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Cyclic AMP-Receptor Protein Activates Aerobactin Receptor IutA Expression in *Vibrio vulnificus*

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The ferrophilic bacterium *Vibrio vulnificus* can utilize the siderophore aerobactin of *Escherichia coli* for iron acquisition via its specific receptor IutA. This siderophore piracy by *V. vulnificus* may contribute to its survival and proliferation, especially in mixed bacterial environments. In this study, we examined the effects of glucose, cyclic AMP (cAMP), and cAMP-receptor protein (Crp) on *iutA* expression in *V. vulnificus*. Glucose dose-dependently repressed *iutA* expression. A mutation in *cya* encoding adenylate cyclase required for cAMP synthesis severely repressed *iut*A expression, and this change was recovered by *in trans* complementing *cya* or the addition of exogenous cAMP. Furthermore, a mutation in *crp* encoding Crp severely repressed *iutA* expression, and this change was recovered by complementing *crp*. Accordingly, glucose deprivation under iron-limited conditions is an environmental signal for *iutA* expression, and Crp functions as an activator that regulates *iutA* expression in response to glucose availability.

Keywords: *Vibrio vulnificus*, iron, aerobactin, glucose, cyclic AMP-receptor protein, siderophore piracy

Introduction

Vibrio vulnificus is a Gram-negative halophilic bacterium capable of causing fatal septicemia and necrotizing wound infections in susceptible individuals. Elevated serum or tissue iron levels are well-known predisposing factors, and the ability to acquire iron is an established virulence factor in the pathogenesis of *V. vulnificus* infections (Jones and Oliver, 2009).

Iron is essentially required for the survival and proliferation of most bacteria. A few bacteria, called ferrophilic or iron-sensitive bacteria, have little ability to acquire iron, and thus, cause diseases primarily in iron-overloaded hosts (Weinberg, 2000). *V. vulnificus* is a ferrophilic bacterium and requires higher levels of readily available iron for growth initiation than other pathogens (Kim *et al.*, 2007), and usually causes diseases in susceptible patients with elevated serum or tissue iron levels (Wright *et al.*, 1981). In addition, iron facilitates the production of some virulence factors in *V. vulnificus* (Kim *et al.*, 2006, 2009).

To establish infection successfully, bacteria must be versatile in terms of acquiring iron from their hosts, and as a result, many bacteria have evolved specific iron uptake systems (IUSs). In fact, *V. vulnificus* possesses multiple IUSs. For example, the vulnibactin receptor (VuuA)-mediated IUS plays a crucial role in the utilization of transferrin-bound iron (Litwin *et al.*, 1996; Webster and Litwin, 2000; Choi *et al.*, 2006; Kim *et al.*, 2006), whereas the heme-receptor (HupA)-mediated IUS plays a role in the direct utilization of heme iron from heme-containing proteins like hemoglobin and hemin (Litwin and Byrne, 1998; Litwin and Quackenbush, 2001; Oh *et al.*, 2009). In addition, *V. vulnificus* can acquire iron via the phenomenon called 'siderophore piracy' (Schubert *et al.*, 1999). More specifically, *V. vulnificus* can utilize heterologous siderophores, such as, *Streptomyces pilosus* deferoxamine, via its cognate receptor DesA (Aso *et al.*, 2002; Takata *et al.*, 2005; Kim *et al.*, 2007) and *Escherichia coli* aerobactin via its cognate receptor IutA (Tanabe *et al.*, 2005). This siderophore piracy may play an important role in the survival and proliferation of *V. vulnificus,* especially in mixed bacterial environments, such as, that of the human large intestine.

Fur (ferric uptake regulator), a transcriptional repressor that responds to iron availability (Hantke, 2001), has been demonstrated to repress the expressions of *vuuA* and *hupA* (Litwin and Calderwood, 1993; Litwin and Byrne, 1998; Webster and Litwin, 2000), and recently, Crp (cyclic AMP-receptor protein), a global regulator primarily responsible for catabolite repression (Deutscher, 2008), was shown to act as an activator of the expressions of *vuuA* and *hupA* (Choi *et al.*, 2006; Oh *et al.*, 2009). A previous study reported that *iutA* expression was negatively regulated by iron and synergistically co-activated by the presence of aerobactin under iron-limited conditions (Tanabe *et al.*, 2005). However, whether Crp acts as an essential activator of *iutA* expression remains to be determined. Therefore, in this study, we examined the effects of glucose, cAMP, and Crp on *iutA* expression.

Materials and Methods

Bacterial strains, plasmids, primers, media, and reagents

The bacterial strains, plasmids and primers used in this study

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

	Relative characteristics and sequences	Sources
Vibrio vulnificus		
M06-24/O	Wild type, highly virulent clinical isolate	Reddy *et al.* (1992)
CMM710	M06-24/O with *crp* deletion mutation	Choi *et al.* (2006)
CMM714	CMM710 with *in trans crp* complementation	Choi *et al.* (2006)
RC386	M06-24/O with *cya* deletion mutation	This study
RC390	RC386 with *in trans cya* complementation	This study
CMM2101	M06-24/O with *lacZ* deletion mutation	Kim *et al.* (2003)
RC100	CMM2101 with *crp* deletion mutation	This study
RC146	CMM2101 with merodiploid P_{iutA}::*lacZ* transcriptional fusion	Kim and Shin (2011)
RC148	RC100 with P_{iutA}::*lacZ* transcriptional fusion	This study
RC256	RC148 with *in trans crp* complementation	This study
Escherichia coli		
SY327 λ*pir*	Δ(*lac pro) argE*(Am) *rif nalA recA56* λ*pir* lysogen; Host for suicide vector	Miller and Mekalanos (1988)
SM10 λ*pir*	*thi thr leu tonA lacY supE recA*::RP4-2-Tc^r::Mu Km^r λ*pir* lysogen; Conjugation donor	Miller and Mekalanos (1988)
Plasmids		
pDM4	Suicide vector with R6K origin; Cm^R	McGee *et al.* (1996)
pRK2013	IncP, Km^R, Tra $Rk2^+$ *repRK2 repE1*	Ditta (1980)
pLAFR3II	pLAFR3 with *bla* inserted at the cos site; Ap^R and Tc^R	Kim *et al.* (2003)
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
pCMM712	pLAFR3II with a 6-kb *Hin*dIII fragment containing *crp*	Choi *et al.* (2006)
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

are listed in Table 1. Heart Infusion (HI; BD, USA) agar or broth containing additional 2.0% NaCl and Thiosulfate-Citrate-Bile Salt-Sucrose (TCBS, BD) agar were used to cultivate *V. vulnificus* strains, and LB medium was used to cultivate *E. 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 (USA).

Limitation and supplementation of iron and glucose

HI broth containing 100 μM α,α′-dipyridyl as an iron chelator was used only for preconditioning *V. vulnificus* strains. HI broth was deferrated using 8-hydroxyquinoline as previously described (Leong and Neilands, 1982). The residual iron concentration of deferrated (DF) HI broth was less than 1 μg/dl according to the method devised by Stookey (1970). To observe the effect of iron on growth and gene expression, various concentrations of ferric chloride (FC) were added to DF-HI broths as an iron source. In a pilot experiment, we found greatest growth and highest *iutA* transcription levels in DF-HI containing 5 μM FC and complete repression of *iutA* transcription in DF-HI containing 25 μM FC. Accordingly, DF-HI broth containing 5 μM FC was used as an iron-deficient medium and DF-HI broth containing 25 μM FC was used as an iron-sufficient medium in subsequent experiments. In addition, DF-HI was used as a glucose-poor medium because it contained 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).

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

V. vulnificus strains were preconditioned by culturing in HI broth containing 100 μM dipyridyl at 37°C overnight for adaptation to iron-limited conditions. Preconditioned strains were inoculated into test broths at a bacterial density of 5×10^6 cells/ml, and cultured with vigorous shaking at 37°C for 12 h. Culture aliquots were withdrawn at appropriate times to measure bacterial growth and gene transcription levels. Bacterial growth levels were determined by measuring the optical densities of culture aliquots at 600 nm (OD_{600}). Gene transcription levels were determined by measuring β-galactosidase activity on a per cell basis in culture aliquots, as previously described (Miller, 1992).

In frame deletion mutation and in trans complementation of genes

RC386 containing a deletion of *cyaA* was constructed as

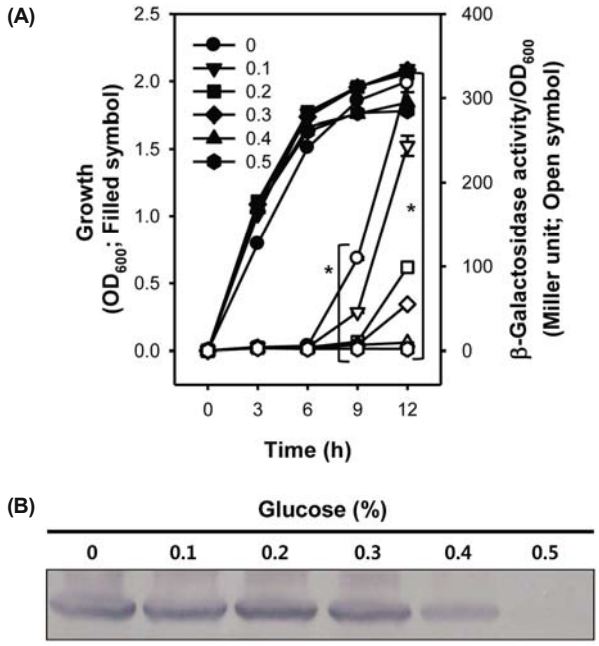

Fig. 1. Effects of glucose on *V. vulnificus* growth and *iutA* expression. (A) RC146 with the P_{iutA}::*lacZ* transcriptional fusion was cultured in TES-DF-HI broths containing 5 μM ferric chloride (FC) plus various concentrations (0–0.5%) of glucose. Bacterial growth was determined by measuring the optical densities of culture aliquots at 600 nm (OD_{600}), and *iutA* transcription was determined by measuring β-galactosidase activity on a per cell basis (Miller unit) in culture aliquots. β-Galactosidase activities are expressed as the means and standard deviations (error bars) of triplicate measurements. The symbol (*) indicates a significant difference at various iron concentrations ($p<0.05$, One Way ANOVA). (B) *V. vulnificus* M06-24/O was cultured under the same conditions. Western blotting was conducted using whole cell lysates containing approximately 1×10^8 cells obtained 12 h after culture initiation. One representative experiment of duplicate experiments is shown.

follows. 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 and 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 *cya*-complemented strain was named RC390.

The *crp*-deleted CMM710 and the *crp*-complemented CMM714 strains were constructed as described previously (Choi *et al.*, 2006).

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

The *lacZ*-fused *iutA* transcription reporter RC146 strain was constructed by introducing the merodiploid P_{iutA}::*lacZ* transcriptional fusion construct into CMM2101 with wild-type *crp* as described previously (Kim and Shin, 2011). Using the same methodology, the merodiploid P_{iutA}::*lacZ* transcription fusion construct was introduced into the *crp*-deleted RC100 strain. Finally, the *crp*-deleted P_{iutA}::*lacZ* transcription reporter strain was named RC148.

To restore wild-type *crp* in RC148, the plasmid pCMM712 containing wild-type *crp* was introduced into RC148 as previously described (Choi *et al.*, 2006), and the resulting *crp*-complemented strain was named RC256. The presence of wild-type *crp* in RC256 was confirmed by PCR using the primers CRP-1 and CRP-7 (data not shown).

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

Recombinant IutA protein and rabbit polyclonal anti-IutA antibody were prepared and Western blotting for IutA was conducted as described previously (Kim and Shin, 2011). Bacterial pellets containing approximately 1×10^8 cells were boiled for 10 min to obtain cell lysates, which were then used for Western blotting.

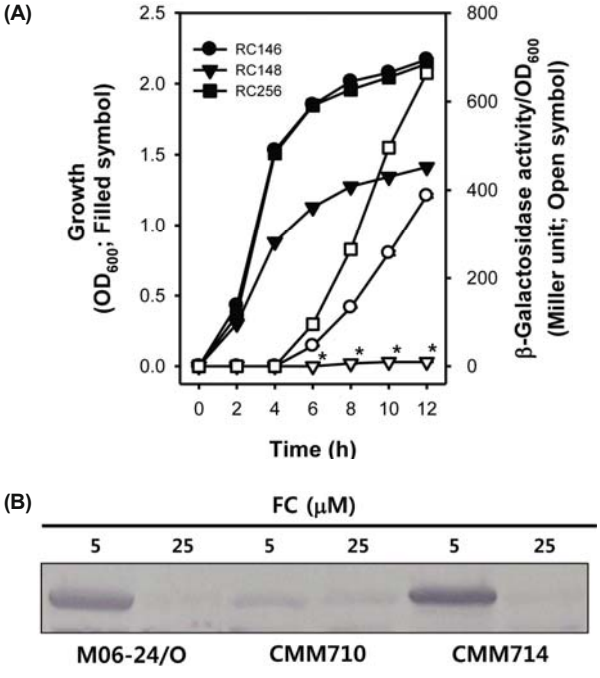

Fig. 2. Effects of Crp on *V. vulnificus* growth and *iutA* expression. (A) The P_{iutA}::*lacZ* transcription reporter strains, RC146 containing wild-type *crp*, RC148 containing mutated *crp*, and RC256 containing *in trans* complemented *crp*, were cultured in DF-HI broths containing 5 μM ferric chloride (FC). Bacterial growth and *iutA* transcription were determined as described in the legend of Fig. 1. The symbol (*) indicates a significant difference between the strains at the same time points ($p<0.05$, Student's *t*-test). (B) The three *V. vulnificus* strains, M06-24/O containing wild-type *crp*, CMM710 containing mutated *crp*, and CMM714 containing *in trans* complemented *crp*, were cultured in DF-HI containing 5 or 25 μM FC. Western blotting was performed as described in the legend of Fig. 1.

Results

Effect of glucose on *iutA* expression

The effect of glucose on *iutA* transcription was determined by culturing RC146 containing the P_{iutA}::*lacZ* transcriptional fusion construct in TES-DF-HI broths containing 5 μM FC and various concentrations (0 to 0.5%) of glucose (Fig. 1A). The growth of RC146 was slightly higher in the presence of glucose, but increases were not proportional to glucose concentration. In contrast, glucose dose-dependently repressed *iutA* transcription, and glucose at >0.4% almost completely repressed *iutA* transcription. The effect of glucose on IutA production was also determined by culturing M06-24/O under the same conditions (Fig. 1B). It was found that glucose dose-dependently repressed IutA production, and at 0.5%, glucose almost completely repressed IutA production.

Effect of *crp* mutation on *iutA* expression

The effect of Crp on *iutA* transcription was determined by culturing the three P_{iutA}::*lacZ* transcription reporters, RC146 containing wild-type *crp*, RC148 containing mutated *crp*, and RC256 containing *in trans* complemented *crp,* in DF-HI broths containing 5 or 25 μM FC (Fig. 3A). The growth of RC146 was severely inhibited in RC148, and this inhibition was completely recovered in RC256. At 5 μM FC, *iutA* transcription was highly induced in RC146 but severely repressed in RC148, and this repression was recovered in RC256. The higher *iutA* transcription observed in RC256 than in RC146 was thought to be due to the presence of multiple copies of *crp*. At 25 μM FC, *iutA* transcription remained completely repressed in all three strains (data not shown). The effect of Crp on IutA production was also determined by culturing the three *V. vulnificus* strains, M06-24/O containing wild-type *crp*, CMM710 containing mutated *crp*, and CMM714 containing *in trans* complemented *crp*, under the same conditions (Fig. 3B). At 5 μM FC, IutA production was highly induced in M06-24/O and severely repressed in CMM710, and this repression was restored in CMM714. At 25 μM FC, IutA production was severely repressed in all three strains.

Effect of *cya* mutation on *hupA* expression

The effect of cAMP on IutA production was determined by culturing the three *V. vulnificus* strains, M06-24/O containing wild-type *cyaA*, RC386 containing mutated *cyaA*, and RC390 containing *in trans* complemented *cyaA*, in DF-HI broths containing 5 μM FC. The growth of M06-24/O was inhibited in RC386, and this inhibition was completely recovered in RC390 (data not shown). At 5 μM FC (Fig. 3), IutA production was highly induced in M06-24/O and severely repressed in RC386, and this repression was prevented in RC390. Furthermore, the exogenous addition of 0.1 and 0.5 mM cAMP dose-dependently increased IutA production in RC386.

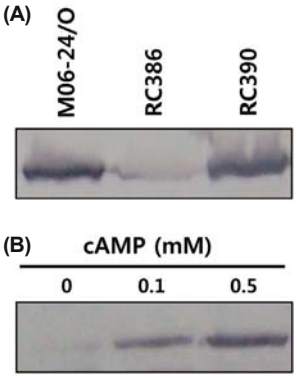

Fig. 3. Effects of cAMP on IutA production. (A) The three *V. vulnificus* strains, M06-24/O containing wild-type *cyaA*, RC386 containing mutated *cyaA*, and RC390 containing *in trans* complemented *cyaA,* were cultured in DF-HI containing 5 μM FC. (B) RC386 containing mutated *cyaA* was cultured in DF-HI containing 5 μM FC plus 0, 0.1 or 0.5 mM cAMP. Western blotting was performed as described in the legend of Fig. 1.

Discussion

Iron plays a crucial role as a cofactor for many proteins and enzymes involved in energy production and bacterial growth. However, the availability of this essential element is severely limited, especially *in vivo*. Most bacteria have adapted to this situation by producing their own siderophores (Andrews *et al*., 2003). On the other hand, some bacteria can acquire iron by utilizing heterologous siderophores produced by other unrelated bacterial or fungal species (Sebulsky *et al*., 2000; Lesic *et al*., 2002). Siderophore piracy is probably highly advantageous to bacteria because it allows them to escape bacteriostatic effects caused by heterologous siderophores without synthesizing principal siderophores, especially in environments with mixed bacterial populations. *V. vulnificus* possesses two IUSs associated with siderophore piracy, the DesA-mediated IUS, which uses deferoxamine, and the IutA-mediated IUS, which uses *E. coli* aerobactin. The presence of these two systems provides *V. vulnificus* with a versatility that is likely to be highly advantageous for its survival and proliferation, especially in environments with mixed bacterial populations, because the organism is ferrophilic and requires more readily available iron than other pathogens (Weinberg, 2000; Kim *et al*., 2007). However, the relationship between the IutA- or DesA-mediated IUS and *V. vulnificus* virulence has yet to be determined.

V. vulnificus can utilize aerobactin via IutA, and the presence of aerobactin under iron-limited conditions can synergistically co-activate *iutA* expression via GntR-like repressor IutR (Tanabe *et al*., 2005). In this study, *iutA* expression was induced at higher levels in the presence of aerobactin than in its absence, and this aerobactin response was abolished in a background of *iutA* mutation (data not shown). Accordingly, it appears that an inducer (probably ferric aerobactin) interacts with IutR to prevent IutR from binding to the *iutA* regulatory region, and thereby, to de-repress *iutA* expression in the presence of aerobactin. These findings also indicate that the presence of aerobactin is not essential for *iutA* expression.

A previous study reported based on Fur titration assay results that iron negatively regulates *iutA* expression (Tanabe *et al*., 2005). In this study, we also found that a *fur* mutation totally abolished the iron-mediated regulation of *iutA* expression (data not shown). As in most bacteria (Hantke, 2001), *V. vulnificus* IUSs are also likely to be under the negative control of Fur, which functions as an iron-dependent

transcriptional repressor (Litwin and Calderwood, 1993). This iron (or Fur)-mediated repression of *iutA* expression implies that iron limitation is an essential signal for *iutA* expression.

The present study shows for the first time that glucose deprivation or cAMP elevation is a signal for *iutA* expression under iron-limited conditions and that Crp functions as a transcription activator of *iutA* expression. Glucose dose-dependently repressed *iutA* expression, a *cya* mutation repressed *iutA* expression, and this change was recovered by the exogenous addition of cAMP, and a *crp* mutation severely repressed *iutA* expression. These findings indicate that *iutA* expression is under the control of Crp, a global regulator, in response to glucose availability. Whether Crp regulates *iutA* expression by direct binding to the *iutA* regulatory region needs to be determined. Our previous study showed that the expression of *vuuA* encoding vulnibactin receptor is under the positive control of Crp (Choi *et al.*, 2006). In addition, a recent study demonstrated that Crp positively regulates the expression of *hupA* encoding heme receptor by directly binding to the regulatory region of *hupA* (Oh *et al.*, 2009). The fact that the expression of *V. vulnificus* IUSs is under the positive control of Crp implies that the acquisition of iron must be increased to stimulate catabolism and to produce energy efficiently under glucose-starved conditions.

Crp is a global regulator that has well established roles in catabolite repression (Deutscher, 2008). Glucose is the preferred energy source for most bacteria. Moreover, the presence of glucose represses the expressions of several genes and catabolite operons; conversely, the absence of glucose stimulates the expressions of several genes and catabolite operons. Iron is essential for activating many catabolite enzymes, especially those involved in the electron transport system, and is also essential for efficient energy production, particularly under glucose-starved conditions. The metabolically coordinated acquisition and effective utilization of iron are likely to be crucial for the successful establishment of infections.

In conclusion, our findings suggest that glucose deprivation under iron-limited conditions is a synergistic signal for *iutA* expression and that Crp functions as an activator or mediator that regulates *iutA* expression in response to glucose availability.

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