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온도변화에 의한 패혈증 비브리오균의 Aerobactin 수용체 유전자 발현 조절

조선대학교대학원

의 학 과

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Temperature Change Modulates the Expression of *iutA* Encoding Aerobactin Receptor in *Vibrio vulnificus*

2015 년 8 월 25 일

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이 논문을 의학 박사학위신청 논문으로 제출함

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최 연 규

지도교수: 의학박사 신 성 희 / 김 성 중

조선대학교 대학원 의학과

패혈증 비브리오균은 필수 영양요소인 철을 획득하기 위하여 다양한 철흡수기전을 이용하며 이러한 철흡수기전은 자체가 잘 알려진 균의 독력인자이다. 패혈증 비브리오균의 철흡수기전 중에는 IutA라 부르는 수용체단백에 의해서 매개되는 철흡수기전이 있다. 패혈증 비브리오균은 이 철흡수기전을 통해 대장균에서 생산된 시데로포아 중 하나인 aerobactin을 이용할 수 있다. 유전자 *iutA* 발현은 철농도와 철농도에 반응하여 유전자 발현을 조절하는 조절인자인 ferric uptake regulator (Fur)에 의해 조절된다. 최근에는 당대사를 조절하는 조절인자인 cyclic AMP-receptor protein (CRP)와 LuxS에 의해 매개되는 쿼럼센싱(quorum sensing; 지배적 조절 인자는 SmcR)에 의해서도 유전자 *iutA*의 발현이 조절됨이 밝혀 졌다. 패혈증 비브리오균이 바닷물에 서식하다가 인체에 들어올 때에는 25°C에서 37°C로 급격한 온도변화를 견뎌내야 하고 이에 적절히 반응할 수 있어야 한다. 본 연구에서는 25°C에서 37°C로 온도변화가 유전자 *iutA, crp*와 *smcR*의 발현에 미치는 영향과 유전자 발현 조절인자인 CRP나 SmcR이 온도변화에

의해 매개된 유전자 *iutA* 발현에 미치는 영향을 조사하여 온도변화가 유전자 *iutA* 발현을 유도할 수 있는 새로운 신호가 될 수 있는가를 규명하고자 하였다. 유전자 발현을 전사수준에서 측정하기 위하여 해당 유전자에 LacZ (β-galactosidase) 융합시킨 전사리포터를 제작하여 사용하였고 해당 유전자 산물인 단백을 측정하기 위해서는 Western blot을 사용하였다. 철농도를 낮추면 패혈증 비브리오균의 증식이 억제되었고 유전자 *iutA* 발현이 유도되었다. 반면, 철농도가 높으면 유전자 *iutA* 발현이 억제되었고 이러한 철에 의한 억제현상은 유전자 *fur*를 돌연변이 시켰을 때는 나타나지 않았다. Aerobactin이 없을 때에 비해, aerobactin이 존재할 때 유전자 *iutA*의 발현이 더 높았다. 유전자 *crp*에 돌연변이를 유발할 경우 유전자 *iutA*의 발현이 현저히 낮아졌지만 유전자 *smcR*에 돌연변이를 유발하였을 경우에는 유전자 *iutA*의 발현에 의미있는 변화가 관찰되지 않았다. 25°C에서 37°C로 온도를 변화시켰을 때 패혈증 비브리오균의 증식이 촉진되었고 유전자 *iutA*, *crp*와 *smcR*의 발현이 모두 증가하였다. 또한, 25°C에서 37°C로 온도변화는 CRP 또는 SmcR이 없는 조건에서도 유전자 *iutA*의 발현을 증가시켰다. 이러한 결과를 종합하여 볼 때, 25°C에서 37°C로 온도변화가 유전자 *iutA, crp*와 *smcR*의 발현을 증가시키는 신호로 작용하며 유전자 발현 조절인자인 CRP나 SmcR은 온도변화에 따른 유전자 *iutA*의 발현 변화에 꼭 필요하지 않음을 알 수 있었다.

중심어: 패혈증 비브리오균, 온도, 철, 철흡수조절인자, Aerobactin 수용체, Cyclic AMP 수용체단백, 쿼럼센싱

Introduction

Vibrio vulnificus is a gram-negative halophilic marine bacterium, and causes rapidly progressing fatal septicemia and necrotizing wound infections mainly in patients with liver disease and hemochromatosis. Most *V. vulnificus* infections actually requires emergency treatment. Several established and potential virulence factors, such as capsular polysaccharides, iron-uptake systems (IUSs), flagella or motility, pili, RTX toxin, and exotoxins such as hemolysins (VvhBA) and proteases (VvpE), are implicated in the pathogenesis of *V. vulnificus* infection (Jones & Oliver, 2009). The combined or concerted expression of these virulence factors is required for *V. vulnificus* pathogenesis and is under the direct or indirect control of global regulators, such as cyclic AMP-receptor protein (CRP) (Kim *et al*., 2005 & 2013), which is primarily responsible for carbon catabolite repression or glucose metabolism (Ishizuka *et al*., 1993), and a master regulator SmcR of the LuxS (synthase for autoinducer-2)-mediated quorum sensing system (Kim *et al*., 2003; Lee *et al*., 2007a).

Iron is an essential element for survival and proliferation of most cells including bacteria such as *V. vulnificus* (Andrews *et al*., 2003; Kim *et al*., 2007). For successful establishment of infection, *V. vulnificus* must be able to acquire iron and to survive or grow within the host. The ability to acquire iron is one of the best-established authentic virulence factors in the pathogenesis of *V. vulnificus* infection (Wright *et al*., 1981). *V. vulnificus* possesses multiple IUS (Shin, 2013). *V. vulnificus* produces two types of siderophore, catechol-type siderophore called vulnibactin and hydroxamate siderophore, for uptake of iron under iron-limited conditions (Simpson & Oliver, 1983). *V. vulnificus* can utilize transferrin-bound iron via vulnibactin or vulnibactin receptor (VuuA)-mediated IUS (Litwin *et al*., 1996; Webster & Litwin, 2000). *V. vulnificus* can also acquire iron from various heme pro-

teins via heme receptor (HupA)-mediated IUS (Litwin & Byrne, 1998). In addition, *V. vulnificus* can assimilate iron via the phenomenon called 'siderophore piracy' (Schubert *et al*., 1999). *V. vulnificus* can utilize heterologous siderophores, such as deferoxamine produced by *Streptomyces pilosus* via the cognate receptor DesA (Kim *et al*., 2007) and aerobactin produced by *Escherichia coli* via the cognate receptor IutA (Kim *et al*., 2012a). This siderophore piracy may play an important role in the survival and proliferation of *V. vulnificus*, especially in mixed bacterial environments, such as human large intestine. These IUSs are all negatively regulated by iron via ferric uptake regulator (Fur), a transcriptional repressor (Litwin & Calderwood, 1993). The most recent studies have documented that these IUSs are all under the positive control of CRP (Choi *et al*., 2006; Kim *et al*., 2012a; Kim *et al*., 2012b; Oh *et al*., 2009). The LuxS-mediated quorum-sensing system positively affects *vuuA* expression and negatively affects *iutA* or *hupA* expressions although it is not an major regulator (Kim and Shin, 2011b).

V. vulnificus resides in estuarine or marine environments and can infect human body. *V. vulnificus* must sense and respond to diverse environmental changes between the two environments for survival in the human body or for successful establishment of infection in humans (Jones and Oliver, 2009; Kim *et al*., 2003b; Lee *et al*., 2007b). Simply speaking, summer estuarine condition and the human body condition can be simulated at a temperature of 25^{o}C and 37^{o}C, respectively. Entering the human body, *V. vulnificus* must sense and respond to temperature change (TC) from 25^{o}C to 37^{o}C. TC is known to modulate the expression of some virulence factors, which is required for survival and for successful infection (Kawase *et al*., 2004; Kim and Shin, 2011a and 2012; McDougald *et al*., 2001; Shao and Hor, 2001).

A recent study reported that *V. vulnificus* can sense and respond to TC by increasing *hupA* expression (Oh et al., 2009). A more recent study documented that TC can also modulate the VuuA-mediated IUS (Yoon, 2013). Therefore, in this study, the author examined the effects of TC on *iutA* expression and the effect of CRP or SmcR on the TC-mediated change of *iutA* expression in an iron-limited condition in order to determine whether TC acts as a CRP or SmcR-independent signal for *iutA* expression.

Materials and Methods

1. Bacterial strains, media and reagents

The *V. vulnificus* strains used in this study are listed in Table 1. Heart Infusion (HI; Becton-Dickinson (BD), Franklin Lakes, NJ, USA) broth and agar containing 2.5% NaCl were used in cultivating *V. vulnificus* strains. As an iron-limited medium, HI broth containing 100 or 200 μM α,α'-dipyridyl (DP) as an iron chelator was used and HI broth not containing DP was used as an iron-sufficient medium. *E. coli* aerobactin was purchased from Genaxxon Bioscience GmbH (Ulm, Germany). Unless otherwise stated, all reagents used were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2. Construction of the P_{iutA}*::lacZ*$_{Ec}$ transcription reporter RC196 strain with mutated *smcR*

A deletion mutation in *smcR* was introduced into the *V. vulnificus* RC146 with P_{iutA}*::lacZ*$_{Ec}$ transcription reporter, and the deletion mutation of *smcR* was confirmed by polymerase-chain reaction, as previously described (Kim and Shin, 2012).

3. Culture conditions, bacterial growth measurement, and β-galactosidase assay

V. vulnificus strains were preconditioned by culturing in HI broth at 25°C overnight in order to adapt them to a condition mimicking their natural habitat. The adapted strains were inoculated into DP-HI broths at 1 x 10^6 cells/mL and cultured with vigorous shaking (220 rpm) at 25°C, 30°C or 37°C for 12 h. Culture aliquots were withdrawn at the appropriate times for measurement of bacterial growth and β-galactosidase activity. Bacterial

growth was measured by determining the optical densities of culture aliquots at a wavelength of 600 nm (OD_{600}). For comparison of gene transcription levels, β-galactosidase activity in culture aliquots obtained 12 h after culture initiation was measured using the Miller method (Miller, 1992). β-Galactosidase activities are expressed as the means and standard deviations (error bars) of triplicate measurements. Statistical analysis was performed using the program SigmaPlot 2001 (version 7.0, SPSS inc.).

4. Preparation of recombinant proteins and rabbit polyclonal antibodies

Recombinant IutA protein and rabbit polyclonal anti-IutA antibody were prepared as described previously (Kim and Shin, 2011b). Recombinant CRP and SmcR proteins and rabbit polyclonal anti-CRP antibody and anti-SmcR antibody were prepared as described in previous studies (Yoon, 2013; Kim *et al*., 2012c).

5. Western blot

Western blot for IutA, were performed as described in our previous studies (Kim *et al*., 2012a; Kim & Shin, 2011 b). In brief, bacterial pellets containing approximately 1 x 10^8 cells were boiled for 10 min, and the resulting cell lysates were electrophoresed on 10% SDS-PAGE gels. Separated proteins were then transferred to nitrocellulose membranes (PROTRAN, Whatman GmbH, Germany). The proprieties of protein loading and transfer among membranes were monitored using a pre-stained protein marker as a control. The membranes were reacted with rabbit polyclonal anti-IutA antibody (1:250) and then with anti-rabbit-IgG antibody conjugated with alkaline phosphatase (1:15,000), and finally visualized using 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium substrate solu-

tion. IutA levels on Western blot were quantitatively digitalized using a densitometer program (ImageJ; http://rsb.info.nih.gov/ij). Using the same methodology, Western blot for CRP and SmcR were also performed. Rabbit polyclonal anti-CRP antibody and anti-SmcR antibody were prepared as described in previous studies (Yoon, 2013; Kim *et al*., 2012c).

Table 1. *V. vulnificus* strains used in this study

Strains	Relative characteristics	References
M06-24/O	Wild-type *cya*	Reddy *et al*., 1992
RC386	Mutated *cya*	Kim *et al*., 2012a
RC390	*In trans* complemented *cya*	Kim *et al*., 2012a
RC242	Merodipliod P_{crp}::$lacZ_{Ec}$ transcriptional reporter	Yoon, 2013
RC146	Merodiploid P_{iutA}::$lacZ_{Ec}$ transcriptional reporter	Kim and Shin, 2011b
RC148	Merodiploid P_{iutA}::$lacZ_{Ec}$ transcriptional reporter, mutated *crp*	Kim *et al*., 2012a
RC150	Merodiploid P_{iutA}::$lacZ_{Ec}$ transcriptional reporter, mutated *fur*	Kim *et al*., 2012a
RC170	Merodiploid P_{iutA}::$lacZ_{Ec}$ transcriptional reporter, mutated *smcR*	This study
RC196	Merodiploid P_{smcR}::$lacZ_{Ec}$ transcriptional reporter	Kim and Shin, 2012

-*Ec*: *Escherichia coli*

Results

1. Effect of iron and Fur on *V. vulnificus* growth and *iutA* expression

The effect of iron on the *V. vulnificus* growth and *iutA* expression levels was observed by culturing RC146 with the merodiploid P_{iutA}*::lacZ*$_{Ec}$ fusion fragment in HI broths containing 0, 100 and 200 μM DP at 37°C for 12 h. DP-induced iron limitation inhibited the growth of RC146 in a dose-dependent manner (Fig. 1A). In β-galactosidase assay using culture aliquots obtained 12 h after culture initiation, DP-induced iron limitation increased the *iutA* transcription levels on a per cell basis in RC146 in a dose-dependent manner ($p<0.05$ in Student's *t*-test) (Fig. 1B). On Western blot analysis, DP-induced iron limitation also resulted in increased the IutA protein levels on a per cell basis in RC146 (Fig. 1C). IutA was not observed in iron-sufficient HI broth containing no DP.

The effects of Fur on the *V. vulnificus* growth and *iutA* expression levels were determined by culturing the P_{iutA}::*lacZ*$_{Ec}$ transcriptional reporter RC150 strain with mutated *fur* in HI broths containing 0, 100, and 200 μM DP at 37°C for 12 h. DP-induced iron limitation inhibited the growth levels of RC150 in a dose-dependent manner (Fig. 2A). In β-galactosidase assay, however, DP-induced iron limitation had no significant effect on the *iutA* transcription levels on a per cell basis in RC150 (Fig. 2B). On Western blot analysis, DP-induced iron limitation had also no remarkable effect on the IutA protein levels on a per cell basis in RC150 (Fig. 2C).

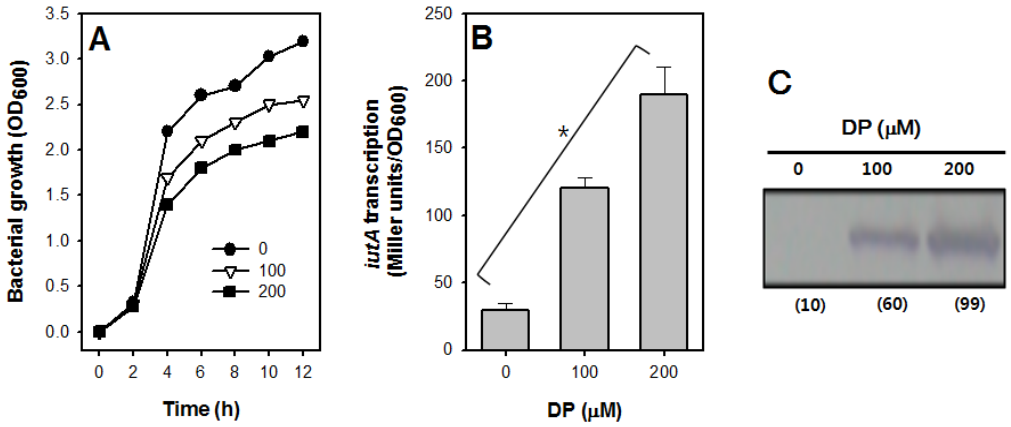

Figure 1. Effect of iron limitation on *V. vulnificus* growth and *iutA* expression. *V. vulnificus* RC146 with the merodiploid P_{iutA}::$lacZ_{Ec}$ transcription reporter was cultured in HI broth containing iron-chelator dipyridyl (DP: 0 (●), 100 (Δ) and 200 (■) μM) for 12 h at 37°C. (A) Bacterial growth levels were determined by measuring the optical densities of culture aliquots at 600 nm (OD_{600}), and (B) *iutA* transcription levels were determined by measuring the β-galactosidase activity on a per cell basis (Miller unit) 12 h after culture initiation. The * symbol indicates a significant statistical difference between the media ($p<0.05$, One Way ANOVA). (C) Bacterial pellets containing approximately 1 x 10^8 cells, which were obtained 12 h after culture initiation, were boiled and the resulting bacterial lysates were electrophoresed and transferred to nitrocellulose membranes, and reacted with rabbit polyclonal anti-IutA antibody. Representative examples of experiments performed in triplicate are shown. The digits in parenthesis indicate the intensities of bands relative to background, which were determined using a computer program.

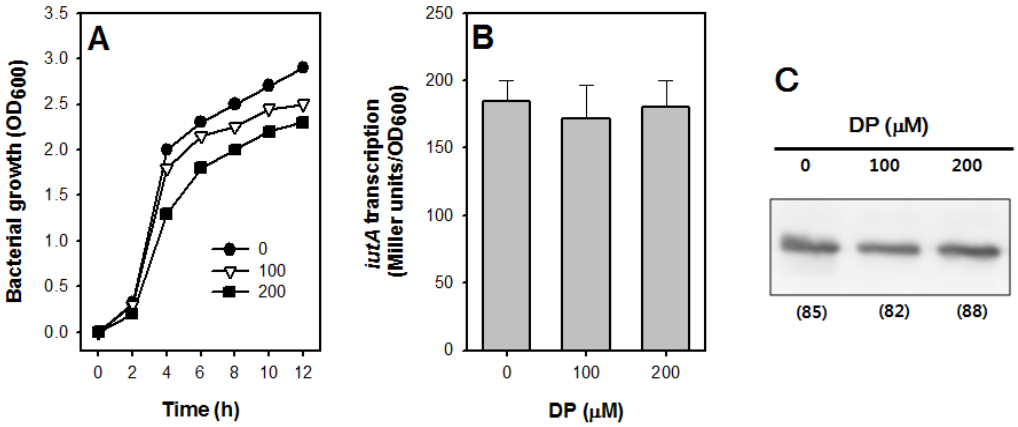

Figure 2. Effect of a *fur* mutation on *V. vulnificus* growth and *iutA* expression. *V. vulnificus* RC150 with the P_{iutA}*::lacZ*$_{Ec}$ transcription reporter and *fur* mutation was cultured in HI broth containing dipyridyl (DP) for 12 h at 37°C. Other figure legends are the same as described in Figure 1.

2. Effect of aerobactin on *V. vulnificus* growth and *iutA* expression

The effects of aerobactin on the *V. vulnificus* growth and *iutA* expression levels were determined by culturing RC146 with the P_{iutA}::$lacZ_{Ec}$ transcriptional fusion in iron-limited HI broths containing 100 μM DP with or without 10 μM aerobactin at 37°C for 12 h. The growth levels of RC146 were slightly increased in the presence of aerobactin, compared with the absence of aerobactin (Fig. 3A). However, in β-galactosidase assay, the *iutA* expression level was significantly higher in the presence of aerobactin than in the absence of aerobactin ($p<0.05$ in Student's *t*-test) (Fig. 3B). On Western blot analysis, the presence of aerobactin also noticeably increased the IutA protein level, compared with the absence of aerobactin (Fig. 3B).

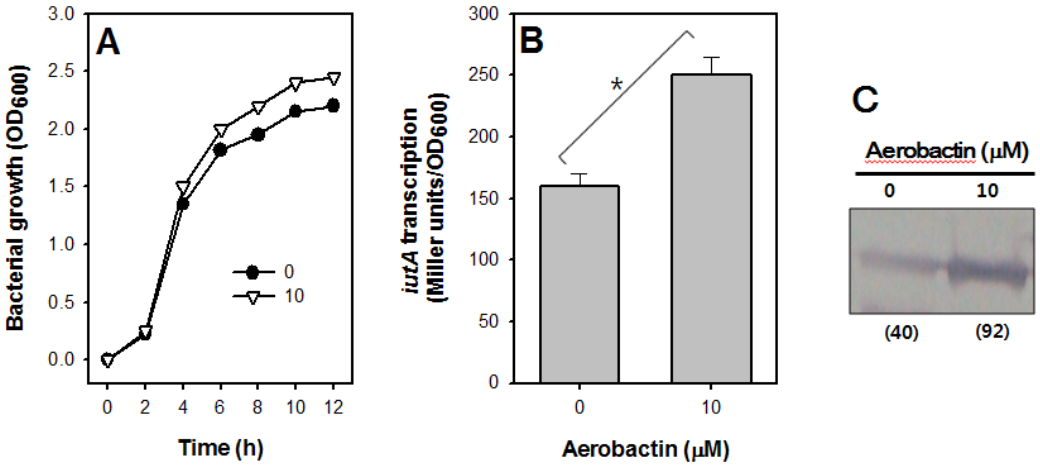

Figure 3. Effect of aerobactin on *V. vulnificus* growth and *iutA* expression in an iron-limited condition. *V. vulnificus* RC146 with the P_{iutA}::$lacZ_{Ec}$ transcriptional fusion was cultured in HI broth containing 100 μM dipyridyl and 0 (●) or 10 (Δ) μM aerobactin for 12 h at 37°C. Other figure legends are the same as described in Figure 1.

3. Effect of cAMP or CRP on *V. vulnificus* growth and *iutA* expression

The effect of CRP on the *V. vulnificus* growth and *iutA* expression levels was determined by culturing the P_{iutA}*::lacZ*$_{Ec}$ transcription reporter RC148 with mutated *crp* in iron-limited HI broths containing 0,100 or 200 μM DP at 37 ^{o}C for 12 h. The growth of RC148 was severely inhibited (Fig. 4A), compared with the growth of RC146 (Fig. 1A). In β-galactosidase assay, the *iutA* transcription level was almost completely decreased in RC148 (Fig. 4B). On Western blot analysis, the IutA protein level was also very low in RC148 (Fig. 4C).

The effect of cAMP on the *V. vulnificus* growth and the IutA protein levels was determined by culturing the three strains, M06-24/O with wild-type *cya*, RC386 with mutated *cya* and RC390 with *in trans* complemented *cya*, in an iron-limited HI broth containing 100 μM DP. The growth of RC 386 was severely inhibited compared with those of M06-24/O and RC390 (data not shown).On Western blot analysis, the IutA protein level was remarkably decreased in RC386, compared with those in M06-24/O and RC390 (Fig. 5A). Moreover, the inhibited growth and decreased IutA protein levels of RC386 were dose-dependently restored by the exogenous addition of cAMP (Fig. 5B).

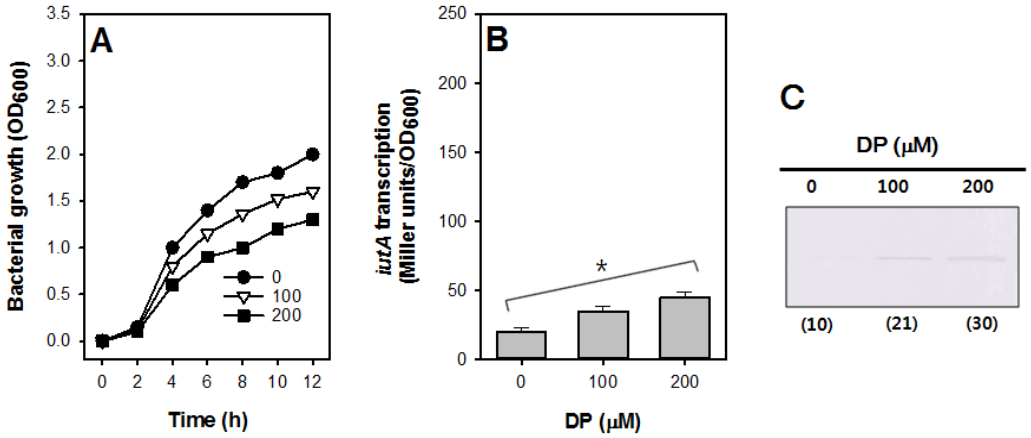

Figure 4. Effect of a *crp* mutation on *V. vulnificus* growth and *iutA* expression. *V. vulnificus* RC148 with the P_{iutA}*::lacZ*$_{Ec}$ transcription reporter and *crp* mutation was cultured in HI broth containing dipyridyl (DP: 0 (●), 100 (Δ) and 200 (■) μM) for 12 h at 37°C. Other figure legends are the same as described in Figure 1.

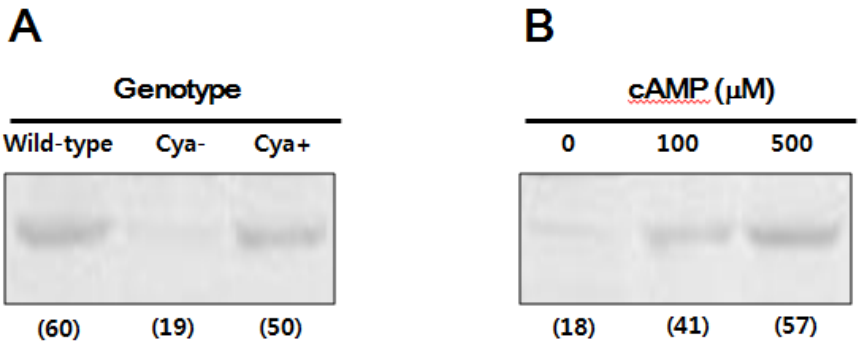

Figure 5. Effect of cAMP on *V. vulnificus* growth and *iutA* expression. (A) *V. vulnificus* M06-24/O with wild-type *cya* (Wild-type), RC386 with mutated *cya* (Cya-) and RC390 with *in trans* complemented *cya* (Cya+) were cultured in HI broth containing dipyridyl (DP) for 12 h at 37°C. (B) *V. vulnificus* RC386 was cultured in HI broth containing 100 μM DP and 0, 100 or 500 μM cAMP for 12 h at 37°C. Other figure legends are the same as described in Figure 1.

4. Effect of TC on *iutA* expression

The effect of TC on the *iutA* expression level was determined by culturing the P_{iutA}::$lacZ_{Ec}$ transcription reporter RC146 strain in an iron-limited HI broth containing 100 μM DP at 25, 30 or 37°C for 12 h. TC stimulated the growth of RC146 during early growth phase, but not during late growth phase (Fig. 6A). In β-galactosidase assay, TC significantly increased the *iutA* transcription levels on a per-cell basis (Fig. 6B). On Western blot analysis, TC also significantly increased the IutA protein levels on a per-cell basis, which was measured 12 h after culture initiation (Fig. 6C).

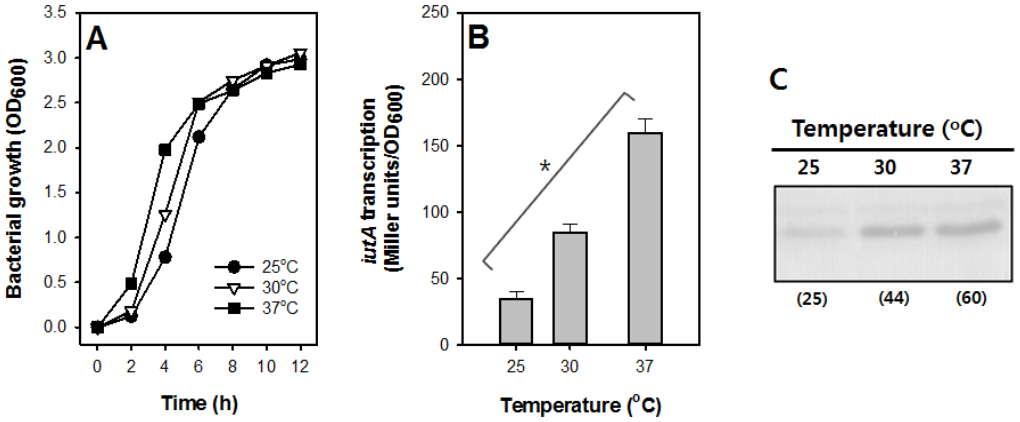

Figure 6. Effect of temperature change on *V. vulnificus* growth and *iutA* expression in an iron-limited condition. *V. vulnificus* RC146 with the merodiploid P_{iutA}*::lacZ*$_{Ec}$ transcription reporter, which was grown overnight at 25°C, was cultured in an iron-limited HI broths containing 100 μM dipyridyl (DP) for 12 h at 25 (●), 30 (Δ) or 37°C (■). Other figure legends are the same as described in Figure 1.

5. Effect of TC on *crp* expression

The effect of TC on the *crp* expression level was determined by culturing the P_{crp}::$lacZ_{Ec}$ transcription reporter RC242 strain in an iron-limited HI broth containing 100 μM DP. TC stimulated the growth of RC242 during early growth phase, but not during late growth phase (Fig. 7A). In β-galactosidase assay, TC significantly increased the *crp* transcription levels on a per-cell basis (Fig. 7B). On Western blot analysis, TC also slightly increased the CRP protein levels on a per-cell basis, which was measured 6 h after culture initiation (Fig. 7C).

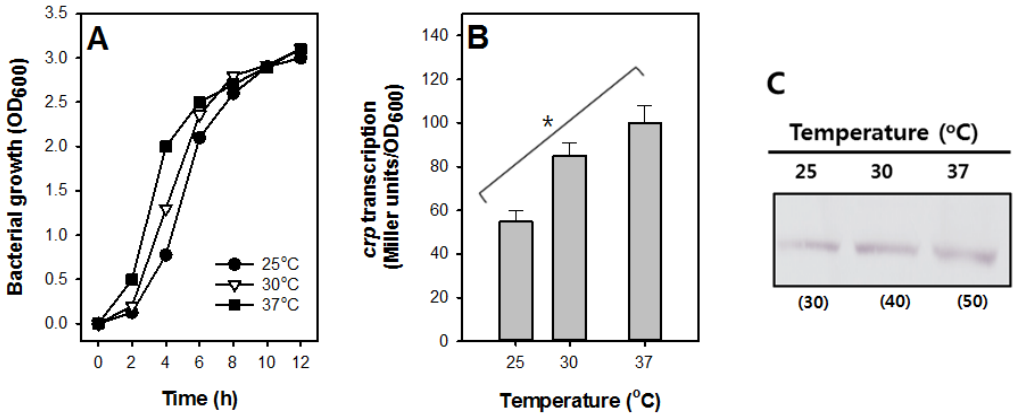

Figure 7. Effect of temperature change on *V. vulnificus* growth and *crp* expression in an iron-limited condition. *V. vulnificus* RC242 with the merodiploid P_{crp}*::lacZ*$_{Ec}$ transcription reporter, which was grown overnight at 25°C, was cultured in an iron-limited HI broth containing 100 μM dipyridyl for 12 h at 25 (●), 30 (Δ) or 37°C (■). Other figure legends are the same as described in Figure 1, except that rabbit polyclonal anti-CRP antibody was used for Western blot.

6. Effect of TC on *smcR* expression

The effect of TC on the *smcR* expression level was determined by culturing the P_{smcR}*::lacZ*$_{Ec}$ transcription reporter RC196 strain in iron-limited HI broth containing 100 μM DP. TC stimulated the growth of RC196 during early growth phase, but not during late growth phase (Fig. 8A). In β-galactosidase assay, TC significantly increased the *smcR* transcription levels on a per-cell basis (Fig. 8B). On Western blot analysis, TC also increased the SmcR protein levels on a per-cell basis, which was measured 6 h after culture initiation (Fig. 8C).

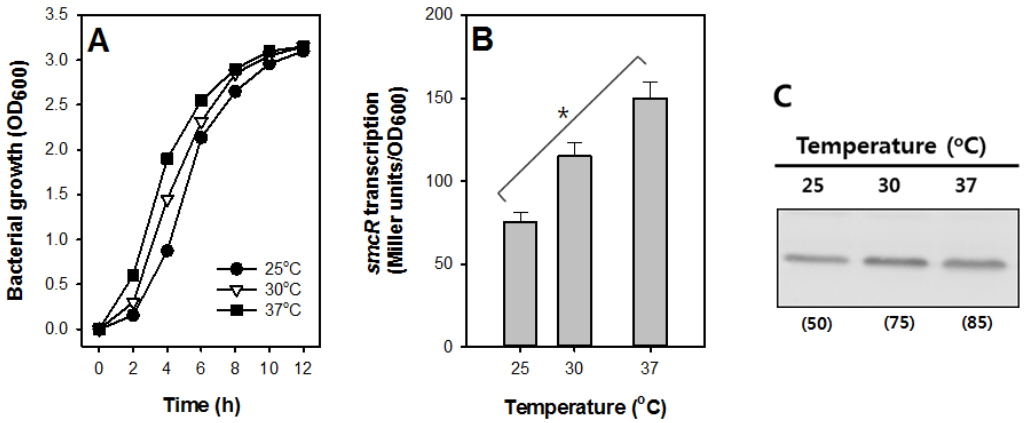

Figure 8. Effect of temperature change on *V. vulnificus* growth and *smcR* expression in an iron-limited condition. *V. vulnificus* RC196 with the merodiploid P_{smcR}*::lacZ*$_{Ec}$ transcription reporter, which was grown overnight at 25°C, was cultured in an iron-limited HI broth containing 100 μM dipyridyl for 12 h at 25 (●), 30 (Δ) or 37°C (■). Other figure legends are the same as described in Figure 1, except that rabbit polyclonal anti-CRP antibody was used for Western blot.

7. Effect of CRP on the TC-mediated increase of *iutA* expression

The effect of TC on *iutA* expression in the absence of CRP was determined by culturing the *iutA* the P_{iutA}*::lacZ*$_{Ec}$ transcription reporter RC148 strain with mutated *crp* in an iron-limited HI broth containing 100 μM DP at 25, 30 or 37°C for 12 h. The *crp* mutation severely decreased the growth and *iutA* expression levels in the iron-limited condition, comparing with those levels in RC146 with wild-type *crp* as shown in Fig. 7. TC increased the growth levels of RC148 especially during early growth phase, but not during late growth phase (Fig. 9A). In β-galactosidase assay, the *iutA* transcription levels in RC148 were very low. Accordingly, whether TC increased the *iutA* transcription levels in RC148 or not was not obvious (Fig. 9B). However, on Western blot analysis, the IutA protein levels in RC148 were able to be discriminated. TC obviously increased the IutA protein levels albeit at low levels even in RC148 (Fig. 9C).

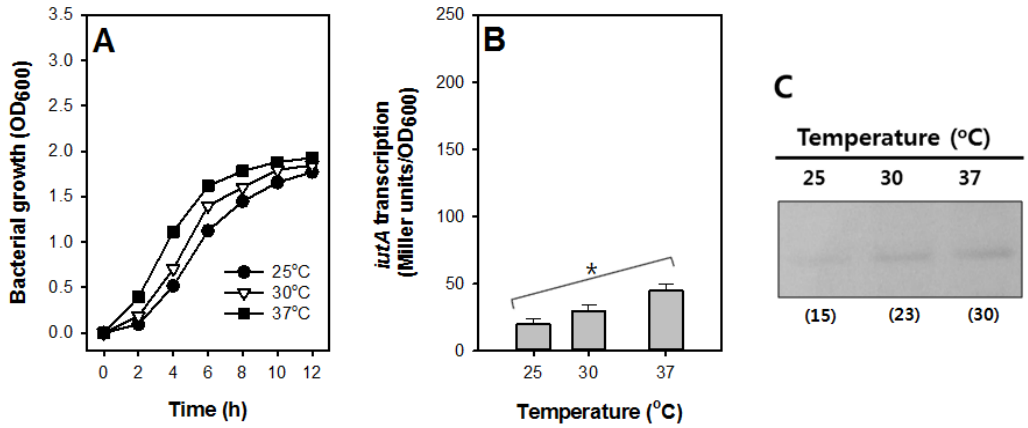

Figure 9. Effect of a *crp* mutation on the TC-mediated increase of *iutA* expression in an iron-limited condition. The P_{iutA}*::lacZ*$_{Ec}$ transcription reporter RC148 strain with mutated *crp*, which was grown in HI broth overnight at 25°C, was inoculated into and cultured in an iron-limited HI broth containing 100 μM dipyridyl at 25 (●), 30 (Δ) and 37°C (■) for 12 h. Other figure legends are the same as described in Figure 1.

8. Effect of SmcR on the TC-mediated increase of *iutA* expression

The effect of TC on *iutA* expression in the absence of SmcR was determined by measuring the *iutA* transcription levels in the P_{iutA}::$lacZ_{Ec}$ transcription reporter RC170 with mutated *smcR* in an iron-limited HI broth containing 100 μM DP at 25, 30 or 37°C for 12 h. The *smcR* mutation had no significant effect on the growth and *iutA* expression levels in the iron-limited condition, comparing with those levels in RC146 with wild-type *crp* as shown in Fig. 7. TC increased the growth levels of RC170 during early growth phase, but not during late growth phase (Fig. 10A). In β-galactosidase assay, TC significantly increased the *iutA* transcription levels in RC170 (Fig. 10B). On Western blot analysis, TC also increased the IutA protein levels in RC170 (Fig. 10C).

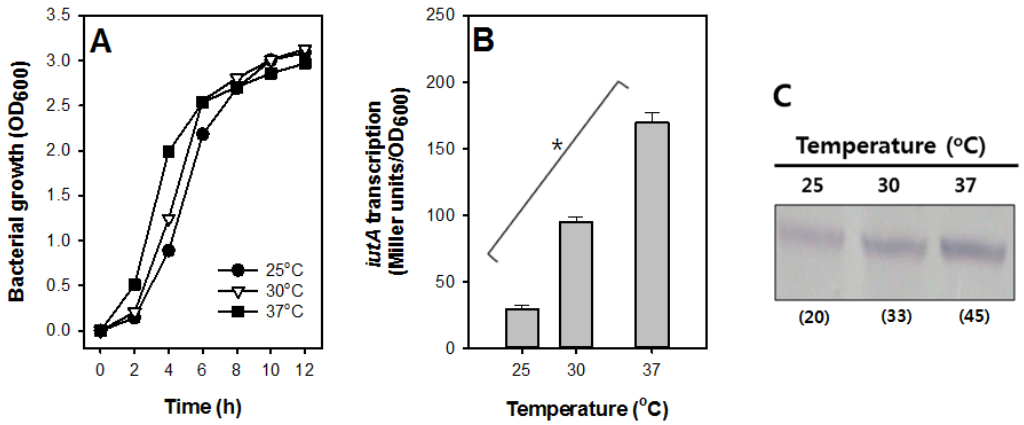

Figure 10. Effect of a *smcR* mutation on the TC-mediated increase of *iutA* expression in an iron-limited condition. The P_{iutA}*::lacZ*$_{Ec}$ transcription reporter RC196 strain with mutated *smcR*, which was grown in HI broth overnight at 25°C, was inoculated into and cultured in an iron-limited HI broth containing 100 μM dipyridyl at 25 (●), 30 (Δ) and 37°C (■) for 12 h. Other figure legends are the same as described in Figure 1.

Discussion

Most bacteria have adapted to iron-limited conditions such as *in vivo* by producing their own siderophores (iron-chelators) (Andrews *et al*., 2003). In addition, some bacteria can assimilate iron by utilizing heterologous siderophores, which are produced by other unrelated bacterial or fungal species (Schubert *et al*., 1999; Sebulsky *et al*., 2000; Lesic *et al*., 2002). This siderophore piracy is probably highly advantageous to bacteria because it allows them to evade bacteriostatic effects caused by heterologous siderophores without synthesizing their own or homologous 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 (Desferal®) (Kim *et al*., 2007), and the IutA-mediated IUS, which uses *E. coli* aerobactin (Kim *et al*., 2012a). *V. vulnificus* is a well-known ferrophilic bacterium, which requires more available iron than other pathogenic bacteria (Kim *et al*., 2007). Accordingly, the presence of these two piratical systems is likely to be highly advantageous for the survival and proliferation of ferrophilic *V. vulnificus*, especially in environments with mixed bacterial populations. *V. vulnificus* possesses multiple IUSs. However, the activities of the IUSs are relatively low, compared with those of other pathogenic bacteria. Accordingly, iron-overloaded mice are used as the most susceptible experimental animals and patients with elevated available iron levels, such as liver diseases and hemosiderosis, are the susceptible persons for *V. vulnificus* (Wright *et al*., 1981; Starks *et al*., 2000).

This study presented that iron limitation inhibited *V. vulnificus* growth, but induced *iutA* expression at the transcription level. This finding clearly indicates that iron limitation is

a primary signal for *iutA* expression as it is well known (Kim *et al*., 2012a; Tanabe *et al*., 2005). Moreover, this study revealed that a *fur* mutation de-repressed *iutA* expression at the transcription level in iron-sufficient conditions, but not in iron-limited conditions. This finding indicates that Fur is a primary local regulator which negatively controls for *iutA* expression in proportion to intracellular iron level, preventing iron overload which is rather toxic to bacterial cells (Litwin & Calderwood, 1993; Kim *et al*., 2012a). In iron-sufficient conditions, iron binds to Fur as a cognate cofactor, and the resulting Fur-iron complex (holo-Fur) binds to the consensus sequences in the promoter regions of the iron-regulated genes called Fur box, repressing the transcription of the genes by preventing the progress of RNA polymerase. In iron-limited conditions, iron does not bind to Fur and Apo-Fur cannot bind the Fur box and prevent the progress of RNA polymerase.

This study showed that *iutA* expression was induced at considerable levels by DP-induced iron limitation alone in the absence of aerobactin. This finding suggests that the cognate siderophore (or inducer) may be produced or that the IutA-mediated IUS may play an innate role in the iron assimilation of *V. vulnificus* in the absence of aerobactin. However, the cognate siderophore has not been identified yet, and the innate role played by the IutA-mediated IUS has not been determined yet. This study also revealed that *iutA* expression was induced at higher levels in the presence of aerobactin than in its absence. This finding indicates that the presence of aerobactin under iron-limited conditions can synergistically co-activate *iutA* expression. A previous study demonstrated that the response of *V. vulnificus* 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 (Tanabe *et al*., 2005). 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.

CRP is a well-known global regulator that controls the expression of various genes including genes associated with carbohydrate (glucose) metabolism. A primary signal for *crp* expression is glucose depletion (Ishizuka *et al*., 1993). Iron is also required for glucose metabolism or energy production, which is essential for bacterial growth. A previous study showed that iron limitation decreased *crp* expression at the transcription level, inhibiting *V. vulnificus* growth (Yoon, 2013). This finding implies that iron limitation represses *crp* expression by inhibiting metabolism and reducing the requirement of glucose for energy production; that is, iron eventually affects *crp* expression. It is likely that iron is required for optimal or coordinated *crp* expression. Conversely, CRP is needed for optimal *iutA* expression, as well as *vuuA* and *hupA* expressions. This study showed that a *crp* mutation severely decreased *iutA* expression at the transcription level. Previous studies using different methods also showed that *iutA*, *vuuA* and *hupA* expressions were under the positive control of CRP (Choi *et al*., 2006; Oh *et al*., 2009; Kim *et al*., 2012a; Kim *et al*., 2012b). Furthermore, this study showed that a mutation of *cya* encoding cAMP synthetase also severely decreased *iutA* expression and the exogenous addition of cAMP dose-dependently recovered *iutA* expression in the background with the *cya* mutation. Overall, these findings indicate that the cAMP-CRP complex is a major positive regulator for *iutA* expression. However, *iutA* expression was evidently observed albeit at very low levels in the absence of CRP, and induced at high levels in the presence of CRP. These findings indicate that CRP is required for metabolically coordinated or optimal *iutA* expression, such that the acquired iron in cells is used for metabolism in the most efficient way. Multiple functional interac-

tions are known to exist between CRP and Fur or iron (Zhang *et al*., 2005; Martinez-Antonio *et al*., 2008). CRP directly modulates *fur* expression at the transcription level in *E. coli* (De Lorenzo *et al*., 1998). In *E. coli*, CRP is also involved in the transcriptional regulation of genes associated with iron uptake (Zhang *et al*., 2005).

SmcR as a master regulator of the LuxS-mediated quorum sensing system is a well-known global regulator that controls the expression of various genes in *V. vulnificus* (Jeong *et al*., 2003; Lee *et al*., 2007a;). A primary signal for *smcR* expression is bacterial population density (Jeong *et al*., 2003; Lee *et al*., 2007a). Accordingly, it is likely that the *smcR* expression levels reflect the *V. vulnificus* growth levels. A previous study showed that iron or CRP positively affected *smcR* expression by stimulating *V. vulnificus* growth (Kim *et al*., 2012c). Conversely, a previous study reported that a *luxS* mutation had a minor effect on *iutA* expression (Kim and Shin, 2011b). However, this study showed that a *smcR* mutation had no significant effect on *iutA* expression at the transcription and IutA protein levels. Overall, these controversial findings indicate that SmcR, if any, acts as a minor or auxiliary regulator for *iutA* expression.

In this study, TC, particularly from 25^{o}C to 37^{o}C, increased *iutA*, *crp* and *smcR* expressions at the transcription and protein levels, stimulating *V. vulnificus* growth under an iron-limited condition. The reason why the TC-mediated increase of *V. vulnificus* is observed only during early growth phase, but not during late growth phase, seems to be because nutrients are depleted during late growth phase. These findings indicate that TC is a signal for *iutA*, *crp* and *smcR* expressions. The growth stimulation of *V. vulnificus* is a final outcome of increased metabolism and energy production. Both iron and glucose are essential for efficient metabolism and energy production, as described above. Accordingly, it is

likely that the TC-mediated increase of metabolism increases the requirement for glucose, inducing *crp* expression, and then the resulting increase of CRP increases *iutA* expression, and that the TC-mediated growth stimulation induces *smcR* expression by increasing bacterial density. However, the possibility that TC may first and directly increase *iutA* or *crp* expression in a yet-undefined way, and the resulting increase of intracellular iron or CRP may increase metabolism and facilitate *smcR* expression and *V. vulnificus* growth cannot be excluded.

The most interesting findings in this study were that TC induced an increase in *iutA* expression even in the absence of CRP or SmcR. These findings imply that TC can increase *iutA* expression independently of CRP or SmcR under iron-limited conditions; that is, TC acts as a new signal for *iutA* expression. The TC-mediated changes of gene expressions have been studied extensively in *Shigella* species (Konkel and Tilly, 2000). *Shigella* infection is spread via the fecal-oral route, and is commonly acquired by drinking water contaminated with human fecal material. TC increases the expression of virulence genes in *Shigella* species, including *ipa* (invasion plasmid antigen), *spa* (surface presentation antigens) and *mxi* (membrane expression of invasion) operons. Changes in DNA supercoiling and the transcription repressor H-NS (VirR) are likely to play important roles in the TC-mediated changes of virulence gene expression. VirR represses transcription of *virB* and *virF* at 30^{o}C, but de-represses at 37^{o}C. VirF positively regulates *virB* transcription and VirB acts as a positive regulator for transcription of the *ipa*, *mxi*, and *spa* operons. TC also activates the VirF-dependent *virB* promoter by changing DNA supercoiling. Although there is no experimental evidence, similar mechanisms may also be involved in the TC-mediated change of *iutA*, *crp* and *smcR* expressions in *V. vulnificus*.

Overall, it is likely that, entering the human body, *V. vulnificus* modulates the expression of some virulence factors, including *iutA* expression, for survival and for successful infection by sensing and responding to TC. Virulence expression is a survival strategy of pathogenic bacteria and an intimate functional link exists between metabolic and virulence expression. Metabolic adaptation is a prerequisite for virulence expression when a pathogen encounters a new environment (Rohmer *et al*., 2011). The TC-mediated increase of *iutA*, *crp* and *smcR* expressions can further change the expression of other virulence factors. Indeed, the expression of several virulence factors is under the control of CRP and SmcR and influenced by intracellular iron levels in *V. vulnificus*. CRP directly regulates the expression of VvhBA and VvpE (Choi *et al*., 2002; Jeong *et al*., 2003), and positively or negatively affects the expression of other virulence factors, including RTX toxin production, capsule formation, and flagella-mediated motility (Kim *et al*., 2005; Kim *et al*., 2013). SmcR is also involved in the regulation of virulence factors (Lee *et al*., 2007a). In addition, iron increases the expression or production of virulence factors including VvhBA and VvpE, stimulating growth of *V. vulnificus* (Kim *et al*., 2009; Kim *et al*., 2012).

In summary, iron limitation is a primary signal for iutA expression and Fur is a principal local regulator that controls *iutA* expression in response to iron levels. The presence of aerobactin is a signal for inducing *iutA* exression in an iron-limited condition. CRP is a positive coordinate regulator that is involved in optimal *iutA* expression in response to metabolism. SmcR, if any, acts as a minor or auxiliary regulator for *iutA* expression. TC increased *iutA*, *crp* and *smcR* expressions along with stimulating *V. vulnificus* growth in an iron-limited condition. Moreover, TC could induce *iutA* expression even in the absence of CRP or SmcR. Accordingly, it is likely that TC acts as a signal for *iutA* expression as well

as *crp* and *smcR* expressions, and that CRP or SmcR is not essential for the TC-mediated change of *iutA* expression.

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Abstract

Temperature Change Modulates the Expression of *iutA* Encoding Aerobactin Receptor in *Vibrio vulnificus*

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Vibrio vulnificus acquires iron as an essential element via multiple iron uptake systems (IUSs), which are the well-known virulence factors. Of them, the aerobactin receptor (IutA)-mediated IUS plays an important role in siderophore piracy. The ferric iron regulator (Fur) or iron-regulated *iutA* expression is also controlled by cyclic AMP-receptor protein (CRP) and the LuxS-mediated quorum sensing system (a master regulator SmcR). Upon entering the human body, *Vibrio vulnificus*, a marine bacterium, must withstand temperature change from 25^{o}C to 37^{o}C (TC). In this study, the author examined the effect of TC on *iutA, crp* and *smcR* expressions and the effect of CRP or SmcR on the TC-mediated change of *iutA* expression in an iron-limited condition to determine whether TC is a signal for *iutA* expression. The LacZ-fused transcription reporter assay were used for determining gene transcription levels and Western blot for comparing protein levels. Iron limitation increased *iutA* expression, along with inhibiting *V. vulnificus* growth. In an iron-sufficient condition, *iutA* expression was repressed and the repression was de-repressed by a *fur* mutation. The presence of aerobactin induced *iutA* exression at higher levels in an iron-limited condition. A *crp* mutation severely decreased *iutA* expression but a *smcR* mutation did not in an iron-limited condition. TC increased *iutA*, *crp* and *smcR* expressions along with stim-

ulating *V. vulnificus* growth in an iron-limited condition. Moreover, TC induced *iutA* expression even in the absence of CRP or SmcR. These results indicate that TC acts as a signal for *iutA* expression as well as *crp* and *smcR* expressions, and that CRP or SmcR is not essential for the TC-mediated change of *iutA* expression.

Key words: *Vibrio vulnificus*, Temperature, Iron, Ferric uptake regulator, Aerobactin receptor, Cyclic AMP-receptor protein, Quorum sensing system