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Original Article

# Coordinate Regulation of *Vibrio vulnificus* Heme Receptor HupA Expression by Cyclic AMP-receptor Protein and Ferric Uptake Regulator

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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 heme receptor protein HupA. This study was undertaken to determine the roles of cyclic AMP-receptor protein (Crp) as an activator and of ferric uptake regulator (Fur) as a repressor in regulating *hupA* expression at various iron and glucose concentrations. Under severely iron-deficient conditions, *hupA* expression in the absence of Crp was induced albeit at low levels and repressed by the addition of iron. In contrast, *hupA* expression in the presence of Crp was increased by the addition of iron. Under moderately iron-deficient and iron-sufficient conditions, iron addition repressed *hupA* expression in the presence of Fur, but not in the absence of Fur. Glucose addition repressed *hupA* expression in the presence of Fur but not in the absence of Fur. Furthermore, a mutation in *cyaA* encoding adenylate cyclase required for cAMP synthesis repressed *hupA* expression, and this repression was prevented by the exogenous addition of cAMP. These results indicate that *hupA* expression is under the coordinate control of cAMP or Crp, which responds to glucose availability, and of Fur, which responds to iron availability, and that Crp is not essential for the constitutional expression of hupA, but is required for the optimal expression of *hupA*, whereas Fur is essential for the prevention of *hupA* over-expression.

**Key Words:** *Vibrio vulnificus*, Iron, Glucose, Heme receptor, cAMP-receptor protein

## INTRODUCTION

*Vibrio vulnificus* is a gram-negative halophilic bacterium that causes life-threatening septicemia and necrotizing wound infections mainly in patients with liver cirrhosis, hemochromatosis, or β-thalassemia. Several established and potential virulence factors have reported to play important roles in the pathogenesis of *V. vulnificus* infections, such as, capsular polysaccharides, lipopolysaccharides, iron-assimilation systems, flagella, pili, RTX toxin, and exotoxins such as cytolysins and proteases. These factors are under the control of global regulators, such as cyclic AMP (cAMP) and cAMP receptor protein (Crp) complex, and ferric uptake regulator (Fur) (1).

Elevated serum iron levels are well-known predisposing

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host factors. In mice, iron treatment reduces the intraperitoneal 50% lethal dose ($LD_{50}$) from $10^6$ cells to only 1 cell (2), and increases the mortality rate to 100% (3). *V. vulnificus* grows better in the blood of patients with hemochromatosis, when transferrin saturation by iron is increased, or if hematin is added (4). Although iron is an essential element for the survival and proliferation of most bacteria, some bacteria called ferrophilic or iron-sensitive bacteria have an impaired ability to acquire iron, and thus, cause diseases primarily in iron-overloaded hosts (5). In particular, *V. vulnificus* is a ferrophilic bacterium that requires higher levels of readily available iron for growth initiation than other pathogens (6).

To establish infection successfully, bacteria must be able to acquire iron from their hosts, and thus, most bacteria have evolved specific iron uptake systems. The ability to acquire iron is a well-established virulence factor in the pathogenesis of *V. vulnificus* infections, and *V. vulnificus* possesses multiple iron-uptake systems (1). Of these, the HupA-mediated iron-uptake system is not essential for survival but probably plays an important role in iron acquisition *in vivo* (7), because most iron within the human body is present as hemoglobin within red blood cells, and *V. vulnificus* produces several cytotoxins, such as, cytolysin (called VvhA) (8) and RTX toxin (9), which can destroy red blood cells, and thus, cause the release of intracellular iron in the form of hemoglobin, hemin, ferritin, and hemosiderin. In fact, a mutation in *hupA* has been reported to increase the intraperitoneal $LD_{50}$ in mice (10).

Fur regulates *hupA* expression at the transcription level in response to iron availability (11, 12), and recently, Crp, a global regulator primarily responsible for catabolite repression (13), was found to act as a transcriptional activator of *hupA* expression (10). However, details of the roles played by Crp as an activator and Fur as a repressor of *hupA* expression have not been observed. It is known that the expressions of many genes are regulated by a combination of global regulators and specific local regulators (14). Therefore, this study was conducted to detail the coordinate roles of Crp as a global regulator and Fur as a local regulator in the regulation of *hupA* expression at various iron and glucose concentrations.

## 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 (15~24). Heart Infusion (HI; BD, Franklin Lakes, NJ, USA) agar or broth containing an additional 2.0% of NaCl, and Thiosulfate-Citrate-Bile Salt-Sucrose (TCBS, BD) agar were used to cultivate *V. vulnificus* strains. Luria-Bertani medium was used to cultivate *Escherichia coli* strains. Unless otherwise stated, all other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

### Limitation and supplementation of iron or glucose

Two types of iron-limited media were used, that is, 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, and HI broth deferrated with 8-hydroxyquinoline as previously described (25) was used for main experiments. The concentration of residual iron in deferrated HI broth was less than 1 μg/dl. To observe the effect of iron on growth and gene expression, various concentrations (0~30 μM) of ferric chloride (FC) were added to deferrated HI broths. In addition, deferrated HI broth was used as a glucose-poor medium because it contains only a low, though undefined, level of glucose. To observe the effect of glucose, various concentrations of glucose were added to deferrated HI broths containing 100 mM N-trismethyl-2-aminoethanesulfonic acid (TES) as a buffering agent (TES-DF-HI), as previously described (18).

### Culture conditions and measurements of bacterial growth and β-galactosidase activity

*V. vulnificus* strains were preconditioned in HI broth containing 200 μM dipyridyl at 37℃ overnight to adapt to iron-limited conditions. Preconditioned strains were then inoculated into test broths at a bacterial density of $5 \times 10^6$ cells/ml and cultured with vigorous shaking at 37℃ for 12 h.

**Table 1.** Bacterial strains, plasmids, and primers used in this study

| Strains/plasmids/primers | Relative characteristics and sequences | References or sources |
|---|---|---|
| *Vibrio vulnificus* | | |
| M06-24/O | Wild type, highly virulent clinical isolate | (15) |
| CMM710 | M06-24/O with *crp* mutation | (16) |
| CMM2303 | M06-24/O with *fur* mutation | (17) |
| RC386 | M06-24/O with mutated *cya* | This study |
| RC390 | RC386 with *in trans* complemented *cya* | This study |
| CMM2101 | M06-24/O with *lacZ* mutation | (18) |
| RC100 | CMM2101 with *crp* mutation | (19) |
| CMM2304 | CMM2101 with *fur* mutation | (20) |
| RC120 | CMM2101 with $P_{hupA}$*::lacZ* transcriptional fusion | (21) |
| RC122 | RC100 with $P_{hupA}$*::lacZ* transcriptional fusion | This study |
| RC124 | CMM2304 with $P_{hupA}$*::lacZ* transcriptional fusion | This study |
| *Escherichia coli* | | |
| SY327 λ*pir* | Host for suicide vector | (22) |
| SM10 λ*pir* | Conjugation donor | (22) |
| Plasmids | | |
| pDM4 | Suicide vector with R6K origin; $Cm^R$ | (23) |
| pLAFR3II | pLAFR3 with *bla* inserted at the cos site; $Ap^R$ and $Tc^R$ | (18) |
| pRK2013 | IncP, $Km^R$, Tra $Rk2^+$ *repRK2 repE1* | (24) |
| pRC316 | pDM4 with 1.63-kb *Xba*I-*Sma*I fragment containing *in-frame* deleted *cyaA* | This study |
| pRC318 | pLAFR3II with 3.16-kb *Bam*HI-*Eco*RI fragment containing wild-type *cyaA* | This study |
| Primers | | |
| cyaA-up-1 | 5'-gctctagaagccagcggccgcgagaatgatc-3' | This study |
| cyaA-up-2 | 5'-cgctttggacatcttctgactttgcaaatccataagcgccag-3' | This study |
| cyaA-down-1 | 5'-gatttgcaaagtcagaagatgtccaaagcggtcaacgtatag-3' | This study |
| cyaA-down-2 | 5'-tcccccgggtgcctactgtgattgctcagattgttg-3' | This study |
| cyaA-comp-1 | 5'-cgggatcctgcacgcccttccagcattgc-3' | This study |
| cyaA-comp-2 | 5'-ggaattcgcgtagctatcgttaagccattaag-3' | This study |

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 previously described (26).

**In frame deletion mutation and in trans complementation of genes**

A *crp*-deleted CMM710 strain and a *fur*-deleted CMM2303 strain were constructed as described previously (16, 17).

RC386 containing a deletion of *cyaA* was constructed as follows. Two pairs of PCR primers cyaA-up-1/cyaA-up-2 and cyaA-down-1/cyaA-down-2 were used to amplify the 5'

and 3' ends of *cyaA*, respectively. The resulting PCR products were used as templates for 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, and the resulting plasmid (pRC316) was transformed into *E. coli* SY327 λ*pir* and SM10 λ*pir*, 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 PCR product so obtained was subcloned into pLAFR3II, and the resulting plasmid pRC318 was transferred into RC386 by triparental mating using pRK2013.

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

The *lacZ*-fused *hupA* transcription reporter RC120 strain was constructed by introducing the merodiploid $P_{hupA}$::*lacZ* construct into CMM2101 with wild-type *crp* and *fur* as described previously (21). Using the same methodology, the merodiploid $P_{hupA}$::*lacZ* construct was introduced into the *crp*-deleted RC100 strain and the *fur*-deleted CMM-2304 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.

**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 blotting for HupA was conducted as described previously (21). In brief, *V. vulnificus* strains were preconditioned and cultured as described above. Bacterial pellets containing approximately $1 \times 10^8$ cells were then boiled for 10 min to obtain cell lysates, which were electrophoresed for Western blot analysis.

## RESULTS

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

The effects of glucose on *hupA* transcription in the

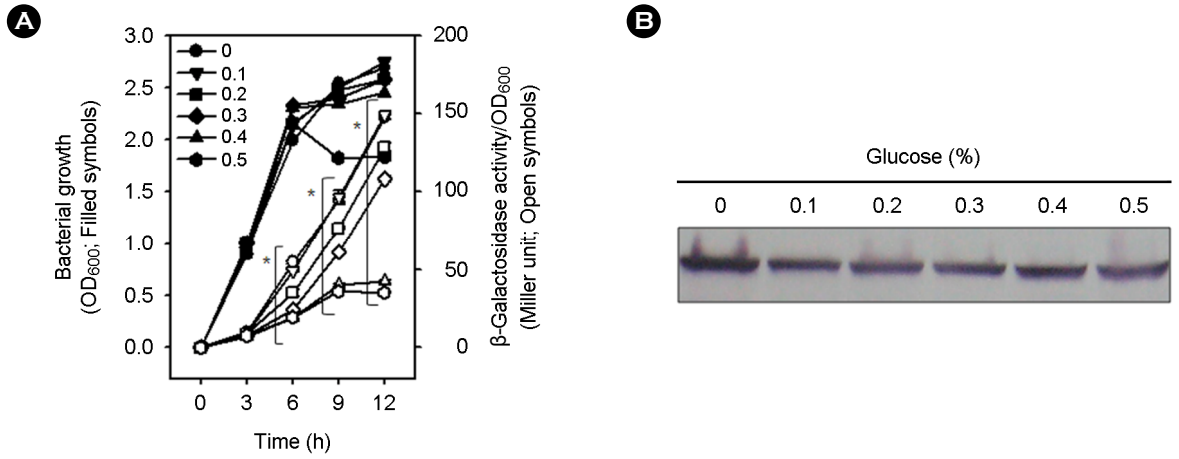


**Figure 1.** Effect of glucose on *Vibrio vulnificus* growth and *hupA* expression in a wild-type *fur* background. (A) RC120 containing wild-type *fur* and the $P_{hupA}$::*lacZ* construct was cultured in TES-DF-HI broths containing 5 μM $FeCl_3$ plus various concentrations (0~0.5%) of glucose. Bacterial growths were determined by measuring the 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 significant differences among glucose concentrations at the same time points ($p < 0.05$, One Way ANOVA). (B) *V. vulnificus* M06-24/O containing wild-type *fur* was cultured under the same conditions. Cell lysates were obtained by boiling bacterial pellets containing approximately $1 \times 10^8$ cells for 10 min, and were electrophoresed on 10% SDS-PAGE gels. Western blotting was conducted using rabbit polyclonal anti-HupA antibody as the primary antibody. A representative experiment of duplicate experiments is shown.

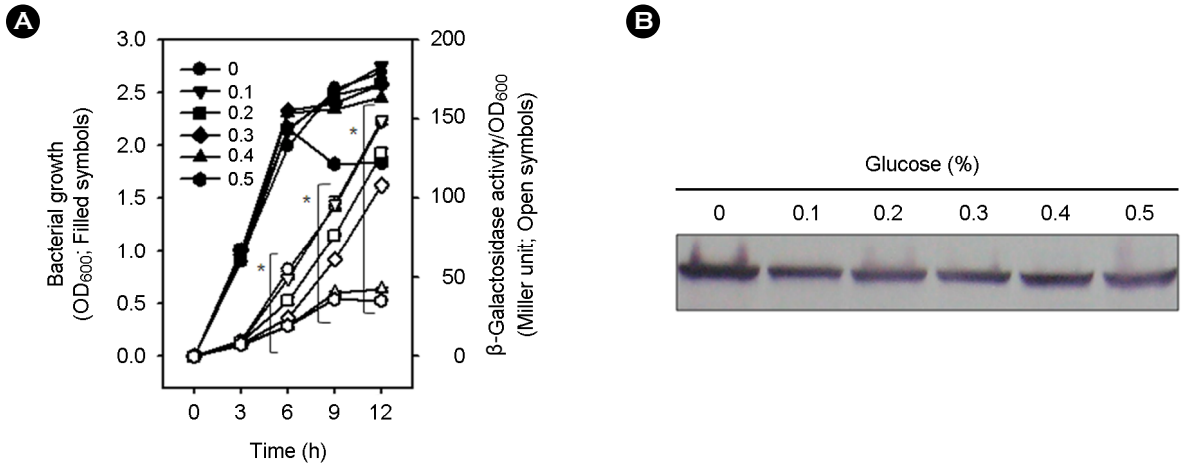


**Figure 2.** Effect of glucose on *Vibrio vulnificus* growth and *hupA* expression in a mutated *fur* background. (A) RC124 containing mutated *fur* and the $P_{hupA}$::*lacZ* construct was cultured in TES-DF-HI broths containing 5 μM $FeCl_3$ plus various concentrations (0~0.5%) of glucose. Growth and *hupA* transcription were determined as described in the legend of Fig. 1. The symbol (*) indicates significant differences among glucose concentrations at the same time points ($p < 0.05$, One Way ANOVA). (B) *V. vulnificus* RC110 containing mutated *fur* was cultured under the same conditions. Western blotting was conducted as described in the legend of Fig. 1. A representative experiment of duplicate experiments is shown.

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%). The growths of the two strains were slightly increased in the presence of glucose, but not in proportion to glucose concentrations (Figs. 1 and 2). However, in RC120, glucose dose-dependently repressed *hupA* transcription and completely repressed it at more than 0.4% ($p < 0.05$ by One Way ANOVA) (Fig. 1A). At 25 μM FC, *hupA* transcription in RC120 was completely repressed, and thus, the effects of glucose on *hupA* transcription could not be observed (data not shown). The effect of glucose on HupA production was also observed under the same conditions. At 5 μM FC (Fig. 1B), glucose dose-dependently repressed HupA production in M06-24/O and completely repressed it at 0.5%. At 25 μM FC, the effect of glucose on HupA production could not be observed in M06-24/O.

In RC124, *hupA* transcription appeared to be dose-dependently repressed by glucose ($p < 0.05$ by One Way ANOVA), but was maintained at considerable levels (approximately 40 Miller units) even in the presence of glucose at > 0.4% (Fig. 2A). Similar results were observed at 25 μM FC (data not shown). In contrast, HupA production in CMM2303 (Fig. 2B) was slightly reduced in the presence of glucose, but was maintained dose-independently at considerable levels in the presence of 0.5% glucose. Similar results were also observed at 25 μM FC (data not shown). A slight discrepancy was observed between *hupA* transcription and HupA protein levels. This discrepancy was probably due to the presence of TES as a pH-buffering agent and the likely low pH resulting from glucose catabolism, which could have synergistically inhibited the measurement of β-galactosidase activity. During a pilot experiment, we found that measured β-galactosidase activities were lower in media containing TES than in media not containing TES (data not shown).

### Effects of cAMP on HupA expression

To find the connection between the effects of glucose and Crp on *hupA* expression, the effects of cAMP on *V. vulnificus* growth and HupA expression were determined by culturing the three strains, M06-24/O containing wild-type *cyaA*, RC386 containing mutated *cyaA*, and RC390 containing *in trans* complemented *cya*A in deferrated HI broths containing 5 μM FC, and then comparing HupA production (Fig. 3A and 3C). The growth and HupA production of M06-24/O were inhibited by mutating *cyaA*,

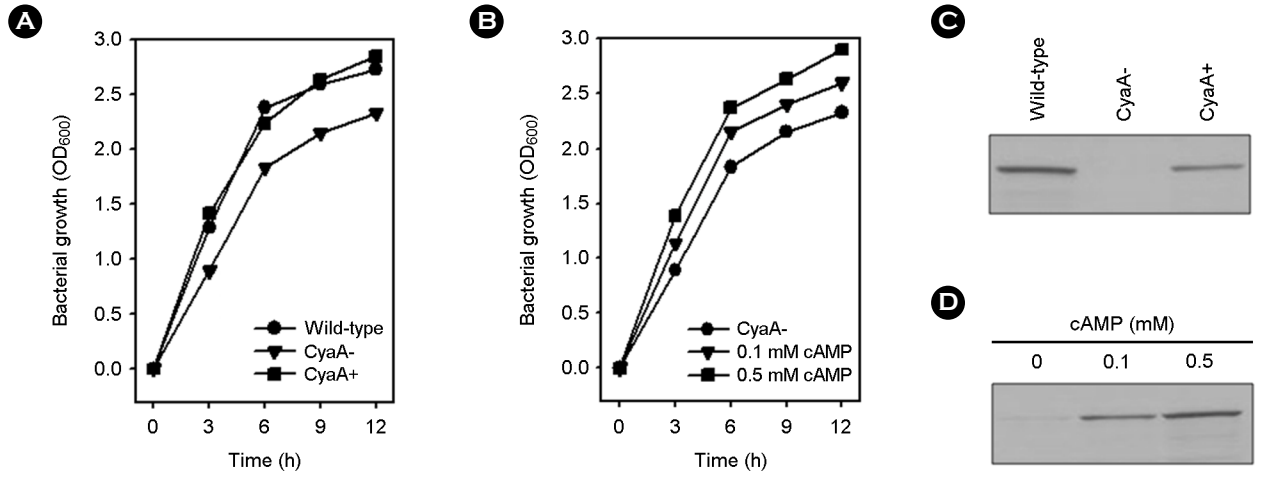


**Figure 3.** Effects of cAMP on *Vibrio vulnificus* growth and HupA production. (A and C) *V. vulnificus* M06-24/O containing wild-type *cyaA* (wild-type), RC386 containing mutated *cyaA* (CyaA-), and RC390 containing *in trans* complemented *cyaA* (CyaA+) were cultured in DF-HI broths containing 5 μM $FeCl_3$ for 12 h. (B and D) *V. vulnificus* RC386 containing mutated *cyaA* (CyaA-) was cultured in DF-HI broths containing 5 μM $FeCl_3$ plus 0, 0.1 or 0.5 mM cAMP for 12 h. Growth and *hupA* transcription were determined and Western blotting was conducted as described in the legend of Fig. 1. A representative experiment of duplicate experiments is shown.

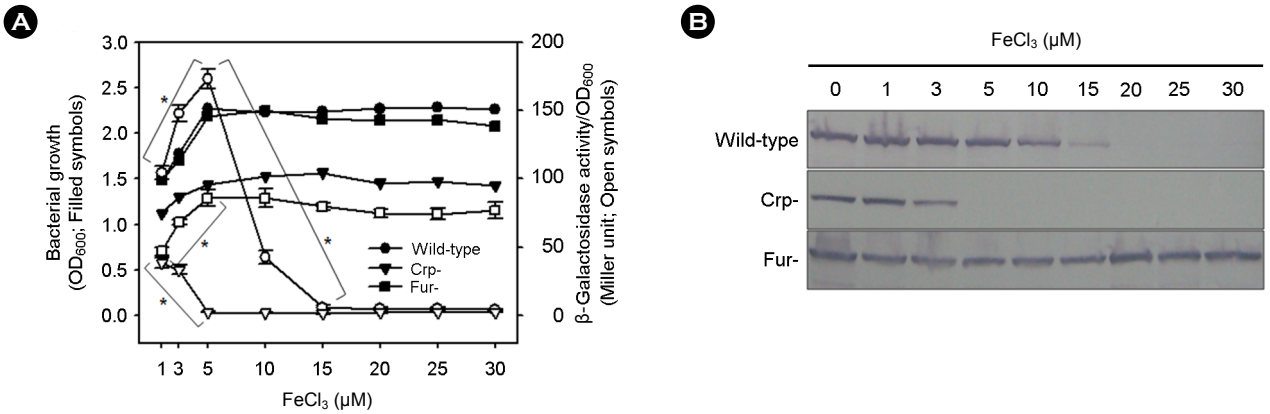


**Figure 4.** Effect of iron on *Vibrio vulnificus* growth and *hupA* expression in a *crp*- or *fur*-mutated background. (A) The three $P_{hupA}$::*lacZ* transcriptional reporter strains, RC20 containing wild-type *crp* and *fur* (wild-type), RC122 containing mutated *crp* (Crp-), and RC124 containing mutated *fur* (Fur-), were cultured in DF-HI broths containing various concentrations (0~30 μM) of $FeCl_3$ for 12 h. Growth and *hupA* transcription were determined as described in the legend of Fig. 1. The symbol (*) indicates significant differences among various iron concentrations ($p < 0.05$, One Way ANOVA). (B) *V. vulnificus* M06-24/O containing wild-type *crp* (wild-type), CMM710 containing mutated *crp* (Crp-) and CMM2303 containing mutated *fur* (Fur-) were cultured under the same conditions. Western blotting was conducted as described in the legend of Fig. 1. A representative experiment of duplicate experiments is shown.

and these effects were prevented by complementing wild-type *cyaA*. In addition, bacterial growth and HupA production in RC386 were dose-dependently increased when exogenous cAMP was added to deferrated HI broths containing 5 μM FC (Fig. 3B and 3D).

### Effects of iron on *hupA* expression in the presence or absence of Crp or 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, RC20 containing wild-type *crp* and *fur*, RC122 containing mutated

*crp*, and RC124 containing mutated *fur*, were cultured in deferrated HI broths containing various concentrations of FC (0~30 μM) for 12 h (Fig. 4A). FC dose-dependently stimulated the growths of the three strains under severely iron-deficient media (< 5 μM FC), but not under moderately iron-deficient media (5 to 15 μM FC) and iron-sufficient media (> 15 μM FC). In RC120, FC dose-dependently increased *hupA* transcription under severely iron-deficient media, but dose-dependently repressed it under moderately iron-deficient media ($p < 0.05$ by One Way ANOVA) and completely repressed it under iron-sufficient media. The level of *hupA* transcription reached a peak at around 5 μM FC. In RC122, FC dose-dependently repressed *hupA* transcription even under severely iron-deficient media ($p < 0.05$ by One Way ANOVA) and completely repressed *hupA* transcription under moderately iron-deficient media. In RC124, FC dose-dependently increased *hupA* transcription under severely iron-deficient media ($p < 0.05$ by One Way ANOVA), but did not repress it under moderately iron-deficient or iron-sufficient media ($p > 0.05$ by One Way ANOVA). Moreover, under severely iron-deficient media, *hupA* transcription levels in RC122 were lower than in RC120 and RC124, and those in RC124 were lower than in RC120 but higher than in RC122 ($p < 0.05$ by Student's *t*-test).

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. 4B). In M06-24/O, FC appeared to have no significant effect on HupA production under severely iron-deficient media, but dose-dependently repressed it under moderately iron-deficient conditions and almost completely repressed it under iron-sufficient media. However, a slight discrepancy was observed between *hupA* transcription and HupA protein levels under severely iron-deficient conditions, suggesting the involvement of post-transcriptional mechanisms. In CMM710, FC dose-dependently repressed HupA production even under severely iron-deficient conditions and completely repressed it under moderately iron-deficient conditions. In CMM2303, FC appeared to have no significant effect on HupA production at any concentration examined. Moreover, HupA production levels under iron-deficient media were lower in CMM710 than in M06-24/O, and higher in CMM2303 than in CMM710.

## DISCUSSION

Iron is essential for activating many catabolite enzymes, especially those involved in the electron transport system, and eventually, for efficient energy production and bacterial growth, although excessive iron is rather toxic to bacteria. This study shows that iron dose-dependently stimulates *V. vulnificus* growth under severely iron-deficient conditions, but not under moderately iron-deficient or iron-sufficient conditions. This iron-dependent biphasic growth suggests that iron availability acts as a double-edged sword in *V. vulnificus*.

Bacteria acquire energy for growth via catabolism, and Crp is a global regulator responsible for catabolite repression. Furthermore, glucose is the preferred energy source in most bacteria including *V. vulnificus* (13). The presence of glucose represses the expressions of a large number of genes associated with catabolism, and conversely, the absence of glucose stimulates the expressions of these genes via Crp. Accordingly, bacterial growth under glucose-deficient contions is certainly Crp-dependent. This study shows that *V. vulnificus* growth is severely inhibited in the absence of Crp regardless of iron concentration, whereas, in a previous study, we found that growth inhibition in the absence of Crp was recovered by glucose supplementation (19). These findings indicate that Crp is essential for energy production and *V. vulnificus* growth, especially under glucose-deficient conditions.

We propose a model for coordinated regulation of *hupA* expression by Crp and Fur, as in Fig. 5. The present study confirms that Crp positively affects *hupA* expression. A previous study demonstrated that Crp regulates *hupA* expression by directly binding to the -186 to -166 positions from the *hupA* transcription start site (10). In the present study, *hupA* expression was observed despite the absence of Crp under severely iron-deficient conditions, and *hupA*

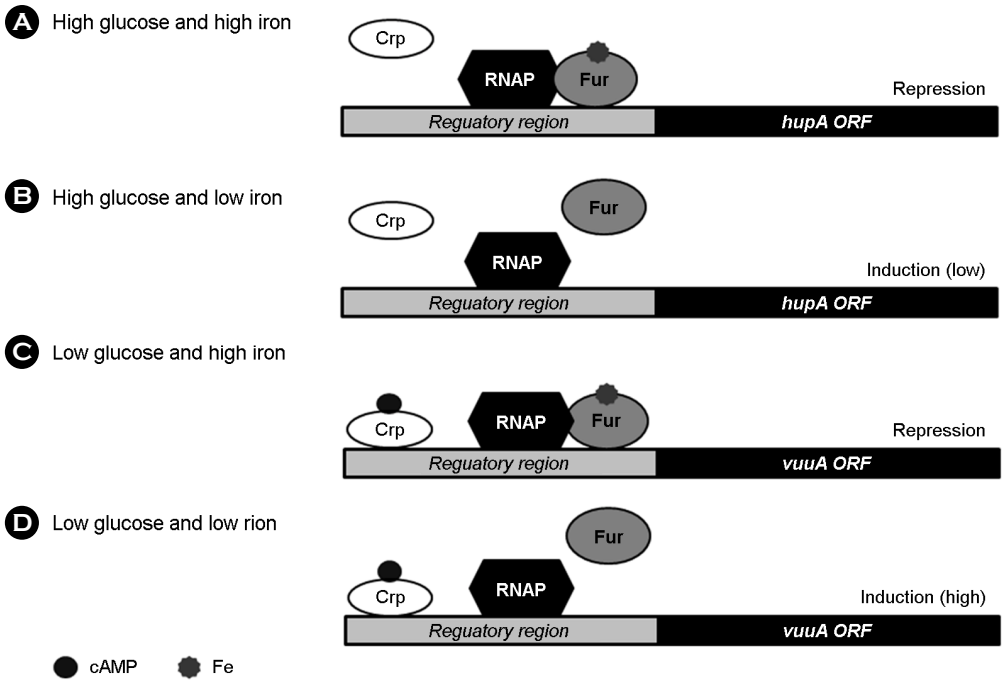


**Figure 5.** Proposed coordinate regulation of *hupA* expression by cyclic AMP-receptor protein (Crp) and ferric uptake regulator (Fur). RNAP (RNA polymerase) and ORF (open reading frame).

expression levels were higher in the presence of Crp than in the absence of Crp. In addition, under the same conditions, iron increased *hupA* expression in the presence of Crp, but repressed it in the absence of Crp. These findings indicate that iron limitation or the absence of Fur-mediated repression is an essential signal for constitutional *hupA* expression, whereas Crp is not essential for constitutional *hupA* expression but is required for optimal *hupA* expression, and that the effect of Crp as an activator is greater than the effect of Fur as a repressor in severely iron-deficient conditions. Furthermore, the present study shows that glucose represses *hupA* expression, and that a mutation in *cyaA* encoding adenylate cyclase required for cAMP synthesis represses HupA production, and this repression is recovered by adding exogenous cAMP. These findings indicate that the glucose- or Crp-mediated regulation of *hupA* expression is mediated by cAMP. Accordingly, it is likely that glucose deprivation increases cAMP by activating adenylate cyclase, cAMP binds to and activates Crp, and cAMP-Crp complex regulates *hupA* expression by directly binding the Crp-inding site, as described above.

Other iron-uptake systems in *V. vulnificu*s are also likely to be under the positive control of Crp. Our recent studies show that Crp positively regulates the expression of *vuuA* encoding vulnibactin receptor (16) and the expression of *iutA* encoding *E. coli* aerobactin receptor (27). Furthermore, it has been reported that Crp also positively regulates the expression of *vvhBA* encoding a cytolysin/hemolysin involved in the release of intracellular iron, including hemoglobin (28, 29). This Crp- or glucose-mediated regulation of iron-uptake systems implies that glucose deprivation is an important signal for the optimal induction of iron-uptake systems, and that Crp is involved in the coordination of iron and carbon metabolisms (14). Accordingly, it is likely that the acquisition of iron should be increased to stimulate catabolism and to produce energy efficiently, especially under glucose-deficient conditions, and that eventually, this metabolically coordinated acquisition and effective utilization of iron is crucial for successful establishment of infection.

In contrast, the absence of Fur can also affect bacterial growth by increasing iron uptake, especially under iron-deficient conditions, because iron is also essential for

efficient energy production. However, in this study, the absence of Fur appeared to have no significant effect on *V. vulnificus* growth. This may be because deferrated HI broths containing < 5 μM FC used in this study represents iron-deficient conditions (iron is deficient but its availability is not limited). Conversely, in our previous study, the absence of Fur could stimulate *V. vulnificu*s growth under iron-limited conditions (sufficient iron is present but its availability is severely limited), for example, in deferrated HI broths containing transferrin-bound iron as the sole iron source [17]. Accordingly, the effect of Fur on *V. vulnificus* growth is likely to be considerably condition-dependent.

It is known that *hupA* expression is negatively controlled by iron or Fur (11, 12). Similarly, in the present study, iron repressed *hupA* expression in the presence of Fur, but de-repressed it in the absence of Fur under moderately iron-deficient and iron-sufficient conditions. Moreover, glucose completely repressed *hupA* expression in the presence of Fur but not in the absence of Fur. These results indicate that Fur or iron is essential for the repression of *hupA* expression regardless of the presence or absence of Crp or glucose availability. However, direct Fur binding to *hupA* promoter has not been demonstrated, although, based on sequence analysis, Fur is likely to bind to the -37 to -19 positions from the *hupA* transcription start site to prevent the binding of RNA polymerase (12). This Fur-mediated complete repression of *hupA* expression implies that iron limitation is an essential signal for the induction of *hupA* expression and that Fur acts as a specific or local regulator that controls *hupA* expression in response to iron levels (14). Similarly, the expressions of other *V. vulnificus* iron-uptake systems are also known to be under the negative control of Fur or iron (30~33). Furthermore, in a previous study, we found that Fur negatively regulated the expression of *vvhBA* encoding a cytolysin/hemolysin, which destroys a variety of cells, including red blood cells, and causes the release of intracellular iron, including hemoglobin (20).

In the present study, *hupA* expression levels under severely iron-deficient conditions were higher in the wild-type background than in the *fur*-mutated background, which suggests that *fur* mutation negatively affects *hupA* expression. Probably, other iron-uptake systems including the VuuA-mediated iron-uptake system would be de-repressed, iron uptake would be increased, and intracellular iron would be increased in the *fur*-mutated strain, and these would partially repress *hupA* expression versus the wild-type strain.

In conclusion, this study shows that *hupA* expression is under the coordinate control of Crp, which responds to glucose availability, and of Fur, which responds to iron availability, and that Crp is not essential for constitutional *hupA* expression but is required for optimal *hupA* expression, whereas Fur is essential for the prevention of *hupA* over-expression.

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