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Iron-mediated Regulation of Metalloprotease VvpE Production in *Vibrio vulnificus*

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단백분해효소 VvpE 의 생산 조절

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이 논문을 의학 박사학위신청 논문으로 제출함

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패혈증 비브리오균은 일종의 호철성 세균(ferrophilic bacterium)으로 다른 병원성 세균과 비교하여 증식을 위해 더 많은 가용철을 필요로 한다. 혈청 또는 조직 내 철농도의 상승은 가장 위험한 숙주인자로서 패혈증 비브리오 감염의 병인에 매우 중요한 역할을 담당한다. 패혈증 비브리오균은 잠정적인 독력인자 중 하나인 단백분해효소(zinc metalloprotease: VvpE)를 생산한다. 정제된 단백분해효소는 다양한 생물학적 활성을 나타내지만 비브리오 패혈증 병인론에서 VvpE가 구체적으로 어떤 역할을 하는지는 규명되어 있지 않다. VvpE의 생산은 유전자 전사수준에서는 catabolite repression에 관여하는 전사인자인 cyclic AMP-receptor protein (Crp)와 autoinducer-2에 의한 quorum sensing system의 전사인자인 SmcR에 의해 조절되며, 세포외 분비단계에서는 type II general secretion system에 의해 조절된다. 최근 연구에 의해 철이 VvpE의 생산을 촉진한다고 알려져 있으나 기전은 밝혀져 있지 않다. 본 연구에서는 그 기전을 규명해 나아가고자 Crp, SmcR와 type II general secretion system이 철에 의해 매개된 VvpE 생산 조절에 관여하는지를 검증하고자 하였다. 철은 용량의존적으로 *vvpE* 유전

자의 전사를 촉진하였고 세포외 VvpE 생산을 증가시켰다. 또한 철은 *crp* 유전자와 *smcR* 유전자의 전사를 증가시켰다. *vvpE* 유전자의 전사는 *crp* 유전자나 *smcR* 유전자에 단일 돌연변이를 유발하거나 두 유전자에 동시에 돌연변이를 유발하였을 경우에 감소하였다. 그러나 이런 돌연변이 유발상태에서도 여전히 철에 의해 *vvpE* 유전자의 전사가 촉진되었다. Type II general secretion system의 필수 요소 중 하나인 PilD 효소를 암호화하고 있는 *pilD* 유전자에 돌연변이를 유발하였을 경우 세포외 VvpE의 세포외 생산이 감소하였고 철은 *pilD* 유전자의 전사를 촉진하였다. 이러한 결과는 철이 Crp와 SmcR 외 다른 인자를 통해 *vvpE* 유전자의 전사를 촉진하며 Type II general secretion system의 활성을 증가시킴으로써 세포외 VvpE 생산을 촉진하는 것을 나타내 준다.

Introduction

1. Restricted iron-availability within the human body

Iron is an essential element for all living things including microorganisms. Iron is rapidly oxidized from Fe^{2+} to Fe^{3+} and exists as insoluble oxyhydroxide (FeOOH) polymers in oxygen-containing environments at neutral pH. In these circumstances, the level of freely available iron is approximately 10^{-18} M. This restricted availability by unusual redox chemistry has various effects on many biological systems, including survival and proliferation of bacterial pathogens in natural environments [Weinberg,1978; Bagg and Neilands, 1987; Neilands, 1991].

Iron availability within the human body is also very low despite large amounts being present. Most iron is sequestered within cells especially red blood cells (RBC). Iron of 99% over indeed exists as hemoglobin, myoglobin or ferritin-hemosiderin within cells. Only a small portion of iron exists in extracellular body fluids such as plasma and mucosal secretions. Moreover, most extracellular iron is tightly bound to the high-affinity iron-binding glycoproteins such as transferrin and lactoferrin, resulting in little free iron being available. During infection, the amount of free available extracellular iron is reduced further by release of lactoferrin from neutrophils and transfer of transferrin-bound iron to intracellular storage in ferritin molecules. Accordingly, the level of freely available iron within the human body is too low to sustain microbial growth [Weinberg,1978; Bagg and Neilands, 1987; Neilands, 1991].

2. Bacterial iron-uptake systems

Microorganisms have adapted to iron-limited environments by developing their specific iron-uptake systems. To satisfy their needs for iron, microorganisms must sense and respond to the environmental cue of restricted iron availability by de-repressing their high-affinity iron-uptake systems.

Bacterial high-affinity iron-uptake systems can be briefly classified into the two ones. First, some bacteria can directly bind host iron-binding proteins including transferrin, lactoferrin and various heme proteins via their corresponding specific receptors [Gray-Owen and Schryvers, 1996; Conrad et al., 1999; Ekins et al., 2004]. For example, *Neisseria* and *Haemophilus* species can utilize only human transferrin and lactoferrin. Some bacteria including *Neisseria* and *Vibrio* species can directly bind hemoglobin via heme-specific receptors [Litwin and Byrne, 1998; Mey and Payne, 2001; Perkins-Balding et al., 2003; Cornelissen, 2003; Oakhill et al., 2005]. Mutations of the genes encoding these receptors suppress *in vivo* bacterial growth and attenuate virulence, which strongly suggests that these iron-uptake systems can be attractive vaccine targets [Rokbi et al., 1997; Myers et al., 1998; Brown et al., 2001; Kuboniwa et al., 2001].

Second, most bacteria acquire (steal) host iron by secreting small molecules called siderophores that can bind iron with a higher affinity than host iron-binding proteins, particularly transferrin and lactoferrin. Except *Neisseria* and *Haemophilus* species, most bacteria produce siderophores with low molecular weight (600-1500 kDa). Siderophores

chelate Fe^{3+} with high-affinity, and then the Fe^{3+}-siderophore complexes are imported back into microbial cells via specific cell-surface receptors [Lankford, 1973; Neilands, 1981; Neilands, 1993; Wooldridge and Williams, 1993]. Many bacteria are capable of producing more than one types of siderophore. In addition, some bacteria are capable of utilizing heterologous (exogenous) siderophores produced by other bacterial or fungal species, and this strategy is called siderophore piracy [Griffiths and Williams, 1999; Tanabe et al., 2005 a and b]. Although the contribution of siderophores to virulence varies among pathogens, mutations of the genes related to the siderophore-mediated iron uptake systems generally suppress *in vivo* bacterial growth and attenuate virulence. This fact strongly suggests that the siderophore-mediated iron-uptake systems can also be attractive vaccine targets.

Extensive studies have been conducted on the siderophore-mediated iron-uptake systems, especially in gram-negative bacteria. The Fe^{3+}-siderophore complexes formed in extracellular milieu are actively transported into the periplasmic space through iron-repressible outer membrane receptors using energy. The outer membrane receptor proteins are responsible for the recognition, binding, and transport into the periplasmic space of ferric-siderophore. Once in the periplasmic space, the periplasmic binding proteins recognize and bind the ferric-siderophore complexes and transfer the complexes to the ATP-binding cassette (ABC) transporters in the cytoplasmic membrane, and then the ATP transporters transfer the complexes into the cytoplasm utilizing energy. After the

ferric-siderophore complexes have reached the cytoplasm, the soluble form of iron (Fe^{2+}) is released from the siderophores probably by either reduction via ferric reductases or by chemical modification or breakdown of ferric siderophore complexes [Neilands, 1993; Braun et al., 1998; Andrews et al., 2003; Massė and Arguin, 2005]. Because excess intracellular iron is toxic due to its tendency to catalyze free radical generation, bacterial iron-uptake systems are tightly regulated via ferric uptake regulator or repressor (Fur) by the intracellular iron level. The Fur represses the expression of the genes associated high affinity iron-uptake systems under iron-sufficient conditions [Escolar et al., 1999].

3. Iron and *V. vulnificus* infections

Vibrio vulnificus is a gram-negative halophilic estuarine bacterium that causes necrotizing wound infection and septicemia. These *V. vulnificus* infections are closely associated with the consumption of raw seafood or the exposure to seawater contaminated with the bacterium in patients with underlying hepatic disease, a heavy alcohol-drinking habit, or another immunocompromised condition. Rapidly progressing *V. vulnificus* infections exhibit a high mortality rate of over 50%, and most of patients with *V. vulnificus* infections die of multi-organ failure within 1 to 2 days after hospitalization despite of aggressive shock and antibiotic therapies [Strom and Paranjpye, 2000; Gulig et al., 2005; Jones and Oliver, 2009].

Several virulence factors such as lipopolysaccharide, capsular polysaccharide, RTX

toxin, hemolysin/cytolysin (VvhA), metalloprotease (VvpE), iron-assimilation systems, flagella or motility, several proteins associated with attachment and adhesion, and so on, have been suggested to be involved in the pathogenesis of *V. vulnificus* infections [Strom and Paranjpye, 2000; Gulig et al., 2005; Jones and Oliver, 2009].

It has been well known that increased iron-availability plays important roles in the pathogenesis of *V. vulnificus* infections. Accumulated experimental data clearly show that the increased iron-availability is directly correlated with increased host susceptibility to *V. vulnificus* [Wright et al., 1981; Brennt et al., 1991.; Hor et al., 1998 and 2000; Starks et al., 2000]. Ferric ammonium citrate treatment can lower the LD_{50} of mouse from approximately 10^6 CFU to 10^0 CFU, and the increased mouse lethality to *V. vulnificus* directly correlates with the serum iron levels elevated following liver damage by injection of CCl_4. Actually, iron-overloaded mice has been widely used as the most susceptible animal model to *V. vulnificus*.

Like other pathogens, *V. vulnificus* also possesses multiple iron-uptake systems as described below. Nevertheless, the fact that *V. vulnificus* preferentially infects patients with elevated serum iron levels indicates that *V. vulnificus* is a ferrophilic bacterium that requires more iron for growth and survival than other pathogens [Kim et al., 2007]. Interestingly, this ferrophilic characteristic of *V. vulnificus* suggests the possibility that its iron-uptake systems would be more effective candidates for the development of vaccine and new chemotherapeutic agents than the iron-uptake systems of other pathogens.

4. *V. vulnificus* iron-uptake systems

1) Siderophore-mediated iron-uptake systems

(1) Vulnibactin-mediated iron-uptake system

V. vulnificus produces catechol (or phenolate) siderophore (also called vulnibactin) in iron-deficient conditions [Simpson and Oliver, 1983]. Generally speaking, virulent isolates of *V. vulnificus* produce vulnibactin, and efficiently utilize transferrin-bound iron, whereas avirulent isolates neither produce vulnibactin nor utilize transferrin-bound iron [Stelma et al., 1992]. Vulnibactin plays more important role in the growth stimulation of *V. vulnificus* under iron-restricted conditions than another siderophore hydroxamate siderophore [Shin et al., 2001]. The *ven* operon consists of the genes essentially required for vulnibactin synthesis [Litwin et al., 1996]. The mutation of the *venB* gene abolishes the ability to produce vulnibactin and to utilize transferrin-bound iron, and significantly attenuates the virulence of *V. vulnificus* in an infant mouse model. In addition, the mutation of the *vis* gene encoding *V. vulnificus* isochorismate synthase also abolishes the ability to produce vulnibactin and to utilize transferrin-bound iron [Kim et al., 2006]. Overall, these results clearly indicate that vulnibactin plays a very important role in iron-uptake from highly saturated transferrin and the virulence expression of *V. vulnificus*.

The *vuuA* gene encoding vulnibactin receptor was identified [Webster and Litwin, 2000]. VuuA, a 72 kDa iron-regulated outer membrane protein, was over-expressed in a *V. vulnificus fur* deletion mutant , and the mutation of the *vuuA* gene abolished the ability of

V. vulnificus to use Fe^{3+}-vulnibactin as an iron source [Litwin and Calderwood,1993].

(2) Hydroxamate siderophore-mediated iron-uptake system

The virulent *V. vulnificus* C7184 strain also produces hydroxamate siderophore with vulnibactin while avirulent *V. vulnificus* strain A1402 produced only hydroxamate siderophore in iron-deficient conditions [Simpson and Oliver, 1983]. However, neither the genes related to the hydroxamate siderophore-mediated iron-uptake system have been cloned, nor the structure of hydroxamate siderophore has been determined until now. Accordingly, the actual function of hydroxamate-siderophore in the virulence of *V. vulnificus* remains totally unknown.

(3) Iron uptake systems capable of utilizing heterologous siderophores

V. vulnificus is able to use deferoxamine or desferrioxamine B (Desferal®) as an exogenous siderophore to reverse iron limitation [Wright et al., 1981]. The 78-kDa iron-repressible outer membrane protein called DesA, which is encoded by the *desA* gene, is induced in iron-starved *V. vulnificus* cells only in the presence of exogenous deferoxamine [Aso et al., 2002]. Interestingly, the *desA* gene is expressed only in the presence of deferoxamine as an effecter molecule together with iron limitation. The AraC-like transcriptional regulator DesR is required for the expression of the *desA* gene in iron-starved cells [Tanabe et al., 2005b]. A recent study cautions that deferoxamine

therapy can be a risk factor for fatal *V. vulnificus* infections because the *desA* gene is widespread in most *V. vulnificus* environmental or clinical strains [Kim et al., 2007]. Other pathogens including *Staphylococcus aureus* and *Yersinia enterocolitica* are also known to be capable of utilizing deferoxamine for iron uptake [Kim and Shin, 2009; Diarra et al., 2002; Lesic et al., 2002].

In addition, *V. vulnificus* can utilize exogenously-supplemented aerobactin under iron-deficient conditions with enhanced production of the 76 kDa iron-repressible ferric-aerobactin receptor (named IutA). The expression of the *iutA* gene is also induced by the exogenous addition of aerobactin into iron-limited conditions, and negatively regulated by the GntR-like transcriptional repressor IutR [Tanabe et al., 2005a].

2) Heme receptor-mediated iron-uptake system

The *hupA* gene encodes an iron-regulated outer membrane protein with molecular mass of 77 kDa called HupA, and the *hupA* mutant failed to utilize haemin or hemoglobin as a source of iron [Litwin and Byrne, 1998]. HupR, a LysR homologue, acts as a positive regulator for the *hupA* gene in the presence of haemin under low-iron conditions [Litwin and Quackenbush, 2001]. A recent study, the contribution of HupA in virulence was also determined [Oh et al., 2009].

5. Iron-mediated increase of *V. vulnificus* virulence

1) Hemolysin or cytolysin VvhA

Serious doubts have been raised about the pathogenic significance of *V. vulnificus* hemolysin since it was demonstrated that the mutation of the *vvhBA* genes encoding *V. vulnificus* hemolysin had no effect on the virulence of *V. vulnificus* [Wright and Morris, 1991]. Nevertheless, it is undeniable that *V. vulnificus* hemolysin is produced *in vivo* albeit a very low level and can lyse human cells including RBC to facilitate the release of intracellular iron even at very low levels [Lee et al., 2004]. *V. vulnificus* is a ferrophilic bacterium that requires high levels of available iron for growth initiation and can utilize the intracellular iron more easily than transferrin-bound iron present in extracellular fluids although it possesses multiple iron-uptake systems, as described in the above. A recent study demonstrated that iron dose-dependently increases extracellular VvhA production via the type II general secretion system although it partially represses *vvhA* transcription via Fur [Kim et al., 2009]. In addition, *VvhA* can elevate available iron by facilitating the release of intracellular iron via hemolysis or cytolysis, it has sometimes been hypothesized as an alternative or additional component of iron-uptake systems [Martinez et al., 1990]. Accordingly, VvhA may be involved in the fulminant or rapid progression of *V. vulnificus* infections by the formation of a vicious cycle release that iron facilitates VvhA production, which subsequently increases iron-availability by releasing intracellular iron.

2) Metalloprotease

Serious doubts have been raised about the pathogenic significance of *V. vulnificus* VvpE since it was demonstrated that the mutation of the *vvpE* gene had no effect on the virulence of *V. vulnificus* [Jeong, 2000]. For long time, it had suggested that VvpE was required for efficient iron utilization from transferrin, lactoferrin and heme proteins via proteolytic cleavage of these proteins, making the bound iron more accessible to the vulnibactin-mediated iron-uptake system [Okujo et al., 1996]. It is evident that VvpE can destroy these iron-binding proteins. Accordingly, the role of VvpE in iron-uptake from various iron-binding proteins cannot be completely ruled out. Different growth phases of *V. vulnificus* can exist simultaneously *in vivo*; at the same time, some could already arrive at plateau, but others could begin to grow. The VvpE produced from *V. vulnificus* cells that already arrived at plateau can affect the iron uptake of *V. vulnificus* cells which begin to grow by destroying iron-binding proteins and releasing available iron from them. In contrast, more recent studies demonstrated that iron can rather facilitate VvpE production via *in vitro* experiments [Shin et al., 2005; Kim et al., 2006; Sun et al., 2006]. The related mechanism remains an open question. Taken together, VvpE may also be involved in the fulminant or rapid progression of *V. vulnificus* infections by forming a vicious cycle release that iron facilitates VvpE production, which subsequently increases iron-availability by destroying iron-withholding proteins.

6. Regulation of virulent factors in *V. vulnificus*

As in other microbial pathogens, the expression of virulence factors is also under the control of several global regulators in *V. vulnificus*. The *toxRS* genes of *V. vulnificus were identified*, and a *toxR* null mutant resulted in decreased expression of VvhA [Lee et al., 2000]. However, the mutant has not been determined for virulence.

RpoS, a well-known alternative sigma factor for RNA polymerase, is involved with gene expression under stationary phase, starvation, and stress conditions. An RpoS mutant was more sensitive to oxidative stress, osmotic and acid stress, and resulted in decreased metalloprotease VvpE production and motility [Hulsmann et al., 2003]. In addition, an RpoS mutant was susceptible to peroxide, starvation, UV light, and acid during exponential growth phase [Park et al., 2004]. None of these studies reported the effects of the *rpoS* mutation on virulence. A recent study identified two promoters expressing *vvpE*: a constitutive log phase (PL) and induced stationary (PS) promoter [Jeong et al., 2001]. The PS promoter was decreased in *crp* and *rpoS* mutants during stationary phase. In addition, RpoS is also necessary for full expression of Fur [Lee et al., 2003].

Catabolite repression mediated by cAMP and cAMP-receptor protein (Crp or also called catabolite-activated protein: CAP) is known to be involved in the virulence expression of *V. vulnificus* [Kim et al., 2005; Dhakal et al., 2006]. An adenylate cyclase (*cya*) mutation or *crp* mutation attenuated virulence of *V. vulnificus*. A *cya* or *crp*

mutation affects the ability of *V. vulnificus* to grow, the production of capsule, VvpE and VvhA, the swarming motility, the expression of the iron-uptake systems, the cytotoxicity, and the mouse lethality [Shin, 2009]. Therefore, the cAMP-CRP system is likely to play an essential role in the global regulation of *V. vulnificus* virulence.

Quorum sensing is defined as the cell density-dependent regulation of gene expression. *V. vulnificus* possesses a *luxS* gene [Kim et al., 2003], involved in the quorum sensing signal molecule (autoinducer-2) production, as well as the *smcR* (*luxR* homologue) gene encoding response regulator that senses the signal molecule [Jeong et al., 2003]. SmcR is required for full expression of VvpE by working synergistically with Crp and RpoS. In addition, a luxS or smcR mutation attenuates the virulence of *V. vulnificus* [Kim et al., 2003; Jeong et al., 2003].

Increased iron levels are also believed to be a factor that affects the virulence expression of *V. vulnificus.* As described in the above, iron is likely to play an important role in *vvhA* and *vvpE* expression. Moreover, iron stimulates *V. vulnificus* growth by affecting metabolism and energy production. Generally speaking, the ability of a pathogen to grow in harsh environments has a significant effect on its virulence [Kim et al., 2005; Choi et al., 2006; Dhakal et al., 2006].

8. The aim of this study

Like other bacteria, *V. vulnificus* is also exposed to a variety of environmental

changes, including temperature, salt concentration, and the supply of essential nutrients and oxygen. The bacterium has developed mechanisms which allow it to withstand these changes [Strom and Paranjpye, 2000; Gulig et al., 2005; Jones and Oliver, 2009]. In particular, *V. vulnificus* is capable of sensing and responding to diverse environmental changes by modulating VvpE production, as a final product [Shao and Hor, 2001; Kawase et al., 2004]. 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, Crp (a carbon availability-responsive global regulator), SmcR (a master regulator of quorum sensing system), and RpoS (a master regulator of general stress response) [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 host factors of *V. vulnificus* septicemia [Wright et al., 1981; Brennt et al., 1991.; Hor et al., 1998 and 2000; 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 also stimulates VvpE production [Shin et al., 2005; Kim et al., 2006; Sun et al., 2006]. However, the mechanisms involved in the iron-mediated regulation of VvpE production remain unidentified. Accordingly, in an attempt to explore the mechanisms involved, we first investigated whether Crp, SmcR, or the type II general secretion system is involved in the iron-mediated regulation of VvpE production.

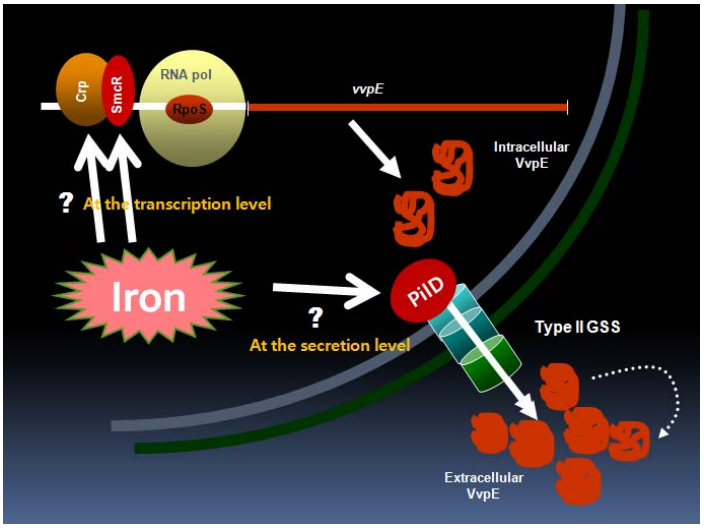

Figure 1. Schematic illustration for VvpE production in *Vibrio vulnificus*. Crp (cAMP-receptor protein) and SmcR (a master regulator of autoinducer-2-quorum sensing system) positively and synergistically regulate *vvpE* transcription by directly binding to the RpoS (sigma factor S)-dependent *vvpE* promoter. The mature VvpE with the molecular size of 45 and 34 kDa are secreted via the type II general secretion system, which is mediated by PilD, an enzyme essential for post-transcriptional protein modification.

Materials and Methods

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

The bacterial strains, plasmids and primers used in this study are listed in Table 1 and 2. Luria-Bertani medium (BD, Franklin Lakes, NJ, USA) and Thiosulfate-Citrate-Bile-Sucrose medium (BD) with or without appropriate antibiotics 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 μg/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. In addition, normal HI broth containing 100 μM of dipyridyl, an iron-chelator, was used only for the preconditioning of *V. vulnificus* strains. Antibiotics (BD) were used at the following concentrations: for *E. coli*, ampicillin 50 μg/mL, kanamycin 50 μg/mL, tetracycline 12.5 μg/mL, chloramphenicol 30 μg/mL; and for *V. vulnificus*, ampicillin 20 μg/mL, kanamycin 200 μg/mL, tetracycline 2 μg/mL, chloramphenicol 2 μg/mL. Unless otherwise stated, all other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2. Culture condition and measurement of bacterial growth and β-galactosidase activity

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. The preconditioned strains were inoculated into test broths at approximately 1 x 10^6 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).

3. Construction of transcriptional reporter strains

A merozygotic P_{crp}::*lacZ* transcriptional fusion was constructed. The 1,057-bp fragment containing the regulatory region of *crp* was amplified using the polymerase chain reaction (PCR) primers, crp-rep-F with a *Bgl*II overhang and crp-rep-R with a *Xba*I overhang. The amplified fragment was subcloned into pQF52 [Farinha and Kropinski, 1990]. From the resulting plasmid pRC182, the *Bgl*II-*Sca*I fragment containing the

P_{crp}::*lacZ* fragment was subsequently subcloned into pDM4 [McGee et al., 1996]. The resulting plasmid pRC184 was transformed into *E. coli* SY327 λ*pir* and SM10 λ*pir* [Miller and Mekalanos, 1988], and transferred to CMM2101 by conjugation. Eventually, a stable transconjugant (RC242) was selected on TCBS agar containing chloramphenicol. The presence of the P_{crp}::*lacZ* fusion in RC242 was confirmed by β-galactosidase assay [Miller, 1992]. The genetic organizations of the merozygotic P_{crp}::*lacZ* transcriptional fusion is shown in Figure 2A.

The same methodology was used to construct the merozygotic P_{smcR}::*lacZ* transcriptional fusion. The 926-bp *Bam*HI-*Hin*dIII fragment containing the regulatory region of *smcR* was amplified using the PCR primers, smcR-rep-F with *Bam*HI overhang and smcR-rep-R with *Hin*dIII overhang. The PCR product was then subcloned into pQF52 [Farinha and Kropinski, 1990]. From the resulting plasmid pRC162, the *Bam*HI-*Sca*I fragment containing the P_{smcR}::*lacZ* fragment was isolated and subcloned into pDM4 [McGee et al., 1996]. The resulting plasmid pC164 was transformed into *E. coli* SY327 λ*pir* and SM10 λ*pir* [Miller and Mekalanos, 1988], and transferred to CMM2101 by conjugation. Eventually, a transconjugant (RC196) was selected on TCBS agar containing chloramphenicol. The presence of the P_{smcR}::*lacZ* fusion in RC196 was confirmed by β-galactosidase assay [Miller, 1992]. The genetic organization of the merozygotic P_{crp}::*lacZ* transcriptional fusion is shown in Figure 2B.

V. vulnificus RC176 with a merozygotic P_{pilD}::*lacZ* fusion was constructed as

previously described [Kim et al., 2009]. The genetic organization of the merozygotic P_{pilD}::*lacZ* transcriptional fusion is shown in Figure 2C.

V. vulnificus CMM2106 with a P_{vvpE}::*lacZ* fusion was constructed as previously described [Kim et al., 2003]. The same methodology was used for the introduction of the P_{vvpE}::*lacZ* fusion into other *V. vulnificus* strains, and their genetic organizations are shown in Figure 3.

4. Deletional mutation and *in trans* complementation of *crp* or *smcR*

A deletion mutation of *crp* and a complementation of *crp* were previously described [Choi et al., 2006]. The same *crp* mutation (RC220) or complementation (RC230) was introduced into CMM2106 with a P_{vvpE}::*lacZ* fusion. The *crp* mutation or complementation in each strain was confirmed by polymerase chain reaction (PCR), as described previously.

A deletion mutation of *smcR* and a complementation of *smcR* were previously described [Kim and Shin, 2010]. The same *smcR* mutation (RC164) or complementation (RC276) was introduced into CMM2106 with a P_{vvpE}::*lacZ* fusion. The *smcR* mutation or complementation in each strain was confirmed by polymerase chain reaction (PCR), as described previously.

A deletion mutation of *pilD* (RC104) and a complementation of *pilD* (RC108) were previously described [Kim et al., 2009]. The *pilD* mutation or complementation was

confirmed by polymerase chain reaction (PCR), as described previously.

5. Western blot for VvpE

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 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 β-mercaptoethanol. Following electrophoresis, proteins were transferred to PROTRAN® nitrocellulose membranes (Whatman GmbH, Germany). After incubation with blocking solution at $4^{o}C$ 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 BCIP-NBT substrate solution.

6. Statistical analysis

Values are the means of three technical replicates per experiment, and each experiment was performed at least twice. Student's *t*-test or one-way RM ANOVA was used to determine the statistical significance of differences between means.

Table 1. Bacterial strains used in this study

Strains	Relative characteristic	Source
M06-24/O	Clinical isolate, highly virulent	Reddy et al., 1992
CMM2101	M06-24/O with a $lacZ_{Vv}$ mutation	Kim et al., 2003
RC242	CMM2101 with a merozygotic P_{crp}::$lacZ_{Ec}$ transcriptional fusion	This study
RC196	CMM2101 with a merozygotic P_{smcR}::$lacZ_{Ec}$ transcriptional fusion	This study
CMM2106	CMM2101 with a P_{vvpE}::$lacZ_{Ec}$ transcriptional fusion	Kim et al., 2003
RC220	CMM2106 with a *crp* mutation	Kim and Shin, 2010
RC230	RC220 with an *in trans crp* complementation	Kim and Shin, 2010
RC164	CMM2106 with a *smcR* mutation	Kim and Shin, 2010
RC234	RC164 with an *in trans smcR* mutation	Kim and Shin, 2010
RC272	CMM2106 with an *crp* and *smcR* double mutation	Kim and Shin, 2010
RC274	RC272 with an *in trans crp* complementation	Kim and Shin, 2010
RC276	RC272 with an *in trans smcR* complementation	Kim and Shin, 2010
RC104	M06-24/O with a *pilD* mutation	Kim et al., 2009
RC108	RC104 with an *in trans pilD* complementation	Kim et al., 2009
RC176	CMM2101 with merozygotic P_{pilD}::$lacZ_{Ec}$ fusion	Kim et al., 2009
SY327 λ*pir*	Host for suicide vector	Miller and Mekalanos, 1988
SM10 λ*pir*	Conjugation donor	Miller and Mekalanos, 1988

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

Table 2. Plasmids and primers used in this study

Plasmids and primers	Relative characteristic or sequence	Source
pDM4	Suicide vector with R6K origin; Cm^R	McGee et al., 1996
pQF52	IncP lacZ transcriptional fusion vector; Ap^R	Farinha and Kropinski, 1990
pRC182	The 1,057-bp *Bgl*II-*Xba*I fragment containing the *crp* promoter region cloned into pQF52	This study
pRC184	The *Bgl*II-*Sca*I fragment containing P_{crp}::*lacZ* fragment from pRC182 cloned into *Bgl*II-*Sma*I cut pDM4	This study
pRC162	The 926-bp *Bam*HI-*Hin*dIII fragment containing the *smcR* promoter region cloned into pQF52	This study
pRC164	The *Bam*HI-*Sca*I fragment containing the P_{smcR}::*lacZ* fragment from pRC162 cloned into the *Bgl*II-*Sma*I-cut pDM4	This study
crp-rep-F	5'-ga**AGATCT**ccaacgatttcccagctctagg-3'	This study
crp-rep-R	5'-gc**TCTAGA**atccttaattagtaccgccacag-3'	This study
smcR-rep-F	5'-cg**GGATCC**caaagccaatccacttcactgg-3'	This study
smcR-rep-R	5'-ccc**AAGCTT**gccacgacgagcaaacacttcc-3'	This study

-Cm^R and Ap^R indicate chloramphenicol-resistance and ampicillin-resistance, respectively

-Capital bold letters indicate the restriction enzyme-recognition sequences: AGATCT (*Bgl*II), TCTAGA (*Xba*I), GGATCC (*Bam*HI), AAGCTT (*Hin*dIII).

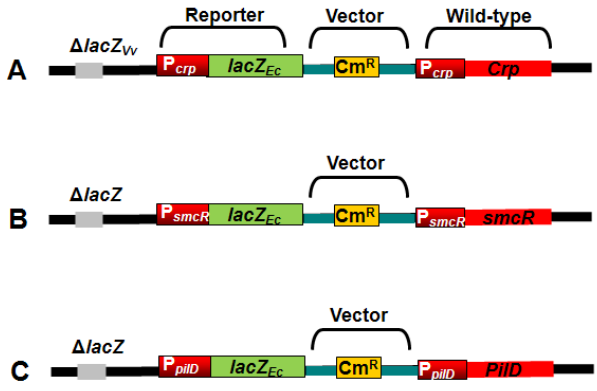

Figure 2. Schematic gene organization of chromosomal *crp* or *smcR* transcriptional reporter. RC242 with a merozygotic P_{crp}*::lacZ* transcriptional fusion (A), RC196 with a merozygotic P_{smcR}*::lacZ* transcriptional fusion (B) and RC176 with a merozygotic P_{pilD}*::lacZ* transcriptional fusion (C). Vv: *Vibrio vulnificus*, Ec: *Escherichia coli*. Δ: deletional mutation, P: promoter , Cm^R: chloramphenicol resistance gene.

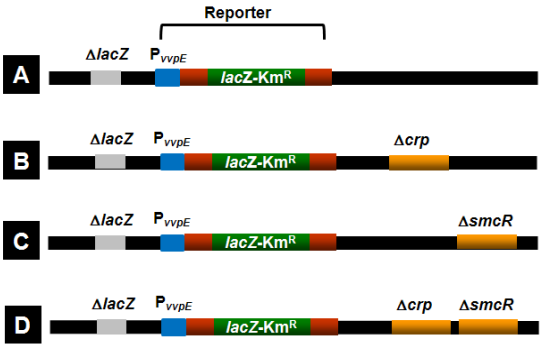

Figure 3. Schematic gene organization of chromosomal *vvpE* transcriptional reporters. CMM2106 with the P_{vvpE}*::lacZ* transcriptional fusion (A), RC220 with the *luxS*-deleted P_{vvpE}*::lacZ* transcriptional fusion (B), RC164 with the *smcR*-deleted P_{vvpE}*::lacZ* transcriptional fusion (C), and RC272 with the *crp*- and *smcR*-deleted P_{vvpE}*::lacZ* transcriptional fusion. Δ: deletional mutation , Km^R: kanamycin resistance gene.

Results

1. Effects of iron on *vvpE* transcription and extracellular VvpE production

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 culture 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. 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.

2. Effect of iron on *crp* or *smcR* expression

The effect of iron on *crp* or *smcR* transcription was observed in deferrated HI broths containing 1.0 or 20.0 μM FC (Figure 2). In RC242 with the P_{crp}*::lacZ* transcriptional fusion, the *crp* transcription level was slightly higher (*ca*. 1.2-fold) at 20.0 μM FC than at

1.0 μM FC ($p<0.05$). Similarly, the *smcR* transcription level in RC196 with the P_{smcR}*::lacZ* transcriptional fusion was also slightly higher (*ca*. 1.3-fold) at 20.0 μM FC than at 1.0 μM FC ($p<0.05$). These results indicate that iron increases *crp* or *smcR* expression to some degree.

3. Effect of a *crp* or *smcR* mutation on the iron-mediated regulation of *vvpE* transcription

The effect of a *crp* 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 (Figure 3). In CMM2106, the *vvpE* expression level was approximately 1.8-fold higher at 20.0 μM FC than at 1.0 μM FC. A *crp* mutation (RC220) severely decreased *vvpE* transcription levels with no effect on the iron-mediated modulation of *vvpE* transcription (*ca*. 1.8-fold). The decreased *vvpE* transcription levels were recovered by an *in trans crp* complementation (RC230). A *smcR* mutation (RC164) also severely decreased *vvpE* transcription levels with no effect on the iron-mediated modulation of *vvpE* transcription (*ca*. 1.6-fold). The decreased *vvpE* transcription levels were recovered by an *in trans smcR* complementation (RC234). These results indicate that iron increases *vvpE* transcription even in the absence of Crp or SmcR.

4. Effect of a *crp* and *smcR* double mutation on the iron-mediated

modulation of *vvpE* transcription

The effect of a *crp* and *smcR* double mutation on the iron-mediated modulation of *vvpE* expression was determined in deferrated HI broths containing 1.0 or 20.0 μM FC (Figure 5). Compared with those in CMM2106, *vvpE* transcription levels were severely decreased by a *crp* and *smcR* double mutation (RC272) regardless of FC concentration. Nevertheless, the *vvpE* transcription level was still approximately 1.9-fold higher at 20.0 μM FC than at 1.0 μM FC ($p<0.05$). An *in trans crp* single complementation (RC274) partially recovered the *vvpE* transcription levels, but did not affect the iron-mediated modulation of *vvpE* transcription. An *in trans smcR* single complementation (RC276) affected neither the *vvpE* transcription levels nor the iron-mediated modulation of *vvpE* transcription. These results indicate that iron increases *vvpE* transcription even in the absence of both Crp and SmcR.

5. Effect of iron on the activity of type II general secretion system

The effect of a *pilD* mutation on extracellular VvpE production was determined in deferrated HI broths containing 1.0 or 20.0 μM FC. Extracellular VvpE production was observed only at 20.0 μM FC (Figure 6A) but not at 1.0 μM 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. When the effect of iron on *pilD* expression was evaluated under the same conditions (Figure 6B), the *pilD* expression level was significantly higher at 20.0 μM FC than at 1.0 μM 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.

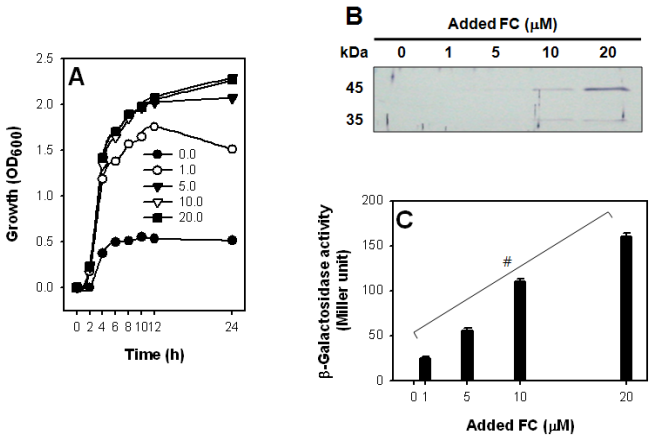

Figure 4. Effect of iron on *Vibrio vulnificus* growth (A), extracellular VvpE production (B) and *vvpE* transcription (C). (A and B) The preconditioned *V. vulnificus* M06-24/O strain was cultured in deferrated Heart Infusion broths containing various concentrations of $FeCl_3$ (FC). Culture aliquots were removed at the indicated times to monitor *V. vulnificus* growth, which was measured by OD_{600}. Culture aliquots removed at 12 h following culture initiation were centrifuged to obtain culture supernatants. Equivalent volumes of culture supernatants, adjusted to their original OD_{600} values, were electrophoresed and transferred to a membrane. Refer to the text for additional procedures. The two forms of VvpE (45 and 35 kDa) are shown. (C) The preconditioned CMM2106 strain was cultured under the same conditions. Culture aliquots were removed at 12 h following culture initiation to monitor the *vvpE* transcription level, which was expressed by accumulated β-galactosidase activity measured by the Miller method [23]. The # symbol stands for $p<0.05$ in one-way RM ANOVA.

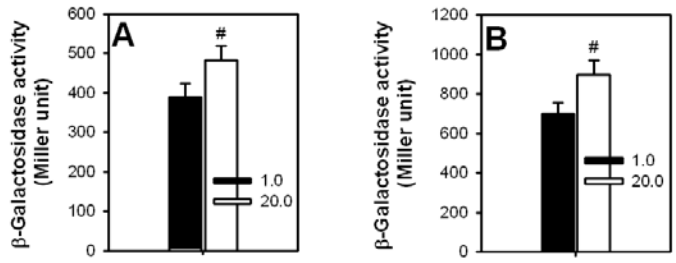

Figure 5. Effect of iron on *crp* (A) and *smcR* (B) transcription. The preconditioned *V. vulnificus* strains, RC242 (A) with a merozygotic P_{crp}*::lacZ* transcriptional fusion and RC196 (B) with a merozygotic P_{smcR}*::lacZ* transcriptional fusion, were cultured in deferrated Heart Infusion broths containing 1.0 and 20.0 μM $FeCl_3$. Culture aliquots were removed at 24 h following culture initiation. Bacterial growth was measured by OD_{600} and β-galactosidase activity was measured by the Miller method [23]. The # symbol stands for $p<0.05$ in Student's *t*-test.

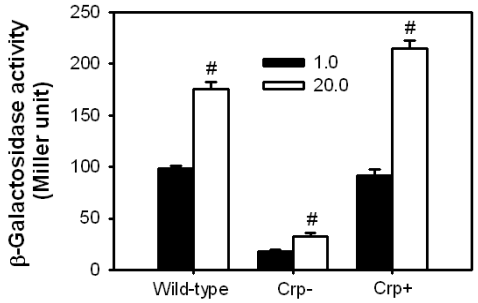

Figure 6. Effect of a *crp* mutation on the iron-mediated regulation of *vvpE* transcription. The preconditioned *V. vulnificus* CMM2106 (wild-type), RC220 (Crp-), and RC230 (Crp+) strains were cultured in deferrated Heart Infusion broths containing 1.0 and 20.0 μM $FeCl_3$. Refer to Figure 5 legend for additional procedure information.

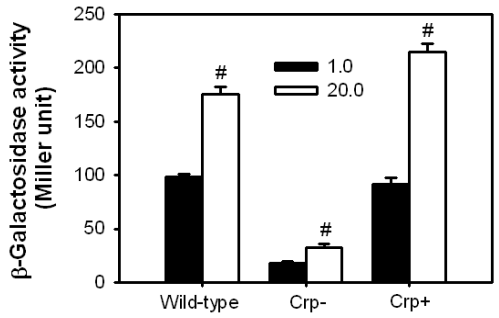

Figure 7. Effect of a *smcR* mutation on the iron-mediated regulation of *vvpE* transcription. The preconditioned *V. vulnificus* CMM2106, RC164 and RC234 strains were cultured in deferrated Heart Infusion broths containing 1.0 and 20.0 μM $FeCl_3$. Refer to Figure 5 legend for additional procedure information.

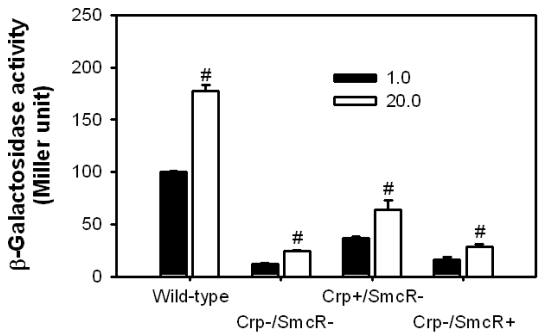

Figure 8. Effect of a *crp* and *smcR* double mutation on the iron-mediated regulation of *vvpE* transcription. The preconditioned *V. vulnificus* strains, CMM2106 (wild-type), RC272 (Crp-/SmcR-), RC274 (Crp+/SmcR-) and RC76 (Crp-/SmcR+), were cultured in deferrated Heart Infusion broths containing 1.0 and 20.0 μM $FeCl_3$. Refer to Figure 5 legend for additional procedure information.

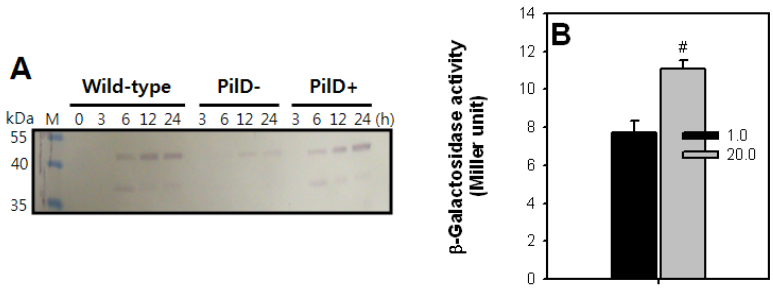

Figure 9. Effect of a *pilD* mutation on extracellular VvpE production (A) and effect of iron on *pilD* transcription (B). (A) The preconditioned *Vibrio vulnificus* M06-24/O (wild-type), RC104 (PilD-) and RC108 (PilD+) strains were cultured in deferrated Heart Infusion broths containing 20.0 μM $FeCl_3$. Culture aliquots removed at 24 h following culture initiation were centrifuged to obtain supernatants. Equivalent volumes of culture supernatants, adjusted to their original OD_{600} values, were electrophoresed and transferred to a membrane. Refer to the text for additional procedures. The standard molecular weight markers (M) are shown on the left side. (B) The preconditioned RC176 strain with the P_{pilD}*::lacZ* transcriptional fusion was cultured under the same conditions. Refer to Figure 5 legend for additional procedure information.

Discussion

Our present and previous studies consistently 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]. However, controversial findings have also been reported [Nishina et al., 1992; Simpson and Oliver, 1993; Okujo et al., 1996; Shao and Hor, 2001] in which VvpE production was required for efficient iron acquisition, or VvpE production could be stimulated by organic iron, but not inorganic iron.

This study presents an unexpected novel finding on the pattern of extracellular VvpE. It has been known that 45 kDa-VvpE is secreted, and 35 kDa-VvpE is generated from 45 kDa-VvpE by the autoproteolysis of the 10-kDa-C-terminal peptide after secretion [Miyoshi et al., 1997]. Our previously study revealed that both 35 kDa-VvpE and 45 kDa-VvpE are simultaneously produced via PilD-mediated type II general secretion system, and 35 kDa-VvpE is the dominant secreted form in the normal HI background [Park et al., 2008]. In contrast, this study shows that both 35 kDa-VvpE and 45 kDa-VvpE are simultaneously produced, but 45 kDa-VvpE is the dominant secreted form in the deferrated HI background. These findings suggest that the proteolytic cleavage of C-terminal peptide may be a controllable reaction rather than a spontaneous reaction which occurs within bacterial cell. However, the extracellular VvpE production pattern was not affected by the addition of iron into deferrated HI broths, suggesting that components or factors other than iron may be involved in the proteolytic cleavage of C-

terminal peptide. Other unexpected components or factors besides iron can also be removed or changed in the middle of deferration using 8-hydroxyquinoline [Leong and Neilands, 1982]. We are searching for these possible components.

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, 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.

Iron stimulates *V. vulnificus* growth, suggesting that iron-mediated growth stimulation may in turn increase *vvpE* transcription. This supposition may also 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 than 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 supplementation 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 [Park et al., 2004; Lee et al., 2003]. We are studying on whether RpoS is involved in the iron-mediated modulation of *vvpE* transcription.

V. vulnificus produces pilins and several exotoxins including VvpE through type II general secretion system, and the activity of type II general secretion system is affected by PilD, an essential component of type II general secretion system [Paranjpye et al., 1998; Park et al., 2008]. 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 may be similar to that responsible for iron-mediated increases in *vvpE* transcription.

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Abstract

Iron-mediated Regulation of Metalloprotease VvpE Production in *Vibrio vulnificus*

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In *Vibrio vulnificus*, the production of metalloprotease VvpE is controlled by cyclic AMP-receptor protein (Crp) and SmcR, the master regulator of the autoinducer-2-mediated quorum sensing system at the transcription level, and by the type II general secretion system at the extracellular secretion level. Iron is known to stimulate VvpE production, but the related mechanisms remain unidentified. This study was conducted to test whether Crp, SmcR, or type II general secretion system is involved in the iron-mediated modulation of VvpE production. Iron dose-dependently increased *vvpE* transcription and extracellular VvpE production together with increased *crp* and *smcR* transcription. Nevertheless, neither a *crp* and *smcR* double mutation nor a *crp* or *smcR* single mutation affected the iron-mediated modulation of *vvpE* transcription although all the mutations repressed *vvpE* expression. A mutation of *pilD* encoding an essential element of type II general secretion system decreased extracellular VvpE production.

Iron stimulated *pilD* transcription. These results indicate that iron increases *vvpE* expression through factor(s) other than Crp and SmcR, and facilitates extracellular VvpE production by increasing the activity of type II general secretion system.

Key words: *Vibrio vulnificus*, Iron, Metalloprotease, Quorum sensing, Cyclic AMP-receptor protein, Type II general secretion system