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박사학위논문

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Vibrio vulnificus Metalloprotease VvpE Has No Direct Effect on Iron-assimilation from Human Hemoglobin

2006년 8월 25일

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이 논문을 의학 박사학위신청 논문으로 제출함

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의 학 과

손 혜 옥

손혜옥의 박사학위 논문을 인준함

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2006년 6월 일

조선대학교 대학원

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사람 혈색소로부터 패혈증 비브리오균의 철획득에 미치는 단백분해효소의 영향

손 혜 옥

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조선대학교 대학원 의학과

본 연구는 패혈증 비브리오균이 사람혈색소로부터 철을 흡수하는 과정에서 단백분해효소에 의한 혈색소의 분해가 동반되어 철흡수가 촉진되는지를 알아보고자 하였다. Vulnibactin을 생산하지 못하는 돌연변이 균주와 heme 수용체를 발현하지 못하는 돌연변이 균주 모두 철공급원으로 혈색소를 첨가한 배지에서 증식이 억제되었다. 이러한 결과는 이 두 가지 철획득 기전 모두가 혈색소로부터 철을 획득하는데 중요한 역할을 담당하고 있음을 나타낸다. 패혈증 비브리오균이 생산하는 단백분해효소 중에서 VvpE는 사람혈색소를 분해할 수 있는 유일한 단백분해효소임에도 불구하고 철결핍배지에서의 VvpE의 발현은 전사수준과 단백수준 모두에서 억제되었다. 또한 전사수준에서 *vvpE* 유전자의 발현은 혈색소 또는 무기철을 철결핍배지에 공급하였을 때 재활성화되나 세포밖으로 VvpE 단백의 생산은 활성화되지 않으며, 전사수준에서 *vvpE* 유전자의 발현 역시 패혈증 비브리오균이 배지에 포함된 철을 이미 소모해 버린 증식 후기에만 일어났다. 더군다나, VvpE를 생산하지 못하는 돌연변이 균주와 이 균주에 다시 *vvpE* 유전자를 삽입하여 VvpE를 생산할 수 있게 보완한 균주 역시 혈색소를 철공급원으로 포함한 철결핍배지와 간경변 환자들에게서 얻은 복수에서 유의한 차이 없이 증식할 수 있었다. 철결핍배지에 첨가된 혈색소는 분해되지 않았고 배양 중

에 눈에 보이는 큰 덩어리를 형성하였으며 이러한 큰 덩어리는 VvpE를 생산하지 못하는 돌연변이 균주나 *vvpE*를 삽입한 보완 균주 모두에서 나타났다. 혈색소 또는 무기철은 *luxS* 유전자 발현을 촉진하였으나 *luxS* 유전자에 돌연변이를 유발시킨 균주에서는 *vvpE* 유전자 발현이 감소되지 않았다. 본 연구결과를 종합하면, 패혈증 비브리오균의 VvpE는 혈색소를 분해하여 철흡수를 촉진하지 않는다. 반대로, 혈색소 또는 무기철이 효과적인 *vvpE* 유전자의 발현을 위해 필요함을 알 수 있다. 그러나 혈색소나 무기철에 의한 *vvpE* 유전자의 발현은 LuxS quorum-sensing system이 아닌 다른 전사조절기전에 의해 조절되고 있으며, *vvpE* 유전자 발현과 단백수준에서 VvpE 생산 사이에도 아직 밝혀지지 않은 조절기전이 있음을 알 수 있다.

중심어: 패혈증 비브리오균, 단백분해효소, 혈색소, 철, Quorum-sensing

1. INTRODUCTION

Vibrio vulnificus is a gram-negative halophilic estuarine bacterium, which can cause fatal and rapidly-progressing septicemia with mortality of 50% or over. This *V. vulnificus* septicemia is closely associated with the consumption of raw seafood contaminated with the bacterium in patients with underlying hepatic diseases, heavy alcohol-drinking habits, or other immunocompromised conditions. Several putative virulence factors including the capsular polysaccharide capsule, lipopolysaccharide, exotoxins including hemolysin/cytolysin, protease and RTX toxin, phopholipase, motility, and iron-assimilation systems have been suggested, but only the capsule and the iron-assimilation systems *of V. vulnificus* have been confirmed to be authentic virulence factors, which is in accordance with the molecular version of Koch's postulates [1-3].

Host iron levels and bacterial iron-assimilation systems play crucial roles in the pathogenesis of *V. vulnificus* infection, which is promoted by elevated serum iron levels [4-7], and crucially requires the assimilation of iron from iron-binding proteins such as transferrin and hemoglobin (HG) by *V. vulnificus* [8-14]. The iron-assimilation systems of *V. vulnificus* can be classified into the two groups (Figure 1): three autologous or heterologous siderophore-mediated iron-assimilation systems [11-14], and one heme receptor-mediated iron-assimilation system [15-18]. The siderophore (especially vulnibactin)-mediated iron-assimilation system is known to play an essential role especially in iron assimilation from transferrin and mouse-lethality by *V. vulnificus*. The heme receptor-mediated iron-assimilation system is known to play an important role in iron assimilation from HG and mouse-lethality by *V. vulnificus*. Moreover, bacterial iron-uptake

systems are themselves virulence factors in many bacterial pathogens, and thus promising vaccine targets [19]. Also, iron-chelation is considered as a prospective therapeutic means of preventing *in vivo* bacterial growth [20]. In these regards, it is important that the roles of iron-assimilation mechanisms and their relating factors in *V. vulnificus* should be elucidated.

A metalloprotease (named VvpE) of *V. vulnificus* has been extensively studied, and is known to exert a variety of biological effects [1-3,21-26]. Purified VvpE induces hemorrhagic damage and dermonecrosis, enhances vascular permeability and edema, and is lethal to mice [23-26]. Moreover, VvpE production is known to be affected by a variety of growth conditions, including temperature, osmolarity, and levels of iron and oxygen [27], and controlled by the stationary sigma factor RpoS [28], the LuxS quorum-sensing system [29-31], and the cyclic AMP (cAMP)-cAMP receptor protein (CRP) complex [29,32].

However, the role of VvpE in the pathogenesis of *V. vulnificus* infection remains unclear, as VvpE-deficient mutants show comparable or higher mouse-lethality than the wild type strains [27,33-35]. Nevertheless, from an evolutional standpoint, it is believed that VvpE is produced because it is required for the survival of *V. vulnificus* in different environments and within human hosts. Accordingly, new paradigms are required to elucidate the veiled pathogenetic roles of VvpE.

Of the various biological activities of VvpE, its roles in facilitating the iron-assimilation of *V. vulnificus* via the proteolytic cleavage of heme proteins, transferrins, and lactoferrins have attracted some attention [21,22]. Our previous study demonstrated that VvpE had no direct effect on the

iron-assimilation of *V. vulnificus* from transferrin [36,37]. However, the role of VvpE in facilitating the iron-assimilation of *V. vulnificus* via the proteolytic cleavage of HG remains to be clarified. The hypothetical role of metalloprotease VvpE in facilitating the iron-assimilation of *Vibrio vulnificus* from hemoglobin is shown in Figure 2.

One group suggested that VvpE could facilitate the iron-assimilation of *V. vulnificus* by releasing heme or iron via the proteolytic cleavage of HG [22]. However, they added purified VvpE to a medium containing HG as an iron source at the starting point of culture, and used VvpE-deficient mutants generated by nonspecific chemical mutagenesis. This exogenous addition of purified VvpE cannot reflect the actual production and role of VvpE, and the VvpE-deficient mutants generated by chemical mutagenesis may have harbored multiple nonspecific mutations on various genes other than the *vvpE* gene. Moreover, without exception, reports issued to date have demonstrated that VvpE is produced in the late growth phase [27-37], by which time most iron in media had already been consumed. These results about VvpE production strongly suggest that VvpE cannot facilitate the iron-assimilation of *V. vulnificus* from HG, which occurs in the early growth phase.

In this study, we attempted to determine which of the two iron-assimilation systems, i.e., vulnibactin- and heme receptor-mediated iron-assimilation systems, plays more important role in the iron-assimilation of *V. vulnificus* from HG, to elucidate the role of VvpE in facilitating the iron-assimilation of *V. vulnificus* from HG in accordance with the molecular version of Koch's postulates [1], and to elucidate the regulatory mechanism of *vvpE* expression by iron or HG. Accordingly, we used (1) a *vis*-inser-

tional mutant which does not produce vulnibactin and a *hupA*-deletional mutant which does not express heme receptor in order to determine which of the two iron-assimilation systems plays more important role in iron-assimilation from HG by *V. vulnificus,* (2) a *V. vulnificus vvpE*-in frame deletion mutant and its *in trans vvpE*-complemented strain in order to observe the effect of *vvpE* mutation on the ability of *V. vulnificus* to utilize HG and to grow on HG in the absence of exogenous VvpE, and (3) a chromosomal P_{vvpE}::*lacZ* transcriptional reporter strain and a *luxS*-deleted P_{vvpE}::*lacZ* transcriptional reporter strain in order to observe *vvpE* transcriptions and to elucidate the regulatory mechanism of *vvpE* transcription by iron or HG. As a result, we found that both vulnibactin- and heme receptor-mediated iron-assimilation systems play important roles in iron-assimilation from HG, that VvpE has no direct effect on *V. vulnificus* iron-assimilation from HG although it is the only protease capable of destroying HG, and that iron or HG is rather required for efficient *vvpE* transcription via unknown transcriptional activator but not the LuxS quorum-sensing system.

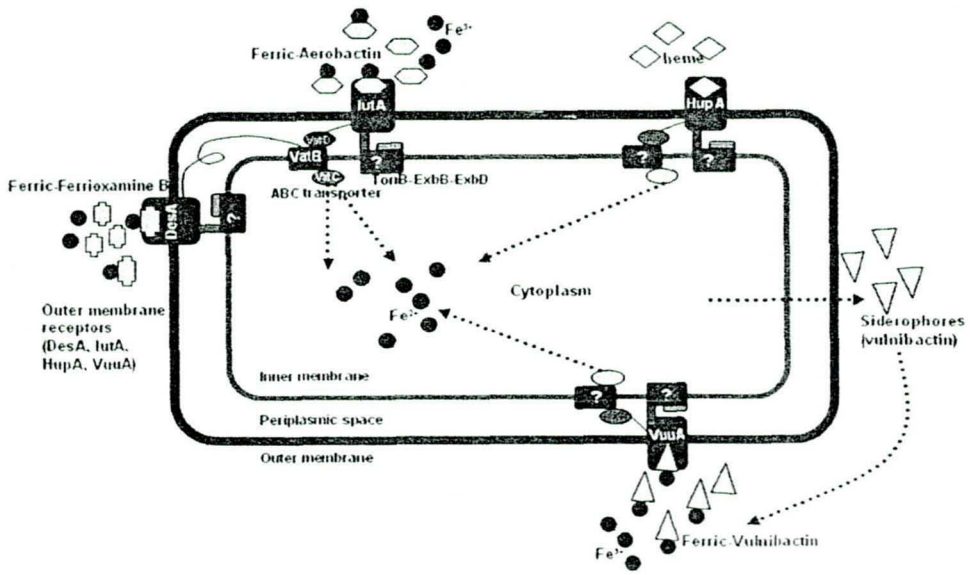


Figure 1. The four hypothetical iron-assimilation systems of *Vibrio vulnificus*: (1) vulnibactin (a catechol or phenolate siderophore)-mediated iron-assimilation system is composed of vulnibactin and its specific outer membrane receptor (VuuA), (2) heme receptor (HupA)-mediated iron-assimilation system can directly bind heme proteins, (3) DesA-mediated iron-assimilation system can bind a heterologous hydroxamate siderophore deferoxamine (ferrioxamine B), and (4) IutA-mediated iron-assimilation system can bind a *E. coli*-deriven hydroxamate siderophore aerobactin. Details in these iron-assimilation systems remains to be clarified (?).

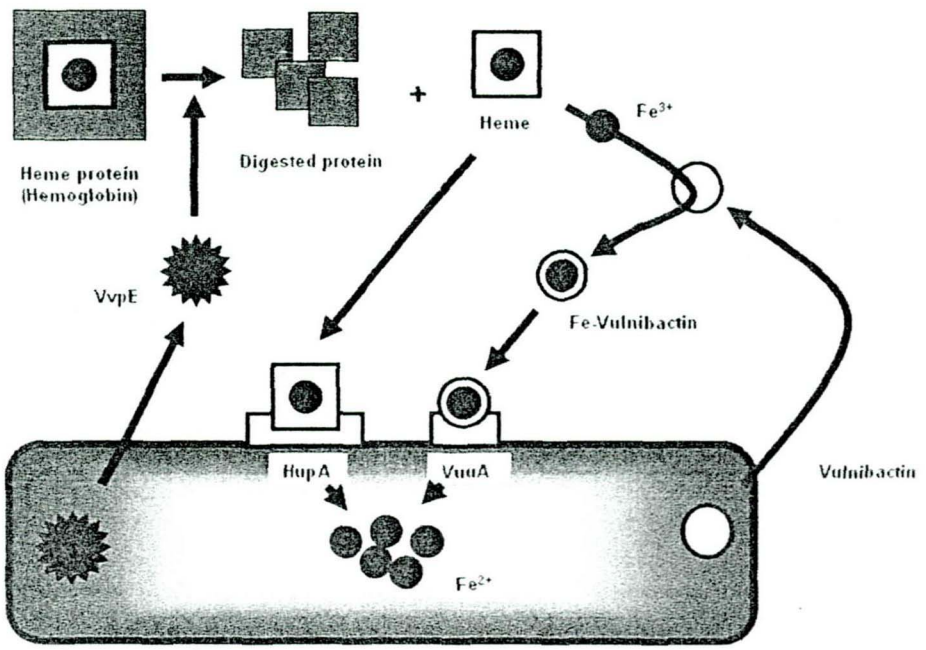


Figure 2. Hypothesis on the role of metalloprotease VvpE in facilitating the iron-assimilation of *Vibrio vulnificus* via the proteolytic cleavage of hemoglobin.

2. MATERIALS AND METHODS

2.1. Bacterial strains, media, cirrhotic ascites and reagents

Bacterial strains, plasmids and primers used in this study were listed in Table 1, 2, and 3. Heart Infusion broth (BD, Sparks, MD, USA) containing an additional 2% NaCl (designated normal (NL)-HI) was used as the basal medium for all experiments in this study. NL-HI broth was deferrated using 8-hydroxyquinoline by the method described by Leong and Neilands [38]. In brief, NL-HI broth was mixed with an equal volume of 3% (w/v) solution of 8-hydroxyquinoline, which was dissolved in chloroform, and vigorously stirred for 2 days. The mixture was allowed to stand at room temperature to be separated into aqueous and chloroform layers. The aqueous extracts were mixed and stirred with pure chloroform for over 2 h in order to remove residual 8-hydroxyquinoline. The residual iron concentration of deferrated HI (DF-HI) broth was 1.0 μg/dl or less. Synbase minimal medium was also used in this study [14,39]. Iron-limited Synbase agars was prepared by adding 75 mg/ml EDDA. Cirrhotic ascites (CA) samples were obtained from five patients with liver cirrhosis as described in our previous study [40]. Equal volumes of the five CA samples were pooled, and this pooled CA was sterilized and freed of human cells using disposable syringe filters (0.45 μm, Sartorius, Germany), and then incubated at 65°C for 30 min to inactivate bactericidal factors such as complement. Human HG (0.5 mg/ml) was added to DF-HI broth or CA, and when required, ferric chloride (FC, 10 μM) or HG (0.5 mg/ml) was added to DF-HI broth. Unless noted otherwise, all reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Table 1. Bacterial strains used in this study.

Strains	Relative characteristics	Sources or references
V. vulnificus		
MO6-24/O	Wild type strain; clinical isolate	J.G. Morris, Jr
CMM2301	MO6-24/O with *vis*-insertional mutation	37
RC110	MO6-24/O with *hupA*-deletional mutant	This study
CMM1049	MO6-24/O with *vvpE*-deletion mutation	37
CMM1502	CMM1049 complemented by wild type *vvpE*	37
CMM2202	CMM2101 with *luxS*-deletional mutation	Kim et al. (2003)
CMM2106	CMM2101 with P*vvpE*::*lacZ* chromosomal reporter	31
CMM2207	CMM2106 with *luxS*-deletional mutation	31
RC138	CMM2101 with P_{luxS}::*lacZ* chromosomal reporter	This study
CMM2101	CMM2100 with *lacZ* deletion mutation	31
CMM2100	MO6-24/O with spontaneous streptomycin resistance	31
E. coli		
SY327 λ*pir*	D(lac pro) argE(Am) rif nalA recA56 λpir lysogen: Host for suicide vector *thi thr leu tonA lacY supE*	42
SM10 λ*pir*	*recA*::RP4-2-Tc^r::Mu Km^r λ*pir* lysogen: Conjugation donor	42

Table 2. Plasmids used in this study.

Plasmids	Relative characteristics	Sources or references
pDM4	Suicide vector with R6K origin:Cm^r	41
pRC110	1.82-kb *Sma*I-*Spe*I fragment containing an in-frame deletion of *V. vulnificus hupA* gene cloned into pDM4	This study
pQF52	IncP *lacZ* transcriptional fusion vector; Ap^r	43
pRC130	1075-bp *Bam*HI-*Hind*III fragment containing the *luxS* promoter region cloned into pQF52	This study
pRC136	*Bam*HI-*Sca*I fragment containing P_{luxS}::*lacZ* fragment from pRC130 cloned into *Bgl*II-*Sma*I- cut pDM4	This study

Table 3. Primers used in this study.

Primers	Sequences	Sources or references
hupA-up-1	5'-tcccccgggtctgactctggttttactcacg-3', *Sma*I overhang	This study
hupA-up-2	5'- gtagttggttacattcccctaatattgatactttg-3'	This study
hupA-down-1	5'- attaggggaatgtaaccaactacaaaaacaaaagc-3'	This study
hupA-down-2	5'- gactagtcaatgttcgccaaagtgagccg-3', *Spe*I overhang	This study
luxS-rep-F	5'- cgggatccgctcatcgtgtgtttgcagagc-3'	This study
luxS-rep-R	5'-cccaagcttcggtaaaactatctaataatggc-3'	This study

2.2. Construction of *hupA*-deletion mutant

An in-frame deletion mutant of the *V. vulnificus hupA* gene was constructed by crossover PCR using R6K origin suicide vector pDM4 [41] (Figures 3 and 4). Two pairs of PCR primers (hupA-up-1/ hupA-up-2 and hupA-down-1/ hupA-down-2) were used for the PCR amplification of the *V. vulnificus hupA* gene. The two PCR products were used as the template for the second PCR amplification using the PCR primers, hupA-up-1 (with *Sma*I overhang) and hupA-down-2 (with *Spe*I overhang). The 1.82-kb *Sma*I-*Spe*I fragment with deleted *hupA* gene was cloned into pDM4. The resulting plasmid pRC110 was transformed into *E. coli* SY327 λ*pir* and SM10 λ*pir* [42], and subsequently transferred to *V. vulnificus* MO6-24/O by conjugation. Transconjugants were selected on TCBS agar containing chloramphenicol, and stable transconjugants were spread onto NL-HI agars containing 10% sucrose to allow the second homologous recombination to occur. The resulting mutation in the *V. vulnificus hupA* gene (RC110) was confirmed by PCR.

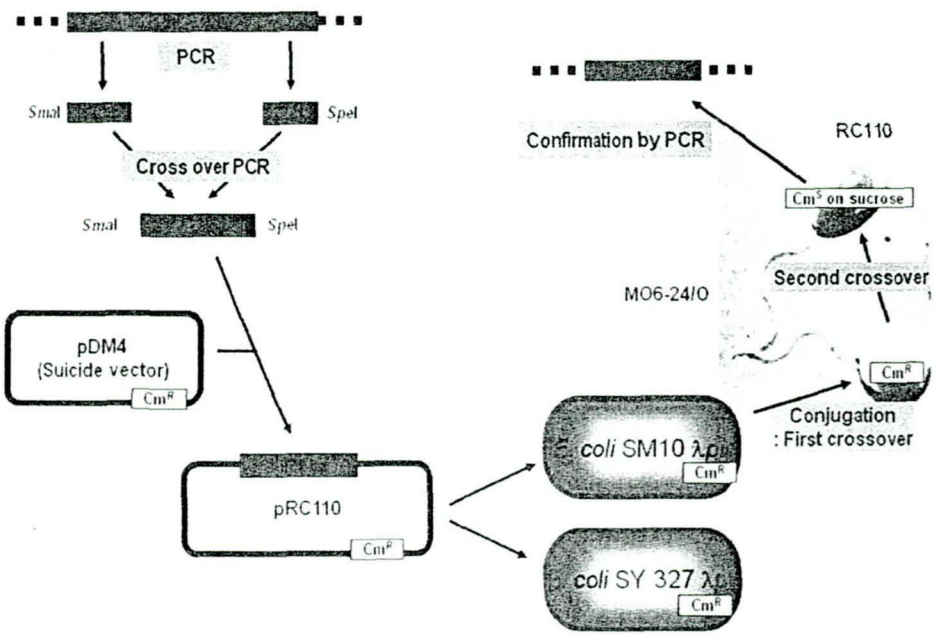


Figure 3. Construction of *Vibrio vulnificus hupA* deletion mutant. PCR; po;ymerase chain reaction, Cm^R, chloramphenicol-resistant; Cm^S, chlor-amphenicol-sensitive, ORF, open reading frame; Chr', chromosome.

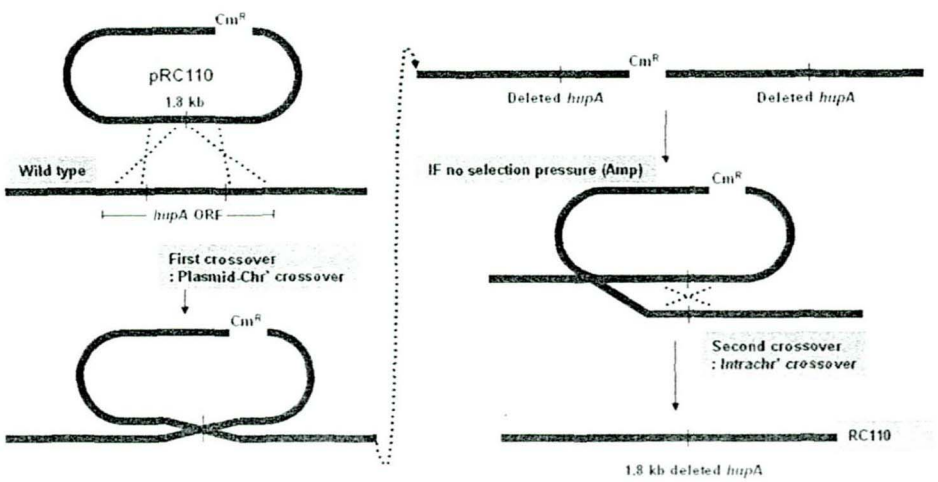


Figure 4. Genetic organization of *Vibrio vulnificus hupA* deletion mutant. Cm^R, chloramphenicol resistance; ORF, open reading frame; Chr', chromosome.

2.3. Construction of P_{luxS}::*lacZ* chromosomal reporter strain

A P_{luxS}::*lacZ* transcriptional fusion was constructed by using a broad-host-range vector containing promoterless *lacZ* gene (pQF52) [43] and R6K origin suicide vector (pDM4) [41] (Figures 5 and 6). A 1075-bp *Bam*HI-*Hind*III fragment containing the promoter region of the *luxS* gene was amplified using PCR primers, luxS-rep-F with *Bam*HI overhang and luxS-rep-R with *Hind*III overhang, and subcloned into pQF52 (named pRC130). A *Bam*HI-*Sca*I fragment containing P_{luxS}::*lacZ* fragment from pRC130 was subsequently subcloned into *Bgl*II-*Sma*I-cut pDM4 (pRC136). The resulting plasmid pRC136 was transformed into *E. coli* SY327 λ*pir* and SM10 λ*pir* [42], and transferred to CMM2101 by conjugation. Transconjugants were selected on TCBS agar containing 2 μg/ml chloramphenicol. Stable transconjugants were also confirmed by β-galactosidase assay [44] and named RC138.

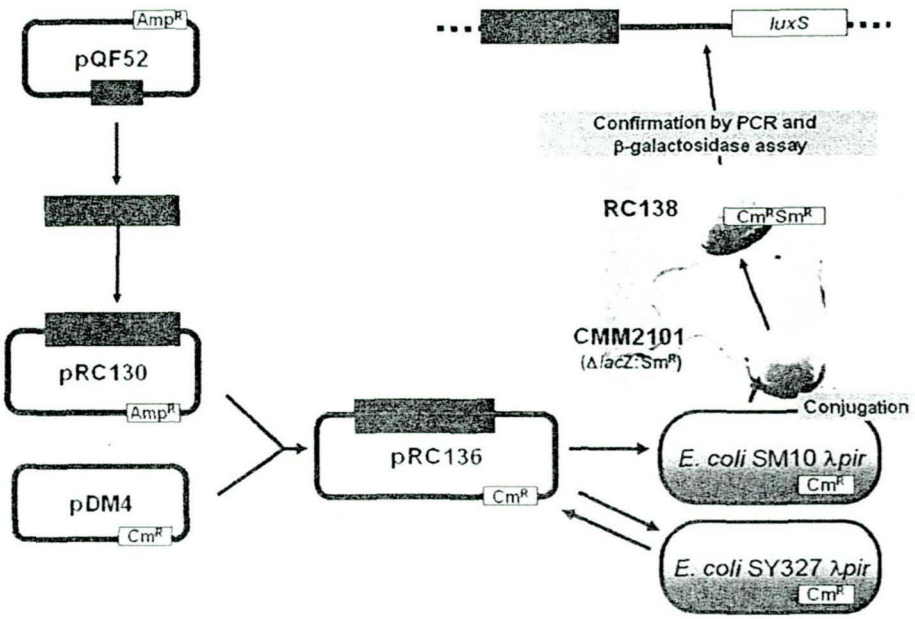


Figure 5. Construction of *Vibrio vulnificus* P_{luxS}::*lacZ* transcriptional reporter strain. Amp^R, ampicillin resistance, Cm^R, chloramphenicol resistance; ORF, open reading frame; Chr', chromosome.

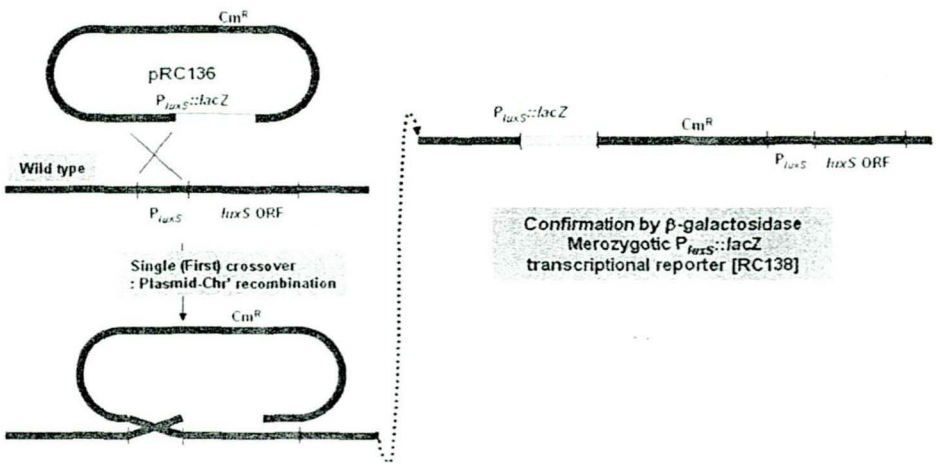


Figure 6. Genetic organization of *Vibrio vulnificus* P_{luxS}::*lacZ* transcriptional reporter strain. Cm^R, chloramphenicol resistance; ORF, open reading frame; Chr', chromosome.

2.4. Bioassay for HG utilization

V. vulnificus strains grown in NL-HI broth overnight were inoculated in NL-HI broth containing 100 μM dipyridyl, an iron chelator, in order to adapt them to iron-limited conditions and to reduce intracellular iron storage, and cultured with vigorous shaking (220 rpm) at 37°C overnight. These preconditioned *V. vulnificus* strains were spread on the surface of iron-limited Synbase agars using 0.5% semisolid top agar at 1 x 10^4 cfu/ml and paper discs containing various amount of HG were placed on the agar surfaces, as described in our previous study [14]. Bacterial growths around discs were determined after incubating the agar plates at 37°C for 24 h.

2.5. Culture conditions, and the measurement of bacterial growth and β-galactosidase activity

V. vulnificus strains grown in NL-HI broth overnight were inoculated in NL-HI broth containing 100 μM dipyridyl, an iron chelator, in order to adapt them to iron-limited conditions and to reduce intracellular iron storage, and cultured with vigorous shaking (220 rpm) at 37°C overnight. These preconditioned *V. vulnificus* strains were then inoculated into test media or CA at 1 x 10^6 cfu/ml, and cultured with vigorous shaking (220 rpm) at 37°C for 24 h. During culture, culture aliquots were removed to measure bacterial growth and β-galactosidase activity. Bacterial growth was determined by measuring the OD_{600} values of culture aliquots. β-Galactosidase activity in culture aliquots was measured using the Miller method [44].

2.6. Measurement of caseinolytic activity and zymography

Culture aliquots were centrifuged at 10,000 rpm for 5 min to obtained culture supernatants. To observe total protease production, caseinolytic activities in culture supernatants were measured using a previously described method [27,30]. To observe protease profile, zymography was performed as described previously [45]. In brief, equal volumes (20 μl) of culture supernatants were electrophoresed on 12% SDS-polyacrylamide gel containing 0.3% skim milk or HG. The gels were then incubated in renaturation buffer containing 2.5% Triton X-100 at room temperature for 1 h to remove SDS and subsequently in developing buffer containing dithiothreitol and $CaCl_2$ at 4°C overnight, and finally they were stained with Coomassie blue.

2.7. Observation of HG aggregates and SDS-PAGE

In order to grossly or microscopically observe insoluble HG aggregates formed during culture, we used culture aliquots obtained after culturing for 6 h in DF-HI or in DF-HI containing HG or FC. Culture aliquots (100 μl) were dropped onto filter paper and photographed. To further observe HG aggregates under a microscope, culture aliquots (100 μl) were smeared and fixed to slide glass, and stained with crystal violet for 1 min. The stained slides were observed in the presence of immersion oil at a magnification of 1,000 x and photographed.

In order to observe the disappearance or the proteolytic cleavage of intact HG molecules remaining in culture supernatants, culture aliquots obtained at appropriate times from cultures in DF-HI containing HG were centrifuged at 10,000 rpm for 5 min to remove HG aggregates, and then

20 μl of culture supernatants were mixed with SDS-sample buffer, heated for 5 min, and electrophoresed on a 6% stacking and 15% running gel. Gels were stained with Coomassie blue.

3. RESULTS

3.1. Both vulnibactin- and heme receptor-mediated iron-assimilation systems play important roles in iron-assimilation from HG by *V. vulnificus*

In order to determine which of the two iron-assimilation systems, vulnibactin- and heme receptor-mediated iron-assimilation systems, plays a more important role in iron-assimilation from HG by *V. vulnificus*, we compared the growths of the two *V. vulnificus* isogenic mutant strains, RC110 CMM2301 (*visA*-insertional mutant) and (*hupA*-deletion mutant), on iron-limited Synbase agars (Figure 7). The growths of the two strains were observed only around paper discs containing HG and stimulated dose-dependently around paper discs containing various amount of HG. However, the growth-enhancing zones of CMM2301 and RC110 strains around paper discs containing small amounts of HG (2 and 10 μg) were smaller than those of the wild type strain MO6-24/O although no noticeable difference in the growths of the three strains was not observed around paper discs containing a large amount of HG (100 μg) These results indicate that both iron-assimilation systems equally play important roles in iron-assimilation from HG by *V. vulnificus*.

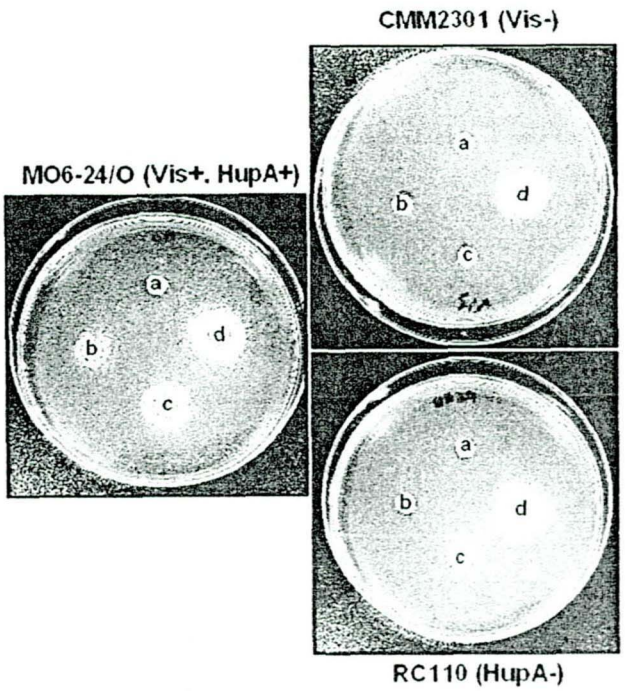
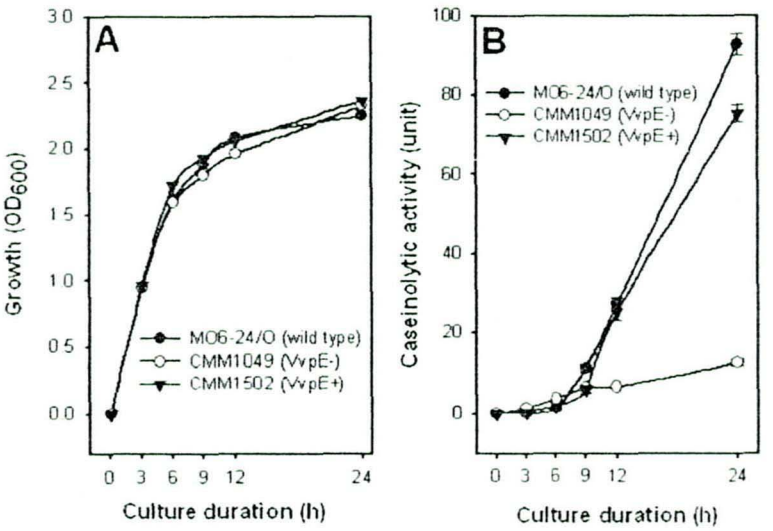


Figure 7. Utilization of HG by *V. vulnificus* as an iron source. The MO6-24/O, CMM2301 (*vis*-insertional mutant) and RC110 (*hupA*-deletional mutant) strains grown in HI broth containing 100 μM dipyridyl overnight were spread at about 1 x 10^4 cfu/ml on the surfaces of iron-limited Synbase agars using top agars, and then paper discs containing 30 μl of HG solutions (a, PBS; b, 2 μg HG; c, 10 μg HG; d, 100 μg HG). were placed on the agar surfaces. The plates were incubated at 37°C overnight.

3.2. VvpE was the only protease capable of destroying HG

In order to compare total protease activity, the three strains, MO6-24/O wild type strain, *vvpE*-deletion mutant (CMM1049) strain, and *in trans vvpE*-complemented (CMM1502) strain, were cultured in NL-HI broth. No differences were observed between the growths of the three strains (Figure 8A). In culture supernatants, CMM1049 strain exhibited far less proteolytic activity than the wild type strain, whereas CMM1502 strain had a proteolytic activity comparable to that of the wild type strain (Figure 8B). In order to observe protease profile, zymography was conducted using Skim milk or HG as protease substrates (Figure 8C). On zymograms using Skim milk, MO6-24/O wild type strain showed a major proteolytic band and at least two minor proteolytic bands. In contrast, CMM1409 strain showed only the minor proteolytic bands and not the major proteolytic band, and CMM1502 strain produced the same proteolytic bands as the wild type strain. On zymograms using HG, both MO6-24/O and CMM1502 strains showed only a proteolytic band, whereas CMM1049 strain did not. These results indicates that; (i) VvpE is a major protease produced by *V. vulnificus*, (ii) VvpE is the only protease capable of destroying HG, and (iii) minor proteases are not involved in the proteolytic destruction of HG. Thus, the roles of minor proteases were not further considered in this study.


A
Growth (OD_{600})
30
25
20
15
10
05
00
0 3 6 9 12 24
Culture duration (h)
MO6-24/O (wild type)
CMM1049 (VvpE-)
CMM1502 (VvpE+)
B
Caseinolytic activity (unit)
100
80
60
40
20
0
0 3 6 9 12 24
Culture duration (h)
MO6-24/O (wild type)
CMM1049 (VvpE-)
CMM1502 (VvpE+)

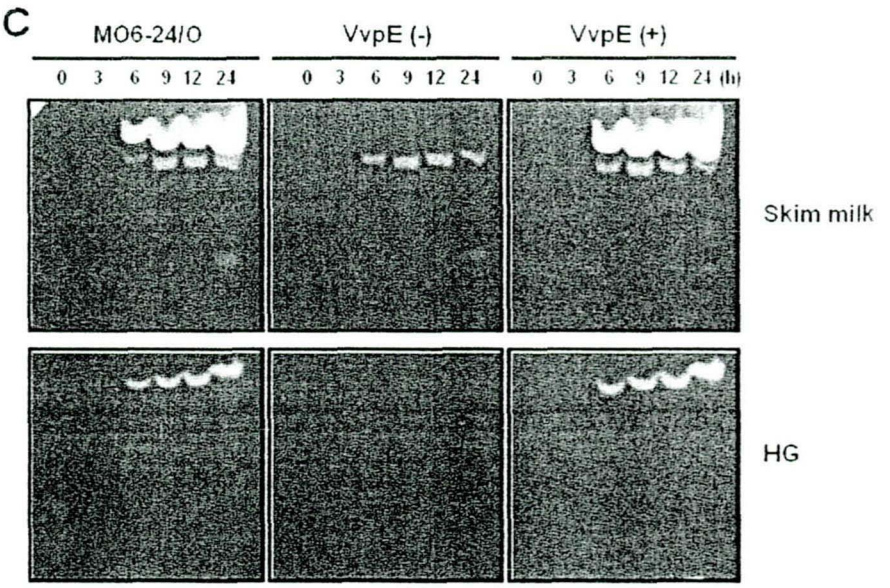

C
MO6-24/O
VvpE (-)
VvpE (+)
0 3 6 9 12 24 (h)
Skim milk
HG

Figure 8. The growths (A), total protease activities (B) and protease profiles (C) of MO6-24/O wild type strain, *vvpE*-deletion mutant CMM1049 strain and *in trans* *vvpE*-complemented CMM1502 strain in normal HI broth. Bacterial growth was monitored by measuring the OD_{600} value of culture aliquots obtained at the indicated times. Culture supernatants were obtained by centrifuging culture aliquots at 10,000 rpm for 5 min. Total protease activities in culture supernatants were measured using azocasein as a substrate. Protease profiles in the culture supernatants were observed by zymography. Equal volumes (20 μl) of the culture supernatants were electrophoresed on SDS-gel containing 0.3% Skim milk or hemoglobin, and the gels were finally stained with Coomassie blue.

3.3. *vvpE* transcription, but not extracellular VvpE production, was stimulated by HG, but this occurred only in the late growth phase

In order to observe *vvpE* transcription, the chromosomal P*vvp::lacZ* reporter strain (CMM2106) was cultured in DF-HI broth only or in DF-HI containing 0.5 mg/ml HG or 10 μM FC, and its growth was found to be stimulated by HG or FC (Figure 9A). Transcription of *vvpE* was also stimulated by HG or FC (Figure 9B). Accordingly, *vvpE* transcription appeared to be stimulated by heme or inorganic iron rather than globin. However, it was evident that all these *vvpE* transcriptions occurred only during the late exponential or stationary growth phases. These results indicate; (i) that *vvpE* transcription begins after *V. vulnificus* growth has plateaued, and thus its final product VvpE cannot facilitate the iron-assimilation of *V. vulnificus* via the proteolytic cleavage of HG, and (ii) that iron is required for efficient *vvpE* transcription, rather than VvpE being required for efficient iron-assimilation of *V. vulnificus* from HG.

Although *vvpE* transcription was stimulated by HG or FC, extracellular VvpE production was not stimulated by either of them (Figures 9B and 10D, or 12B and 13D). This discrepancy suggests that extracellular VvpE production is additionally controlled by unknown post-transcriptional events. Nevertheless, these results indicate that *V. vulnificus* is able to utilize HG as an iron source for its growth without the extracellular production of VvpE.

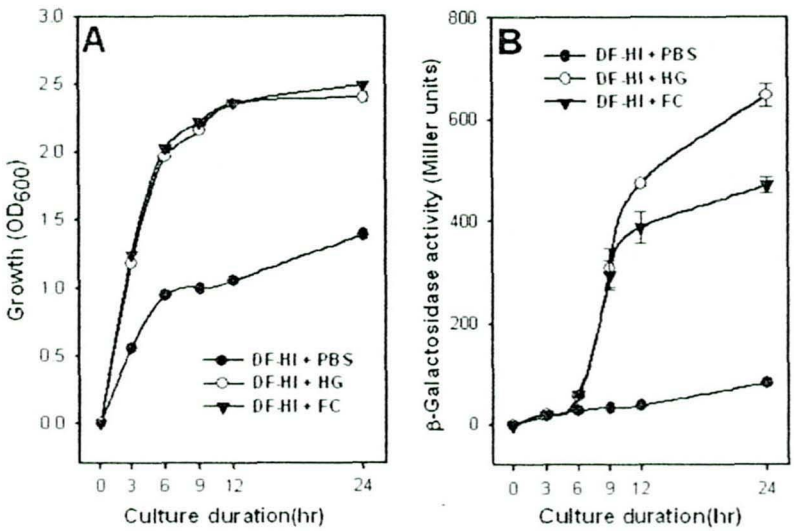
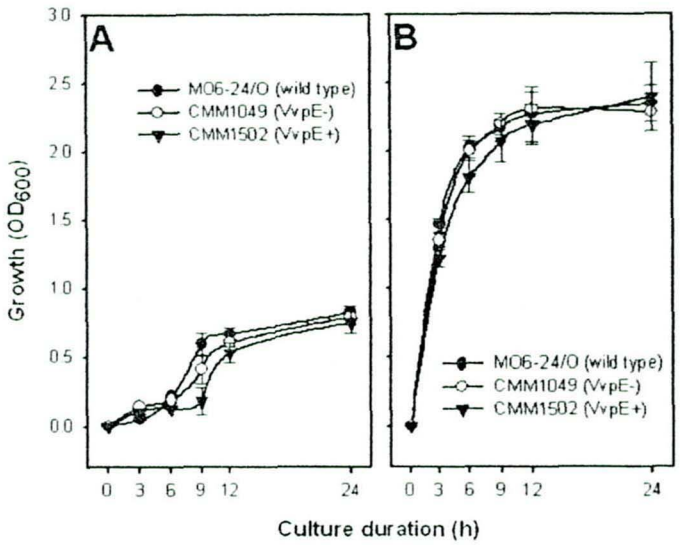


Figure 9. Transcription of the *vvpE* gene in deferrated (DF)-HI broth containing phosphate-buffered saline (PBS), 0.5 mg/ml of hemoglobin (HG) or 10 μM of ferric chloride (FC). The growth (A) of chromosomal P*vvpE::lacZ* reporter CMM2106 strain was monitored by OD_{600} of culture aliquots at the indicated times. β-Galactosidase activity (B) in the culture aliquots was measured by the Miller method [44].

3.4. The *vvpE* mutation did not affect the growth of *V. vulnificus* in DF-HI broth containing HG as an iron source

The three strains, MO6-24/O wild type strain, *vvpE*-deleted mutant (CMM1049) strain and *in trans vvpE*-complemented (CMM1502) strain, were cultured in DF-HI broth or DF-HI broth containing 0.5 mg/ml HG. The growths of all three strains were stimulated by HG, but without noticeable inter-strain differences (Figures 10A and 10B). In contrast, extracellular VvpE productions by the three strains were not stimulated by HG (Figures 10C and 10D). These results indicate that *V. vulnificus* is able to utilize HG and to grow on HG without the assistance of VvpE, the final product of the *vvpE* gene.


A
B
MO6-24/O (wild type)
CMM1049 (VvpE-)
CMM1502 (VvpE+)
Growth (OD_{600})
Culture duration (h)

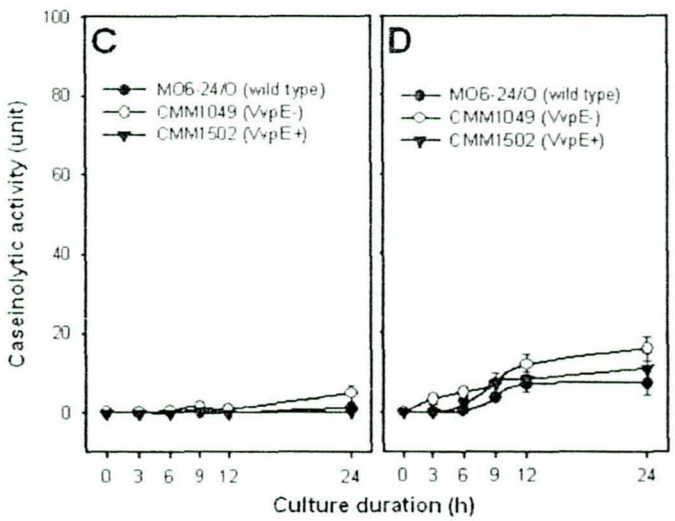
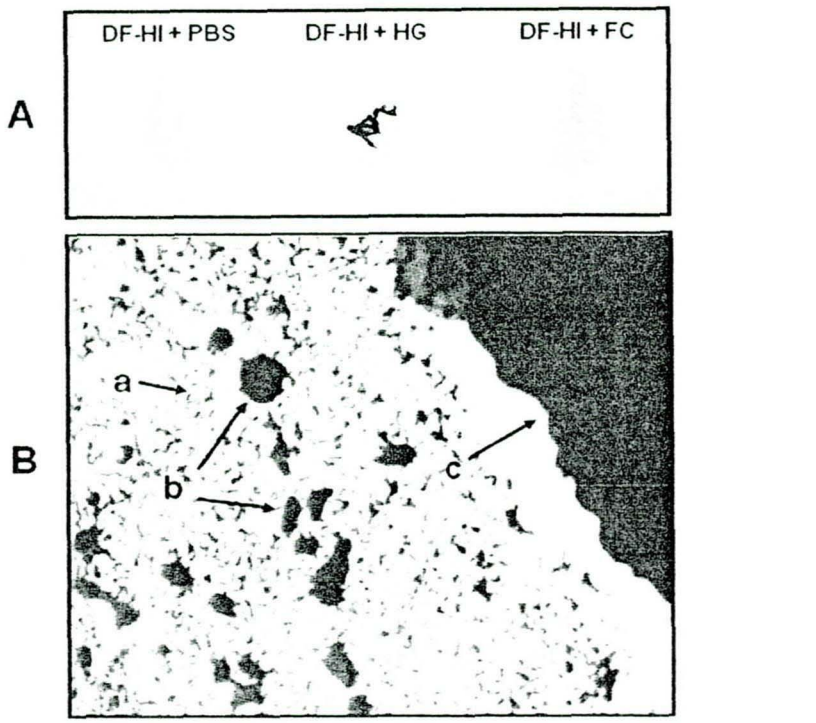

C
D
MO6-24/O (wild type)
CMM1049 (VvpE-)
CMM1502 (VvpE+)
Caseinolytic activity (unit)
Culture duration (h)

Figure 10. Growths (A and B) and total protease productions (C and D) by MO6-24/O wild type strain, *vvpE*-deleted CMM1049 strain and *in trans* *vvpE*-complemented CMM1502 in deferrated (DF) HI broth containing phosphate-buffered saline (PBS) and 0.5 mg/ml of hemoglobin (HG). Bacterial growths were measured by OD_{600} of culture aliquots at the indicated times. Culture supernatants were obtained by centrifuging culture aliquots at 10,000 rpm for 5 min. Total protease activities in culture supernatants were measured using azocasein as a substrate.

3.5. HG was not destroyed but aggregated to insoluble forms during culture

Interestingly, bizarre insoluble aggregates were observed in exponential growth phase culture fluids. These aggregates were observed only during culture in DF-HI broth containing HG, but not in DF-HI only or in DF-HI broth containing FC (Figure 11A), regardless of *vvpE* mutation or complementation (data not shown). By microscopy, these insoluble aggregates appeared to be formed from HG, and not from *V. vulnificus* cells; typical comma-shaped *V. vulnificus* cells, and small and large HG aggregates were observed simultaneously (Figure 11B). These findings suggested that HG was not destroyed but rather that it aggregated during culture. Accordingly, in order to observe whether or not HG molecules were destroyed, SDS-PAGE was conducted using culture supernatants in which insoluble aggregates were removed by centrifugation. Residual HG molecules were separated into the four forms with molecular sizes of about 16, 17, 35 and 70 kDa (Figure 11C), and none of these HG molecules were destroyed, but rather they gradually disappeared. Moreover, this disappearance of HG was observed regardless of *vvpE* mutation or complementation. Overall, these results also indicate that *V. vulnificus* is able to utilize HG and to grow on HG without the proteolytic cleavage of HG.


A
DF-HI + PBS
DF-HI + HG
DF-HI + FC
B
a
b
c

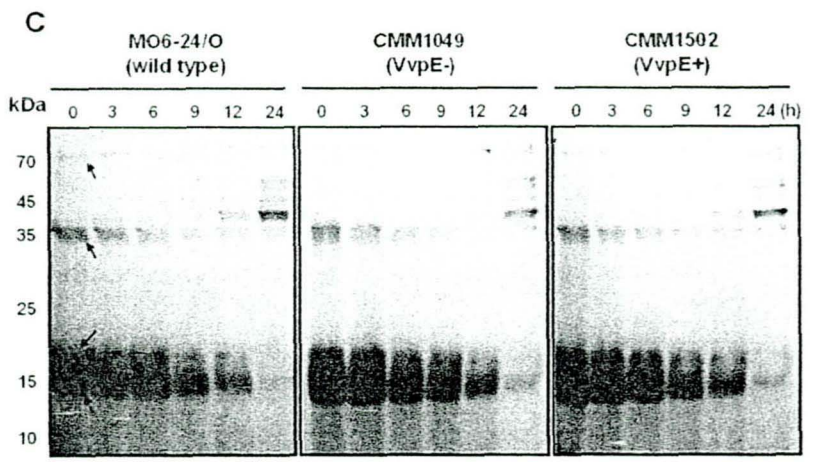

C
MO6-24/O
(wild type)
CMM1049
(VvpE-)
CMM1502
(VvpE+)
kDa
0 3 6 9 12 24
0 3 6 9 12 24
0 3 6 9 12 24 (h)
70
45
35
25
15
10

Figure 11. Nonspecific aggregation (A and B) and disappearance (C) of hemoglobin during culture. MO6-24/O wild type strain, *vvpE*-deleted CMM1049 strain, and *in trans vvpE*-complemented CMM1502 strain were cultured in deferrated (DF) HI broth containing phosphate-buffered saline (PBS) and 0.5 mg/ml of hemoglobin (HG) and 10 μM ferric chloride (FC). Culture aliquots were obtained at 6 h. (A) 100 ml of culture aliquots were dropped onto filter paper. (B) 100 μl of culture aliquot containing HG aggregates was smeared on slide glass, stained with crystal violet for 1 min, and then observed under a microscope (1,000 X). Typical comma-shaped *V. vulnificus* cells (a), small forms (b) and large (c) HG aggregates occurred simultaneously. (C) 20 μl of the culture supernatants were electrophoresed on 15% SDS-gels. The arrows indicate the four HG forms (about 16, 17, 35 and 70 kDa).

3.6. The *vvpE* mutation did not affect the growth of *V. vulnificus* in CA containing HG as an iron source

In order to determine the role of VvpE in a human *ex vivo* background, we used CA obtained from liver cirrhosis patients, who are known to be highly susceptible to *V. vulnificus* infection [2,3]. The four strains, MO6-24/O wild type strain, *vvpE*-deleted mutant (CMM1049) strain, *in trans* *vvpE*-complemented (CMM1502) strain, and *vvpE*-reporter (CMM2106) strain, were cultured in CA or CA containing 0.5 mg/ml HG. The growths of the four strains were stimulated by adding HG to CA. It was found that neither *vvpE* mutation nor *vvpE* complementation affected the growth of *V. vulnificus* in CA or CA containing HG (Figures 12A and 13AB). Transcription of *vvpE* in the CA background was also stimulated by HG (Figure 12B), as in DF-HI broth (Figure 9B), but this occurred during the late growth phase when *V. vulnificus* growth had already plateaued. Moreover, although *vvpE* transcription was stimulated by HG, no protease activity was detected in culture supernatants (Figures 13CD). These results indicate that the discrepancy between *vvpE* transcription and extracellular VvpE production is also present in the CA background. Nevertheless, these results clearly indicate that *V. vulnificus* is also able to utilize HG and to grow on HG without the assistance of VvpE in a human *ex vivo* background.

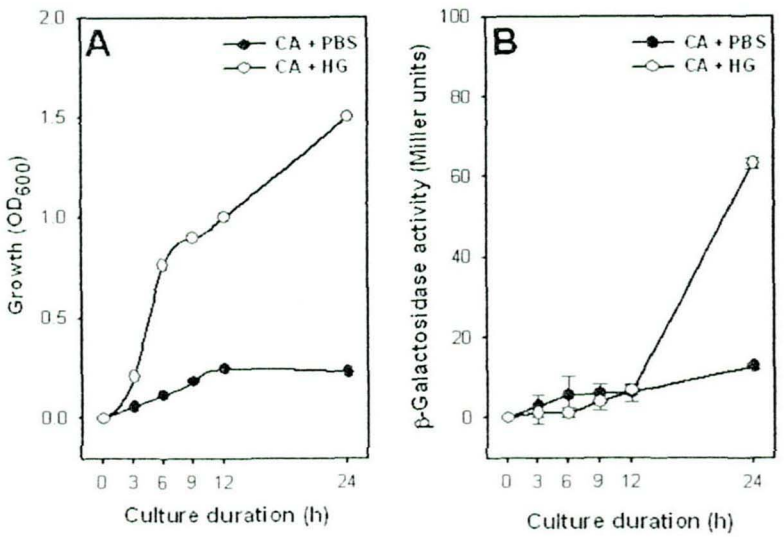
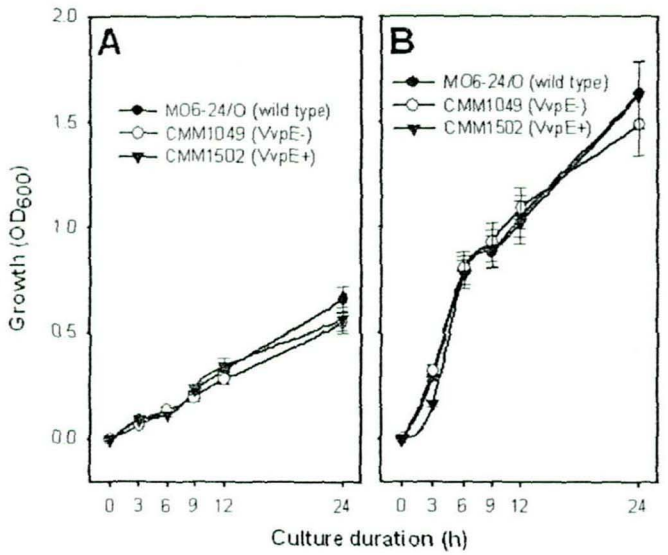


Figure 12. Transcription of the *vvpE* gene in cirrhotic ascites (CA) containing phosphate-buffered saline (PBS) or 0.5 mg/ml of hemoglobin (HG). (A) The growth of chromosomal P*vvpE::lacZ* reporter CMM2106 strain was monitored by OD_{600} of culture aliquots at the indicated times. (B) β-Galactosidase activity in culture aliquots was measured by the Miller method [44].


A
B
MO6-24/O (wild type)
CMM1049 (VvpE-)
CMM1502 (VvpE+)
Growth (OD600)
2.0
1.5
1.0
0.5
0.0
0 3 6 9 12 24
Culture duration (h)

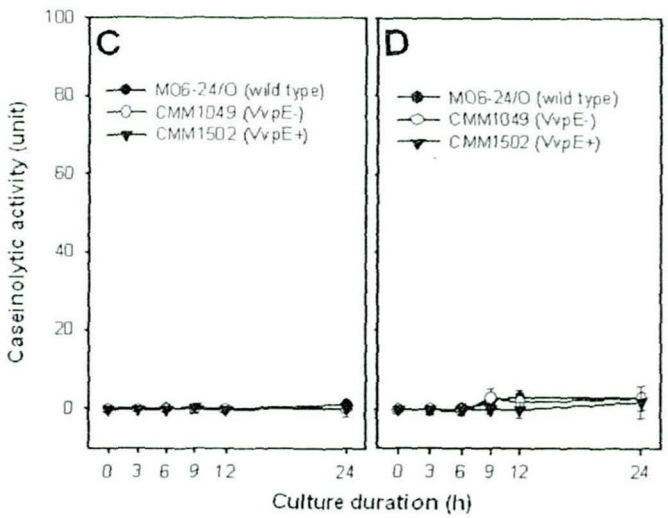

C
D
MO6-24/O (wild type)
CMM1049 (VvpE-)
CMM1502 (VvpE+)
Caseinolytic activity (unit)
100
80
60
40
20
0
0 3 6 9 12 24
Culture duration (h)

Figure 13. Growths (A and B) and total protease productions (C and D) by MO6-24/O wild type strain, *vvpE*-deleted CMM1049 strain, and *in trans* *vvpE*-complemented CMM1502 in cirrhotic ascites (CA) containing phosphate-buffered saline (PBS) and 0.5 mg/ml of hemoglobin (HG). Bacterial growths were measured by OD_{600} of culture aliquots at the indicated times. Culture supernatants were obtained by centrifuging culture aliquots at 10,000 rpm for 5 min. Total protease activities in culture supernatants were measured using azocasein as a substrate.

3.7. Iron or HG up-regulates *luxS* expression

In order to determine the effect of iron or HG on the transcription of the *luxS* gene encoding an enzyme for the synthesis of a quorum-sensing molecule, autoinducer-2, we cultured the chromosomal P_{luxS}::*lacZ* transcriptional reporter strain (RC138) in DF-HI broths containing PBS as a control, and containing 10 μM FC or 0.5 mg/ml of HG as an iron-source (Figure 14). The growth of RC 138 strain was stimulated by the addition of FC or HG. The expression level of the *luxS* gene was stimulated by the addition of FC or HG and consistent with its growth level. No noticeable difference was observed between the two iron sources, FC and HG. These results indicate that iron stimulates the expression of the LuxS quorum-sensing system by facilitating *V. vulnificus* growth.

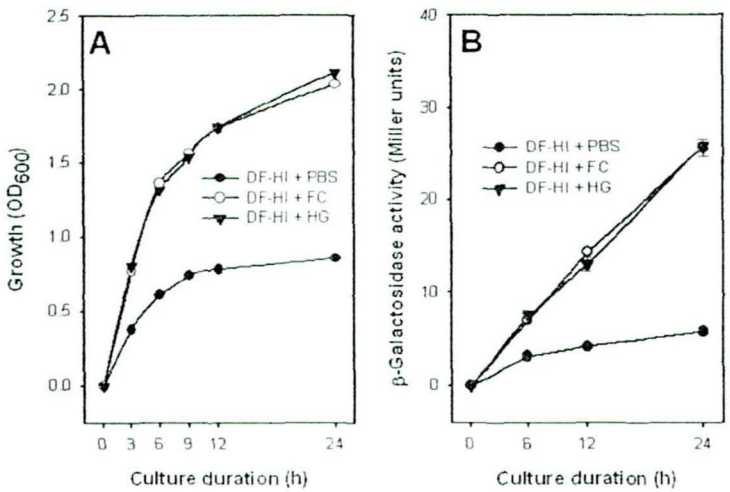


Figure 14. Effect of iron or hemoglobin on the expression of the *luxS* gene. *V. vulnificus* RC138 strain (P_{luxS}::*lacZ*) grown in HI broth containing 100 μM dipyridyl overnight was inoculated into DF-HI broths containing PBS as a control, and containing 10 μM FC or 0.5 mg/ml HG as an iron source, at about 1 x 10^6 cfu/ml, and then cultured with vigorous shaking (220 rpm) at 37°C for 24 h. At the indicated times, culture aliquots were withdrawn to measure bacterial growth (A) and β-galactosidase activity (B). Bacterial growth was monitored by measuring the OD_{600} value and β-galactosidase activity was measured by the Miller method [44].

3.8. Effect of the *luxS* mutation on the iron- or HG-induced *vvpE* expression

In order to determine the effect of the *luxS* mutation on the iron- or HG-regulated vvpE expression, we used the two transcriptional reporter strains, P_{vvpE}::*lacZ* reporter (CMM2106) and *luxS*-deleted P_{vvpE}::*lacZ* reporter (CMM2207) strains. We cultured the two reporter strains in DF-HI broths containing PBS as a control, and containing 10 μM FC or 0.5 mg/ml HG as an iron source (Figure 15). The growths of the two strains were stimulated by the addition of FC or HG. The *vvpE* expression levels were also stimulated by the addition of FC or HG. No noticeable difference was observed between the two iron sources, FC and HG. However, interestingly, the *vvpE* expression levels were not decreased but slightly increased by the *luxS* mutation. These results indicate that iron stimulates *vvpE* expression via unknown transcription regulator (s) but not via the LuxS quorum-sensing system.

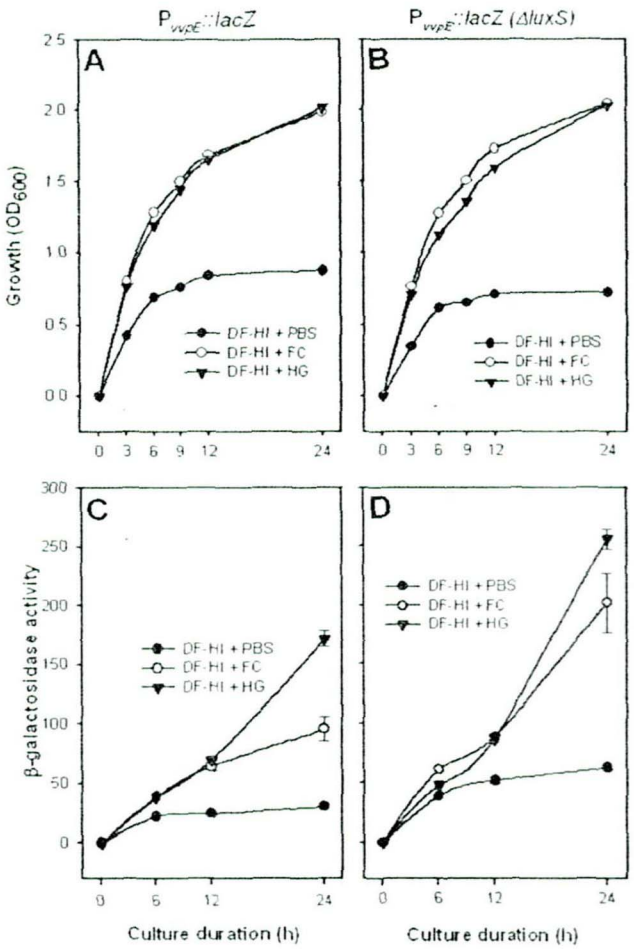

P vvpE::lacZ
P vvpE::lacZ (ΔluxS)
A
B
C
D
Growth (OD600)
β-galactosidase activity
DF-HI + PBS
DF-HI + FC
DF-HI + HG
Culture duration (h)
Culture duration (h)

Figure 15. Effect of the *luxS* mutation on the iron- or HG-induced *vvpE* expression. *V. vulnificus* CMM2106 (P_{vvpE}::*lacZ;* A and C) and CMM2207 (*luxS*-deleted P_{vvpE}::*lacZ;* B and D) strains grown in HI broth containing 100 μM dipyridyl overnight was inoculated into DF-HI broths containing PBS as a control, and containing 10 μM FC or 0.5 mg/ml HG as an iron source, at about 1 x 10^6 cfu/ml, and then cultured with vigours shaking (220 rpm) at 37°C for 24 h. At the indicated times, culture aliquots were withdrawn to measure bacterial growth (A and B) and β-galactosidase activity (C and D). Bacterial growth was monitored by measuring the OD_{600} value and β-galactosidase activity was measured by the Miller method [44].

4. DISCUSSION

4.1. Both the vulnibactin- and heme recceptor-mediated iron-assimilation systems are involved in iron-assimilation from HG by *V. vulnificus*

In this study, when we compared the roles of the vulnibactin- and heme receptor-mediated iron-assimilation systems in the iron-assimilation of *V. vulnificus* from HG (Figure 7), both iron-assimilation systems played important roles in iron-assimilation from HG by *V. vulnificus*. This findings were consistent with results of other researchers [11,13,15]. Litwin et al. reported that the mutation of the *vuuA* gene encoding vulnibactin-specific receptor resulted in the loss of the ability of *V. vulnificus* to utilize HG as well as transferrin-bound iron. In addition, they also reported that the mutation of the *hupA* gene resulted in the loss of the ability to utilize only HG, but not transferrin-bound iron. Moreover, vulnibactin was found to be essentially required for the iron-assimilation of *V. vulnificus* from holotransferrin in our previous study [37]. Taken together, the ability of *V. vulnificus* to utilize transferrin-bound iron appears to be dependent on the vulnibactin-mediated iron-assimilation system, but the ability to utilize HG to be dependent on both iron-assimilation systems.

4.2. VvpE can cleave HG but has no direct effect on the iron-assimilation of *V. vulnificus* from HG

According to our results, VvpE is a major protease produced by *V. vulnificus* and the only protease capable of destroying HG (Figure 8). Other researchers have also observed that purified VvpE is capable of

destroying HG [22]. However, this fact alone is insufficient to prove that VvpE facilitates the iron-assimilation and growth of *V. vulnificus* via the proteolytic cleavage of HG. In order for VvpE to facilitate iron-assimilation of *V. vulnificus* from HG, VvpE must be produced robustly during the exponential growth phase when *V. vulnificus* consumes most of the iron required for its active growth, especially under iron-limited conditions containing HG as an iron source. *Pseudomonas aeruginosa* proteases are known to facilitate iron-assimilation from iron-binding proteins, such as HG and transferrin, via their proteolytic cleavage [46-49]. *P. aeruginosa* produces proteases in the early growth phase, and thus, these proteases can facilitate the pyoverdin-mediated iron-assimilation of *P. aeruginosa* via the proteolytic cleavage of supplemented iron-binding proteins. In a previous study, we found that a *Bacillus subtilis* clinical strain produces proteases concomitantly with the production of siderophores in the early growth phase, and thus, is able to facilitate siderophore-mediate iron-assimilation from transferrin [50]. In contrast, almost all reports to date have shown that *V. vulnificus* produces proteases only in the late exponential or stationary growth phases [26-31,33,35-37], and especially in iron-sufficient conditions rather than in iron-limited conditions [24-26,30,31,33, 35-37,39,40]. For these reasons, we previously concluded that VvpE did not facilitate the iron-assimilation of *V. vulnificus* via the proteolytic cleavage of transferrin [36,37]. The findings of the above reports strongly suggest that VvpE cannot facilitate the iron-assimilation of *V. vulnificus* via the proteolytic cleavage of HG even though only VvpE appears to have the ability to destroy HG.

Nishina et al. first suggested that VvpE can facilitate the iron-assim-

ilation of *V. vulnificus* via the proteolytic cleavage of HG [22]. However, they added purified VvpE to a HG-containing medium at the start of culture. We believe that this exogenous addition of purified VvpE is unlikely to reflect the actual production and role of VvpE. Accordingly, we considered it necessary to determine whether or not VvpE can facilitate the iron-assimilation of *V. vulnificus* in the presence of HG as an iron-source, but in the absence of exogenous VvpE.

In the present study, *vvpE* transcription was stimulated by HG or inorganic iron (FC), rather than globin. However, this transcription obviously occurred only in the late growth phase when most of the iron in media had already been consumed and bacterial growth had already plateaued (Figure 9). Our previous work also revealed that *vvpE* transcription is stimulated by various iron sources, and that it occurred during the late growth phase [36]. Without exception, reports to date have shown that *vvpE* transcription occurs only in the late exponential or stationary growth phases [26-31,33,35-37]. In addition, it was recently reported that iron was required for efficient VvpE production [51,52]. Accordingly, our results indicate; (1) that because *vvpE* transcription occurs after the growth of *V. vulnificus* has already plateaued, its final product VvpE cannot facilitate iron-assimilation by *V. vulnificus* via the proteolytic cleavage of HG, and (2) that iron is required for efficient *vvpE* transcription.

Moreover, *vvpE* transcription, but not extracellular VvpE production, was stimulated in DF-HI containing HG or FC as an iron source (Figures 2, 3, 5 and 6). A similar discrepancy was also observed in DF-HI containing holotransferrin or FC as iron source in our previous work [26]. This discrepancy suggests that extracellular VvpE production is additionally

controlled by unknown posttranscriptional events. Moreover, another discrepancy also remains to be clarified, as Simpson and Oliver [18] reported that extracellular protease production by *V. vulnificus* is stimulated by HG or heme, but not by inorganic iron or globin. We do not know the reason for this difference between the findings of the two research groups. Nevertheless, the fact that extracellular VvpE production was not stimulated in iron-limited conditions also supports our opinion, namely that VvpE cannot facilitate the iron-assimilation of *V. vulnificus* via the proteolytic cleavage of HG. In contrast, *P. aeruginosa* proteases are more robustly produced under iron-limited conditions than under iron-sufficient conditions, and thus, these proteases facilitate the pyoverdin-mediated iron-assimilation of *P. aeruginosa* via the proteolytic cleavage of iron-binding proteins such as HG and transferrin, even under iron-limited conditions such as in the human body [46-49].

In order to confirm our opinion that *V. vulnificus* can assimilate iron from HG without the assistance of VvpE, the three strains, MO6-24/O wild type strain, *vvpE*-deleted mutant (CMM1049) strain and *in trans* *vvpE*-complemented (CMM1502) strain, were cultured in DF-HI broth or DF-HI broth containing 0.5 mg/ml HG. The growths of all three strains were stimulated by HG, without noticeable differences, whereas extracellular VvpE productions by these three strains were not stimulated by HG (Figure 10). These results indicate that *V. vulnificus* is able to utilize HG and to grow on HG without the assistance of VvpE. Simpson and Oliver [18] reported that all *V. vulnificus* protease-deficient mutants, generated by chemical or transposon mutagenesis, were able to utilize HG as an iron source. On the other hand, Nishina et al. [22] reported that all *V.*

vulnificus protease-deficient mutants, generated by chemical mutagenesis, were unable to utilize HG as an iron source. This difference may be caused by non-specific mutagenesis. Protease-deficient mutants generated by chemical or transposon mutagenesis may have multiple nonspecific mutations and exhibit unexpected phenotypic changes. In contrast, in the CMM1049 strain used in the present study, only the *vvpE* gene was specifically mutated by site-directed mutagenesis. Accordingly, we believe that our results are more confirmative than the results reported by the two other research groups.

Interestingly, we found that insoluble HG aggregates were formed during culture in DF-HI broth containing HG, regardless of *vvpE* mutation or complementation (data not shown), and that this occurred concomitantly with the disappearance of intact HG molecules (Figure 11). Moreover, no cleaved HG products were observed. These findings indicate that HG is not destroyed but rather that it aggregated during culture. HG molecules appear to be denatured and aggregated due to their loss of heme or iron, or by metabolites other than VvpE generated by *V. vulnificus* during culture. Accordingly, this finding also supports our opinion that VvpE does not facilitate the iron-assimilation of *V. vulnificus* via the proteolytic cleavage of HG.

Finally, we determined the role of VvpE in a CA background, a human *ex vivo* system, and results obtained in the DF-HI background were also reproducibly obtained in this background (Figures 12 and 12). Accordingly, these findings also indicate that *V. vulnificus* is able to utilize HG and to grow on HG regardless of VvpE production even in a CA background.

4.3. Iron stimulates *vvpE* expression via unknown transcriptional regulators but not via the LuxS quorum-sensing system

According to our results, the expressions of the *luxS* and *vvpE* genes were increased by the addition of FC or HG, but the *luxS* mutation did not affect the FC- or HG-induced *vvpE* expression. These results indicate that iron stimulates *vvpE* expression via unknown transcriptional regulator (s) but not via the LuxS quorum-sensing system.

Quorum-sensing is the cell-density-dependent regulation of gene expression by bacteria using the accumulation of small molecules called autoinducers to affect gene expression. *V. vulnificus* possesses the *luxS* gene involved in autoinducer-2 production, as well as the *luxR* (also called SmcR) gene encoding the regulator that senses the autoinducer [53]. Shao and Hor reproted that the mutation of the LuxR or SmcR gene decreased VvpE production [30]. Also, Jeong *et al.* reported that SmcR was required for full expression of *vvpE* by working synergistically with CRP and RpoS [29]. Consistently with these results, Kim *et al.* showed that the mutation of the *luxS* gene decreased *vvpE* expression [31]. However, this regulation was observed only in normal HI broth [31] or Luria-Bertani broth [29,30,52], In addition, Kawase et al. most recently reproted that *vvpE* was upregulated with the *luxS* expression during growth of *V. vulnificus* in Luria-Bertani broth, but in serum, *vvpE* expression was related to amounts of iron, not *luxS* expression. These findings indicate that the iron-induced *vvpE* expression occurred regardless of the LuxS quorum-sensing system in serum containing iron, and are consistent with

results in the present study.

RpoS and cAMP-CRP complex are known as transcriptional activators capable of upregulating *vvpE* expression other than the LuxS quorum-sensing system [28,29,32]. More recently, the expressions of the two transcriptional activators have been known to be regulated by iron levels [54,55]. Lee *et al.* reported that the mutation of the *rpoS* gene down regulated the expression of the *fur* gene encoding Fur protein, which regulates ferric uptake by bacteria [54]. Choi et al. reproted that the mutation of the crp gene downregulated *V. vulnificus* vulnibactin-mediated iron-assimilation system [55]. However, no reports are available on the roles of RpoS and CRP in regulating the iron-induced *vvpE* expression. Further studies on these regulations are necessary.

5. CONCLUSION

In this study, we determined which of the two iron-assimilation systems, *i.e.*, vulnibactin- and heme receptor-mediated iron-assimilation systems, plays more important role in the iron-assimilation of *V. vulnificus* from HG, elucidated the role of VvpE in facilitating the iron-assimilation of *V. vulnificus* from HG in accordance with the molecular version of Koch's postulates, and attempted to elucidate the mechanism of *vvpE* expression induced by iron or HG.

Results obtained in the present study are as follows. The growths of both a *V. vulnificus* vulnibactin-deficient mutant and a heme receptor-deficient mutant were impaired in ironlimited media containing HG as an iron source, indicating that both iron-uptake systems play significant roles in *V. vulnificus* iron-assimilation from human HG. Of the proteases produced by *V. vulnificus*, VvpE was found to be a major protease as well as the only protease capable of destroying HG. However, VvpE expressions at both the transcriptional and protein levels were suppressed in iron-limited media. Only *vvpE* transcription, and not extracellular VvpE production, was reactivated when HG or inorganic iron was added to iron-limited media, but *vvpE* transcription obviously occurred only in the late growth phase when *V. vulnificus* had already consumed most iron for growth. Moreover, neither *vvpE* mutation nor *in trans vvpE* complementation affected the ability of *V. vulnificus* to assimilate iron or to grow in iron-limited media containing HG or in cirrhotic ascites containing HG. Moreover, HG added into iron-limited media was not destroyed but gradually aggregated as insoluble forms during culture, and this HG aggregation occurred regardless of *vvpE* mutation or complementation. Iron

or HG up-regulated *luxS* transcription, but *luxS* mutation did not down-regulate the iron-induced *vvpE* transcription.,

Overall, our results indicate that VvpE is not required for efficient iron-assimilation from HG by *V. vulnificus*, on the contrary, HG or iron is required for efficient *vvpE* transcription via unknown transcriptional regulators but not the LuxS quorum-sensing system. In addition, a discrepancy exists between *vvpE* transcription and extracellular VvpE production in iron-limited media containing inorganic iron or HG as an iron source, which suggests that unknown posttranscriptional events are additionally involved in the extracellular production of VvpE. Subsequent studies on the unknown transcriptional regulators and posttranscriptional events are necessary.

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ABSTRACT

Vibrio vulnificus Metalloprotease VvpE Has No Direct Effect on Iron-assimilation from Human Hemoglobin

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We attempted to determine whether or not *Vibrio vulnificus* metalloprotease VvpE can promote iron-assimilation via the proteolytic cleavage of human hemoglobin (HG). The growths of both a *V. vulnificus* vulnibactin-deficient mutant and a heme receptor-deficient mutant were impaired in ironlimited media containing HG as an iron source, indicating that both iron-uptake systems play significant roles in *V. vulnificus* iron-assimilation from human HG. Of the proteases produced by *V. vulnificus*, VvpE was found to be a major protease as well as the only protease capable of destroying HG. However, VvpE expressions at both the transcriptional and protein levels were suppressed in iron-limited media. Only *vvpE* transcription, and not extracellular VvpE production, was reactivated when HG or inorganic iron was added to iron-limited media, but *vvpE* transcription obviously occurred only in the late growth phase when *V. vulnificus* had already consumed most iron for growth. Moreover, neither *vvpE* mutation nor *in trans vvpE* complementation affected the ability of *V. vul-*

nificus to assimilate iron or to grow in iron-limited media containing HG or in cirrhotic ascites containing HG. Moreover, HG added into iron-limited media was not destroyed but gradually aggregated as insoluble forms during culture, and this HG aggregation occurred regardless of *vvpE* mutation or complementation. Iron or HG up-regulated *luxS* transcription, but *luxS* mutation did not downregulate *vvpE* transcription. These results indicate that VvpE is not required for efficient iron-assimilation from HG by *V. vulnificus*, on the contrary, HG or iron is required for efficient *vvpE* transcription via unknown transcriptional regulators but not the LuxS quorum-sensing system. In addition, a discrepancy exists between *vvpE* transcription and extracellular VvpE production in iron-limited media containing inorganic iron or HG as an iron source, which suggests that unknown posttranscriptional events are additionally involved in the extracellular production of VvpE.

Key words: *Vibrio vulnificus*, Metalloprotease, Hemoglobin, Iron, Quorum-sensing

감사의 글

저 혼자만의 노력으로는 여기까지 올 수 없었을 겁니다. 저의 오늘의 성공이 있기까지 여러 가지로 도움을 주신 모든 분들께 고개 숙여 깊이 인사드립니다.

처음 실험실에 발을 들여놓아서부터 지금까지 한결같이 듬직한 모습으로 아낌없는 지도를 해 주신 신성희 교수님께 감사드립니다. 그리고 실험실 생활을 처음 하는 저한테 많은 조언을 주시고 저의 영어공부 같은 세세한 부분까지 자상하게 신경을 써주신 미생물학교실의 양남웅 교수님께도 감사드립니다. 본 논문 심사과정에서 많은 관심을 보여주시고 또 깊은 배려까지 해 주신 해부학교실의 정윤영 교수님께 감사드립니다. 그리고 바쁘신 일상에도 저의 논문 심사를 기꺼이 맡아주신 해부학교실의 문정석 교수님과 김종중 교수님 그리고 예방의학교실의 류소연 교수님께도 감사드립니다.

고향을 멀리 떠나 힘든 유학생활을 하는 저희 중국동포유학생들에게 설날 어머님께서 끓여주신 따뜻한 떡국을 마련해 주신 전호종 교수님께 감사합니다. 그리고 힘든 유학생활 도중 뜻하지 않게 아팠을 때 저를 다시 걸을 수 있게 치료해 주시고 말없이 경제적 부담을 덜어주시느라 애쓰신 조선대학교병원 신경외과 신호 교수님께도 감사드립니다.

실험실에서 가장 많은 시간을 함께 보냈고 나한테 많은 도움을 주었던 박라영씨, 역시 한 실험실에서 많은 도움을 준 착한 최미화씨에게 감사드립니다. 모르는 것 물어보면 너무나도 알기 쉽게 가르쳐 주시고 논문발표 때에도 사심 없이 도와주신 김춘미 박사님은 만난 시간이 짧은 것이 아쉽도록 감사합니다.

나한테 공부할 수 있는 기회를 마련해 주시고 용기를 주신 현용배 선배님한테 감사드립니다. 내가 힘들고 지칠 때마다 나한테 활력소가 되어준 밝고 명랑한 후배 김연씨, 유난히도 자주 아팠던 나를 가족같이 따뜻하게 보살폈던 든든한 동료 김복씨에게도 감사드리고, 그리고 힘든 유학생활을 함께 했던 유학생친구들, 내가 병마와 싸워 이기고 나머지 공부를 마저 마치도록 힘이 되어주었지만 미처 고맙다는 인사조차 제대로 못한 것 같아 항상 아쉽고 영원히 잊지 못 할 고마운 후배들 너무너무 감사합니다.

내가 부담없이 공부할 수 있도록 건강하게 자라주고 아직 어린나이에 엄마하고 함께 어려움을 나누었던 씩씩한 아들한테 고맙고, 함께 고생한 남편과 도움을 준 모든 가족에게 감사합니다.

내가 처음 이 공부를 시작하기로 어려운 결정을 할 때, 그때 이미 말기 암 환자로서 어쩌면 엄마의 임종을 지켜드리지 못 할지도 모르는 이 딸의 등을 밀어주시던, 지금은 하늘나라에 계시는 사랑하는 어머니께 이 논문을 가장 먼저 바칩니다.