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Regulatory Mechanism of the *iutA* Gene Encoding Aerobactin Receptor Protein in *Vibrio vulnificus*

조선대학교대학원

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암호화하는 *iutA* 유전자의 발현 조절

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지도교수 김 성 중

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의 학 과

김 우 형

김우형의 박사학위 논문을 인준함

위원장: 조선대학교 교수 조 남 수 (인)

위 원: 전남대학교 교수 민 용 일 (인)

위 원: 조선대학교 교수 조 수 형 (인)

위 원: 조선대학교 교수 신 성 희 (인)

위 원: 조선대학교 교수 김 성 중 (인)

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조선대학교 대학원

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김 우 형

지도교수: 김 성 중, 의학박사

조선대학교 대학원 의학과

호철성 세균(ferrophilic bacterium)인 패혈증 비브리오균은 철 획득을 위해 대장균이 생산한 시데로포아(siderophore)인 aerobactin을 특이 수용체인 IutA를 통해 이용할 수 있다. 이러한 시데로포아 해적행위는 패혈증 비브리오균이 여러 가지 세균들이 혼재되어 있는 환경에서 생존하고 증식하는데 중요한 역할을 할 것으로 생각된다. 본 연구에서는 유전자 *iutA* 발현을 조절하는데 있어 조절신호 또는 조절인자로서 포도당/Crp (cAMP-receptor protein), aerobactin/IutR (Gnt-like repressor), 철/Fur (ferric uptake regulator)의 역할과 이들의 상호작용을 규명하고자 하였다. 염기서열을 결손시키는 방법으로 *crp* 유전자에 돌연변이를 유발하였을 때 철이 결핍된 배지에서 *iutA*의 발현은 현저히 억제되었고 이러한 변화는 야생형(wild-type) *crp* 유전자를 보충하여 주었을 때 다시 회복되었다. 철을 더욱 결핍시켰을 때 Crp가 없는 상태에서도 *iutA*의 발현을 관찰할 수 있었으며, Crp가 없는 상태에서 *iutA* 발현은 철 농도에 비례하여 억제되었지만 Crp가 있는 상태에서는 철 농도에 비례하여 증가하였다. 유전자 *fur*에 돌연변이를 유발하였을 때 철이 풍부한 환경에서 철에

의해 억제되었던 *iutA* 유전자 발현이 탈억제(derepression)되었고 이러한 변화는 야생형 *fur* 유전자를 보충하였을 때 회복되었다. 철은 Fur가 존재하는 상황에서만 *iutA* 유전자 발현을 완전히 억제할 수 있었다. 포도당도 Fur 존재 하에서만 *iutA* 유전자의 발현을 완전히 억제할 수 있었다. Aerobactin의 존재는 철 농도와 상관없이, 그리고 Crp 또는 Fur의 존재와 무관하게 *iutA* 발현을 유도하였다. 이러한 결과를 종합하여 보면, 첫째로, Crp는 *iutA* 유전자 발현에 필수적이지는 않지만 이 유전자가 최적으로 발현되는데 필요하며, 철 농도와 aerobactin 유무에 영향을 받지 않으며 오직 포도당 농도에 의해 영향을 받는다. 둘째로, IutR 또한 *iutA* 유전자 발현에 필수적이지 않으며 철 농도나 포도당 농도에 상관없이 aerobactin 유무에 영향을 받는다. 셋째로, Fur는 *iutA* 유전자 발현을 억제하는데 필수적이며 포도당 농도나 aerobactin 유무에 영향을 받지 않고 철 농도에만 반응한다. 따라서 *iutA* 유전자 발현은 Crp, IutR 그리고 Fur에 의해 독립적이지만 상호협조적으로 조절되고 있다.

중심어

패혈증 비브리오균, 철, Aerobactin, 포도당, Ferric uptake regulator, Cyclic AMP-receptor protein, 시데로포아 해적행위

1. Introduction

1.1. Background

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 host factors, and the ability of *V. vulnificus* to acquire iron is an established virulence factor in the pathogenesis of *V. vulnificus* infections [1].

Iron is essentially required for the survival and proliferation of most bacteria. Particularly, there are a few bacteria, called ferrophilic or iron-sensitive bacteria, with little ability to acquire iron that cause diseases primarily in iron-overloaded hosts [2]. *V. vulnificus* is a ferrophilic bacterium and requires higher levels of readily-available iron for growth initiation than other pathogens [3], and usually causes diseases in susceptible patients with elevated serum or tissue iron levels [4]. In addition, iron facilitates the production of some virulence factors in *V. vulnificus* [5,6].

To establish infection successfully, bacteria must possess a versatile ability to acquire iron from their hosts. As a result, many bacteria have evolved specific iron uptake systems (IUSs). In fact, *V. vulnificus* also possesses multiple IUSs (Fig. 1). For example, the vulnibactin receptor (VuuA)-mediated IUS plays a crucial important role in the utilization of transferrin-bound iron [7-9], whereas the heme-receptor (HupA)-mediated IUS plays a

role in the direct utilization of heme irons from heme-containing proteins like hemoglobin and hemin [10-12]. In addition, *V. vulnificus* can acquire iron via the phenomenon called 'siderophore piracy' [13]. *V. vulnificus* can utilize heterologous siderophores, such as, *Streptomyces pilosus* deferoxamine via the cognate receptor DesA [14-16] and *Escherichia coli* aerobactin via the cognate receptor IutA [17]. 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 human large intestine.

Fur (ferric uptake regulator), a transcriptional repressor that responds to iron availability [18], has been demonstrated to repress the expressions of *vuuA* and *hupA* [8,10,19]. Recently, Crp (cyclic AMP-receptor protein), a global regulator primarily responsible for catabolite repression [20], was shown to act as an essential activator of the expressions of *vuuA* and *hupA* [9,12]. In addition, the presence of heme (a co-inducer) under iron-limited conditions synergistically co-activates *hupA* expression, and this response is mediated by the LysR-like activator HupR [10,11]. The presence of deferoxamine under iron-limited conditions is an only signal for the induction of *desA* expression under the mediation of the AraC-like activator DesR [16]. Furthermore, the presence of *E. coli* aerobactin under iron-limited conditions synergistically co-activates *iutA* expression, and this response is mediated by the GntR-like repressor IutR [17]. However, the Fur-mediated negative regulation of iron-repressible *iutA* or *desA* expression,

suggested by the Fur titration assay, remains to be proved by direct mutational studies. Moreover, it also remains to be determined whether Crp acts as an essential activator of *iutA* or *desA* expression.

1.2. The aim of this study

In the present study, the author first attempted to confirm the involvement of IutA in the utilization of *E. coli* aerobactin for *V. vulnificus* iron uptake. Secondly, the author also attempted to experimentally determine the negative effect of Fur on *iutA* expression at the transcription and protein levels. Thirdly, the author newly attempted to demonstrate the involvement of cAMP or Crp in the positive regulation of *iutA* expression at the transcription and protein levels. Finally, the author attempted to detail the coordinate roles of IutR, Fur and Crp in the transcriptional regulation of *iutA* expression at various aerobactin, iron and glucose concentrations. For these, an *iutA*-inactivated mutant strain, a *fur*-inactivated mutant strain, a *crp*-inactivated mutant strain, and a *cya*A-inactivated mutant strain, were constructed. In addition, *lacZ*-fused *iutA* transcriptional reporter strains were constructed in the wild-type *fur* and *crp*, *fur*-inactivated, and *crp*-inactivated backgrounds. Finally, polyclonal anti-IutA antibody was prepared and Western blot was conducted to measure *iutA* expression at the protein level.

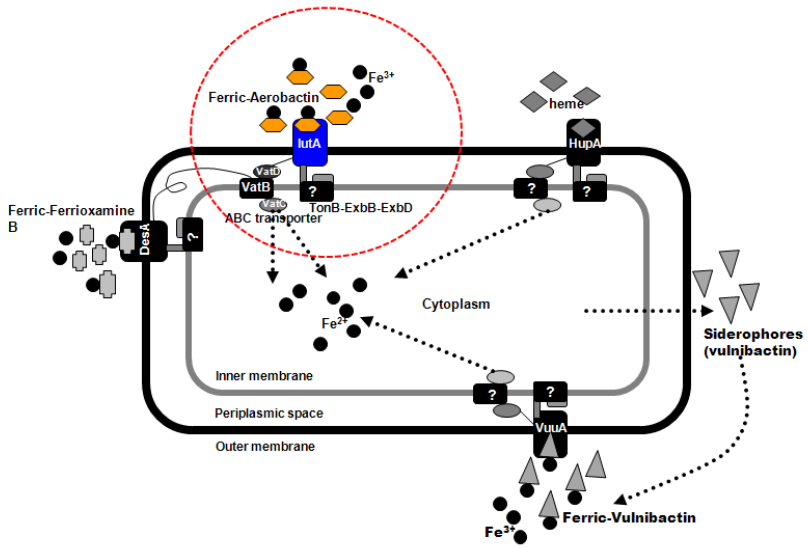

Figure 1. The four iron-uptake systems of *Vibrio vulnificus*. Four iron-repressible outer membrane receptors of *V. vulnificus* have been identified. IutA is a receptor that can bind *Escherichia coli* aerobactin. DesA is a receptor that can bind desferrioxamine which is produced by *Streptomyces*. HupA is a receptor that can directly bind heme proteins. VuuA is a receptor that can bind vulnibactin produced by *V. vulnificus*. The question marks indicate yet-unidentified mediators. ABC: ATP-binding cassette.

2. Materials and Methods

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

The bacterial strains, plasmids and primers used in this study are listed in Tables 1. Heart Infusion (HI; BD, Franklin Lakes, NJ, 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. 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. *E. coli* aerobactin was purchased from Genaxxon Bioscience GmbH (Ulm, Germany). Unless otherwise stated, all other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. DNA manipulations

Restriction and DNA-modifying enzymes were used according to the instruction of 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.

2.3. Limitation and supplementation of iron and glucose

Two types of iron-limited media were used in this study. First, HI broth containing 100 μM α,α'-dipyridyl as an iron chelator was used only for preconditioning *V. vulnificus* strains. Second, HI broth was deferrated using 8-hydroxyquinoline, as previously described [29]. The residual iron concentration of deferrated (DF) HI broth was less than 1 μg/dL according to the method devised by Stookey [30]. 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 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 [22].

Table 1. Bacterial strains used in this study

Strains	Relative characteristics[a]	Sources
Vibrio vulnificus		
M06-24/O	Wild type, highly virulent clinical isolate	[21]
RC154	M06-24/O with *iutA* deletion mutation	This study
RC158	RC154 with *in trans iutA* complementation	This study
CMM710	M06-24/O with *crp* deletion mutation	[9]
CMM714	CMM710 with *in trans crp* complementation	[9]
CMM2303	M06-24/O with *fur* deletion mutation	[6]
RC144	CMM2303 with *in trans fur* complementation	This study
RC386	M06-24/O with *cyaA* mutation	This study
RC390	RC386 with *in trans cyaA* complementation	This study
CMM2101	M06-24/O with *lacZ* deletion mutation	[22]
CMM2304	CMM2101 with *fur* deletion mutation	[5]
RC100	CMM2101 with *crp* deletion mutation	This study
RC146	CMM2101 with merodiploid P_{iutA}*::lacZ* transcriptional fusion	[23]
RC148	RC100 with P_{iutA}*::lacZ* transcriptional fusion	This study
RC256	RC148 with *in trans crp* complementation	This study
RC150	CMM2304 with P_{iutA}*::lacZ* transcriptional fusion	This study
RC310	RC150 with *in trans fur* complementation	This study
Escherichia coli		
SY327 λ*pir*	Δ(*lac pro) argE*(Am) *rif nalA recA56* λ*pir* lysogen; Host for suicide vector	[24]
SM10 λ*pir*	*thi thr leu tonA lacY supE recA*::RP4-2-Tc^r::Mu Km^r λ*pir* lysogen; Conjugation donor	[24]
DH5a	F- *recA1*; restriction negative	Laboratory stock
BL21	F-, *ompT*, *hsdS* (r_B-, m_B-), *gal*	Laboratory stock

[a]: P, promoter; Tc^r, tetracycline-resistant; Km^r, kanamycin-resistant.

Table 2. Plasmids used in this study

Plasmids	Characteristics[a]	Sources
pDM4	Suicide vector with R6K origin; Cm^R	[25]
pQF52	IncP lacZ transcriptional fusion vector; Amp^R	[26]
pRK2013	IncP, Km^R, Tra $Rk2^+$ *repRK2 repE1*	[27]
pLAFR3	IncP cosmid vector; Tc^R	[28]
pLAFR3II	pLAFR3 with *bla* inserted at the cos site; Amp^R and Tc^R	[22]
pRC146	pDM4 with a 1.59-kb *Bgl*II-*Spe*I fragment containing the in-frame deleted *iutA*	This study
pRC150	pLAFR3II with a 3.72-kb *Bam*HI-*Eco*RI fragment containing wild-type *iutA*	This study
pRC140	pQF52 with a 800-bp *Bgl*II-*Hin*dIII fragment containing the *iutA* promoter region	This study
pRC142	pDM4 with a *Bgl*II-*Sca*I fragment containing P_{iutA}::*lacZ* fragment from pRC140	This study
pRC132	pLAFR3II with a 861-bp *Bam*HI-*Hin*dIII fragment containing wild-type *fur*	This study
pCMM712	pLAFR3II with a 6-kb *Hin*dIII fragment containing *crp*	[9]
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
pRC188	pET-30a(+) with 2,034-bp *Bam*HI-*Eco*RI fragment containing the *iutA* gene	This study
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

[a]: Cm^R, chloramphenicol-resistant; Amp^R, ampicillin-resistant; Tc^R, teteracycline-resistant; Km^R, kanamycin-resistant.

Table 3. Primers used in this study

Primers	Sequences[a]	
iutA-up-1	5′-ga**agatct**tcaatgcattagagcagtgtcg-3′	This study
iutA-up-3	5′-gagcgtgtatgttgctgtcattattttctctatg-3′	This study
iutA-down-3	5′-ataatgacagcaacatacacgctcaattaccaagtg-3′	This study
iutA-down-4	5′-g**actagt**ggttcacaccatgcgtttgacc-3′	This study
iutR-comp-1	5′-cg**ggatcc**ggtctcttggcctctttgattgg-3′	This study
iutA-comp-2	5′-g**gaattc**gagagcggttccatctttaaacc-3′	This study
CRP1	5′-tacctactggcgatgatcgatg-3′	[9]
CRP7	5′-cggaatctgagagggtttagt-3′	[9]
fur-comp-F	5′-cg**ggatc**cccgttaaagagaaaatactgcc-3′	This study
fur-comp-R	5′-ccc**aagctt**tatcgagcgtcgatattagttc-3′	This study
cyaA-up-1	5′-gc**tctaga**agccagcggccgcgagaatgatc-3′	This study
cyaA-up-2	5′-cgctttggacatcttctgactttgcaaatccataagcgccag-3′	This study
cyaA-down-1	5′-gatttgcaaagtcagaagatgtccaaagcggtcaacgtatag-3′	This study
cyaA-down-2	5′-tcc**cccggg**tgcctactgtgattgctcagattgttg-3′	This study
cyaA-comp-1	5′-cg**ggatcc**tgcacgcccttccagcattgc-3′	This study
cyaA-comp-2	5′-g**gaattc**gcgtagctatcgttaagccattaag-3′	This study
His-IutA-F	5′-cg**ggatcc**agccgcactccaaaagcgatcag-3′	This study
His-IutA-R	5′-**ggaatt**cttagaacttcacttggtaattgagcg-3′	This study

[a]: Bold letters indicate the restriction enzyme-recognition sequences: agatct (*Bgl*II), actagt (*Spe*I), ggatcc (*Bam*HI), gaattc (*Eco*RI), and aagctt (*Hin*dIII).

2.4. 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 x 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 [31].

2.5. In frame deletion mutation and in trans complementation of genes

The *iutA*-deleted RC154 strain and the *iutA*-complemented RC158 strain were constructed as follows (Figs. 2 and 3). Two pairs of polymerase chain reaction (PCR) primers iutA-up-1/iutA-up-3 and iutA-down-3/iutA-down-4 were used to amplify the 5' and 3' fragments of *iutA*, respectively. The resulting two PCR products were used as templates for the second PCR amplification using the PCR primers iutA-up-1 with a *Bgl*II overhang and iutA-down-4 with a *Spe*I overhang. The *Bgl*II-*Spe*I fragment with deleted *iutA* was then cloned into pDM4 [25]. The resulting pRC146 was transformed into *E. coli*

SY327 λ*pir* and SM10 λ*pir* [24], and subsequently transferred to *V. vulnificus* M06-24/O by conjugation. A stable transconjugant (RC154) was first selected on TCBS agar containing chloramphenicol, and then on HI agar containing 10% sucrose. The *iutA* deletion in RC154 was confirmed by PCR using the primers iutA-up-1 and iutA-down-4 (data not shown).

To restore wild-type *iutA* in the *iutA*-deleted RC154 strain, a 3.72-kb *Bam*HI-*Eco*RI fragment containing intact wild-type *iutA* was amplified using the PCR primers iutR-comp-1 with a *Bam*HI overhang and iutA-comp-2 with an *Eco*RI overhang. The resulting PCR product was subsequently subcloned into pLAFR3II [22]. The resulting pRC150 was transferred into RC154 by triparental mating using pRK2013 [27]. A stable transconjugant (RC158) was selected on TCBS agar containing ampicillin and tetracycline, and the presence of wild-type *iutA* in RC158 was confirmed by PCR using the primers iutA-up-1 and His-iutA-R (data not shown).

The *crp*-deleted CMM710 strain, the *crp*-complemented CMM714 strain and the *fur*-deleted CMM2303 strain were constructed as described previously was constructed as described previously [6,9]. The *fur*-complemented RC144 strain was constructed as follows. To restore wild-type *fur* in the *fur*-deleted CMM2303 strain, a 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 follows. 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 [25]. The resulting plasmid (pRC316) was transformed into *E. coli* SY327 λ*pir* and SM10 λ*pir* [24], 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 [22], and the resulting plasmid pRC318 was transferred into RC386 by triparental mating using pRK2013 [27]. The resulting *cyaA*-complemented strain was named RC390.

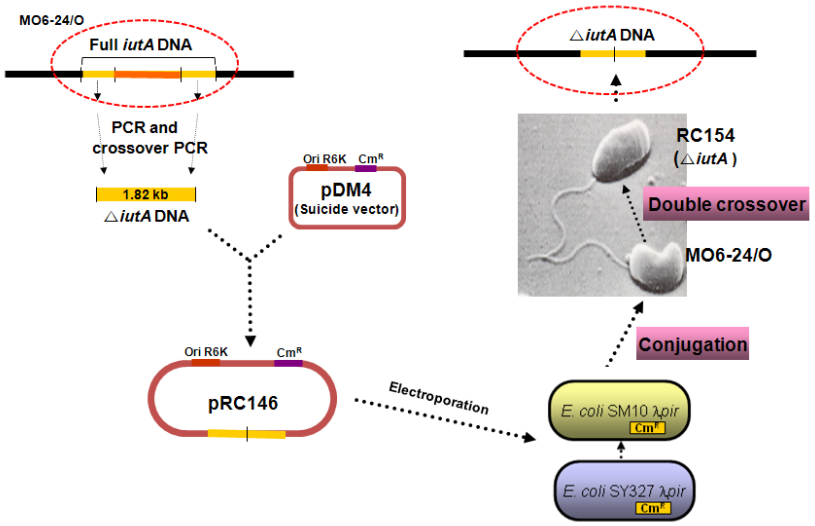

Figure 2. The construction of the *Vibrio vulnificus* RC154 strain containing an *in frame* deletion of the *iutA* gene. Details are described in the text.PCR (polymerase chain reaction), Cm^R: chloramphenicol-resistant cassette, Δ: deletion.

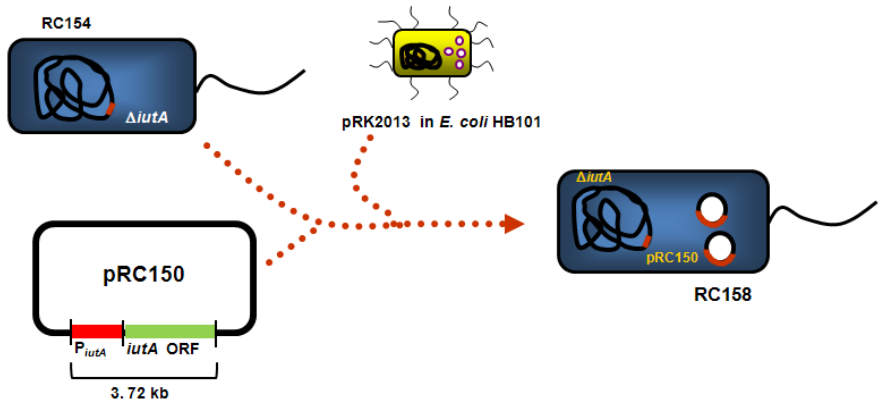

Figure 3. The construction of the *Vibrio vulnificus* RC158 strain containing an *in trans*-complementation of the *iutA* gene. Details are described in the text. P_{iutA}: the promoter region of *iutA*, ORF: open reading frame, Δ: deletion.

2.6. 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* and *fur,* as shown in Fig. 4. In brief, an 800-bp fragment from -744 to +39 bp from the *iutA* start codon was amplified using PCR primers, iutA-up-1 with *Bgl*II overhang and iutA-rep-R with *Hin*dIII overhang. The amplified fragment was subcloned into pQF52. From the resulting plasmid pRC140, a *Bgl*II-*Sca*I fragment containing a P_{iutA}::*lacZ* fusion was subsequently subcloned into pDM4 yielding pRC142. The plasmid pRC142 containing a P_{iutA}::*lacZ* transcriptional fusion was introduced into CMM2101, yielding RC146.Using the same methodology, the merodiploid P_{iutA}::*lacZ* transcription fusion construct was introduced into the *crp*-deleted RC100 strain and the *fur*-deleted CMM2304 strain. Finally, the *crp*-deleted P_{iutA}::*lacZ* transcription reporter strain and the *fur*-deleted P_{iutA}::*lacZ* transcription reporter strain were named RC148 and RC150, respectively.

To restore wild-type *crp* in RC148, the plasmid pCMM712 containing wild-type *crp* was introduced into RC148 as previously described [9], 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). Using the same methodology, wild-type *fur* on pRC132 plasmid was introduced into RC150, and the

resulting *fur*-complemented strain was named RC310. The presence of wild-type *fur* in RC310 was confirmed by PCR using the fur-comp-F and fur-comp-R primers (data not shown).

2.7. 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 blot for IutA was conducted as shown in Fig. 5. In brief, the coding region of *iutA* was amplified using the PCR primers His-IutA-F with *Bam*HI overhang and His-IutA-R with *Eco*RI overhang. The resulting 2,034-bp *Bam*HI-*Eco*RI fragment was cloned into the pET-30a(+) expression vector (Novagen), yielding pRC188. The resulting plasmid pRC188 was transformed into *E. coli* DH5α and BL21 (DE3). His-tagged IutA 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 IutA fusion protein was purified using Ni-NTA agarose columns (Qiagen).

His-tagged IutA 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 IutA-deficient RC154 strain. The specificity of the antibody was confirmed using the M06-24/O, RC154, and RC158 strains, as shown in Fig. 6.

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-IutA 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.

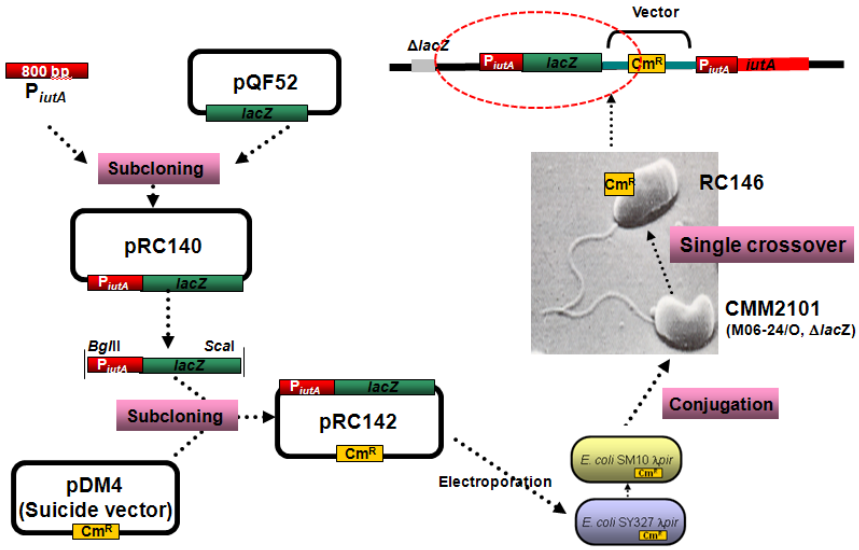

Figure 4. The construction of the *Vibrio vulnificus* RC146 strain containing the *lacZ*-fused *iutA* transcription reporter. Details are described in the text. P_{iutA}: the promoter region of *iutA*, Cm^R: chloramphenicol-resistant cassette, Δ: deletion.

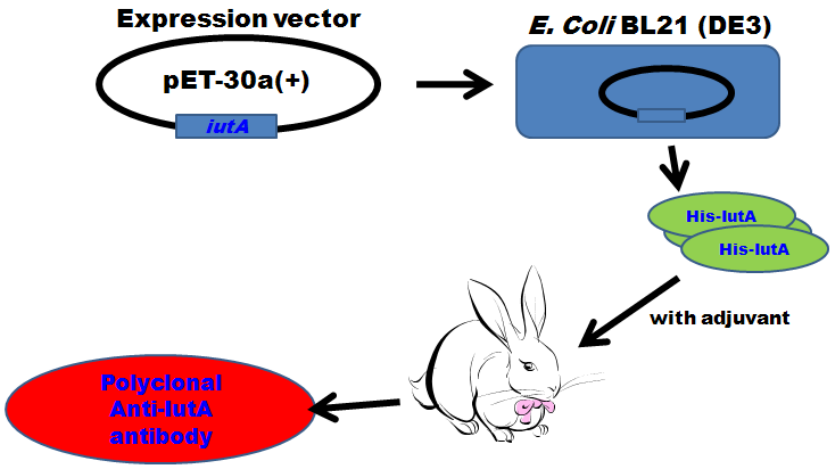

Figure 5. The preparation of recombinant IutA protein and rabbit polyclonal anti-IutA antibody. Details are described in the text. His-IutA: His-tagged IutA fusion protein.

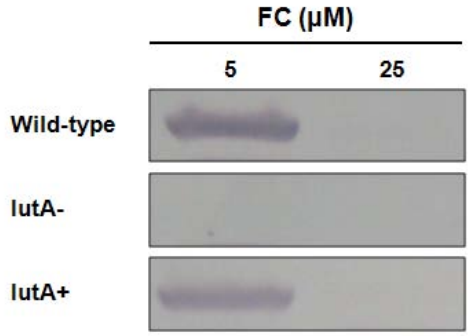

Figure 6. The specificity of rabbit polyclonal anti-IutA antibody. The three *V. vulnificus* strains, M06-24/O with wild type *iutA* (Wild-type), RC154 with mutated *iutA* (IutA−), RC158 with *in trans* complemented *hupA* (IutA+), were cultured in DF-HI containing 5 or 25 μM FC. Whole cell lysates containing 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-IutA antibody as the primary antibody was conducted as described in Materials and Methods. A representative experiment of duplicate experiments is shown.

3. Results

3.1. Effect of aerobactin on *iutA* expression

The presence of aerobactin is known to affect *iutA* expression via the GntR-like repressor IutR [17]. The effects of aerobactin (or IutR) on *V. vulnificus* growth and *iutA* transcription were determined by culturing RC146 with the P_{iutA}::*lacZ* transcriptional fusion in DF-HI broths containing 5 μM FC with or without 10 μM aerobactin (Fig. 7A). The presence or absence of aerobactin had no significant effect on the growth of RC146. However, *iutA* transcription was significantly higher in the presence of aerobactin ($p<0.05$ in Student's *t*-test). The effect of aerobactin on IutA production was also determined by culturing M06-24/O with wild-type *iutA*, RC154 with mutated *iutA*, and RC158 with complemented *iutA* under the same conditions (Fig. 7B). M06-24/O and RC158 produced IutA at low levels in the absence of aerobactin, whereas RC154 did not. The addition of aerobactin noticeably increased IutA production in M06-24/O and RC158, but not in RC154.

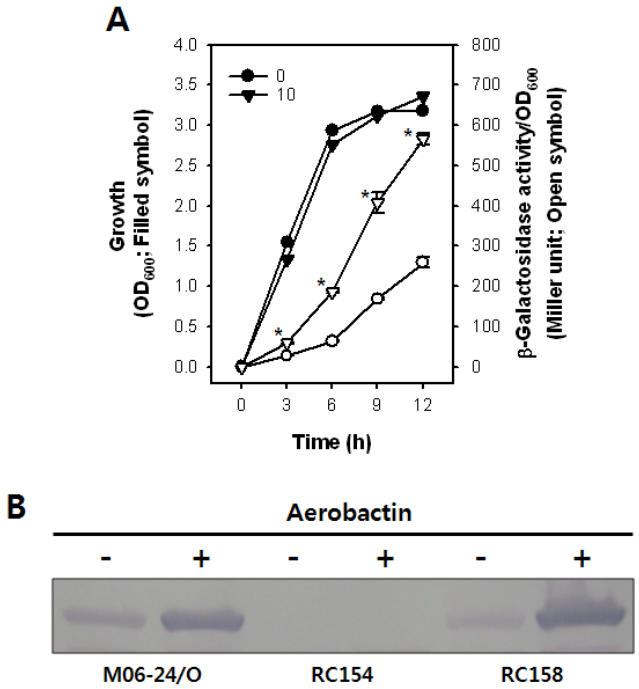

Figure 7. Effects of aerobactin on *Vibrio vulnificus* growth and *iutA* expression. (A) *V. vulnificus* RC146 with the P_{iutA}-*lacZ* transcriptional fusion was cultured in deferrated Heart Infusion broths containing 5 μM ferric chloride with (+) or without (-) 10 μM aerobactin. Bacterial growths were determined by measuring optical densities of culture aliquots at 600 nm (OD_{600}), and *iutA* 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 that a significant difference for with or without aerobactin at the same time points ($p<0.05$, Student's *t*-test). (B) The three *V. vulnificus* strains, M06-24/O with wild-type *iutA*, RC154 with mutated *iutA*, and RC158 with *in trans* complemented *iutA*, were cultured under the same conditions. Western blotting was performed as described in the legend of Fig. 6. A representative experiment of duplicate experiments is shown.

3.2. Effect of Fur on *iutA* expression

The effects of Fur on *V. vulnificus* growth and *iutA* transcription were determined by culturing the three P_{iutA}::*lacZ* transcriptional reporter strains, RC146 with wild-type *fur,* RC150 with mutated *fur*, and RC310 with complemented *fur,* in DF-HI broths containing 5 or 25 μM FC (Fig. 8A). At 5 μM FC, the *fur* mutation in RC150 and *fur* complementation in RC310 had no significant effect on *iutA* transcription (data not shown). However, at 25 μM FC, *iutA* transcription in RC146 was almost completely repressed, and this repressed *iutA* transcription in RC146 was de-repressed in RC150, and this de-repression was almost completely repressed in RC310. In addition, the *fur* mutation in RC150 slightly inhibited growth at an FC concentration of 25 μM, especially during the late growth phase. Excessive intracellular iron resulting from the *fur* mutation was thought to be rather toxic for *V. vulnificus*. The effect of the *fur* mutation on IutA production was also determined by culturing M06-24/O with wild-type *fur,* CMM2303 with mutated *fur,* and RC144 with 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 effect on IutA production. However, at 25 μM FC, IutA production in M06-24/O was almost completely repressed. This repressed IutA production in M06-24/O was de-repressed in CMM2303, and this de-repression was again repressed in RC144.

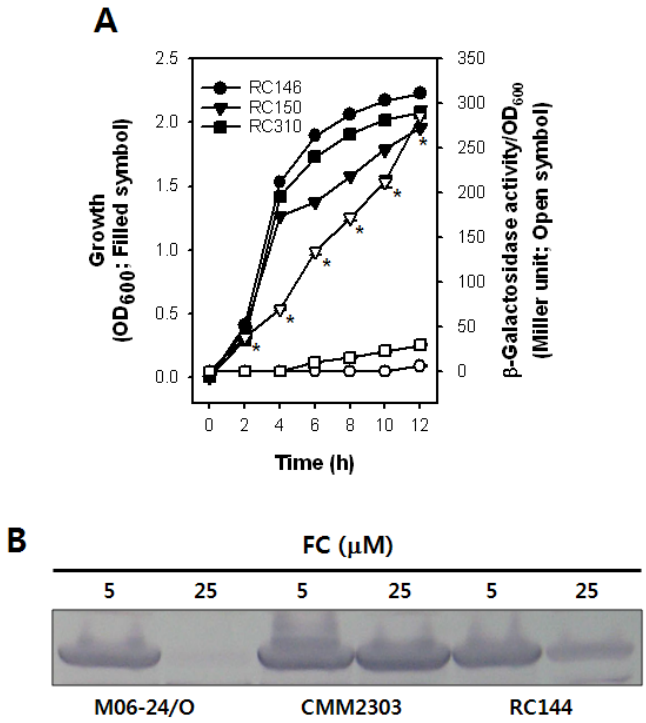

Figure 8. Effects of Fur on *Vibrio vulnificus* growth and *iutA* expression. (A) The P_{iutA}*::lacZ* transcription reporter strains, RC146 with wild type *fur*, RC150 with mutated *fur*, and RC310 with *in trans* complemented *fur*, were cultured in deferrated Heart Infusion containing 25 μM ferric chloride (FC). Bacterial growth and *iutA* transcription were determined as described in the legend of Fig. 7. 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 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 blotting was performed as described in the legend of Fig. 6. A representative experiment of duplicate experiments is shown.

3.4. Effect of Crp on *iutA* expression

The effect of *crp* mutation on *iutA* transcription was determined by culturing the three P_{iutA}*::lacZ* transcription reporters, that is, RC146 with wild-type *crp*, RC148 with mutated *crp*, and RC256 with complemented *crp*, in DF-HI broths containing 5 or 25 μM FC (Fig. 9A). The growth of RC146 was severely inhibited in RC148, and this inhibition was completely restored in RC256. At 5 μM FC, *iutA* transcription was highly induced in RC146 but almost completely repressed in RC148, and this repression was recovered in RC256. The higher *iutA* transcription observed in RC256 than in RC146 may have been due to the presence of multiple copies of *crp*. At 25 μM FC, *iutA* transcription remained severely repressed in all three strains (data not shown). The effect of the *crp* mutation on IutA production was also determined by culturing the three *V. vulnificus* strains, M06-24/O with wild-type *crp*, CMM710 with mutated *crp*, and CMM714 with complemented *crp*, under the same conditions (Fig. 9B). At 5 μM FC, IutA production was highly induced in M06-24/O and severely repressed in CMM710, and this repression in CMM710 was restored in CMM714. At 25 μM FC, IutA production was severely repressed in all three strains.

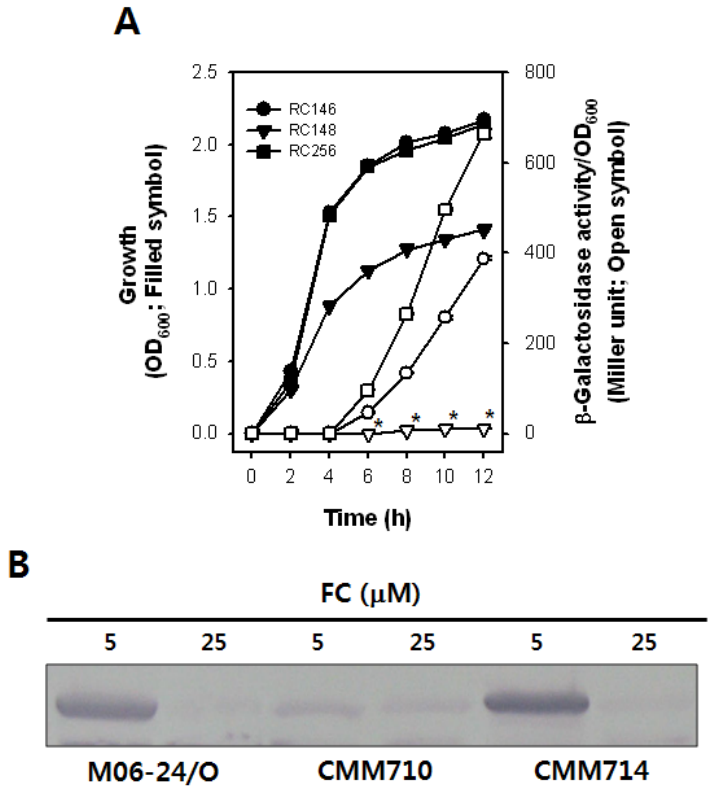

Figure 9. Effects of Crp on *Vibrio vulnificus* growth and *iutA* expression. (A) The P_{iutA}*::lacZ* transcription reporter strains, RC146 with wild type *crp*, RC148 with mutated *crp*, and RC256 with *in trans* complemented *crp*, were cultured in deferrated Heart Infusion containing 5 μM ferric chloride (FC). Bacterial growth and *iutA* transcription were determined as described in the legend of Fig. 7. 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 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 blotting was performed as described in the legend of Fig. 6. A representative experiment of duplicate experiments is shown.

3.3. Effect of cAMP on *iutA* expression

The biosynthesis of cAMP is controlled by adenylate cyclase, which is encoded by *cyaA* [32]. The effect of cAMP on IutA production was also examined by culturing the three *V. vulnificus* strains, M06-24/O with wild-type *cyaA*, RC386 with mutated *cyaA*, RC390 with *in trans* complemented *cyaA*, in DF-HI broths containing 5 or 25 μM FC. At 5 μM FC, the growth of M06-24/O was inhibited in RC386, and this inhibition was completely recovered in RC390 (data not shown). IutA production was highly induced in M06-24/O, but repressed in RC386. This repression in RC386 was prevented in RC390 (Fig. 10A). Furthermore, the exogenous addition of 0.1 and 0.5 mM cAMP dose-dependently stimulated bacterial growth and increased IutA production in RC386 (Fig. 10B).

3.5. A putative Crp binding sequence in the regulatory region of *iutA*

To locate a putative Crp binding site, DNA sequences in the regulatory region of *iutA* were compared with the *E. coli* Crp binding consensus sequence [21] using the DNAssist program (Fig. 11). The consensus sequence (aattgtgatctagatcacattt) was matched with two putative Crp binding sites, which were located at the -235 to-214 and -51 to -29 nucleotide positions upstream from the transcription start site of *iutA* [17]. Of the 22 bases, 12 and 11, respectively, were matched.

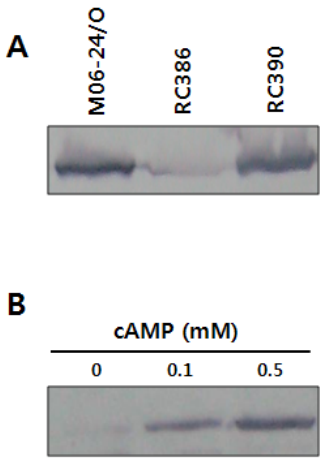

Figure 10. Effects of cAMP on IutA production. (A) The three *V. vulnificus* strains, M06-24/O containing wild-type *cyaA*, RC386 containing mutated *cyaA*, and RC390 containing *in trans* complemented *cyaA*, were cultured in DF-HI containing 5 μM FC. (B) RC386 containing mutated *cyaA* was cultured 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. 6. A representative experiment of duplicate experiments is shown.

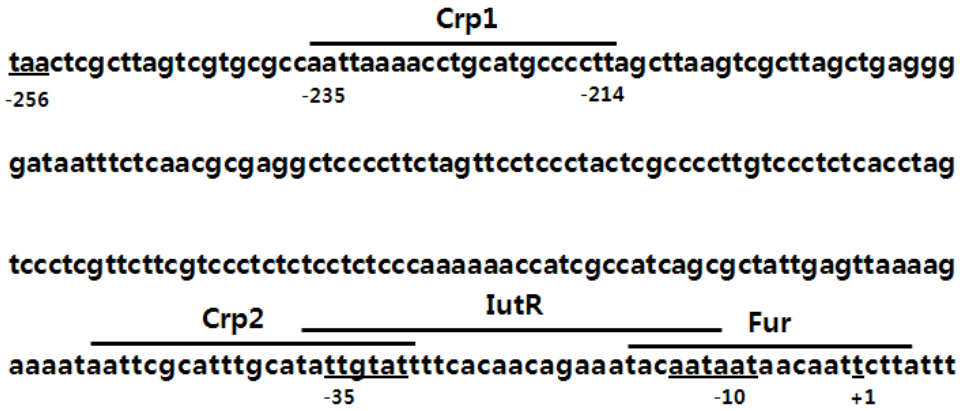

Figure 11. Putative Crp binding sequences in the regulatory region of *iutA*. The Crp binding consensus of *Escherichia coli* was used for sequence analysis (DNAssist ver.2.2). Two putative Crp binding sites were found at the -235 to -214 (Crp1) and -51 to -29 (Crp2) positions from the transcription start site (+1) of *iutA*. A putative IutR binding site and a putative Fur binding site are also shown. The sequence (taa) indicates the stop codon of *iutR*, which encodes the transcriptional repressor IutR.

3.6. Effects of iron on *iutA* expression in the presence or absence of Crp or Fur

To observe the effects of iron on *V. vulnificus* growth and *iutA* transcription in the presence or absence of Crp or Fur, the three P_{iutA}*::lacZ* transcriptional reporter strains, RC146 with wild-type *crp* and *fur*, RC148 with mutated *crp*, and RC150 with mutated *fur*, were cultured in DF-HI broths containing various concentrations (0.5 to 30 μM) of FC for 12 h. FC dose-dependently stimulated the growths of the three strains at below 5 μM, but not at above 5 μM (Fig. 12A). In RC120, FC dose-dependently increased *iutA* transcription at below 5 μM ($p<0.05$), but dose-dependently repressed *iutA* transcription at above 5 μM ($p<0.05$) and almost completely repressed *iutA* transcription at more than 15 μM (Fig. 12B). The *iutA* transcription level peaked at 5 μM FC. In RC122, FC dose-dependently repressed *iutA* transcription even at below 5 μM and almost completely repressed *iutA* transcription at above 5 μM. In RC124, FC dose-dependently increased *iutA* transcription at below 5 μM, but had no significant effect at above 5 μM.

The effects of iron on IutA 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. 12C). In M06-24/O, FC at below 5 μM had no significant effect on IutA production, but FC at above 5 μM dose-dependently repressed IutA production, and FC at above 20 μM almost completely repressed Iut production. A discrepancy between *iutA* transcription and IutA

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

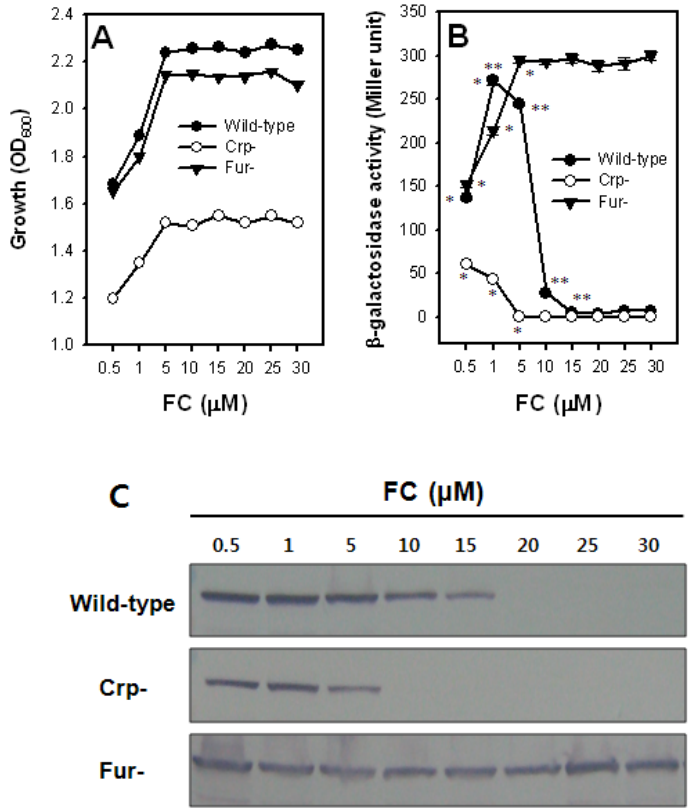

Figure 12. Effect of iron on *Vibrio vulnificus* growth and *iutA* expression in the absence of Crp or Fur. (A) The three P_{iutA}*::lacZ* transcriptional reporter strains, RC146 with wild-type *crp* and *fur*, RC148 with mutated *crp* (Crp-), and RC150 with mutated *fur* (Fur-), were cultured in DF-HI broths containing various concentrations of ferric chloride (FC) for 12 h. Growth and *iutA* transcription were determined as described in the legend of Fig. 7. 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. 6. A representative experiment of duplicate experiments is shown.

3.7. Effects of glucose on *iutA* expression in the presence or absence of Fur

The effects of glucose on *iutA* transcription in the presence or absence of Fur were examined by culturing the two P_{iutA}*::lacZ* transcriptional reporter strains, RC146 with wild-type *fur* and RC150 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 RC146 and RC150 were slightly increased in the presence of glucose, but not in proportion to glucose concentrations (Figs. 13A and 13B). However, in RC146 (Fig. 13A), glucose dose-dependently repressed *iutA* transcription and almost completely repressed *iutA* transcription at 0.4% over ($p<0.05$, One Way ANOVA). In contrast, *iutA* transcription in RC150 (Fig. 13B) 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 IutA 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. 13C), glucose dose-dependently repressed IutA production and almost completely repressed IutA production at 0.5%. In contrast, IutA production in CMM2303 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, *iutA* transcription in RC146 was severely repressed, and thus, the effects of glucose on *iutA* transcription and IutA production could not be observed (data

not shown). In contrast, *iutA* transcription in RC124 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, IutA production in CMM2303 was not lowered by glucose.

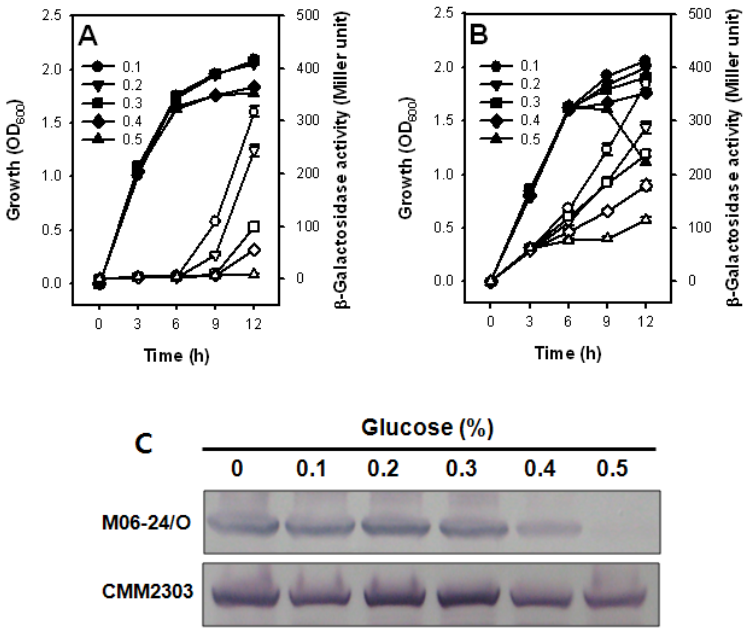

Figure 13. Effect of glucose on *Vibrio vulnificus* growth and *iutA* expression in the presence or absence of Fur. (A and B) The P_{iutA}*::lacZ* transcriptional reporter strains, RC146 with wild-type *fur* (A) and RC150 with mutated *fur* (B) were cultured in TES-DF-HI broths containing 5 μM ferric chloride plus various concentrations (0-0.5%) of glucose. Growth and *iutA* transcription were determined as described in the legend of Fig. 7. (C) *V. vulnificus* M06-24/O with wild-type *fur* and CMM2303 with mutated *fur* were cultured under the same conditions. Western blot was conducted as described in the legend of Fig. 6. A representative experiment of duplicate experiments is shown.

3.8. Effects of aerobactin on *iutA* expression in the absence of Crp and Fur

To determine the effect of aerobactin on *iutA* expression in the absence of Crp or Fur, *V. vulnificus* CMM710 with mutated *crp* and CMM2303 with mutated *fur* were cultured in DF-HI broths containing 5 or 25 μM FC with or without 10 μM aerobactin for 12 h. At both FC concentrations, the presence or absence of aerobactin had no significant effect on *V. vulnificus* growth levels (data not shown). At 5 μM FC, the presence of aerobactin increased IutA production in both CMM710 and CMM2303 (Figs. 14A and 14B). In contrast, at 25 μM FC, IutA production was observed in CMM2303 but not in CMM710, and the presence of aerobactin increased IutA production in CMM2303 but not in CMM710. Similar results were also observed when the two transcription reporter strains, RC140 with mutated *crp* and RC150 with mutated *fur*, were cultured under the same conditions, and *iutA* transcription levels were compared between the two strains (data not shown).

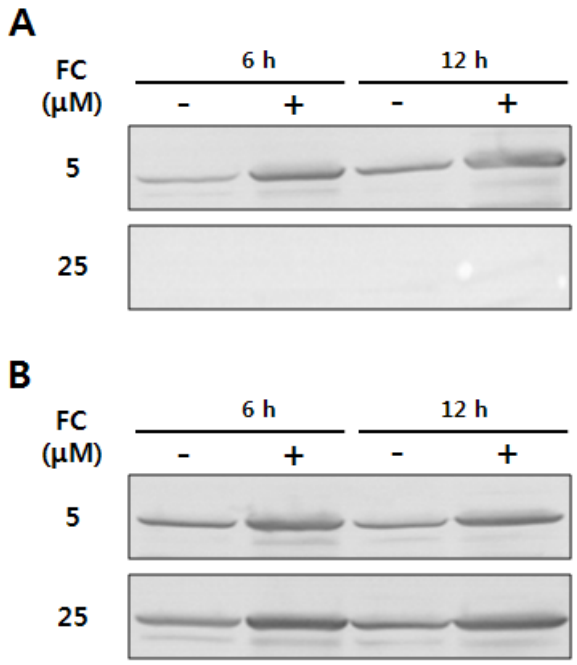

Figure 14. Effect of aerobactin on *iutA* expression in the absence of Crp or Fur. The two *V. vulnificus* strains, CMM710 with wild-type *crp* and CMM2303 with mutated *fur*, were cultured in DF-Heart Infusion broths containing 5 μM ferric chloride with (+) or without (-) 10 μM aerobactin. Western blotting was performed as described in the legend of Fig. 6. A representative experiment of duplicate experiments is shown.

4. 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 [33]. In addition, some bacteria can acquire iron by utilizing heterologous siderophores produced by other unrelated bacterial or fungal species [34,35]. Siderophore piracy is probably highly advantageous to bacteria because it allows them to escape bacteriostatic effects caused by heterologous siderophores without synthesizing their principal siderophores, especially in environments with mixed bacterial populations, such as, human large intestine. *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 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 available iron than other pathogens [2,3].

The present study shows that *iutA* expression was induced at considerable levels only by iron limitation in the absence of aerobactin (Fig. 7). This finding suggests that the cognate siderophore (or inducer) is produced or that the IutA-mediated IUS plays an innate

role in the iron assimilation of *V. vulnificus* in the absence of aerobactin. However, the cognate siderophore has not been identified and the innate role played by the IutA-mediated IUS has not been determined. In contrast, DesA is induced only in the presence of deferoxamine under iron-limited conditions [14-16], which suggests that the DesA-mediated IUS contributes only to siderophore piracy.

V. vulnificus can utilize aerobactin via IutA, and the presence of aerobactin under iron-limited conditions can synergistically co-activate *iutA* expression. In this study, *iutA* expression was found to be induced at higher levels in the presence of aerobactin than in its absence, and this response was abolished in a background of *iutA* mutation (Fig. 7). In a previous study [17], it was demonstrated that response to aerobactin is mediated by the GntR-like repressor IutR, and that a putative IutR binding site is located at the -37 to -9 position from the *iutA* transcription start site (Fig. 11). Accordingly, it appears that an inducer (probably ferric aerobactin) interacts with IutR to prevent IutR from binding to the *iutA* regulatory region, and thereby, de-represses *iutA* expression in the presence of aerobactin.

As in most bacteria [18], *V. vulnificus* IUSs are also likely to be under the negative control of Fur, which functions as an iron-dependent transcriptional repressor [19]. In a previous study, it was reported based on Fur titration assay results that iron negatively regulates *iutA* expression [17]. The present study shows that *fur* mutation totally abolished

iron-repressible *iutA* expression, and that this was recovered by complementing wild-type *fur* (Fig. 8). According to sequence analysis, Fur is likely to function by binding to the -15 to +4 position from the *iutA* transcription start site to block the progress of RNA polymerase (Fig. 11). This Fur-mediated regulation 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 is an essential signal for *iutA* expression under iron-limited conditions and that Crp functions as an essential transcription activator for *iutA* expression. Glucose dose-dependently repressed *iutA* expression (Fig. 13) and *crp* mutation severely repressed *iutA* expression at the transcription and protein levels (Fig. 9). In addition, a *cyaA* mutation repressed IutA production, and the repression was prevented by *in trans* complementing wild-type *cyaA* or the addition of exogenous cAMP (Fig. 10). Furthermore, one putative Crp binding site was found at the -235 to -214 position from the transcription start site of *iutA* (Fig. 11). This position is unusually distant for direct activation by Crp, which suggests that another factor may be involved in the activation of *iutA* promoter [36]. Another putative Crp binding site was found at the -51 to -29 position from the *iutA* transcription start site. This position overlaps the -35 promoter element, and thus, may provide an opportunity for direct interaction between Crp and the sigma factor of RNA polymerase [36]. Accordingly, this Crp-mediated regulation of *iutA* expression implies that Crp is an important activator that

controls *iutA* expression in response to glucose availability and that glucose starvation is an important signal for the induction of *iutA* expression.

In a previous study [9], the expression of *vuuA* encoding vulnibactin receptor was also found to be under the positive control of Crp, and recently, it was reported that Crp positively regulates the expression of *hupA* encoding haem receptor by directly binding to the regulatory region of *hupA* [12]. The finding that the expression of *V. vulnificus* IUSs is under the positive control of Crp implies that the acquisition of iron should be increased to stimulate catabolism and to produce energy efficiently under conditions of glucose-starvation. Furthermore, Crp is a global regulator that has well established roles in catabolite repression [20]. 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 or catabolite operons. Iron is essential for activating many catabolite enzymes, especially those involved in the electron transport system, and is essential for efficient energy production, especially under glucose-starved conditions. Furthermore, the presence of delicate functional interactions between Crp and Fur have been demonstrated in *E. coli* [37], and is also likely to be true in *V. vulnificus* (our unpublished data). This metabolically coordinated acquisition and effective utilization of iron are likely to be crucial for the successful establishment of infections.

The present study also discriminates the roles of iron/Fur as an essential or principal signal/regulator, of aerobactin/IutR as an additional regulator, and of glucose/Crp as a coordinator or mediator of *iutA* expression. In the presence of Fur, glucose dose-dependently and completely repressed both *iutA* transcription and IutA production (Fig. 13). However, in the absence of Fur, glucose only partially or slightly repressed IutA production. These findings indicate that the presence of Fur is essential for the glucose-mediated repression of *iutA* expression. In addition, the present study shows that severe iron limitation alone can induce *iutA* expression albeit at relatively low levels in the absence of Crp (Fig. 12), which indicates that *iutA* is constitutively expressed albeit at relatively low levels and that Crp is not essential for the induction of *iutA* expression under iron-limited conditions. Furthermore, the Fur-mediated repression of *iutA* 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. IutR is likely to act independently of Crp and Fur. The presence of aerobactin increased IutA production even in the absence of Crp or Fur, or regardless of iron levels (Fig. 14). As shown in Fig. 11, a putative IutR binding site is separated from Crp or Fur-binding sites. Overall, Fur is likely to act as a principal or local regulator of the induction of *iutA* expression in response to iron availability, Crp is likely to act as a coordinator or mediator that regulates *iutA*

expression in response to carbon availability or metabolic status, and IutR is likely to play a crucial role in siderophore piracy; that is, competition with other bacteria for iron acquisition in mixed bacterial environments.

In the present study, a noticeable discrepancy was observed between the glucose-mediated repression of *iutA* transcription and IutA production in the *fur*-mutated background. Glucose dose-dependently repressed *iutA* transcription, which was measured by the *lacZ*-fused transcription reporter assay, whereas it only slightly repressed IutA production, which was measured by Western blotting (Fig. 13). 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 *iutA* transcription levels in the *lacZ*-fused transcription reporter assay and IutA production by Western blotting, especially under severely iron-limited conditions. FC at less than 5 μM dose-dependently increased *iutA* transcription, but tended to slightly decrease IutA production in the presence of Crp (Fig. 12). However, in the absence of Crp, *iutA* transcription levels paralleled IutA

production levels under the same conditions. One plausible interpretation of these findings is that the *lacZ*-fused transcriptional reporter assay may not represent actual *iutA* 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 *iutA* transcription but not IutA production, and post-transcriptional mechanisms may be involved. To test these assumptions, more accurate quantitative analysis at the *iutA* mRNA level is necessary.

CONCLUSION

The present study shows that (i) Crp is not essential for constitutional *iutA* expression, but is required to optimally induce *iutA* expression in response to glucose deprivation regardless of iron or aerobactin availability, (ii) IutR is not essential for *iutA* repression, but responds to aerobactin availability regardless of iron or glucose levels, (iii) Fur functions as a repressor of *iutA* expression in proportion to iron levels. Taken together, *iutA* expression is likely to be under the independent but coordinate control of Crp, IutR and Fur.

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ABSTRACT

Regulatory Mechanism of the *iutA* Gene Encoding Aerobactin Receptor Protein in *Vibrio vulnificus*

Kim Woo-Hyung

Advisor: Prof. Kim Seong-Jung, M.D.

Department of Medicine,

Graduate School of Chosun University

The ferrophilic bacterium *Vibrio vulnificus* can utilize the siderophore aerobactin of *Escherichia coli* for iron acquisition via the specific receptor IutA. This siderophore piracy by *V. vulnificus* may contribute to its survival and proliferation, especially in mixed bacterial environments. This study was undertaken to determine the effects of glucose/Crp (cAMP-receptor protein), aerobactin/IutR (Gnt-like repressor) and iron/Fur (ferric uptake regulator) on *iutA* expression, and to detail the roles of Crp, IutR and Fur in the regulation of *iutA* expression under iron-deficient (ID) and iron-sufficient (IS) conditions. An *in frame* deletion mutation in *crp* severely repressed *iutA* expression under moderately ID conditions, and this change was recovered by *in trans* complementing wild-type *crp*. Under severely ID conditions, *iutA* expression was induced even in the absence of Crp, and iron increased *iutA* expression in the presence of Crp but repressed it in the absence of Crp.

The presence of aerobactin increased *iutA* expression under moderately ID conditions. An *in frame* deletion mutation in *fur* de-repressed iron-repressible *iutA* expression under IS conditions, and this change was recovered by *in trans* complementing wild-type *fur*. Iron repressed *iutA* expression under moderately ID and IS conditions only in the presence of Fur but de-repressed it in the absence of Fur. Glucose repressed *iutA* expression under moderately ID conditions in the presence of Fur, but not in the absence of Fur. Aerobactin induced *iutA* expression under both ID and IS conditions and regardless of the absence or presence of Crp or Fur. These results indicate that (i) Crp is not essential for constitutional *iutA* expression, but is required to optimally induce *iutA* expression in response to glucose deprivation regardless of iron or aerobactin availability, (ii) IutR is not essential for *iutA* repression, but responds to aerobactin availability regardless of iron or glucose levels, (iii) Fur functions as a repressor of *iutA* expression in proportion to iron levels. Taken together, iutA expression is likely to be under the independent but cooperative control of Crp, IutR and Fur.

Key words

Vibrio vulnificus, Iron, Aerobactin, Glucose, Ferric uptake regulator, Cyclic AMP-receptor protein, Siderophore piracy