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Iron-mediated regulation of metalloprotease VvpE Production in *Vibrio vulnificus*

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SUMMARY

In *Vibrio vulnificus,* the production of metalloprotease VvpE is controlled by Crp (cAMP-receptor protein) and SmcR (a quorum sensing regulator) at the transcription level, and by PilD-mediated type II general secretion system (TTGSS) at the extracellular secretion level. Iron is known to stimulate VvpE production but the related mechanisms remain unidentified. Iron stimulated *vvpE* transcription and extracellular VvpE production even in the background with a *crp* and/or *smcR* mutation. Iron stimulated the transcription of *pilD* encoding an essential element of TTGSS. Therefore, iron seems to stimulate *vvpE* transcription through factor(s) other than Crp and SmcR, and to facilitate extracellular VvpE production by increasing the activity of TTGSS.

KEY WORDS: Vibrio vulnificus, Iron, Metalloprotease, Quorum sensing, Cyclic AMP-receptor protein.

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INTRODUCTION

Vibrio vulnificus, a gram-negative halophilic bacterium, causes fatal septicemia or necrotizing wound infections in susceptible patients, particularly those with underlying hepatic diseases. A wide variety of virulence factors, including acid neutralization, capsular polysaccharide expression, iron acquisition, cytotoxicity, motility and expression of proteins associated with attachment and adhesion, are involved in the pathogenesis of *V. vulnificus* (Jones and Oliver, 2009). The bacterium has developed mechanisms which allow it to withstand a variety of environmental changes (Jones and Oliver, 2009). In particular, *V. vulnificus* is capable of sensing and responding to diverse environmental changes by modulating the production of the metalloprotease VvpE (Shao and Hor, 2001; Kawase *et al.*, 2004). Although the pathogenetic role of VvpE in *V. vulnificus* infections remains enigmatic (Shao and Hor, 2000), VvpE production or *vvpE* expression is useful in studying how *V. vulnificus* senses, integrates, and responds to several environmental signals. It is the best known phenotype directly regulated by the three global regulators, a carbon availability-responsive global regulator cyclic AMP-receptor protein (Crp), a master regulator of quorum sensing system SmcR, and a master regulator of general stress response RpoS (Jeong *et al.,* 2003). Crp and SmcR are simultaneously required and cooperatively function in RpoS-dependent *vvpE* expression (Jeong *et al.,* 2003; Kim and Shin, 2010). In addition, extracellular VvpE production is controlled by the type II general secretion system, which is mediated by PilD, a type IV leader peptidase/N-methyltransferase (Paranjpye *et al.,* 1998; Park *et al.,* 2008).

V. vulnificus is a ferrophilic bacterium that requires higher levels of available iron for growth initiation than do other pathogenic bacteria (Kim *et al.,* 2007). Elevation of serum or tissue iron levels is one of the best-known predisposing

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host factors of *V. vulnificus* septicemia (Wright *et al.,* 1981; Starks *et al.,* 2000). In addition, iron facilitates the extracellular production of hemolysin VvhA by increasing the activity of the PilD-mediated type II general secretion system, although it decreases *vvhA* transcription via the ferric uptake regulator Fur, which functions as a transcriptional repressor (Kim *et al.,* 2009). Moreover, iron is also known to stimulate VvpE production (Shin *et al.,* 2005; Kim *et al.,* 2006; Sun *et al.,* 2006), but the related mechanisms remain unidentified. Accordingly, we first investigated whether Crp, SmcR, or the type II general secretion system is involved in the iron-mediated regulation of VvpE production.

The bacterial strains, plasmids and primers used in this study are listed in Table 1. Luria-Bertani medium (BD, Franklin Lakes, NJ, USA) and Thiosulfate-Citrate-Bile-Sucrose medium (TCBS, BD) with or without appropriate antibiotics (BD) were used for selection and subculture of recombinant strains. Heart Infusion (HI; BD) agar or broth containing an additional 2.0% NaCl was used as the basal medium for cultivation of *V. vulnificus* strains. HI broth was deferrated using 8-hydroxyquinoline, as described previously (Leong and Neilands, 1982). The residual iron concentration of deferrated HI broths was 1.0 g/dL or less, which was measured by the method described by Stookey (1970). This iron concentration could not support *V. vulnificus* growth; thus, various concentrations of ferric chloride (FC) were added to deferrated HI broths to support *V. vulnificus* growth. Unless otherwise stated, all other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

To adapt *V. vulnificus* strains to iron-restricted conditions and to reduce intracellular iron storage, they were preconditioned by culturing for 12 h in HI broth containing 100 μM dipyridyl, an iron-chelator. The preconditioned strains were inoculated into test broths at approximately $1x10^6$ cells/ml, and cultured with vigorous shaking at 37°C. At appropriate times, culture aliquots were withdrawn to measure bacterial growth and gene expression levels. Bacterial growth levels were measured by the optical density of the culture aliquots at a wavelength of 600 nm (OD_{600}), and gene expression levels were determined on a per-cell basis by measuring β-galactosidase activity in the culture aliquots as previously described (Miller, 1992). The computer program SigmaStat for Window (version 1.0) was used for statistical analysis (Jandel Scientific, San Rafael, CA, USA).

VvpE production levels were determined by the Western blot method using rabbit polyclonal anti-VvpE antibody (Park *et al.,* 2008). In brief, culture aliquots were centrifuged to obtain culture

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

Strains	*Relative characteristics*	*Sources*
M06-24/O	Clinical isolate, highly virulent	Reddy *et al.*, 1992
CMM2101	M06-24/O with a $lacZ_{Vv}$ mutation	Kim *et al.*, 2003
CMM2106	CMM2101 with a P_{vvpE}: $lacZ_{Ec}$ transcriptional fusion	Kim *et al.*, 2003
RC220	CMM2106 with a *crp* mutation	Kim & Shin, 2010
RC230	RC220 with an *in trans crp* complementation	Kim & Shin, 2010
RC164	CMM2106 with a *smcR* mutation	Kim & Shin, 2010
RC234	RC164 with an *in trans smcR* mutation	Kim & Shin, 2010
RC272	CMM2106 with an *crp* and *smcR* double mutation	Kim & Shin, 2010
RC274	RC272 with an *in trans crp* complementation	Kim & Shin, 2010
RC276	RC272 with an *in trans smcR* complementation	Kim & Shin, 2010
RC104	M06-24/O with a *pilD* mutation	Park *et al.*, 2008
RC108	RC104 with an *in trans pilD* complementation	Park *et al.*, 2008
RC176	CMM2101 with merozygotic P_{pilD}: $lacZ_{Ec}$ fusion	Kim *et al.*, 2009

Vv and *Ec* stand for *Vibrio vulnificus* and *Escherichia coli*, respectively.

supernatants. Equivalent volumes of culture supernatants adjusted to their original OD_{600} values were electrophoresed with 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels after denaturation by heating and -mercaptoethanol. Following electrophoresis, proteins were transferred to PROTRAN® nitrocellulose membranes (Whatman GmbH, Germany). After incubation with blocking solution at 4ºC overnight, the membranes were reacted with rabbit polyclonal anti-VvpE antibody (1:1,000 diluted in washing buffer) as the primary antibody. After washing, the membranes were reacted with anti-rabbit-IgG antibody conjugated with alkaline phosphatase (1:15,000 diluted in washing buffer) as the secondary antibody. After washing, the membranes were finally visualized with 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium substrate solution.

The effects of iron on extracellular VvpE production and *vvpE* transcription were determined in deferrated HI broths containing various FC concentrations. At 10 µM or less, FC stimulated the growth of M06-24/O in a dose-dependent manner (Figure 1A). At 10 µM or more, FC had no effect on the growth of M06-24/O. Extracellular VvpE production was determined in culture supernatants obtained 12 h after cul-

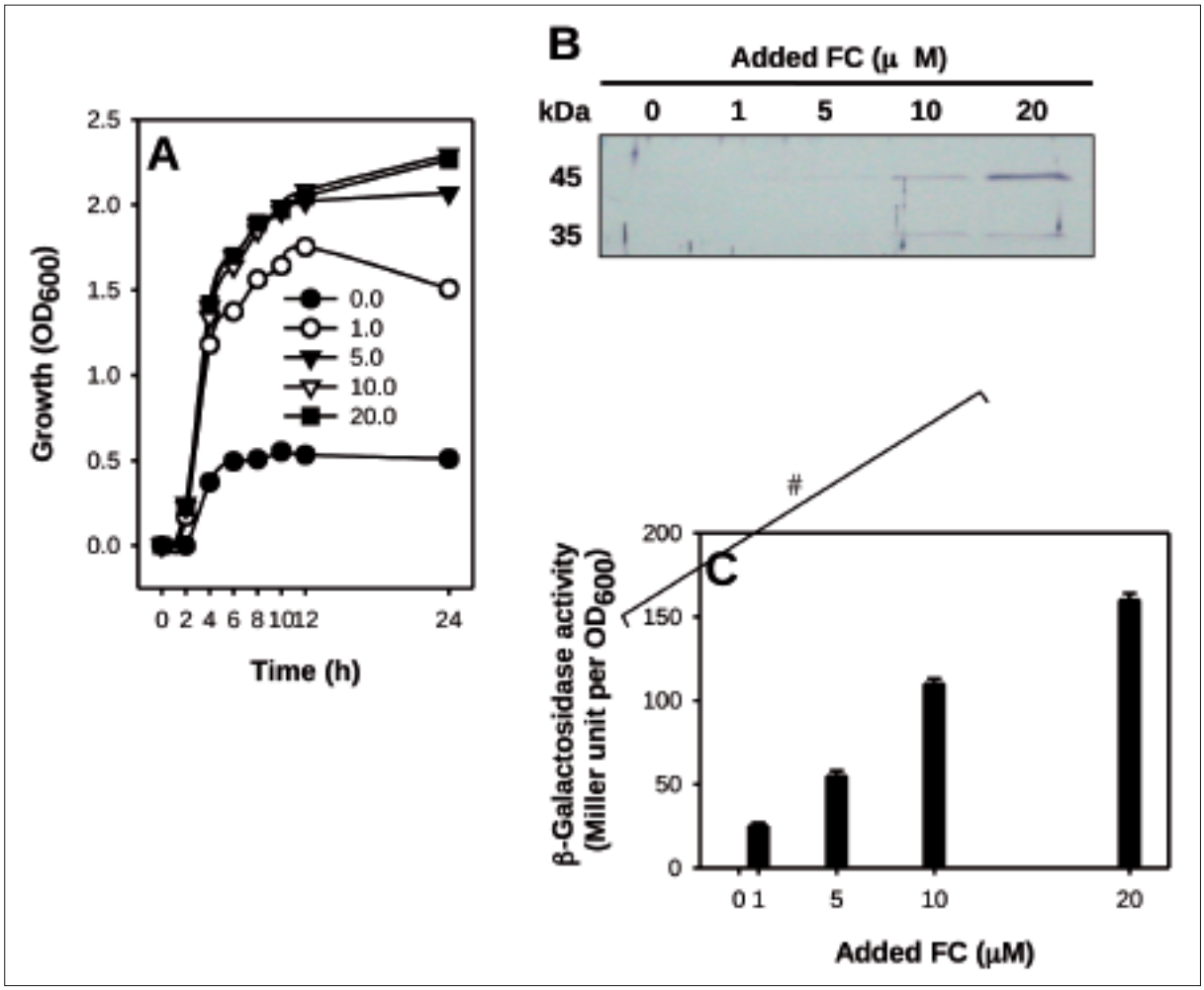

FIGURE 1 - *Effect of iron on Vibrio vulnificus growth (A), extracellular VvpE production (B) and vvpE transcription (C). (A and B) V. vulnificus M06-24/O was used and (C) V. vulnificus CMM2106 with the P_{vvpE}::lacZ transcriptional fusion was used. The numbers indicate the concentration of $FeCl_3$ (FC; µM) and the # symbol stands for $p<0.05$ in one-way RM ANOVA.*

ture initiation. FC increased extracellular VvpE production in a dose-dependent manner (Figure 1B). FC increased the production of both 45 kDa-VvpE and 35 kDa-VvpE and, of the two forms, the 45 kDa-VvpE was dominantly produced. Under the same condition, the level of *vvpE* transcription was monitored using the CMM2106 with the P_{vvpE}:*lacZ* transcription fusion (Figure 1C). FC dose-dependently increased the *vvpE* transcription level on a per-cell basis ($p<0.05$). These results indicate that iron increases *vvpE* transcription and extracellular VvpE production. Our previous studies also showed that iron increases *vvpE* transcription and extracellular VvpE production regardless of iron sources (Shin *et al.,* 2005; Kim *et al.,* 2006; Sun *et al.,* 2006). Iron stimulates *V. vulnificus* growth, suggesting that iron-mediated growth stimulation may in turn increase *vvpE* transcription. This supposition may be misjudged based on our previous results which showed that glucose stimulated *V. vulnificus* growth but decreased *vvpE* transcription in the absence of both Crp and SmcR (Kim and Shin, 2010). However, iron may provide a signal different from glucose for *vvpE* transcription, e.g. iron increases *vvpE* transcription but glucose decreases *vvpE* transcription. Accordingly, iron-mediated growth stimulation may have a different effect on *vvpE* transcription compared to glucose-mediated growth stimulation. The supplemen-

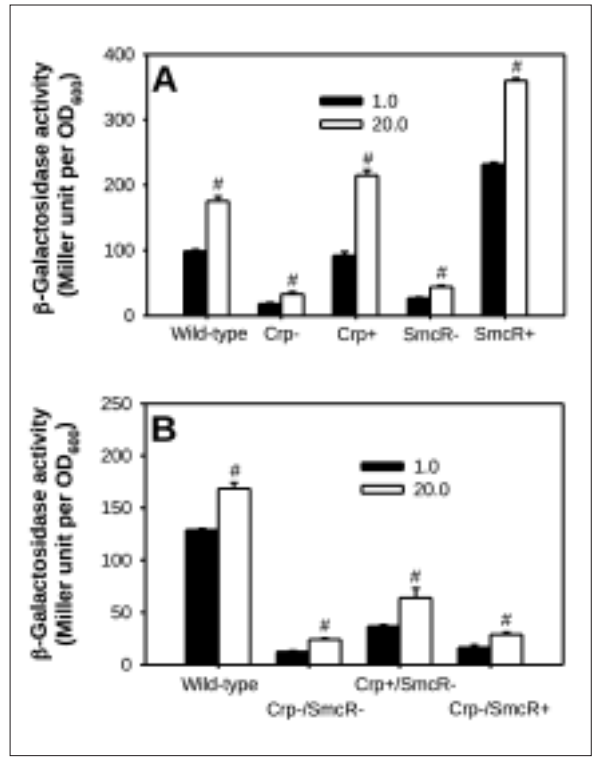

FIGURE 2 - *Effect of a crp or smcR single mutation (A) and a crp-smcR double mutation (B) on the iron-mediated regulation of vvpE transcription. V. vulnificus CMM2106 (wild-type), RC220 (Crp-), RC230 (Crp+), RC164 (SmcR-), RC234 (SmcR+), RC272 (Crp-/SmcR-), RC274 (Crp+/SmcR-) and RC76 (Crp-/SmcR+) were used. The numbers indicate the concentration of $FeCl_3$ (μM) and the # symbol $p<0.05$ in Student's t-test.*

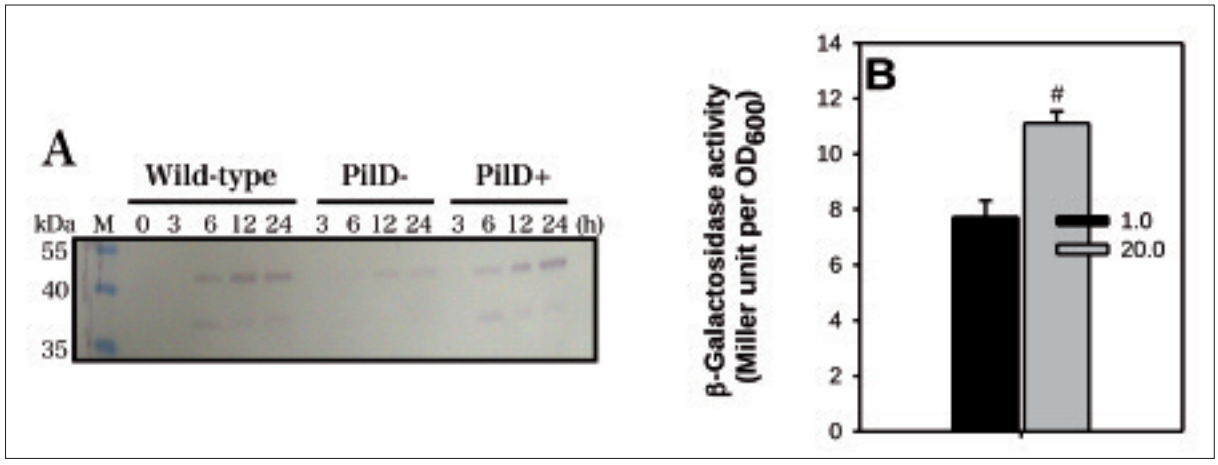

FIGURE 3 - *Effect of a pilD mutation on extracellular VvpE production (A) and effect of iron on pilD transcription (B). (A) V. vulnificus M06-24/O (wild-type), RC104 (PilD-) and RC108 (PilD+) were used. The standard molecular weight markers (M) are shown on the left side. (B) V. vulnificus RC176 strain with the P_{pilD}::lacZ transcriptional fusion was used. The numbers indicate the concentration of $FeCl_3$ (μM) and the # symbol $p<0.05$ in Student's t-test.*

tation of iron with no addition of other energy sources including glucose may increase metabolic or oxidative stress in the absence of Crp and/or SmcR. The increased stress may secondarily affect other factors including RpoS, which may subsequently affect *vvpE* transcription. Recently, RpoS has been reported to play an important role in the response of *V. vulnificus* to different environmental stresses (Lee *et al.,* 2003; Park *et al.,* 2004; Rosche *et al.,* 2005).

The effect of a *crp* and/or *smcR* mutation on the iron-mediated regulation of *vvpE* transcription was determined in deferrated HI broths containing 1.0 or 20.0 μM FC. In CMM2106, the *vvpE* expression level was approximately 1.8-fold higher at 20.0 μM FC than at 1.0 M FC (Figure 2A). A *crp* mutation (RC220) had no significant effect on the iron-mediated modulation of *vvpE* transcription (*ca*. 1.8-fold) although it severely decreased *vvpE* transcription levels. The decreased *vvpE* transcription levels were recovered by an *in trans crp* complementation (RC230). An *smcR* mutation (RC164) also had no significant effect on the iron-mediated modulation of *vvpE* transcription (*ca*. 1.6-fold) although it severely decreased *vvpE* transcription levels. The decreased *vvpE* transcription levels were recovered by an *in trans smcR* complementation (RC234). Moreover, a *crp* and *smcR* double mutation (RC272) had no effect on the iron-mediated modulation of *vvpE* transcription (*ca*. 1.9-fold) although it severely decreased *vvpE* transcription levels (Figure 2B). The decreased *vvpE* transcription levels were partially recovered by an *in trans crp* single complementation (RC274), but not at all by an *in trans smcR* single complementation (RC276). These results indicate that iron increases *vvpE* transcription regardless of the presence or absence of Crp and/or SmcR. Crp and SmcR are known to synergistically coactivate RpoS-dependent *vvpE* transcription (Jeong *et al.,* 2003). In addition, we recently found that Crp functions as an essential activator for *vvpE* transcription, whereas SmcR functions as a modulator capable of responding to increasing bacterial density, synergistically cooperating with Crp for full *vvpE* transcription (Kim and Shin, 2010). In this study, iron slightly increased both *crp* transcription and *smcR* transcription (data not shown), suggesting that iron may increase intracellular Crp and SmcR levels, which may subsequently affect *vvpE* transcription. However, this suggestion is refuted by the results that neither a *crp* or *smcR* mutation nor a *crp-smcR* double mutation affected the iron-mediated regulation of *vvpE* transcription. Conversely, a possible alternate suggestion is that iron increases *vvpE* transcription probably via factors other than Crp and SmcR.

The effect of a *pilD* mutation on extracellular VvpE production was determined in deferrated HI broths containing 1.0 or 20.0 M FC. Extracellular VvpE production was observed only at 20.0 M FC (Figure 3A) but not at 1.0 M FC (data not shown). In M06-24/O, extracellular VvpE production began to be observed 6 h after culture initiation and increased thereafter. A *pilD* mutation delayed extracellular VvpE production and decreased the level of extracellular VvpE production. This decreased and delayed extracellular VvpE production was recovered by an *in trans pilD* complementation. The effect of iron on *pilD* expression was evaluated under the same conditions (Figure 3B). The *pilD* expression level was significantly higher at 20.0 M FC than at 1.0 M FC ($p<0.05$). These results indicate that iron can facilitate extracellular VvpE production by increasing the activity of PilD-mediated type II general secretion system. Our previous study revealed that iron facilitates the secretion of another exotoxin, VvhA, by increasing *pilD* expression (Kim *et al.*, 2009). This study confirms that VvpE is secreted via PilD-mediated type II general secretion system, and iron increases *pilD* expression. Accordingly, iron is likely to facilitate extracellular VvpE production by increasing *pilD* expression. The related mechanism might be similar to that responsible for iron-mediated increases in *vvpE* transcription.

In conclusion, iron is likely to increase *vvpE* expression through factor(s) other than Crp and SmcR, and to facilitate extracellular VvpE production by increasing the activity of type II general secretion system.

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