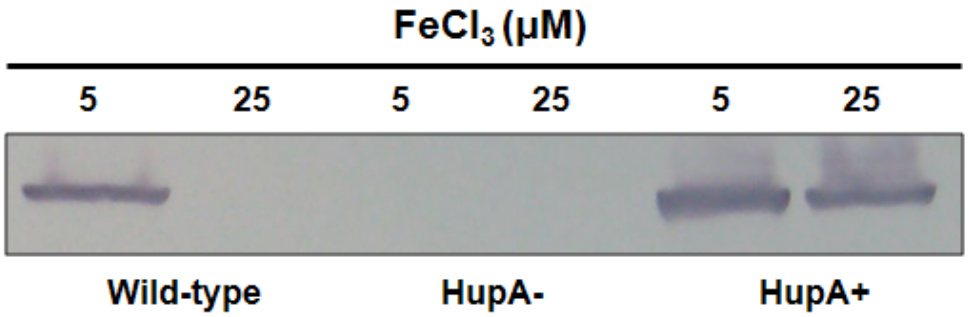

Figure 5. The confirmation of *hupA* mutation and complementation, and the specificity of rabbit polyclonal anti-HupA antibody. The three *V. vulnificus* strains, M06-24/O with wild type *hupA* (Wild-type), RC110 with mutated *hupA* (HupA-), RC114 with *in trans* complemented *hupA* (HupA+), were cultured in DF-HI containing 5 or 25 μM FC. Whole cell lysates containing approximately 1 x 10^8 cells were obtained 12 h after culture initiation and electrophoresed on 10% SDS-PAGE gels. Western blot using rabbit polyclonal anti-HupA antibody as the primary antibody was conducted as described in Materials and Methods.

Results

The HupA-mediated utilization of hemoglobin

The HupA-mediated utilization of hemoglobin was determined using the three strains M06-24/O containing wild-type *hupA*, RC110 containing mutated *hupA*, and RC114 containing *in trans* complemented *hupA* (Fig. 6). In all three strains, the growth-enhanced zones were found to be dose-dependently increased by 0.5 or 2.0 μg hemoglobin ($p<0.05$, One Way ANOVA). However, the growth-enhanced zones in M06-24/O were significantly reduced by the *hupA* mutation in RC110 ($p<0.05$, Student's *t*-test). This reduction in RC110 was recovered by the *in trans hupA* complementation in RC114 ($p<0.05$, Student's *t*-test). No significant difference was observed among the growths of these three strains in DF-HI broths containing hemoglobin (Fig. 7). These results indicate that HupA is involved the direct utilization of hemoglobin by *V. vulnificus*, especially under the condition that other IUSs does not operate.

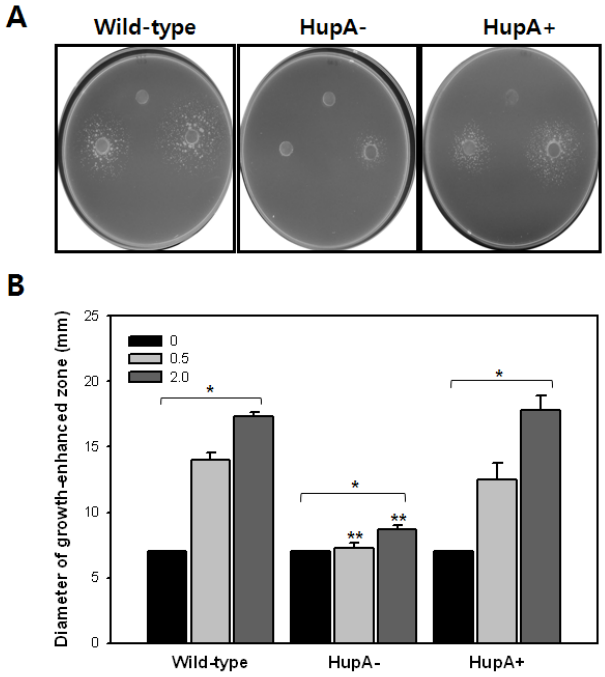

Figure 6. Effect of *hupA* mutation on the utilization of hemoglobin by *Vibrio vulnificus* on the surface of iron-deficient agars. The three *V. vulnificus* strains, M06-24/O containing wild-type *hupA* (Wild-type), RC110 containing mutated *hupA* (HupA-) and RC114 containing *in trans*-complemented *hupA* (HupA+), were used. (A) An inoculum of 10^4 cells was spread on and then paper discs containing 0, 0.5 or 2.0 mg/ml of hemoglobin, placed on the surface of deferrated Synbase agar, and incubated for 24 h at 37^{o}C. (B) The diameters of growth-enhanced zones were measured after incubation for 24 h. Means and standard deviations (error bars) were obtained from triplicate measurements. The symbols (* and **) indicate statistically significant differences among hemoglobin concentrations ($p<0.05$ in One Way ANOVA) and among the three strains ($p<0.05$ in Student's t-test), respectively.

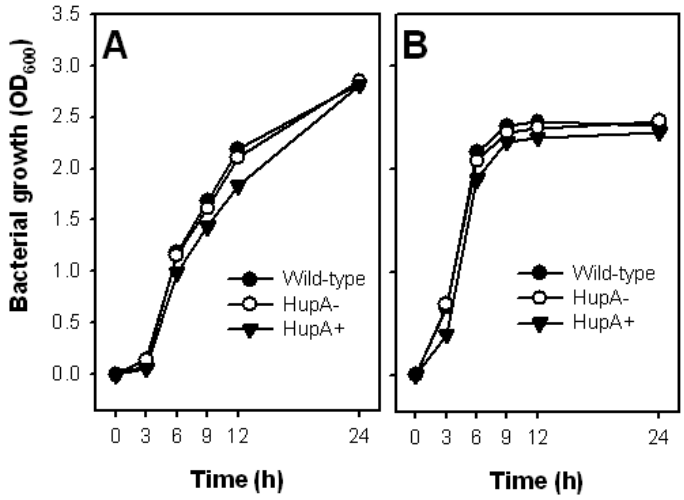

Figure 7. Effect of *hupA* mutation on the utilization of hemoglobin by *Vibrio vulnificus* in iron-limited broths. The three *V. vulnificus* strains, M06-24/O containing wild-type *hupA* (Wild-type), RC110 containing mutated *hupA* (HupA-) and RC114 containing *in trans*-complemented *hupA* (HupA+), were cultured in DF-HI broth (A) or DF-HI broth containing 0.5 mg/ml hemoglobin (B). Bacterial growths were determined by measuring optical densities of culture aliquots at 600 nm (OD_{600}).

The prevalence of the HupA-mediated IUS

To assess the presence of the HupA-mediated IUS in *V. vulnificus* strains, we investigated the presence of *hupA* DNA and HupA protein in *V. vulnificus* isolates using PCR and Western blot (Table 4). Of our laboratory stock strains, 10 environmental strains and 10 clinical strains including M06-24/O were randomly selected. Of these strains, 17 strains possessed *hupA* DNA and expressed HupA protein. The remaining three strains (one environmental and two clinical strains) did not possess *hupA* DNA or express HupA protein. These results indicate that the HupA-mediated IUS is widespread in *V. vulnificus* isolates.

Table 4. Prevalence of HupA in *Vibrio vulnificus* isolates

Strains	Number of positive *hupA* DNA[a]	Number of positive hupA protein[b]
Clinical isolates (n=10)	8	8
Environmental isolates (n=10)	9	9

[a]: Polymerase-chain reaction using the primers hupA-1 and -2
[b]: Western blot using rabbit polyclonal anti-HupA antibody

Effect of *fur* mutation on *hupA* expression

The effects of Fur on *V. vulnificus* growth and *hupA* transcription were determined by culturing the three P_{hupA}::*lacZ* transcriptional reporter strains, RC120 containing wild-type *fur,* RC124 containing mutated *fur* and RC312 containing *in trans* complemented *fur,* in DF-HI broths containing 5 or 25 μM FC. At 5 μM FC, the *fur* mutation in RC124 and *fur* complementation in RC312 had no significant effects on *V. vulnificus* growth or *hupA* transcription (data not shown). At 25 μM FC (Fig. 8A), no noticeable difference was observed among the growths of the three strains. In contrast, *hupA* transcription in RC120 was almost completely repressed. This repression in RC120 was de-repressed by the *fur* mutation in RC124 ($p<0.05$, Student's *t*-test), and this de-repression in RC124 almost completely repressed by the *in trans fur* complementation in RC312 ($p<0.05$, Student's *t*-test).

The effect of a *fur* mutation on HupA production was determined by culturing M06-24/O containing wild-type *fur*, CMM2303 containing mutated *fur,* and RC144 containing *in trans* complemented *fur*, under the same conditions (Fig. 8B). At 5 μM FC, the *fur* mutation in CMM2303 and *fur* complementation in RC144 had no significant effects on HupA production. However, at 25 μM FC, HupA production in M06-24/O was almost completely repressed. This repression in M06-24/O was de-repressed by the *fur* mutation

in CMM2303, and this de-repression in CMM2303 repressed by the in trans *fur* complementation in RC144.

Overall, these results indicate that *hupA* expression is under the negative control of Fur, the iron-responsive transcriptional repressor.

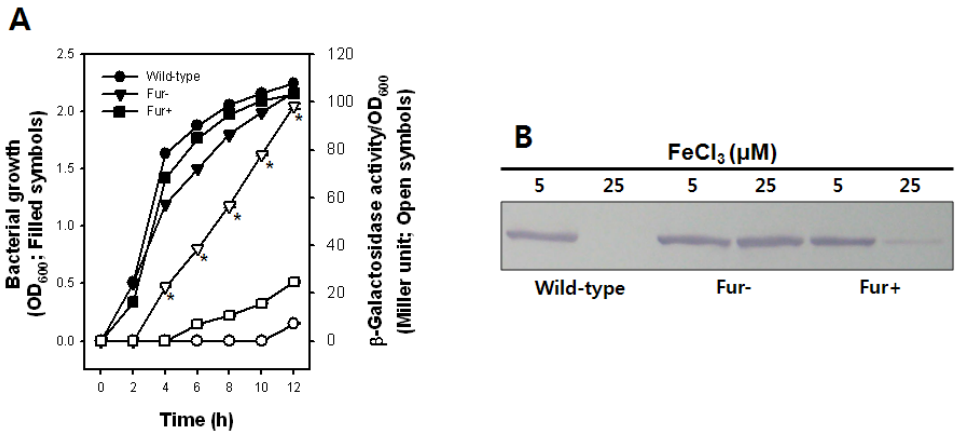

Figure 8. Effect of Fur on *Vibrio vulnificus* growth and *hupA* expression. (A) The P_{hupA}*::lacZ* transcription reporter strains, RC120 with wild-type *fur*, RC124 with mutated *fur*, RC312 with *in trans* complemented *fur*, were cultured in deferrated Heart Infusion (DF-HI) broths containing 25 μM ferric chloride (FC). Bacterial growths were determined by measuring optical densities of culture aliquots at 600 nm (OD_{600}), and *hupA* transcription was quantified by measuring β-galactosidase activity on a per cell basis (Miller units) in culture aliquots. β-Galactosidase activities are the means and standard deviations (error bars) of triplicate measurements. The symbol (*) indicates a statistically significant difference among the strains at the same time points ($p<0.05$ in Student's *t*-test). (B) The three *V. vulnificus* strains, M06-24/O with wild-type *fur*, CMM2303 with mutated *fur*, and RC144 with *in trans* complemented *fur,* were cultured in DF-HI containing 5 or 25 μM FC. Western blot was conducted as described in the legend of Fig. 5. A representative experiment of duplicate experiments is shown.

Effect of *crp* mutation on *hupA* expression

The effect of Crp on *hupA* transcription was determined by culturing the three P_{hupA}*::lacZ* transcription reporters, RC120 with wild-type *crp*, RC122 with mutated *crp* and RC258 with complemented *crp*, in DF-HI broths containing 5 or 25 μM FC. At both FC concentrations (Fig. 9A), the growth of RC120 was severely inhibited by the *crp* mutation in RC122, and this growth inhibition in RC122 was completely restored by the in trans *crp* complementation in RC256. At 5 μM FC, *hupA* transcription was highly induced in RC120, but almost completely repressed in RC122. This repression in RC148 was prevented in RC258 ($p<0.05$, Student's *t*-test). At 25 μM FC (data not shown), *hupA* transcription was severely repressed in all three strains.

The effect of Crp on HupA production was also examined by culturing the three *V. vulnificus* strains, M06-24/O with wild-type *crp*, CMM710 with mutated *crp*, CMM714 with *in trans* complemented *crp*, under the same conditions (Fig. 9B). At 5 μM FC, HupA production was highly induced in M06-24/O, but severely repressed in CMM710. This repression in CMM710 was prevented in CMM714. At 25 μM FC, HupA production was severely repressed in all the three strains.

Furthermore, hemoglobin utilization was determined using the three strains M06-24/O with wild-type *crp*, CMM710 with mutated *crp* and CMM714 with complemented *crp* (Fig. 10). The growth-enhanced zones of M06-24/O were dose-dependently increased

by 0.5 or 2.0 μg hemoglobin ($p<0.05$, One Way ANOVA). However, the growth-enhanced zones of CMM710 were not increased dose-dependently by hemoglobin and were significantly smaller than those of M06-24/O ($p<0.05$, Student's *t*-test). These decreases in CMM710 were recovered by the *in trans crp* complementation in CMM714 ($p<0.05$, Student's *t*-test).

In addition, the effect of cAMP on HupA production was also examined by culturing the three *V. vulnificus* strains, M06-24/O with wild-type *crp*, RC386 with mutated *cyaA*, RC390 with *in trans* complemented *cyaA*, under the same conditions. At 5 μM FC, the growth of M06-24/O was inhibited in RC386, and this inhibition was completely recovered in RC390 (Fig. 11A). HupA production was highly induced in M06-24/O, but severely repressed in RC386. This repression in RC386 was prevented in RC390 (Fig. 11C). Furthermore, the exogenous addition of 0.1 and 0.5 mM cAMP dose-dependently stimulated bacterial growth and increased HupA production in RC386 (Figs. 11B and 11D).

Overall, these results indicate that iron-repressible *hupA* expression is under the positive control of cAMP-Crp complex.

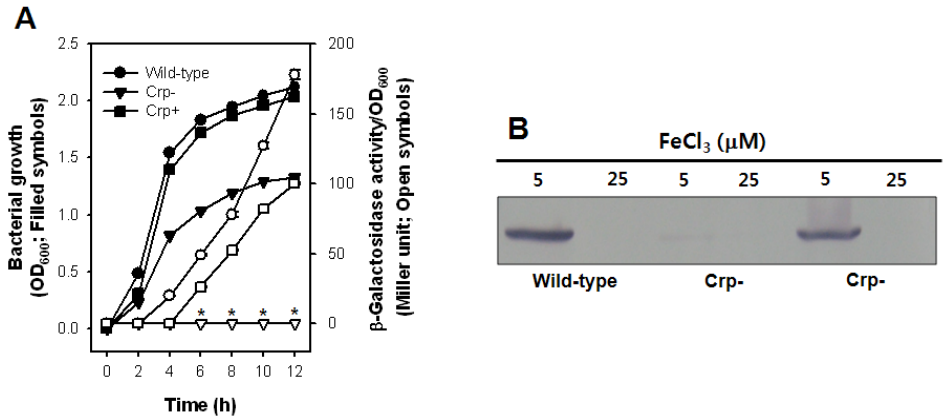

Figure 9. Effect of Crp on *Vibrio vulnificus* growth and *hupA* expression. (A) The P_{hupA}*::lacZ* transcription reporter strains, RC120 with wild type *crp*, RC122 with mutated *crp*, and RC258 with *in trans* complemented *crp*, were cultured in deferrated Heart Infusion (DF-HI) broths containing 5 μM ferric chloride (FC). Growth and *hupA* transcription were determined as described in the legend of Fig. 8. The symbol (*) indicates significant differences among the strains at the same time points ($p<0.05$ in Student's *t*-test). (B) The three *V. vulnificus* strains, M06-24/O with wild type *crp* and *fur*, CMM710 with mutated *crp*, and CMM714 with *in trans* complemented *crp*, were cultured in DF-HI containing 5 or 25 μM FC. Western blot was conducted as described in the legend of Fig. 5. A representative experiment of duplicate experiments is shown.

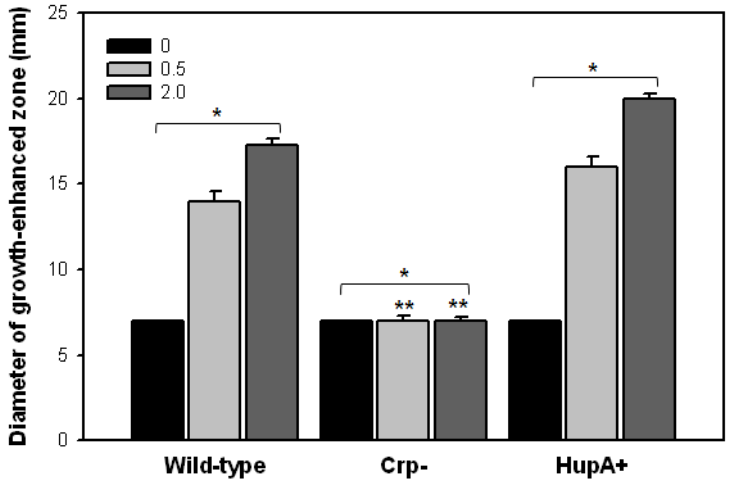

Figure 10. Effect of *crp* mutation on the utilization of hemoglobin by *Vibrio vulnificus* on the surface of iron-limited agars. The three *V. vulnificus* strains, M06-24/O with wild-type *crp* (Wild-type), CMM710 with mutated *crp* (Crp-) and CMM714 with *in trans*-complemented *crp* (Crp+), were used. Symbols are as described in the legend of Figure 6.

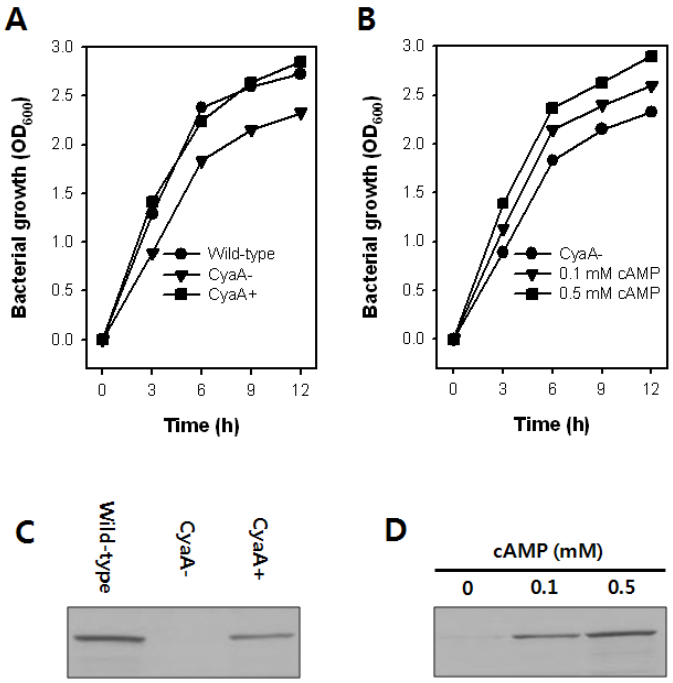

Figure 11. Effect of cAMP on *Vibrio vulnificus* growth and *hupA* expression. (A and C) *V. vulnificus* M06-24/O containing wild-type *cyaA*, RC386 containing mutated *cyaA*, and RC390 containing *in trans* complemented *cyaA* were cultured in deferrated Heart Infusion (DF-HI) broths containing 5 μM ferric chloride (FC) for 12 h. (B and D) *V. vulnificus* RC386 was cultured in DF-HI broths containing 5 mM FC plus 0, 0.1 or 0.5 mM cAMP for 12 h. Growth was determined as described in the legend of Fig. 8. (B) Western blot was conducted as described in the legend of Fig. 5. A representative experiment of duplicate experiments is shown.

Effects of glucose on *hupA* expression in the presence or absence of Fur

The effects of glucose on *hupA* transcription in the presence or absence of Fur were examined by culturing the two P_{hupA}*::lacZ* transcriptional reporter strains, RC120 with wild-type *fur* and RC124 with mutated *fur*, in TES-DF-HI broths containing 5 or 25 μM FC at various glucose concentrations (0 to 0.5%). At 5 μM FC, the growths of RC120 and RC124 were slightly increased in the presence of glucose, but not in proportion to glucose concentrations (Figs. 12A, 13A, 14A and 15A). However, in RC120 (Fig. 12A), glucose dose-dependently repressed *hupA* transcription and almost completely repressed *hupA* transcription at 0.4% over ($p<0.05$, One Way ANOVA). In contrast, *hupA* transcription in RC124 (Fig. 13A) was dose-dependently repressed by glucose ($p<0.05$), but maintained at low levels (about 40 Miller units) even in the presence of glucose of 0.4% over. The effect of glucose on HupA production in the presence or absence of Fur was also examined by culturing M06-24/O and CMM2303 under the same conditions. In M06-24/O (Fig. 12B), glucose dose-dependently repressed HupA production and almost completely repressed HupA production at 0.5%. In contrast, HupA production in CMM2303 (Fig. 13B) was slightly decreased in the presence of glucose of below 0.3%, but not further decreased at concentrations of above 0.4%.

At 25 μM FC, *hupA* transcription in RC120 was severely repressed, and thus, the effects of glucose on *hupA* transcription and HupA production could not be observed (Figs.

14A and 14B). In contrast, *hupA* transcription in RC124 (Fig. 15A) was dose-dependently repressed by glucose ($p<0.05$), but maintained at low levels (about 40 Miller units) even at glucose levels of above 0.4%. Nevertheless, HupA production in CMM2303 (Fig. 15B) was only slightly decreased in the presence of glucose of below 0.3%, but appeared to be slightly increased at glucose levels of above 0.4%.

Overall, these results indicate that glucose deprivation is also an important signal for the induction of iron-repressible *hupA* expression and that the presence of Fur is essential for the glucose-mediated repression of *hupA* expression.

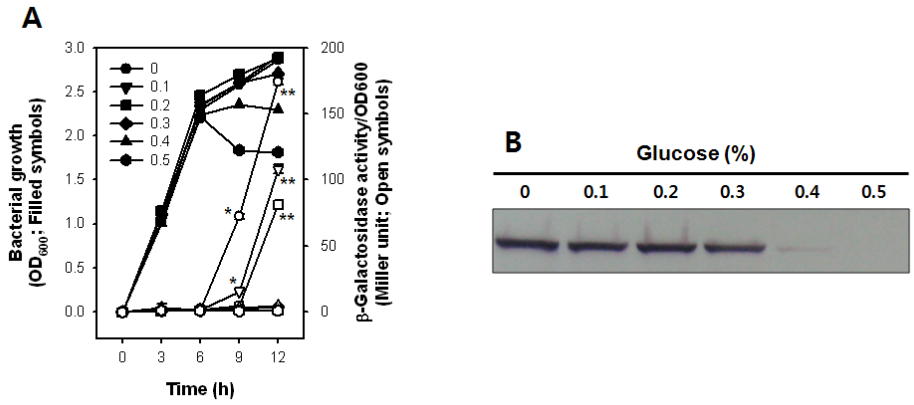

Figure 12. Effect of glucose on *Vibrio vulnificus* growth and *hupA* expression in a wild-type *fur* background under an iron-deficient condition. (A) RC120 with the wild-type *fur* and P_{hupA}*::lacZ* construct was cultured in TES-DF-HI broths containing 5 μM ferric chloride (FC) plus various concentrations (0-0.5%) of glucose. Growth and *hupA* transcription were determined as described in the legend of Fig. 8. The symbols (* and **) indicate significant differences among glucose concentrations at the same time points ($p<0.05$ in One Way ANOVA). (B) *V. vulnificus* M06-24/O with wild-type *fur* was cultured under the same conditions. Western blot was conducted as described in the legend of Fig. 5. A representative experiment of duplicate experiments is shown.

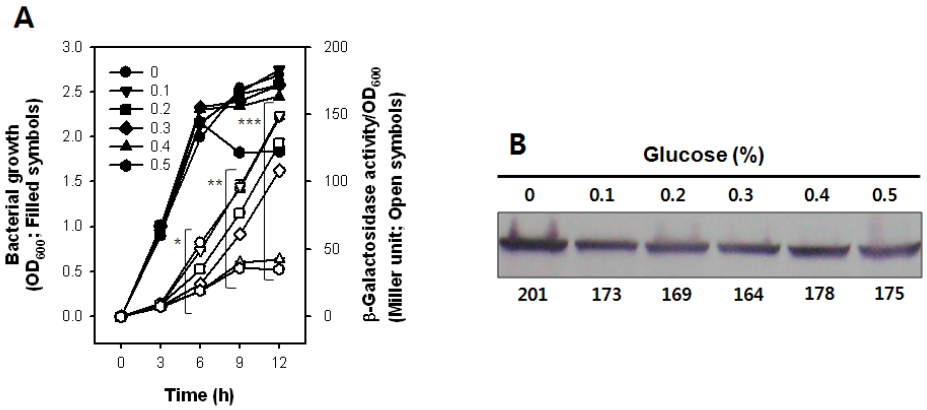

Figure 13. Effect of glucose on *Vibrio vulnificus* growth and *hupA* expression in a mutated *fur* background under an iron-limited condition. (A) RC124 with the mutated *fur* and P_{hupA}*::lacZ* construct was cultured in TES-DF-HI broths containing 5 μM ferric chloride (FC) plus various concentrations (0-0.5%) of glucose. Growth and *hupA* transcription were determined as described in the legend of Fig. 8. The symbols (*, ** and ***) indicate significant differences among glucose concentrations at the same time points ($p<0.05$ in One Way ANOVA). (B) *V. vulnificus* RC110 with mutated *fur* was cultured under the same conditions. Western blot was conducted as described in the legend of Fig. 5. A representative experiment of duplicate experiments is shown.

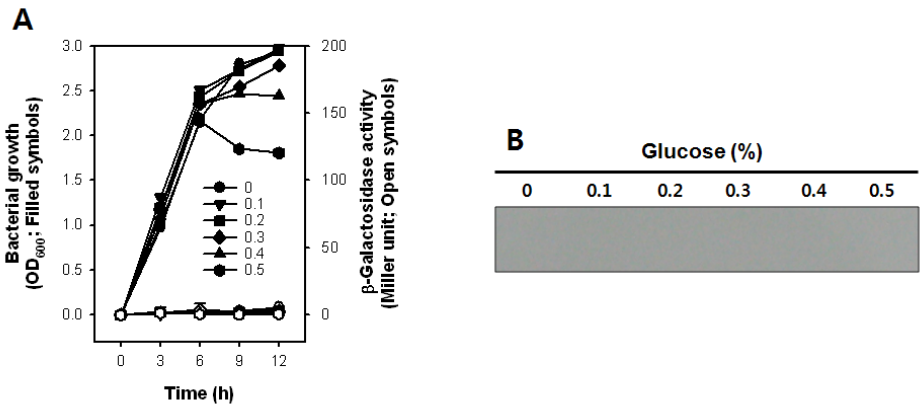

Figure 14. Effect of glucose on *Vibrio vulnificus* growth and *hupA* expression in a wild-type *fur* background under an iron-sufficient condition. (A) RC120 with the wild-type *fur* and P_{hupA}*::lacZ* construct was cultured in TES-DF-HI broths containing 25 μM ferric chloride (FC) plus various concentrations (0-0.5%) of glucose. Growth and *hupA* transcription were determined as described in the legend of Fig. 8. (B) *V. vulnificus* M06-24/O with wild-type *fur* was cultured under the same conditions. Western blot was conducted as described in the legend of Fig. 5. A representative experiment of duplicate experiments is shown.

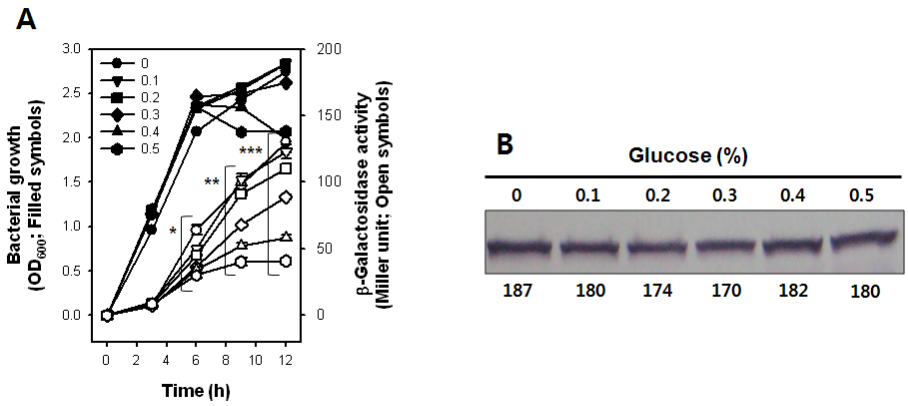

Figure 15. Effect of glucose on *Vibrio vulnificus* growth and *hupA* expression in a mutated *fur* background under an iron-sufficient condition. (A) RC124 with the mutated *fur* and P_{hupA}*::lacZ* construct was cultured in TES-DF-HI broths containing 25 μM ferric chloride (FC) plus various concentrations (0-0.5%) of glucose. Growth and *hupA* transcription were determined as described in the legend of Fig. 8. The symbols (*, ** and ***) indicate significant differences among glucose concentrations at the same time points ($p<0.05$ in One Way ANOVA). (B) *V. vulnificus* RC110 with mutated *fur* was cultured under the same conditions. Western blot was conducted as described in the legend of Fig. 5. A representative experiment of duplicate experiments is shown.

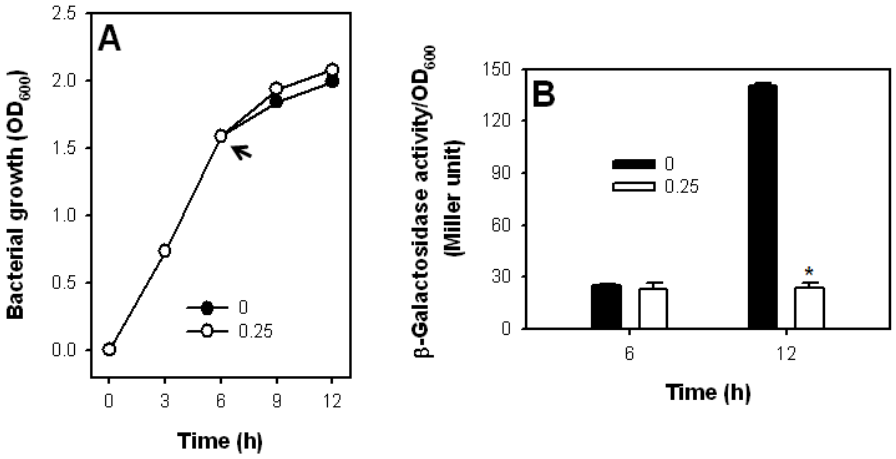

Figure 16. Effect of glucose added in the middle of culture on *Vibrio vulnificus* growth and *hupA* expression under an iron-deficient condition. RC120 with the wild-type *fur* and P_{hupA}*::lacZ* construct was cultured in TES-DF-HI broths containing 5 μM ferric chloride (FC), and 0.25% glucose was added 6 h after culture initiation. Growth (A) and *hupA* transcription (B) were determined as described in the legend of Fig. 8. The symbol (*) indicate significant differences between the presence and absence of glucose at the same time points ($p<0.05$ in Student's *t*-test).

Effects of iron on *hupA* expression in the presence or absence of Crp and Fur

To observe the effects of iron on *V. vulnificus* growth and *hupA* transcription in the presence or absence of Crp or Fur, the three P_{hupA}*::lacZ* transcriptional reporter strains, RC120 with wild-type *crp* and *fur*, RC122 with mutated *crp*, and RC124 with mutated *fur*, were cultured in DF-HI broths containing various concentrations (0.5 to 30 μM) of FC for 12 h (Fig. 17A). FC dose-dependently stimulated the growths of the three strains at below 5 μM, but not at above 5 μM. In RC120, FC dose-dependently increased *hupA* transcription at below 5 μM ($p<0.05$), but dose-dependently repressed *hupA* transcription at above 5 μM ($p<0.05$) and almost completely repressed *hupA* transcription at more than 15 μM. The *hupA* transcription level peaked at 5 μM FC. In RC122, FC dose-dependently repressed *hupA* transcription even at below 5 μM and almost completely repressed *hupA* transcription at above 5 μM. In RC124, FC dose-dependently increased *hupA* transcription at below 5 μM, but had no significant effect at above 5 μM.

The effects of iron on HupA production in the presence or absence of Crp or Fur were also determined by culturing M06-24/O with wild-type *crp* and *fur*, CMM710 with mutated *crp*, and CMM2303 with mutated *fur*, under the same conditions (Fig. 17B). In M06-24/O, FC at below 5 μM had no significant effect on HupA production, but FC at above 5 μM dose-dependently repressed HupA production, and FC at above 20 μM almost completely repressed HupA production. A discrepancy between *hupA* transcription and

HupA production levels was observed at below 5 μM FC. In CMM710, FC at below 5 μM dose-dependently repressed HupA production and FC at above 5 μM almost completely repressed HupA production. In CMM2303, FC had no significant effect on HupA production at any concentration examined.

Overall, these results indicate that the Fur-mediated repression of *hupA* expression occurs at lower iron concentrations in the absence of Crp than in the presence of Crp.

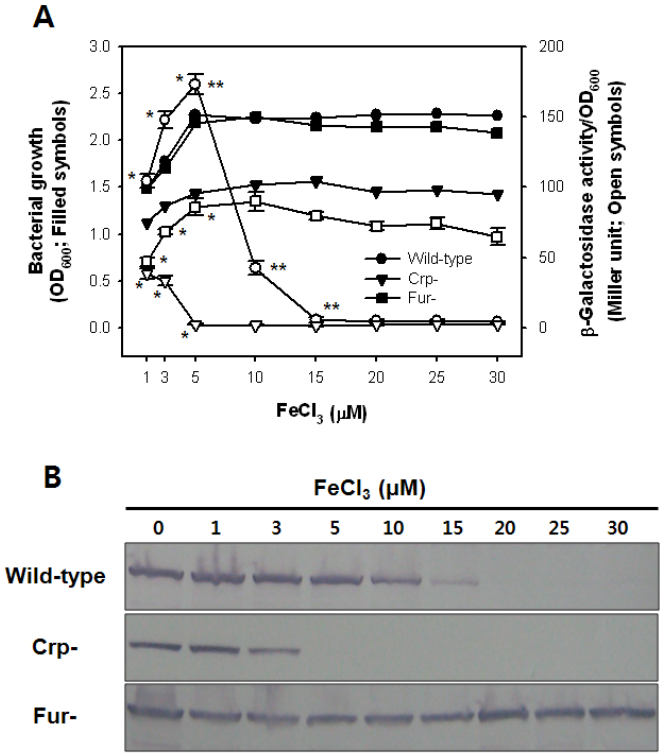

Figure 17. Effect of iron on *Vibrio vulnificus* growth and *hupA* expression in a *crp*- or *fur*-mutated background. (A) The three P_{hupA}*::lacZ* transcriptional reporter strains, RC20 with wild-type *crp* and *fur*, RC122 with mutated *crp* and RC124 with mutated *fur*, were cultured in DF-HI broths containing various concentrations of ferric chloride (FC) for 12 h. Growth and *hupA* transcription were determined as described in the legend of Fig. 8. The symbols (* and **) indicate significant differences at various iron concentrations ($p<0.05$, One Way ANOVA). (B) *V. vulnificus* M06-24/O (wild-type), CMM710 (Crp-) and CMM2303 (Fur-) were cultured under the same conditions. Western blotting was conducted in described in the legend of Fig. 5. A representative experiment of duplicate experiments is shown.

Discussion

In most bacteria, iron is essential for energy production and growth. In the human body, most intracellular iron is present in hemoglobin, heme, ferritin, and hemosiderin. The trace quantities of iron present extracellularly are bound to high-affinity iron-binding proteins, such as, transferrin and lactoferrin. Therefore, iron availability for most bacteria is severely limited *in vivo*. Accordingly, most bacteria have evolved specific IUSs to adapt to this limited iron availability [Weinberg, 1978 and 2000; Andrews et al., 2003]. *V. vulnificus* possesses multiple IUSs [Litwin et al., 1996; Litwin and Byrne, 1998; Webster and Litwin, 2000; Aso et al., 2002; Tanabe et al., 2005]. The presence of multiple IUSs enables *V. vulnificus* to survive and proliferate *in vivo* and is highly advantageous to *V. vulnificus*, a ferrophilic bacterium that requires more iron than other pathogens [Kim et al., 2007].

In the present study, the *V. vulnificus hupA* mutant RC110 strain was successfully constructed and rabbit polyclonal anti-HupA antibody was prepared (Fig. 5). When a plasmid containing the open reading frame and upstream region of *hupA* was transferred into the *hupA* mutant strain, HupA production was recovered and found to be regulated by iron, which suggests that the upstream DNA is sufficient for regulation of the gene by Fur or iron. However, complete repression of HupA expression was not observed even at 25 μM FC. Presumably, this was due to the presence of multiple copies of *hupA* saturating the

limited quantities of Fur expressed in a single copy on the chromosome. A similar phenomenon was also observed in another study using the same plasmid [Litwin and Byrne, 1998].

The presence of the HupA-mediated IUS allows *V. vulnificus* to utilize haem iron directly. The TonB-dependent outer membrane heme receptor HupA in *V. vulnificus* was first described by Litwin and Byrne. The present study also shows that HupA is involved in the direct utilization of haem iron (Fig. 6). The wild-type strain was found to be able to use hemoglobin as an iron source, whereas a *hupA* mutant strain could not. This defective hemoglobin utilization by the *hupA* mutant strain was recovered by *in trans* complementing wild-type *hupA*.

The HupA-mediated IUS seems to be active in the absence of vulnibactin-or VuuA-mediated IUS; that is, HupA-mediated IUS may not be essential for the iron uptake and growth of *V. vulnificus* under iron-deficient or limited conditions. *V. vulnificus* produces vulnibactin, a siderophore with high affinity for iron that can capture iron from heme proteins including hemoglobin, as well as from transferrin and lactoferrin, which are high affinity iron-withholding proteins [Litwin et al., 1996]. Accordingly, *V. vulnificus* can also utilize heme iron via the vulnibactin- or VuuA-mediated IUS. Previous studies have shown that mutant strains that do not produce vulnibactin or express the vulnibactin receptor VuuA do not grow well even in agars or broths containing hemoglobin as a sole

iron source [Litwin et al., 1996, Webster and Litwin, 2000; Sun et al., 2006]. This implies that it may be difficult to assess the direct utilization of heme iron by *V. vulnificus* in the presence of vulnibactin. In the present study, *hupA* mutation impaired hemoglobin utilization by *V. vulnificus* in agar plates, but did not in broths (Fig. 7). Presumably, these are because vulnibactin diffused out and *V. vulnificus* could not use vulnibactin for iron uptake on agar plates containing hemoglobin as a sole iron source. Thus, the *in vivo* significance of the HupA-mediated IUS would be obvious in local environments lacking vulnibactin, although a *hupA* mutation alone was reported to lower the cytotoxicity and mouse lethality of *V. vulnificus* [Oh et al., 2009]. In *V. cholerae*, a heme utilization mutant showed only a slight reduction in virulence versus the wild-type or a vibriobactin (siderophore) synthesis mutant [Henderson and Payne, 1994; Rey and Payne, 2001].

The present study shows that HupA is widespread in *V. vulnificus* isolates (Table 4). Of the 20 *V. vulnificus* isolates examined in the present study, only three strains did not possess *hupA* and express HupA protein under iron-limited conditions. No significant difference was observed between clinical and environmental strains in terms of the possession of *hupA* and the expression of HupA. Further characterization of these hupA- or HupA-deficient strains was not conducted during this study. Similar outer membrane receptor proteins responsible for heme utilization have also been identified in various Vibrio species *V. cholera* [Mey and Payne, 2001], *V. parahaemolyticus* [Yamamoto et al.,

1995], *V. anguillarum* [Mourino et al., 2006], *V. fluvialis* [Ahn et al., 2005], and *V. fischeri* [Septer et al., 2011]. Accordingly, heme utilization receptor proteins are likely to be widespread among Vibrio species and among *V. vulnificus* clinical and environmental strains. This ubiquity suggests a common ancestral origin and these proteins play critical roles in the physiology of Vibrio species.

The present study shows that iron limitation is a principal or essential signal for *hupA* expression. This indicates that Fur acts as a principal or local regulator that negatively regulates *hupA* expression in response to iron availability. As in most bacteria [Andrews et al., 2003], all *V. vulnificus* IUSs, including the HupA-mediated IUS, are under the negative control of Fur, which functions as the well-established iron-responding transcriptional repressor [Litwin and Calderwood, 1993]. The present study confirms that *hupA* expression is under the negative control of iron or Fur (Fig. 8). Iron dose-dependently repressed *hupA* expression at the transcriptional and protein levels in the wild-type strains, and this iron-responsiveness of *hupA* expression was completely abolished in the *fur*-mutated strains. According to sequence analysis of the *hupA* regulatory region (Fig. 1), Fur is likely to function by binding to the -37 to -19 position from the *hupA* transcription start site to prevent the binding of RNA polymerase [Litwin and Byrne, 1998]. This Fur-mediated regulation of iron-repressible *hupA* expression implies that iron limitation is an

essential signal for the induction of *hupA* expression and that Fur is a principal or local regulator that primarily controls *hupA* expression in response to iron levels.

The present study confirms that *hupA* expression is under the positive control of Crp. This suggests that Crp functions a coordinator or mediator that positively regulates *hupA* expression in response to carbon availability or metabolic status. A *crp* mutation severely repressed *hupA* expression at the transcriptional and protein levels, and the repression of *hupA* expression by the *crp* mutation was prevented by *in trans* complementing wild-type *crp* (Figs. 9 and 10). Furthermore, a *cyaA* mutation repressed HupA production, and the repression was prevented by *in trans* complementing wild-type *cyaA* or the addition of exogenous cAMP (Fig. 11). A recent study also found that Crp directly and positively regulates *hupA* expression by binding to the -186 to -166 position from the *hupA* transcription start site [Oh et al., 2009], although this position is unusually distant for direct activation by Crp, which suggests that another factor (s) is involved in the activation of *hupA* promoter [Brown and Busby, 2004]. Accordingly, this Crp-mediated regulation of *hupA* expression implies that Crp is an important activator that controls *hupA* expression in response to glucose availability and that glucose starvation is an important signal for the induction of *hupA* expression.

Furthermore, the present study also discriminates the roles of iron/Fur as an essential or principal signal/regulator and the roles of glucose/Crp as a coordinator or mediator of

hupA expression. In the presence of Fur, glucose dose-dependently and completely repressed both *hupA* transcription and HupA production (Figs. 12, 13 and 16). However, in the absence of Fur, glucose only partially or slightly repressed HupA production (Figs. 14 and 15). These findings indicate that the presence of Fur is essential for the glucose-mediated repression of *hupA* expression. In addition, the present study shows that severe iron limitation alone can induce *hupA* expression albeit at relatively low levels in the absence of Crp (Fig. 17), which indicates that *hupA* is constitutively expressed albeit at relatively low levels and that Crp is not essential for the induction of *hupA* expression under iron-limited conditions. Furthermore, the Fur-mediated repression of *hupA* expression occurred at lower iron concentrations in the absence of Crp than in its presence, which indicates that the presence of Crp is essential for optimal *hupA* expression, but not for constitutional *hupA* expression, under iron-limited conditions. Overall, Fur is likely to act as a principal or local regulator of the induction of *hupA* expression in response to iron availability, and Crp is likely to act as a coordinator or mediator that regulates *hupA* expression in response to carbon availability or metabolic status.

The expressions of other IUSs in *V. vulnificus* are also under the positive control of Crp. Our recent studies showed that Crp positively regulates the expression of *vuuA* encoding vulnibactin receptor [Choi et al., 2006] and the expression of *iutA* encoding *E. coli* aerobactin receptor [Kim et al., 2012]. Furthermore, Crp also positively regulates the

expression of *vvhBA* encoding a cytolysin/hemolysin, which destroys a variety of cells, including red blood cells, and releases intracellular iron [Choi et al., 2002; Kim et al., 2005; Choi et al., 2006], and positively regulates the expression of *vvpE*, which encodes a metalloprotease that destroys iron-binding proteins, such as, transferrin and hemoglobin, to release freely-available iron [Nishina et al., 1992; Okujo et al., 1996; Jeong et al., 2003; Kim & Shin, 2010]. This Crp-or glucose-mediated regulation of IUSs implies that glucose starvation is an important signal for the induction of IUSs and that Crp functions as a coordinator or mediator that regulates IUSs expression in response to glucose-availability and so connects iron metabolism and carbon metabolism.

Crp is a global regulator with well known roles in catabolite repression [Deutscher, 2008]. Glucose is the preferred energy source in most bacteria, and the presence of glucose represses the expressions of a large number of genes associated with catabolism, conversely, the absence of glucose stimulates the expressions of these genes. Iron is essential for activating many catabolite enzymes, especially those involved in the electron transport system. Furthermore, iron is essential for efficient energy production especially under glucose deprivation. The fact that the expressions of IUSs are under the positive control of Crp implies that, under glucose-poor stressful conditions, the acquisition of iron should be increased for efficient catabolism and efficient energy production. That is, it is likely that optimal IUS expression is under the coordinate control of Crp (glucose

availability) and Fur (iron availability), as illustrated in Fig. 17. Eventually, this metabolically coordinated acquisition and effective utilization of iron is likely to be crucial for successful establishment of infection.

According to the results in the present study, *hupA* expression reached a peak (about 170 Miller units in β-galactosidase activity) at 5 μM FC in the wild-type strains, but was maintained at about 80 Miller units in the *fur*-mutated strains (Fig. 16), which suggests that *fur* mutation may indirectly represses *hupA* expression under iron-limited conditions. This may occur because other IUSs, including the VuuA-mediated IUS, would be de-repressed, iron uptake would be increased, intracellular iron would be increased, and thus, *hupA* transcription would be partially repressed in the *fur*-mutated strain versus the wild-type strain.

In the present study, a noticeable discrepancy was observed between the glucose-mediated repression of *hupA* transcription and HupA production in the *fur*-mutated background. Glucose dose-dependently repressed *hupA* transcription, which was measured by the *lacZ*-fused transcription reporter assay, whereas it only slightly repressed HupA production, which was measured by Western blotting (Figs. 12 and 14). This discrepancy is probably due to the presence of TES as a pH-buffering agent and the likely low pH resulting from glucose catabolism, both of which may synergistically inhibit the measurements of β-galactosidase activity. During a pilot experiment, we found that the

presence of TES interferes with the measurement of β-galactosidase activities. This fact has not been previously known. Thus, we are now examining the effect of TES and/or pH on β-galactosidase activity.

Another noticeable difference was also observed between *hupA* transcription levels in the *lacZ*-fused transcription reporter assay and HupA production by Western blotting, especially under severely iron-limited conditions. FC at less than 5 μM dose-dependently increased *hupA* transcription, but tended to slightly decrease HupA production in the presence of Crp (Fig. 16). However, in the absence of Crp, *hupA* transcription levels paralleled HupA production levels under the same conditions. One plausible interpretation of these findings is that the *lacZ*-fused transcriptional reporter assay may not represent actual *hupA* transcription levels under severely iron-limited conditions in which *V. vulnificus* growth was severely impaired. If so, the maintenance of appropriate bacterial growth may be a prerequisite for quantitative measurements and comparisons of gene transcription levels when the *lacZ*-fused transcription reporter assay is used. Another possible explanation is that iron may increase intracellular Crp or cAMP levels in concert with *V. vulnificus* growth, and thereby, stimulate *hupA* transcription but not HupA production, and post-transcriptional mechanisms may be involved. To test these assumptions, more accurate quantitative analysis at the *hupA* mRNA level is necessary.

In *Vibrio cholerae*, heme utilization involves multiple TonB-dependent heme receptors, such as HutA, HutR and HasR, although HutA plays a dominant role [Henderson and Payne, 1994; Rey and Payne, 2001]. A recent study reported that an additional TonB-dependent hemin receptor HvtA is needed with HupA for optimal hemin utilization in *V. vulnificus* [Datta and Crosa, 2011]. On analysis of *hvtA*, it is located in the second chromosome where it is predicted to be the second open reading frame in a putative five-member operon. This operon is significantly similar to the operon in *V. cholerae* containing the *hutR* hemin receptor gene [Rey and Payne, 2001], which suggests vertical transmission from *V. cholerae* to *V. vulnificus*. The *hvt* operon includes *ptrB* (a type II protease), *hvtA*, and other three genes, the functions of which remain to be determined. A putative Fur-bindng site is located upstream of *ptrB* and the expression of *hvtA* is known to be negatively regulated by iron. However, in the present study, *hvtA* mutation did not impair the ability of *V. vulnificus* to utilize hemin as compared with that observed for the *hupA* mutant strain. Probably this is because the level of *hvtA* expression is substantially lower than the level of *hupA* expression. The effects of Crp and glucose on the expression of the *hvt* operon need to be determined.

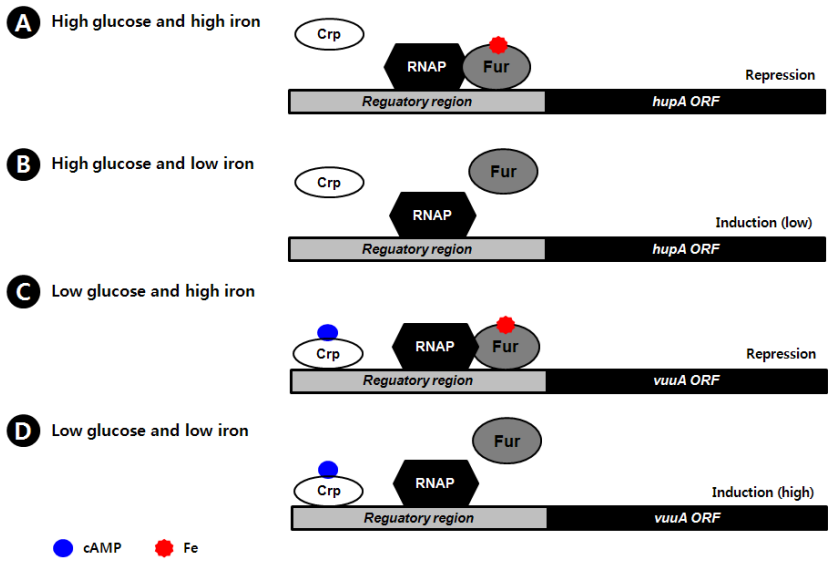

Figure 18. Proposed coordinate regulation of *hupA* expression. Crp (cyclic AMP-receptor protein), RNAP (RNA polymerase), Fur (Ferric uptake regulator), ORF (open reading frame).

Conclusion

The present study showed that (i) HupA is involved in the direct utilization of heme iron by *V. vulnificus*, (ii) HupA is widespread among *V. vulnificus* strains, (iii) iron limitation is a principal or essential signal for *hupA* expression, (iv) glucose starvation is a synergistic signal for optimal *hupA* expression under iron-limited conditions, (v) Fur is essentially required to prevent *hupA* over-expression in response to iron availability, and (vi) Crp is an activator required for optimal *hupA* expression in response to glucose availability.

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Abstract

Regulatory Mechanism of the *hupA* Gene Encoding Heme Receptor Protein in *Vibrio vulnificus*

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Vibrio vulnificus causes rapid progressing fulminant infections in susceptible individuals, especially those with elevated serum iron levels. This ferrophilic bacterium can directly acquire iron from heme-containing proteins, such as hemoglobin, via its outer membrane heme receptor HupA. The expression of *hupA* has been known to be under the negative control of Fur (Ferric uptake regulator) which functions as a transcription repressor in response to iron sufficiency, and recently, to be under the positive control of Crp (cAMP-receptor protein) which functions as an transcription activator in response to glucose deprivation. The purpose of this study was to confirm the role of HupA in the direct utilization of hemoglobin, to examine the distribution of HupA in *V. vulnificus* clinical and environmental isolates, and finally, to detail the coordinate roles played by Fur and Crp in the regulation of *hupA* expression. An *in frame* deletion mutation in *hupA* abolished the ability of *V. vulnificus* to utilize hemoglobin as an iron source under iron-

deficient (ID) conditions and this defect was rescued by *in trans* complementing wild-type *hupA*. Only two of ten clinical isolates and one of ten environmental isolates did not possess *hupA* DNA or expressed HupA protein under ID conditions. An *in frame* deletion mutation in *crp* repressed *hupA* expression under ID conditions, and this defect was recovered by *in trans* complementing wild-type *crp.* In addition, an *in frame* deletion mutation in *cyaA* encoding adenylate cyclase required for cAMP synthesis repressed *hupA* expression under ID conditions and this defect was recovered by *in trans* complementing wild-type *cyaA.* An *in frame* deletion mutation in *fur* de-repressed *hupA* expression under iron-sufficient (IS) conditions and this change is recovered by *in trans* complementing wild-type *fur*. Under severely ID conditions, *hupA* expression was induced at higher levels in the presence of Crp than in the absence of Crp. Glucose dose-dependently and completely repressed *hupA* expression only in the presence of Fur, but not in the absence of Fur. These results indicate that (i) HupA is involved in the utilization of heme iron, (ii) HupA is widespread among *V. vulnificus* strains, (iii) glucose deprivation under iron limitation synergistically activates *hupA* expression; (iv) Fur is essentially required to prevent *hupA* over-expression in response to iron availability; and (v) Crp is an activator required for optimal *hupA* expression in response to glucose availability.

Key words: *Vibrio vulnificus*, Iron, Glucose, Heme receptor, cAMP-receptor protein, Ferric uptake regulator