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Vibrio vulnificus Metalloprotease VvpE has no Direct Effect on Iron-uptake from Human Hemoglobin

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This study was designed to determine whether or not *Vibrio vulnificus* metalloprotease VvpE can promote iron uptake via the proteolytic cleavage of human hemoglobin. We found that *V. vulnificus* utilized hemoglobin as an iron source more efficiently via the vulnibactin-mediated iron-uptake system than via the HupA-mediated iron-uptake system and, of the proteases produced by *V. vulnificus*, VvpE was found to be the only protease capable of destroying hemoglobin. However, VvpE expression, on both the transcriptional and protein levels, was suppressed in iron-limited media. However, *vvpE* transcription, but not extracellular VvpE production, was reactivated by the addition of hemoglobin or inorganic iron into iron-limited media. Moreover, *vvpE* transcription began only in the late growth phase when *V. vulnificus* had already consumed most of the iron for growth. In addition, neither *vvpE* mutation nor *in trans vvpE* complementation affected the ability of *V. vulnificus* to acquire iron or to grow in iron-limited media or in cirrhotic ascites containing hemoglobin. Hemoglobin added into iron-limited media was not destroyed, but gradually formed an insoluble aggregate during culture; this aggregation of hemoglobin occurred regardless of *vvpE* mutation or complementation. These results indicate that VvpE is not required for efficient iron uptake from hemoglobin. On the contrary, hemoglobin or iron is required for efficient *vvpE* transcription. In addition, a discrepancy exists between *vvpE* transcription and extracellular VvpE production in iron-limited media containing inorganic iron or hemoglobin, which suggests that additional unknown posttranscriptional events may be involved in the extracellular production of VvpE.

Keywords: *Vibrio vulnificus*, metalloprotease, hemoglobin, iron

Vibrio vulnificus is a gram-negative halophilic estuarine bacterium that can cause fatal and rapidly-progressing septicemia with a mortality of greater than 50%. *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 factors, including polysaccharide capsule, extracellular hemolysin and protease, RTX toxin and iron-uptake systems (IUSs), have been associated with *V. vulnificus* virulence (Gulig *et al.*, 2005).

Host serum or tissue iron levels are known to be the most predisposing host factor for *V. vulnificus* infection. *V. vulnificus* infection is promoted by elevated serum iron levels (Wright *et al.*, 1981; Brennt *et al.*, 1991; Hor *et al.*, 1998, and 2000) and requires crucial iron uptake from host iron-binding proteins, such as transferrin and hemoglobin, by *V. vulnificus* (Morris *et al.*, 1987; Starks *et al.*, 2000; Webster and Litwin, 2000). *V. vulnificus* has two types of IUSs: siderophore (especially vulnibactin)-mediated IUS (Simpson and Oliver, 1983; Litwin *et al.*, 1996; Shin *et al.*, 2001) and haem receptor (named HupA)-mediated IUS (Helms *et al.*, 1984; Miyoshi *et al.*, 1997; Litwin and Byrne, 1998). IUSs are themselves considered virulence factors in many bacterial pathogens including *V. vulnificus*, and thus promising vaccine targets (Brown *et al.*, 2001). Also, iron chelation is considered prospective therapeutic means of preventing *in vivo* bacterial growth (Lesic *et al.*, 2002). In this regard, it is important to elucidate the roles of IUSs and their relating factors in *V. vulnificus*.

Hemoglobin is the most sufficient iron-binding

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haem protein although it is sequestered within red blood cells. This intracellular hemoglobin can be released by various bacterial cytolysins or host-derived inflammatory mediators, and then utilized as a good iron source by pathogens. *V. vulnificus* can also utilize hemoglobin as a preferential iron source for its growth. Hemoglobin as an iron source is utilized in a different manner from transferrin-bound iron. *V. vulnificus* can utilize transferrin-bound iron only via vulnibactin-mediated IUS, whereas it can utilize hemoglobin via both HupA- and vulnibactin-mediated IUSs (Litwin *et al.*, 1996; Litwin and Byrne, 1998; Webster and Litwin, 2000). Which of these IUSs plays a dominant or essential role in the iron uptake of *V. vulnificus* from hemoglobin has not been determined yet.

A metalloprotease (named VvpE) from *V. vulnificus* has been extensively studied and is known to exhibit a variety of biological effects (Gulig *et al*, 2005). However, the role of VvpE in the pathogenesis of *V. vulnificus* infection remains to be clarified, as VvpE-deficient mutants showed comparable or higher virulence than their wild type strains in experimental mouse models (Jeong *et al.*, 2000; Shao and Hor, 2000; Fan *et al.*, 2001). Of the various biological activities of VvpE, its role in facilitating iron uptake by *V. vulnificus* via the proteolytic cleavage of hemoglobin, transferrin and lactoferrin have attracted some attention (Nishina *et al.*, 1992; Okuzo *et al.*, 1996). However, recent reports indicate that VvpE had no direct effect on iron uptake from human transferrin, and that iron or the high growing ability of *V. vulnificus* conferred by iron was required for the efficient VvpE production (Kawase *et al.*, 2004; Watanabe *et al.*, 2004; Shin *et al.*, 2005; Kim *et al.*, 2006). These findings strongly suggest that VvpE cannot facilitate iron uptake from hemoglobin by *V. vulnificus*.

The Shinoda group first suggested that VvpE can facilitate iron uptake by *V. vulnificus* via the proteolytic cleavage of hemoglobin (Nishina *et al.*, 1992). However, they added purified VvpE to a hemoglobin-containing medium at the start of culture in order to induce the artificial proteolysis of hemoglobin. We believe that this exogenous addition of purified VvpE does not reflect the actual production and role of VvpE. In order for VvpE to directly facilitate iron-uptake of *V. vulnificus* from hemoglobin, 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 in which hemoglobin is the only iron source.

Accordingly, in this study, we first determined which IUS, the HupA- or vulnibactin-mediated IUS, plays a dominant or essential role in iron uptake from hemoglobin by *V. vulnificus* and whether or not VvpE can destroy hemoglobin. We then determined whether inorganic iron can activate VvpE expression on both the transcriptional and protein levels as well as hemoglobin can, whether VvpE is required for efficient iron uptake by *V. vulnificus* via the proteolytic cleavage of hemoglobin, or whether iron or hemoglobin is required for efficient VvpE production, using *in vitro* and human *ex vivo* experimental systems, in accordance with the molecular version of Koch's postulates (Falkow, 1988).

Materials and Methods

Media, cirrhotic ascites and reagents

Heart Infusion broth (BD, 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 (1982). In brief, NL-HI broth was mixed with an equal volume of 3% (w/v) solution of 8-hydroxyquinoline dissolved in chloroform and vigorously stirred for 2 days. The mixture was allowed to stand at room temperature to separate into aqueous and chloroform layers. The aqueous extracts were mixed and stirred with pure chloroform for more than 2 h in order to remove residual 8-hydroxyquinoline. The residual iron concentration of deferrated HI (DF-HI) broth, as measured by the method described by Stookey (1970), was 1.0 μg/dl or less. Synbase minimal medium was also used in this study (Simpson and Oliver, 1983). Iron-limited Synbase agars were prepared by adding 75 μg/ml ethylenediamine-di(o-hydroxyphenyl) acetic acid. Cirrhotic ascites (CA) samples were obtained from five patients with liver cirrhosis, as described in our previous study (Choi *et al.*, 2006a, 2006b). Equal volumes of the five CA samples were pooled; this pooled CA was sterilized and freed of human cells using disposable syringe filters, and then incubated at 65°C for 30 min to inactivate bactericidal factors such as complement. Human hemoglobin (0.5 mg/ml) was added to DF-HI broth or CA, and when required, ferric chloride (10 μM) was added to DF-HI broth. Unless otherwise noted, all reagents were purchased from Sigma-Aldrich (USA).

Bacterial strains, plasmids and PCR primers

Bacterial strains, plasmids and PCR primers used in this study are listed in Table 1. A *vis*-insertional mutant (CMM2301) and a *vvpE*-deletional mutant (CMM1049) were constructed from the *V. vulnificus* MO6-24/O strain, which is a highly virulent clinical isolate, as described in our previous study (Kim *et al.*, 2006). A P_{vvpE}::*lacZ* transcriptional reporter strain (CMM2106) was constructed from a *lacZ*-deleted *V. vulnificus* strain, which originated from the MO6-24/O

Table 1. Bacterial strains, plasmids and primers used in this study

Strains, plasmids and primers	Relative characteristics and sequences	Sources or references
V. vulnificus		
MO6-24/O	Wild type strain; clinical isolate	J.G. Morris, Jr (Lab. Collection)
CMM2301	MO6-24/O with *vis*-insertion mutation; vulnibactin-deficient	Kim *et al.*, 2006
CMM1049	MO6-24/O with *vvpE*-deletion	Kim *et al.*, 2006
CMM1502	CMM1049 with *vvpE* complementation	Kim *et al.*, 2006
CMM2106	CMM2101 with P*vvpE::lacZ* chromosomal reporter	Kim *et al.*, 2003
RC110	MO6-24/O with *hupA*-deletion	This study
E. coli		
SY327 λ*pir*	Host for suicide vector	Miller & Mekalanos, 1988
SM10 λ*pir*	Conjugation donor	Miller & Mekalanos, 1988
pDM4	Suicide vector with R6K origin:Cm^r	McGee *et al.*, 1996
pRC110	1.82 kb *Sma*I-*Spe*I fragment containing an in-frame deletion of *V. vulnificus hupA* gene cloned into pDM4	This study
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

strain as described previously (Kim *et al.*, 2003). The *vvpE'::lacZ::vvpE'* transcriptional fusion was constructed by inserting promoterless *lacZ-*Km^R *cassette* into the open reading frame of the *vvpE* gene without changing the *vvpE* promotor region.

V. vulnificus hupA mutation

General genetic procedures were carried out as described by Sambrook *et al.* (1989). Restriction and DNA-modifying enzymes were used as recommended by manufacturers. PCR amplification was performed using a Mastercycler (Eppendorf, Germany) and standard protocols. An in-frame deletion mutant of the *V. vulnificus hupA* gene was constructed by crossover PCR using the suicide vector pDM4 with an R6K origin (McGee *et al.*, 1996). 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 *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* was cloned into pDM4. The resulting pRC110 was transformed into *E. coli* SY327 λ*pir* and SM10 λ*pir* (Miller and Mekalanos, 1988), 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.

Plate bioassay for hemoglobin utilization

V. vulnificus strains grown in NL-HI broth overnight were inoculated into NL-HI broth containing 100 μM dipyridyl and cultured with vigorous shaking (220 rpm) at 37°C overnight in order to adapt them to iron-limited conditions and to reduce intracellular iron storage. These preconditioned *V. vulnificus* strains were pour-plated onto the surface of iron-limited Synbase agars using 0.5% semisolid top agar at 1×10^4 cfu; paper discs containing various amounts of hemoglobin were placed on the agar surfaces, as described in our previous study (Shin *et al.*, 2001). After the agar plates had incubated at 37°C for 24 h, the diameters of the growth-enhanced areas around the paper discs were measured.

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

Preconditioned *V. vulnificus* strains, as described above, were inoculated into test media or CA at 1×10^6

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 as described by Miller (1992).

Measurement of caseinolytic activity and zymography Culture aliquots were centrifuged at 10,000 rpm for 5 min to obtain culture supernatants. To measure total protease production, caseinolytic activities in culture supernatants were measured using a previously described method (Jeong *et al.*, 2000). To determine the protease profile, zymography was performed as described previously (Raser *et al.*, 1995). In brief, equal volumes (20 μl) of culture supernatants were electrophoresed on 12% SDS-polyacrylamide gel containing 0.3% Skim milk or hemoglobin. Gels were then incubated in renaturation buffer containing 2.5% Triton X-100 at room temperature for 1 h and subsequently in developing buffer containing dithiothreitol and $CaCl_2$ at 4°C overnight, and finally stained with Coomassie blue.

Observation of hemoglobin aggregates and SDS-PAGE In order to grossly or microscopically observe insoluble hemoglobin aggregates formed during culture, culture aliquots were obtained 6 h after culture initiation in DF-HI broth or in DF-HI broth containing hemoglobin or ferric chloride. Culture aliquots (100 μl) were dropped onto filter paper and photographed. To further observe hemoglobin aggregates under a microscope, culture aliquots (100 μl) were smeared and fixed to slide glass, and then stained with crystal violet for 1 min. The stained samples 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 hemoglobin molecules remaining in culture supernatants, culture aliquots obtained at appropriate times from DF-HI broth containing hemoglobin were centrifuged at 10,000 rpm for 5 min to remove hemoglobin aggregates; 20 μl of culture supernatants were then 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.

Results and Discussion

HupA-mediated IUS is involved in, but not essentially required for, iron uptake from hemoglobin by V. vulnificus In order to determine whether the vulnibactin- or HupA-mediated IUS plays a more dominant or essential role in iron uptake from hemoglobin by *V. vulnificus*, we compared the growths of the RC110 (*hupA*-deletion mutant) and CMM2301 (*visA*-insertion mutant; vulnibactin-deficient) strains with that of the wild-type MO6-24/O strain on iron-limited Synbase agars (Table 2). The growth of the MO6-24/O strain was observed only around paper discs containing hemoglobin and was stimulated dose-dependently by 2 and 10 μg hemoglobin. The growth of the RC110 strain was slightly stimulated by 10 μg hemoglobin, but not by 2 μg hemoglobin. The growth of the CMM2301 strain was not stimulated by 2 or 10 μg hemoglobin. These results indicate that both the vulnibactin- and HupA-mediated IUSs are involved in iron uptake from hemoglobin by *V. vulnificus*, but that the vulnibactin-mediated IUS plays a more dominant role in iron uptake from hemoglobin than the HupA-mediated IUS.

Table 2. Hemoglobin utilization by *V. vulnificus*

Hemoglobin (μg/20 μl)	Growth-enhancing area (mm) of		
	MO6-24/O (wild type)	RC110 (HupA-)	CMM2301 (Vis-)
0	0	0	0
2	16 ± 1	0	0
10	20 ± 3	12 ± 2*	0*

MO6-24/O, CMM2301 (*vis*-insertion mutant) and RC110 (*hupA*-deletion mutant) strains grown overnight in HI broth containing 100 μM dipyridyl were pour-plated at about 1×10^4 cfu on the surfaces of iron-limited Synbase agars using top agars, and then paper discs containing 20 μl of phosphate-buffered saline (0) or hemoglobin solutions (2 and 10 μg) were placed on the agar surfaces. The agar plates were incubated at 37°C overnight. Diameters of growth-enhancing area around paper discs were measured. This experiment was repeated in triplicate and the diameters were expressed as mean ± standard error values. The symbol * indicates that there is a statistical significance of $p<0.05$ between RC110 and CMM2301 strains.

Litwin *et al.* (1996) reported that vulnibactin-deficient or vulnibactin receptor (called VuuA)-deficient mutants could acquire iron from hemoglobin, but less effectively than its wild-type strains (Litwin *et al.*, 1996; Webster and Litwin, 2000), indicating that the vulnibactin-mediated IUS is required for efficient iron uptake from hemoglobin. In addition, they also reported that a HupA-deficient mutant was unable to acquire iron from hemoglobin, but the ability of the mutant to utilize hemoglobin was recovered to its wild type level by the exogenous addition of vulnibactin (Litwin and Byrne, 1998). These findings indicate that the HupA-mediated IUS is not essentially required for iron uptake from hemoglobin in the presence of functional vulnibactin-mediated IUS although it can play a role in iron uptake from hemoglobin in the absence of functional vulnibactin-mediated IUS.

VvpE is a major protease and the only protease capable of destroying hemoglobin

In order to compare the ability of these strains to produce protease, MO6-24/O wild-type, *vvpE*-deleted CMM1049 and *in trans vvpE*-complemented CMM1502 strains were cultured in NL-HI broths. No differences were observed between the growths of the three strains (Fig. 1A). In culture supernatants, caseinolytic activity was observed beginning in the early stationary growth phase. The CMM1049 strain exhibited far less caseinolytic activity than the wild-type strain, whereas the CMM1502 strain exhibited caseinolytic activity comparable to that of the wild-type strain (Fig. 1B). In order to observe the protease profile, zymography was conducted using Skim milk or hemoglobin as protease substrates (Fig. 1C). Proteolytic activity on zymograms was observed beginning in the late exponential growth phase. On zymograms using Skim milk, the MO6-24/O wild-type strain exhibited a major proteolytic band and at least two minor proteolytic bands. In contrast, the CMM1409 strain showed only the minor proteolytic bands, and the CMM1502 strain showed the same proteolytic bands as the wild-type strain. On zymograms using hemoglobin, both the MO6-24/O and CMM1502 strains showed a proteolytic band, whereas the CMM1049 strain did not. These results indicate that: (1) VvpE is a major protease produced by *V. vulnificus*, (2) VvpE is the only protease capable of destroying hemoglobin, and (3) minor proteases are not involved in the proteolytic destruction of hemoglobin. Thus, the roles of minor proteases were not further considered in this study.

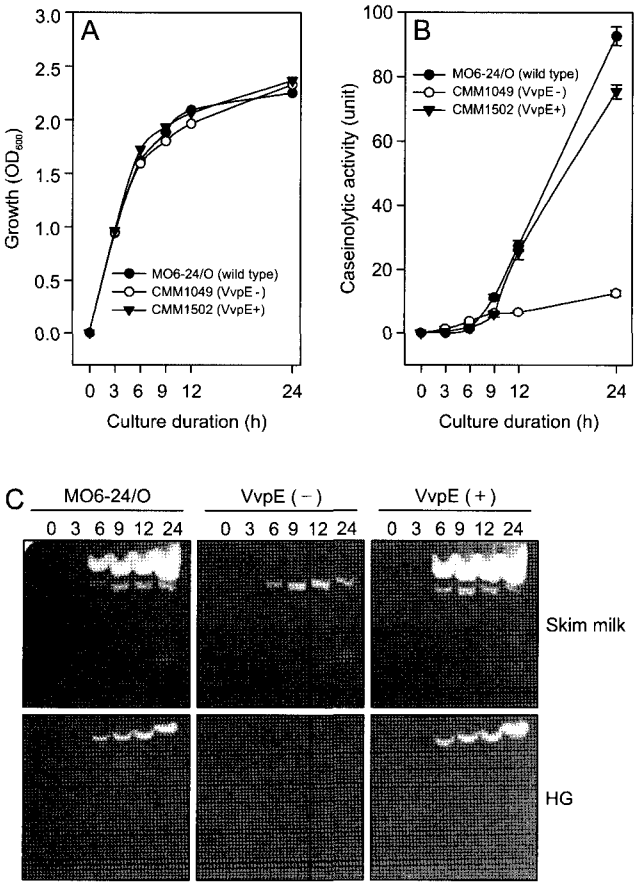

Fig. 1. The growth (A), total protease production (B) and protease profiles (C) of wild type MO6-24/O, *vvpE*-deleted CMM1049 and *in trans vvpE*-complemented CMM1502 strains in normal HI broth. Bacterial growth was monitored by measuring the OD_{600} values of culture aliquots obtained at the indicated times. Culture supernatants were obtained by centrifuging culture aliquots at 10,000 rpm for 5 min. Total protease production in culture supernatants was 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-polyacrylamide gel containing 0.3% Skim milk or hemoglobin (HG), and the gels were subsequently stained with Coomassie blue.

According to our results, VvpE is the only protease capable of destroying hemoglobin. Other researchers also reported that purified VvpE was capable of destroying hemoglobin (Nishina *et al.*, 1992). However, these findings were only observed with purified VvpE or when VvpE was sufficiently produced under optimal conditions and the VvpE was allowed to react with hemoglobin as in the present study. Moreover, this fact alone is insufficient to prove that VvpE facilitates the iron uptake and growth of *V. vulnificus* via the proteolytic cleavage of hemoglobin. In order for VvpE to directly facilitate iron uptake from hemoglobin by *V. vulnificus*, 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 when hemoglobin is the only iron source.

The Shinoda group first suggested that VvpE can facilitate iron uptake of *V. vulnificus* via the proteolytic cleavage of hemoglobin (Nishina *et al.*, 1992). However, they added purified VvpE to a hemoglobin-containing medium at the start of culture. We believe that this exogenous addition of purified VvpE does not reflect the actual production and role of VvpE. Accordingly, in order to determine whether or not VvpE can directly facilitate iron uptake by *V. vulnificus* in the presence of hemoglobin as an iron-source, but in the absence of exogenous VvpE, we conducted the following experiments.

Transcription of vvpE is stimulated by iron or hemoglobin, but only in the late growth phase

In order to determine whether iron or hemoglobin stimulates *vvpE* transcription, the chromosomal P_{vvp}::*lacZ* reporter strain (CMM2106) was cultured in DF-HI broth or in DF-HI broth containing 0.5 mg/ml hemoglobin or 10 μM ferric chloride. Its growth was stimulated by hemoglobin or ferric chloride (Fig. 2A). Transcription of *vvpE* was also stimulated by hemo-

globin or ferric chloride (Fig. 2B). However, *vvpE* transcription was evidently stimulated only during the late exponential or stationary growth phase. Accordingly, these results indicate that: (1) *vvpE* transcription begins after *V. vulnificus* growth has plateaued, and thus its final product VvpE cannot directly facilitate iron uptake by *V. vulnificus* via the proteolytic cleavage of hemoglobin, and (2) iron is required for efficient *vvpE* transcription rather than VvpE being required for efficient iron uptake from hemoglobin by *V. vulnificus*. Transcription of *vvpE* is known to be affected by a variety of growth conditions, including temperature, osmolarity and levels of iron and oxygen (Shao and Hor, 2001), and positively regulated by the stationary sigma factor RpoS (Jeong *et al.*, 2001, and 2003; Hülsmann *et al.*, 2003), the cyclic AMP (cAMP)-cAMP receptor protein complex (Jeong *et al.*, 2003), and the LuxS-quorum sensing system (Jeong *et al.*, 2003; Kim *et al.*, 2003). Without exception, all of these reports showed that *vvpE* transcription is activated only during the late exponential or stationary growth phases. Moreover, it was recently reported that iron or the high growing ability conferred by iron is required for efficient *vvpE* transcription (Kawase *et al.*, 2004; Watanabe *et al.*, 2004). Our previous work also revealed that *vvpE* transcription is stimulated by inorganic iron and transferrin-bound iron, and that it evidently occurs only during the late growth phase (Shin *et al.*, 2005; Kim *et al.*, 2006). In addition, Kawase *et al.* (2004) suggested that the iron-induced *vvpE* transcription might be mediated by the LuxS-quorum sensing system. It is well-documented that the LuxS-quorum sensing system regulates *vvpE* expression during the late exponential or stationary growth phase through RpoS-dependent promotor (Jeong *et al.*, 2001, 2003). Accordingly, all these findings support our opinion that VvpE cannot directly facilitate iron uptake by *V. vulnificus* via the proteolytic cleavage of hemoglobin because *vvpE* expression begins after *V. vulnificus* growth has plateaued; rather, iron or hemoglobin is required for efficient *vvpE* transcription.

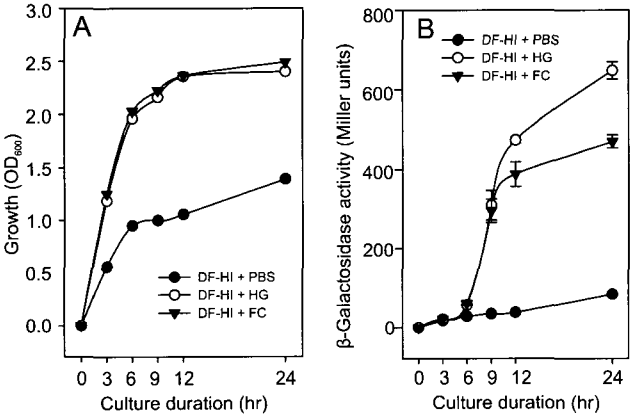

Fig. 2. Transcription of the *vvpE* gene in deferrated (DF)-HI broth containing phosphate-buffered saline (PBS), 0.5 mg/ml hemoglobin (HG) or 10 μM ferric chloride (FC). The growth (A) of chromosomal P*vvpE::lacZ* reporter CMM2106 strain was monitored by measuring the OD_{600} values of culture aliquots at the indicated times. β-Galactosidase activity (B) in the culture aliquots was measured by the Miller method (1992).

Extracellular VvpE production is not stimulated by iron or hemoglobin

Interestingly, although *V. vulnificus* growth and *vvpE* transcription were stimulated by hemoglobin or ferric chloride (Fig. 2A, B and 3A, B), extracellular VvpE production was not stimulated by either (Fig. 3C, D). The extracellular VvpE production in DF-HI broth containing hemoglobin or ferric chloride was far lower than that in NL-HI broth (Fig. 1B). This discrepancy suggests that extracellular VvpE production is addition-

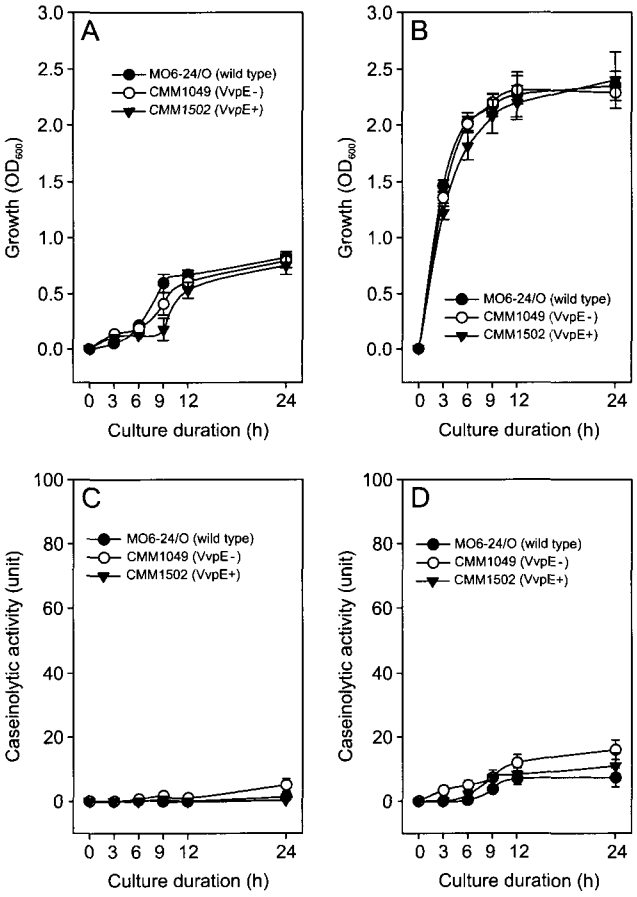

Fig. 3. Growth (A and B) and total protease production (C and D) of wild type MO6-24/O, *vvpE*-deleted CMM1049 and *in trans vvpE*-complemented CMM1502 strains in deferrated (DF) HI broth containing phosphate-buffered saline (A and C) and 0.5 mg/ml hemoglobin (B and D). Bacterial growth was monitored by measuring the OD_{600} values of culture aliquots at the indicated times. Culture supernatants were obtained by centrifuging culture aliquots at 10,000 rpm for 5 min. Total protease production in culture supernatants was measured using azocasein as a substrate.

ally controlled by unknown posttranscriptional events. A similar discrepancy was also observed in DF-HI broth containing holotransferrin as an iron source in our previous work (Shin *et al.*, 2005; Kim *et al.*, 2006). The mechanism of this discrepancy remains to be clarified and further studies are needed. Nevertheless, these results indicate that *V. vulnificus* is able to utilize hemoglobin as an iron source for its growth without the assistance of VvpE.

Mutation of the vvpE gene does not affect the growth of V. vulnificus in DF-HI broth containing hemoglobin as an iron source

In order to confirm that *V. vulnificus* can assimilate iron from hemoglobin without the assistance of VvpE, MO6-24/O wild-type, *vvpE*-deleted CMM1049 and *in trans* *vvpE*-complemented CMM1502 strains were cultured in DF-HI broth or DF-HI broth containing 0.5 mg/ml hemoglobin or 10 μM ferric chloride. The

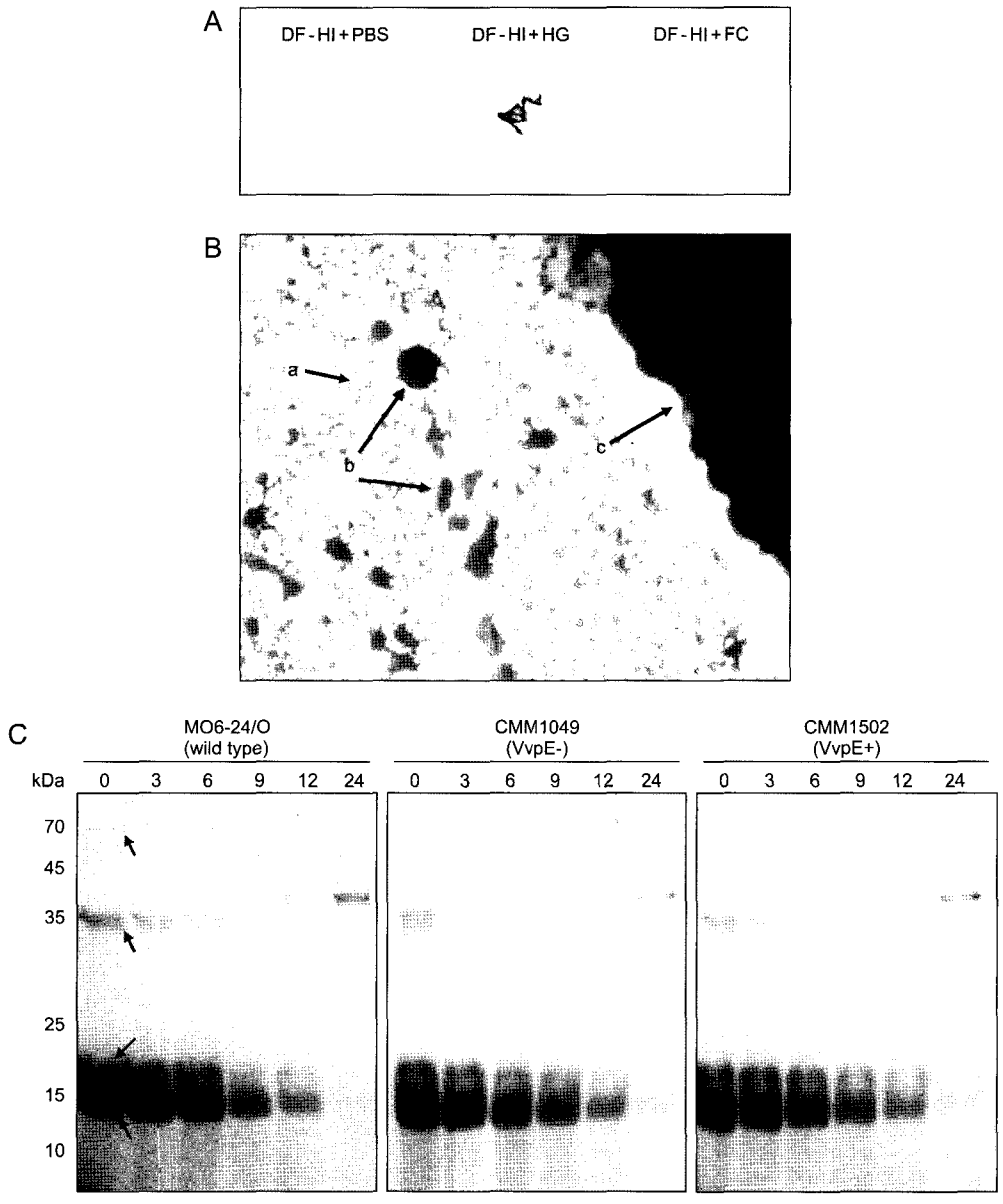

Fig. 4. Nonspecific aggregation (A and B) and disappearance (C) of hemoglobin during culture. Wild type MO6-24/O strain was cultured in deferrated (DF) HI broth containing phosphate-buffered saline (PBS) and 0.5 mg/ml hemoglobin (HG) and 10 μM ferric chloride (FC). Culture aliquots were obtained at 6 h. (A) 100 μl of culture aliquots were dropped onto filter paper. Hemoglobin aggregates are grossly visible. (B) 100 μl of culture aliquot containing hemoglobin aggregates was smeared on slide glass, stained with crystal violet for 1 min, and then observed under microscope (1,000 x). Typical comma-shaped *V. vulnificus* cells (a), small (b) and large (c) hemoglobin aggregates are simultaneously seen. (C) 20 μl of the culture supernatants in which insoluble aggregates were removed by centrifugation were electrophoresed on 15% SDS-polyacrylamide gels. Residual hemoglobin molecules gradually disappear without proteolytic cleavage. The arrows indicate the four hemoglobin forms (about 16, 17, 35, and 70 kDa).

growths of all three strains were stimulated by hemoglobin or ferric chloride, but no significant differences were observed among the growths of the three strains (Fig. 3A, B). These results clearly indicate that *V. vulnificus* is able to utilize hemoglobin and to grow on hemoglobin without the assistance of VvpE.

Simpson and Oliver (1993) reported that all *V. vulnificus* protease-deficient mutants generated by chemical or transposon mutagenesis were able to utilize hemoglobin as an iron source. In contrast, Nishina *et al.* (1992) reported that a *V. vulnificus* protease-deficient mutant generated by chemical mutagenesis was unable to utilize hemoglobin as an iron source. This difference may result from non-specific mutagenesis. Protease-deficient mutants generated by chemical or transposon mutagenesis may have multiple nonspecific mutations and exhibit unexpected phenotypic changes. In contrast, we specifically mutated only the *vvpE* gene by in-frame deletion of the internal sequences of the *vvpE* open-reading frame as described in our previous studies (Kim *et al.*, 2006).

Hemoglobin is not destroyed, but forms insoluble aggregates during culture

Interestingly, when the MO6-24/O strain was cultured in DF-HI broth containing hemoglobin, bizarre insoluble aggregates were observed 3 h after culture initiation; these aggregates were large enough to be grossly distinguished 6 h after culture initiation as shown in Fig. 4A, and continued to gradually increase throughout culture. Under the microscope, typical comma-shaped *V. vulnificus* cells and small and large hemoglobin aggregates were observed simultaneously (Fig. 4B). These findings suggested that hemoglobin was not destroyed, but rather aggregated during culture. Therefore, in order to further determine whether or not the hemoglobin molecules were destroyed, SDS-PAGE was conducted using culture supernatants from which insoluble aggregates had been removed by centrifugation. Residual hemoglobin molecules were separated into the four forms with molecular sizes of approximately 16, 17, 35 and 70 kDa (Fig. 4C); none of these hemoglobin molecules were destroyed, but rather they gradually disappeared. This novel phenomenon was observed regardless of *vvpE* mutation or complementation (data not shown). Accordingly, these findings indicate that hemoglobin is not destroyed but only disappears due to aggregation during culture. We believe that hemoglobin molecules may be denatured due to their loss of heme or iron, or by metabolites other than VvpE generated by *V. vulnificus* during culture. Furthermore, these findings also support our opinion that VvpE does not directly facilitate iron uptake by *V. vulnificus* via the proteolytic cleavage of hemoglobin.

VvpE does not affect the iron uptake of V. vulnificus in a human ex vivo system containing hemoglobin 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 (Gulig *et al*, 2005; Choi *et al.*, 2006). MO6-24/O wild type, *vvpE*-deleted CMM1049, *in trans vvpE*-complemented CMM1502 and P_{vvpE}::*lacZ* transcription reporter CMM2106 strains were cultured in CA or CA containing 0.5 mg/ml hemoglobin. The growths of the four strains were stimulated by adding hemoglobin to CA (Fig. 5A and 6A, B). However, neither *vvpE* mutation nor *vvpE* complementation affected the growth of *V. vulnificus* in CA or CA containing hemoglobin (Fig. 6A, B). Transcription of *vvpE* in the CA background was also stimulated by hemoglobin, but this evidently occurred during the late growth phase when *V. vulnificus* growth had already plateaued (Fig. 5B). Moreover, although *vvpE* transcription was stimulated by hemoglobin, no protease activity was detected in culture supernatants (Fig. 6C, D). We found that a 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 hemoglobin and to grow on hemoglobin without the assistance of VvpE in a human *ex vivo* background.

Pseudomonas aeruginosa proteases are known to facilitate iron uptake from iron-binding proteins, such as hemoglobin and transferrin, via proteolytic cleavage (Britigan *et al.*, 1993; Wolz *et al.*, 1994). *P. aeruginosa* produces proteases in the early growth phase and does

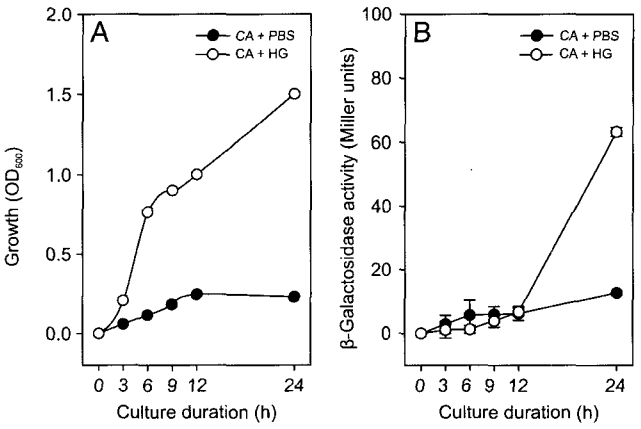

Fig. 5. Transcription of the *vvpE* gene in cirrhotic ascites (CA) containing phosphate-buffered saline (PBS) or 0.5 mg/ml hemoglobin (HG). (A) The growth of chromosomal P*vvpE*::*lacZ* reporter CMM2106 strain was monitored by measuring the OD_{600} values of culture aliquots at the indicated times. (B) β-Galactosidase activity in culture aliquots was measured by the Miller method (1992).

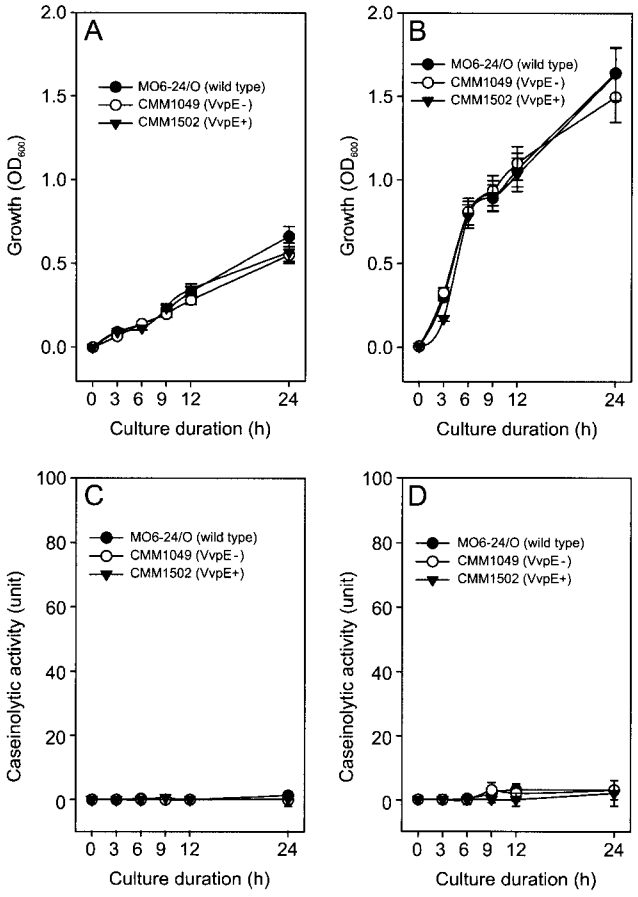

Fig. 6. Growth (A and B) and total protease production (C and D) of wild type MO6-24/O, *vvpE*-deleted CMM1049 and *in trans* *vvpE*-complemented CMM1502 strains in cirrhotic ascites containing phosphate-buffered saline (A and C) and 0.5 mg/ml of hemoglobin (B and D). Bacterial growth was monitored by measuring the OD_{600} values of culture aliquots at the indicated times. Culture supernatants were obtained by centrifuging culture aliquots at 10,000 rpm for 5 min. Total protease production in culture supernatants was measured using azocasein as a substrate.

so more robustly under iron-limited conditions than under iron-sufficient conditions; these proteases facilitate the pyoverdin-mediated iron uptake by *P. aeruginosa* via the proteolytic cleavage of iron-binding proteins. In addition, we recently reported that *P. aeruginosa* produces alkaline proteases concomitantly with the production of siderophores in the early growth phase, and these proteases can facilitate siderophore-mediated iron uptake from transferrin via proteolytic cleavage (in press). In another study, we also found that a *Bacillus subtilis* clinical strain produces proteases in parallel with the production of siderophores in the early growth phase, and these proteases can facilitate siderophore-mediated iron uptake from transferrin via proteolytic cleavage (Park *et al.*, 2006). Overall, all these reports show that a protease must be produced robustly during the exponential growth phase, especially under iron-limited conditions when iron-binding proteins are the primary iron sources, in order for the protease to directly facilitate iron uptake from the iron-binding proteins.

In summary, *V. vulnificus* is able to utilize hemoglobin for growth more efficiently via the vulnibactin-mediated IUS than via the HupA-mediated IUS, and VvpE is the only protease capable of destroying hemoglobin. Transcription of *vvpE* is stimulated by hemoglobin or inorganic iron. However, *vvpE* transcription occurs only in the late exponential or stationary growth phases by which time *V. vulnificus* growth has already plateaued and most of the iron has already been consumed. Moreover, extracellular VvpE production is not stimulated by hemoglobin or inorganic iron, and hemoglobin is not destroyed by VvpE, but disappears following aggregation during culture. Finally, *vvpE* mutation and complementation have no effect on the ability of *V. vulnificus* to utilize hemoglobin and to grow on hemoglobin. Therefore, VvpE appears to have no direct effect on iron uptake from hemoglobin by *V. vulnificus* although it is the only protease capable of destroying hemoglobin.

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