Evidence That Temporally Alternative Expression of the *Vibrio vulnificus* Elastase Prevents Proteolytic Inactivation of Hemolysin

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Abstract   Numerous secreted and cell-associated virulence factors have been proposed to account for the fulminating and destructive nature of *Vibrio vulnificus* infections. Among the putative virulence factors are an elastase, elastolytic protease, and a cytolytic hemolysin. Effects of the elastase on the hemolysin were assessed by evaluating changes of hemolytic activities either in the presence or absence of the protease. Although hemolytic activity in the culture supernatant was lowered by the purified elastase added *in vitro*, the cellular level of hemolytic activity was unaffected by the mutation of *vvpE* encoding the elastase. Growth kinetic studies revealed that hemolysin reached its maximum level in the exponential phase of growth, and the elastase appeared at the onset of the stationary phase. These results have provided insight into the regulation of virulence factors: temporally coordinate regulation of virulence factors is essential for the overall success of the pathogen during pathogenesis.

Key words: *Vibrio vulnificus*, elastase, hemolysin

The pathogenic marine bacterium *Vibrio vulnificus* occurs in raw seafoods, and has been identified as the causative agent of foodborne diseases such as gastroenteritis and life-threatening septicemia in immunocompromised individuals. Mortality from septicemia is quite high (exceeding 50%), and death may occur as fast as within 1 to 2 days after ingestion. Disease caused by infection with *V. vulnificus* is remarkable for the invasive nature of the infection, ensuing severe tissue damage, and a rapidly fulminating course [for recent reviews, see 6, 18, 20, 35]. The characterization of somatic as well as secreted products of *V. vulnificus* has

yielded a large list of putative virulence attributes, whose known functions are easily accepted as explaining the pathology of the disease. These putative virulence factors include a carbohydrate capsule, lipopolysaccharide, a cytolysin/hemolysin, elastolytic metalloprotease, iron sequestering systems, and lipase [4, 20, 32, 35, 39].

Among the putative virulence factors is the cytolytic hemolysin encoded by the *vvhA* gene. Hemolysin can lyze red blood cells (RBC) from a variety of animals by forming small pores in the cytoplasmic membrane, and it also shows cytolytic activity against cultured cell lines [34, 37]. The purified hemolysin with an estimated 51 kDa molecular mass is heat labile, lytic for mammalian erythrocytes, and toxic for Chinese hamster ovary (CHO) cells [9, 17, 36]. When injected intravenously, the purified toxin was lethal in mice at levels of ca. 3 μg per kg body weight. The hemolysin binds possibly to cholesterol and induces the release of K^+ ions and to a lesser extent Na^+ ions from liposome membranes [38]. A 3.4 kilobase (kb) DNA fragment of *V. vulnificus* strain EDL174, which encodes VvhA, has been cloned and its nucleotide sequence has been sequenced [37]. It has been reported that hemolysin production in *V. vulnificus* is repressed by adding glucose and restored by adding cyclic AMP (cAMP) to the culture broth [1]. In a previous report, we showed that *vvhA* expression is activated by CRP (cAMP receptor protein), and CRP exerts its effects by directly binding to the promoter region of *vvhA* [5].

Elastase, an elastolytic metalloprotease, has been suggested as an important virulence factor of various human pathogenic bacteria [10]. The well characterized elastase of *Pseudomonas aeruginosa* is capable of degrading or inactivating elastin, collagen, immunoglobulins, serum complement factors, and some plasma proteins [8, 14, 31, 33]. There have been several different lines of evidence leading to the hypothesis

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that elastase is an important, if not essential, component of the virulence of *V. vulnificus* during the infection of animals. Injection of purified elastase could reproduce many of the observed aspects of disease caused by *V. vulnificus*, including dermonecrosis, destruction of tissues, edema, and ulceration [reviewed in reference 27]. These diverse activities are believed to be caused by the proteolytic degradation or inactivation of biologically important host proteins and immune system components such as collagen, fibrin, and complement [16, 24, 25, 26, 30]. Recently, the gene that encodes a *V. vulnificus* elastase was cloned and sequenced [3, 7, 12]. The deduced gene product was predicted to be a 609-amino acid polypeptide with the mature protease having a 45-kDa molecular mass consisting of 413 amino acids generated by deletion of the N-terminal 196 amino acids [28]. More recently, we demonstrated that expression of *vvpE*, encoding elastase, is differentially directed by two different promoters, and elevated by RpoS and CRP [11].

The broad substrate specificity of the *V. vulnificus* elastase observed in the proteolytic degradation or inactivation of diverse host molecules raises the question of whether elastase can affect the activity of hemolysin. Accordingly, the influence of elastase on hemolytic activity was examined in the present study. It was apparent that the hemolytic activity in the culture supernatant was decreased by adding purified elastase. The effect of a *vvpE* null mutation on hemolytic activity was also examined. Finally, the implication of the timing of the genes expression encoding the two virulence factors in the pathogenesis of *V. vulnificus* was discussed.

The bacterial strains and plasmids used in this study are listed in Table 1. Unless noted otherwise, *V. vulnificus* strains were grown in LB medium supplemented with 2.0% (w/v) NaCl (LBS). Cultures of *V. vulnificus* strains were grown at 30°C with aeration. Five ml samples were removed at the indicated times for determination of cell densities, hemolysin activities, elastase activities, cellular level of *vvhA* or *vvpE* transcripts, and cellular protein concentrations. Growth was monitored by measuring the OD_{600} of the cultures, and the elastase activities were determined according to the procedures described previously [12]. Hemolytic activities in culture supernatant were determined by the method described by Shinoda *et al.* [34], and a hemolytic unit was defined as the reciprocal of the maximal dilution showing 50% hemolysis of human RBC solution (1%, v/v). Protein concentrations were determined by the method of Bradford [2], with bovine serum albumin as the standard. Northern blot analysis involved the procedures described previously [5, 11]. The coding region of *vvhA* on pHK0202 or *vvpE* on pKC980 [5, 12] (Table 1) was labeled with [α-^{32}P]dCTP and used to probe *vvpE* and *vvhA* transcripts. Averages and standard errors of the mean (SEM) were calculated from at least three independent experiments.
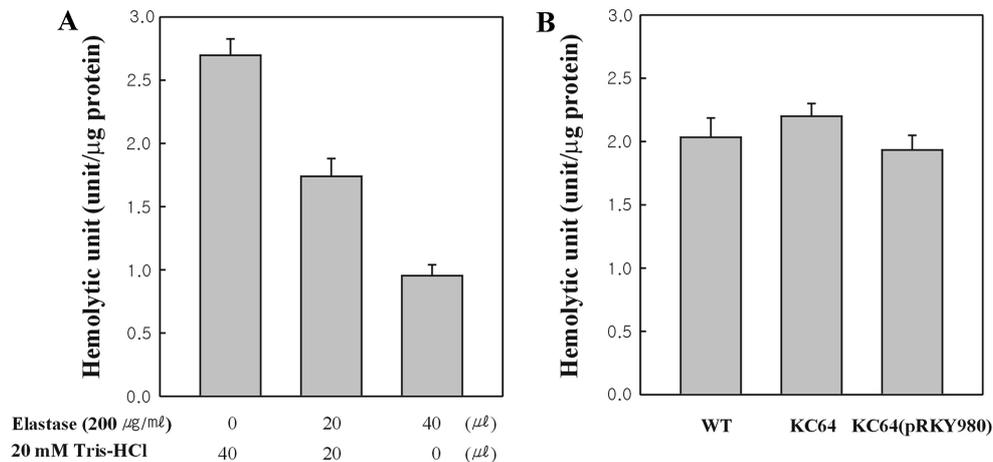
Influence of Elastase on the Hemolytic Activity

To examine whether hemolysin of *V. vulnificus* is inactivated by elastase, hemolytic activities in the supernatant of the *V. vulnificus* culture were analyzed with or without the addition of purified elastase. For this purpose, *V. vulnificus* elastase was purified and used as described by Miyoshi *et al.* [29]. Hemolytic activities of strain KC64, an isogenic mutant of *V. vulnificus* ATCC29307 in which *vvpE* was disrupted by allelic exchange [12, 21], appeared at the beginning of growth and reached a maximum during the exponential phase. The elastase was added to the supernatant of KC64 culture grown to the exponential phase and incubated at 37°C for 1 h. The addition of elastase resulted in a significant decrease in hemolytic activity (Fig. 1A). Hemolytic activity in the supernatant of KC64 culture grown to the exponential phase was about

Table 1. Plasmids and bacterial strains used in this study.

Strain or plasmid	Relevant characteristics[a]	References
Bacterial strains		
E. coli		
DH5α	*supE*44 Δ*lacU169* (*φ80 lacZ* ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi-1 relA1*	Laboratory collection
SM10λ*pir*	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu λ*pir, oriT* of RP4, Km^r; conjugational donor	[22]
V. vulnificus		
ATCC29307	Clinical isolate	Laboratory collection
KC64	ATCC 29307, *vvpE::nptI*	[12]
Plasmids		
pRK415	IncP *ori*; broad host range vector; *oriT* of RP4; Tc^r	[13]
pKC980	pUC18 with 2.5-kb partial *Sau*3A fragment encoding *vvpE*; Ap^r	[12]
pHK0202	pUC19 with *vvhBA*; Ap^r, Km^r	[5]
pRKY980	pRK415 with 2.5-kb *Eco*RI-*Sal*I fragment carrying *vvpE* from pKC980; Tc^r	[12]

[a]: Ap^r, ampicillin resistant; Km^r, kanamycin resistant; Tc^r, tetracycline resistant.

Fig. 1. Influence of elastase on the hemolytic activity *in vitro* and in the culture.
(A) KC64 was grown to exponential phase, and culture supernatant was harvested. The supernatant (200 μl) was mixed with purified elastase and with 20 mM Tris-HCl (pH 7.6, as a negative control) as indicated and incubated for 1 h at 37°C before hemolytic activities were measured. (B) Hemolytic activity of wild-type, KC64, and KC64(pRKY980) as indicated. Samples removed at the exponential phase from cultures were analyzed for determination of hemolytic activities. For both panels, human RBC was used for determination of hemolytic activities. Error bars represent SEM.

3.0 units without the addition of elastase. When the amount of added elastase increased to 8 μg in the supernatant, the level of hemolytic activity decreased to approximately 1.0 unit. This level of elastase added to the supernatant *in vitro* corresponds to the maximum level of elastase activity observed in the culture supernatant of wild-type *V. vulnificus* (data not shown). This dose-dependent decrease of hemolytic activity by elastase suggested that elastase inactivates hemolysin or at least inhibits hemolytic activity *in vitro*.

However, this *in vitro* reduction of hemolytic activity by added elastase does not necessarily imply that the activity of hemolysin in the culture is affected by elastase, which is produced by *V. vulnificus*'s own cells. To further confirm the effect of elastase on hemolysin in the culture, the hemolytic activities of ATCC29307 and KC64, which lack elastase activity, were compared. When cultures of ATCC29307 and KC64 were grown to the exponential phase, hemolytic activities in the supernatant were similar in the two strains (Fig. 1B). It was also investigated whether the reintroduction of recombinant *vvpE* could affect the hemolytic activity of KC64. For this purpose, the coding region of *vvpE* was subcloned into a broad host-range vector pRK415 to result in pRKY980 as described previously [12, 16]. *Escherichia coli* SM10 λ*pir*, *tra* [22] was transformed with pRKY980 and used as a conjugal donor to KC64. When the plasmid pRKY980 was mobilized into KC64, the hemolytic activity of KC64(pRKY980) was comparable to the level of KC64. It was, therefore, apparent that the elastase production is unlikely to affect the cellular level of hemolytic activity in the culture. This is opposite to the result observed in the studies described above, in which hemolytic activity was reduced by the purified elastase added *in vitro* into the culture supernatant.

Growth Kinetics of Hemolysin and Elastase Activity
The major problem to be addressed is the discrepancy between the results obtained from the elastase addition experiment and the *vvpE* disruption experiment. One possible explanation is that the timing of the expression of the elastase was different from that of hemolysin, therefore, the level of elastase was too low to affect hemolysin, when the level of hemolysin was substantial. To examine this possibility, changes in the level of hemolysin and elastase activities were monitored in the supernatant of ATCC29307 cells taken at different stages of growth (Fig. 2A). Hemolysin activity appeared at the beginning of growth, reached a maximum in the exponential phase, and then decreased in the stationary phase. In contrast, most of the elastase activity appeared at the onset of the stationary phase of growth. At the exponential phase, when hemolytic activity reached a maximum (6 h), the level of elastase activity of the wild-type was very low and almost identical to that of KC64 (Figs. 2A and 2B). After that, elastase activity increased approximately 20-fold, when hemolysin production ended. Therefore, it was apparent that hemolytic activity in the exponential phase was not affected by elastase, which was mostly expressed in the stationary phase.

However, since the elastase activity apparently increased in the stationary phase, it is still possible that elastase was the major cause for the stationary-phase decrease in hemolytic activity. The disruption of *vvpE* in KC64 resulted in a significant loss of elastase activity, but the level and general pattern of hemolytic activity of KC64 was similar to that of the wild-type parent. It was noteworthy that hemolytic activity still decreased in the stationary phase in KC64 (Fig. 2B), indicating that the loss of hemolysin in the stationary phase was not due to proteolytic inactivation
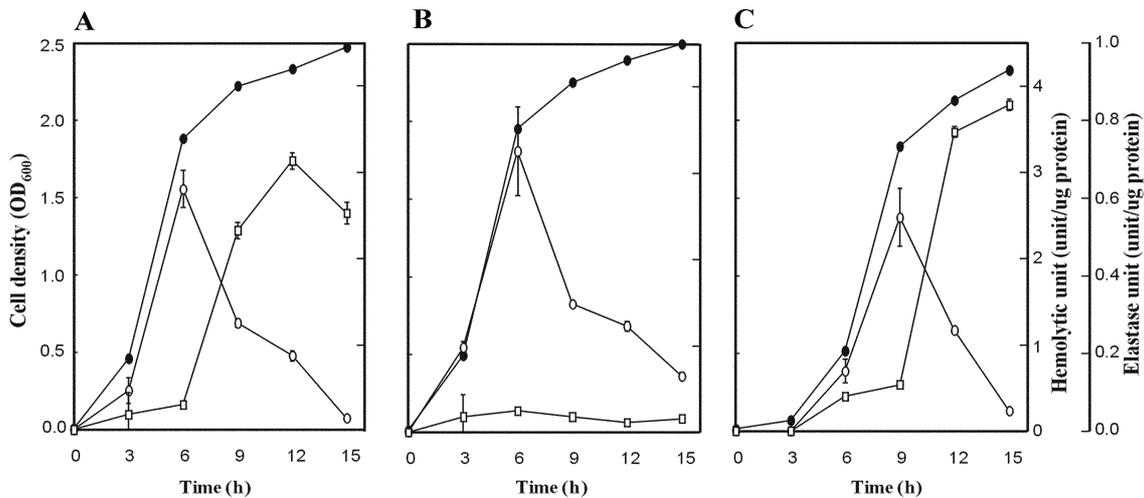
Fig. 2. Growth kinetics and activities of elastase and hemolysin in *V. vulnificus* strains.
V. vulnificus strains were grown in heart infusion broth at 37°C with aeration. Samples were removed from the cultures of ATCC29307 (A), KC64 (B), and KC64(pRKY980) (C) at indicated times, and were analyzed for growth (●), elastase activity (□), and hemolytic activity (○). Hemolytic activities were measured by using human RBC. OD_{600}, optical density at 600 nm.

caused by elastase. The elastase activity in KC64 containing pRKY980 was restored to a level comparable to that in the wild-type parent. Again, when compared with hemolytic activities either in the wild-type or in KC64, the level and kinetic variation of hemolytic activity in KC64(pRKY980) did not differ significantly (Fig. 2C).

These results together led us to the hypothesis that the lack of significant difference in hemolytic activity between the *vvpE* mutant and wild-type parent strain is due to temporally distinct expression of the *vvhA* and *vvpE* genes. In addition, the data also suggested that the stationary-phase decrease in hemolytic activity is not due to the presence of a high level of elastase.

Temporally Differential Expression of *vvpE* and *vvhA* Genes

Although it was apparent that the decrease in hemolytic activity in the stationary phase was not due to the elastase, it was still possible that the decrease resulted from the presence of unknown factors that degraded and/or inactivated hemolysin. To examine this possibility, levels of *vvhA* mRNA were monitored during growth. The same amount of total RNA was isolated from ATCC29307 cells at different stages of growth and analyzed. The data revealed that *vvhA* mRNA levels decreased as the bacterial culture entered the stationary phase, and the pattern of the growth-phase dependent decrease of *vvhA* mRNA was similar to that of the hemolytic activity, which was determined directly (Fig. 3). This result suggested that the growth-phase dependent decrease in hemolytic activity was caused by down-regulation of the *vvhA* transcription, rather than by the appearance of hemolysin-inactivating factor(s) in the stationary phase.

Similarly, the growth phase variation of elastase might possibly occur either at the transcriptional level or post-transcriptional level of *vvpE* expression. To distinguish these two possibilities, changes in the level of *vvpE* mRNA were monitored. The relative levels of the *vvpE* mRNA were much lower in the exponential phase and increased as
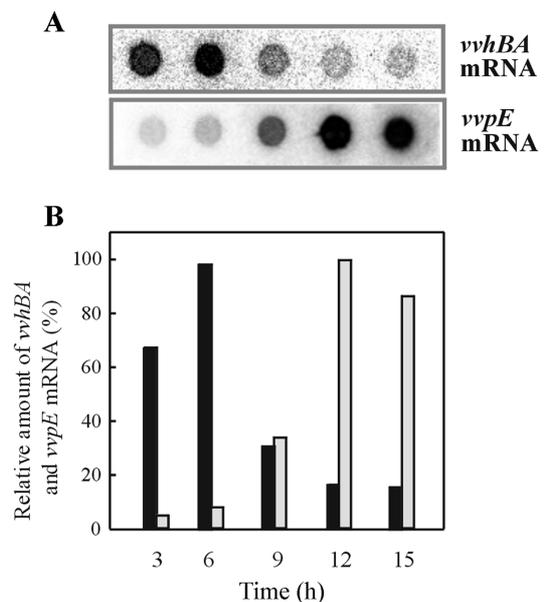
Fig. 3. Temporally distinct expression of *vvhA* and *vvpE* genes. (A) Samples removed in different growth phases as indicated from culture of strain ATCC29307 were analyzed by Northern dot blot analysis. (B) Relative amounts of the transcripts of each dot were presented using the amount of the *vvpE* transcript at 12 h and *vvhA* transcript at 6 h as 100%, respectively. Filled bars represent *vvhA* transcript and open bars represent *vvpE* transcript.

the bacterial culture entered the stationary phase (Fig. 3). Again, the pattern of variation of the *vvpE* transcript in different growth phases was similar to that of elastase activity. These results indicated that a decrease in the level of *vvpE* transcription was the major mechanism whereby elastase activity remained at a low level in the exponential phase.

Consequently, it appeared that the lack of substantial adverse effects by elastase on the activity of hemolysin in the culture is mainly, if not solely, due to the temporally distinct expression of *vvpE* and *vvhA* genes. Because the expressions of the two genes are temporally separated, a significant portion of elastase is not produced until most of the hemolysin is down-regulated to a low level.

It has been reported previously that the hemolysin purified from *V. vulnificus* CDC B3547, a biotype 2, was also cleaved into the 40-kDa fragment and other small fragment(s) by the addition of the purified elastase *in vitro* [29]. However, the results revealed that the hemolytic activity of the 40-kDa fragment was comparable to that of intact hemolysin, and that the nicked-hemolysin fragment become more hydrophilic and stable. This result is opposite to the present finding that, when added to supernatant *in vitro*, elastase inactivated the hemolysin of *V. vulnificus* ATCC29307, a biotype 1 strain. A plausible explanation for this difference in the susceptibility of the biotype 2 hemolysin to elastase would be that the compositions and/ or sequences of the amino acids of the biotype 2 hemolysin differ from those of biotype 1 hemolysin. Indeed, substantial differences in the nucleotide sequences of the *vvhA* gene from CDC B357 and ATCC29307 have been found (Miyoshi, personal observation).

The multifaceted nature of the host-pathogen interaction indicates that more than one virulence factor is typically involved in pathogenesis, and the multifactorial nature of pathogenicity has been supported by numerous studies, showing that specific virulence factors, such as adhesions, invasions, toxins, and capsules, contribute to unique stages of infection and disease [21]. Most of these virulence factors act cooperatively to obtain maximum effectiveness in the pathogenesis, and their expression is controlled by a common regulatory system in response to environmental signals [23]. However, it is likely that an additional level of regulation may be needed to precisely regulate the timing of expression of the virulence factors. The temporally coordinated regulation would facilitate cooperation of the virulence factors during pathogenesis. Indeed, although the elastase showed adverse effects on hemolysin *in vitro*, the present study revealed that the genes encoding hemolysin and elastase are expressed sequentially as temporally separable events, such that elastase has no substantial adverse effect on hemolysin in the culture. It is proposed that hemolysin is involved in a stage of pathogenicity in which elastase is not required, and that expression of elastase occurs after

hemolysin's role has been diminished. Since the energy and resources in cells should be used in the most efficient way possible during pathogenesis, this sequential expression of genes encoding virulence factors is not unexpected.

Acknowledgment

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