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High growing ability of Vibrio vulnificus biotype 1 is essential for production of a toxic metalloprotease causing systemic diseases in humans

Hirofumi Watanabe* Shin-ichi Miyoshi† Tomoka Kawase‡
Ken-ichi Tomochika** Sumio Shinoda††

*Okayama University
†Okayama University, miyoshi@pharm.okayama-u.ac.jp
‡Okayama University
**Okayama Univeristy
††Okayama University

High growing ability of *Vibrio vulnificus* biotype 1 is essential for production of a toxic metalloprotease causing systemic diseases in humans

Hirofumi Watanabe, Shin-ichi Miyoshi*, Tomoka Kawase, Ken-ichi Tomochika, Sumio Shinoda

Faculty of Pharmaceutical Sciences, Okayama University, Tsushima-Naka, Okayama 700-8530, Japan

Short Title: Protease production by *V. vulnificus*

Correspondent Footnote

Corresponding author: Shin-ichi Miyoshi

Postal address: Faculty of Pharmaceutical Sciences, Okayama University, Tsushima-Naka, Okayama 700-8530, Japan

Phone/Fax: +81-86-251-7967

E-mail: miyoshi@pharm.okayama-u.ac.jp

Abstract

Vibrio vulnificus biotype 1, a causative agent of fatal septicemia or wound infection in humans, is known to produce a toxic metalloprotease as an important virulence determinant. *V. vulnificus* biotype 2 (serovar E), a primary eel pathogen, was found to elaborate an extracellular metalloprotease that was indistinguishable from that of biotype 1. The potential of *V. vulnificus* biotype 1 for production of the metalloprotease was compared with biotype 2 and other human non-pathogenic *Vibrio* species (*V. anguillarum* and *V. proteolyticus*). When cultivated at 25 ^{o}C in tryptone-yeast extract broth supplemented with 0.9 % NaCl, all bacteria multiplied sufficiently and secreted significant amounts of the metalloprotease. However, at 37 ^{o}C with 0.9 % NaCl, *V. anguillarum* neither grew nor produced the metalloprotease. In human serum, only *V. vulnificus* biotype 1 revealed a steady multiplication accompanied with production of the extracellular metalloprotease. This prominent ability of biotype 1 in growth and protease production may contribute to cause serious systemic diseases in humans.

Key words: *Vibrio vulnificus*, Metalloprotease, Protease

Introduction

Vibrio vulnificus biotype 1 is a human pathogen causing fatal septicemia or wound infection, and the infection is characterized by formation of hemorrhagic and edematous lesions on the limbs [1]. This bacterium produces a 45-kDa thermolysin-like metalloprotease (*V. vulnificus* protease [VVP]) as an important virulence determinant, which increases vascular permeability and causes serious hemorrhagic damage [2,3]. *V. vulnificus* biotype 2 that is also named as serovar E [4] is a primary causative agent of epizooticus in eel farms [5,6], while it has isolated occasionally from human clinical sources [7]. Our preliminary experiments using partially purified preparations indicated that an extracellular protease produced by biotype 2 might be indistinguishable from VVP. In spite of their being avirulent to humans, other vibrios such as *V. anguillarum* [8,9] and *V. proteolyticus* [10] also produce closely related metalloproteases those exhibit 70-80 % identities in the primary structures and have comparable biological activities. Additionally, these proteolytic enzymes are neutralized with the antibody against VVP [11].

In various microorganisms, production of extracellular proteases, as well as other factors, is coordinated by the quorum sensing or cell density-dependent regulation system [12,13]. Two kinds of quorum-sensing systems have been reported in *V. anguillarum*. One system is operated by a small substance, *N*-(3-oxodecanoyl)-L-homoserine lactone [14], while another system is linked to a substance related to *V. harveyi* autoinducer 2 (AI-2) [15]. In *V. vulnificus* biotype 1, VVP production is positively regulated by the system dependent on the AI-2-like substance [16,17,18]. Namely, expression of the *vvp* gene is augmented at stationary growth phase because of sufficient bacterial multiplication followed by accumulation

of the AI-2-like substance.

The present study showed that *V. vulnificus* biotype 1 was able to multiply in human plasma. Therefore, although both biotypes have equal capability of producing toxic metalloproteases *in vitro*, only biotype 1 is capable of growing and therefore secreting the metalloprotease in humans.

Results and Discussion

Extracellular protease produced by biotype 2

For purification of a metalloprotease (E86 protease), the supernatant of the 24-h culture of *V. vulnificus* E86, a biotype 2 strain, was fractionated by ammonium sulfate precipitation. Thereafter, the preparation was subjected to gel filtration on a HiLoad 26/60 Sephacryl S-200 HR column and hydrophobic interaction chromatography on a HiLoad 10/2 Phenyl Sepharose column. The final preparation thus obtained revealed homogeneity on SDS-PAGE. Namely, in the presence or absence of a reducing agent, a single protein band with a molecular mass of 45-kDa, the same size as VVP from biotype 1, was formed.

The purified E86 protease (1.0 nmol) showed sufficient proteolytic activities toward both azocasein (490 PU) and elastin-Congo red (95 EU), which were very close to those of recombinant VVP (520 PU and 89 EU) used as a positive control. Likewise VVP [2,3], E86 protease was inactivated by incubation with phosphoramidon or zincov, the competitive peptide inhibitor for thermolysin-like zinc metalloproteases. Additionally, in the immunodiffusion test using the IgG antibody against VVP, E86 protease formed a clear precipitation line fused completely with the line to VVP. When injected into a guinea pig skin, it showed the comparable permeability-enhancing and

hemorrhagic activity (data not shown).

In order to study genetic identity, PCR amplification using primers for the *vvp* gene was carried out. As shown in Fig. 1, the 668-bp product was detected from *V. vulnificus* E86, as well as biotype 1 strains. Additionally, a total of 12 *V. vulnificus* strains including 5 strains in the biotype 2 were tested for PCR amplification. The results revealed that all strains produced the same size amplicon (data not shown). However, 6 strains from other *Vibrio* species including *V. anguillarum* and *V. proteolyticus* did not produce the amplicon (data not shown).

Taken together, it may be concluded that an extracellular metalloprotease elaborated by *V. vulnificus* biotype 2 is closely related to or identical with VVP produced by biotype1.

Bacterial growth and protease production in a complex medium

When cultivated at 25 or 37 ^{o}C in TYE broth (0.5 % tryptone, 0.25 % yeast extract, 25 mM K_2HPO_4 [pH 7.5]) supplemented with 0.9 or 3.0 % NaCl, *V. vulnificus* L-180 (a biotypes 1 strain), *V. vulnificus* E86, and *V. proteolyticus* ATCC 15338 showed sufficient growth under all experimental conditions (Fig. 2). However, *V. anguillarum* FY-8701 multiplied very poorly in the broth containing 0.9 % NaCl when cultivated at 37 ^{o}C (Fig. 2). Temperature and/or salinity were found to modulate protease production (Fig. 3). *V. vulnificus* L-180 could produce sufficient amounts of the protease at 25 ^{o}C, however, much less protease was produced at 37 ^{o}C. Protease production by *V. vulnificus* E86 was also regulated, but less effectively. By contrast, *V. proteolyticus* ATCC 15338 could elaborate the protease independent of the cultivation conditions. On the other hand, *V. anguillarum* FY-8701 produced a little amounts of

the protease even when cultured at 25 ^{o}C. The extracelluar protease secreted by each vibrio was completely neutralized with the antibody against VVP, indicating the thermolysin-like metalloprotease is a single protease secreted.

It should be noted that, all vibrios elaborated significant amounts of the metalloprotease only at the stationary phase. These findings strongly suggest that protease production correlates to the bacterial cell density. In fact, the quorum-sensing system has been shown to regulate the protease production [14, 15,16,17,18]. However, in spite of enough multiplication, *V. vulnificus* and *V. anguillarum* showed decreased protease production at 37 ^{o}C. Therefore, efficiency of the quorum-sensing system is thought to be dependent on the cultivation conditions. Our preliminary experiments indicated that secretion of the AI-2-like substance by *V. vulnificus* biotype 1 was detected at mid-logarithmic phase and increased several times at stationary phase when cultivated at 25 ^{o}C, while its secretion reached maximum around mid-logarithmic phase at 37 ^{o}C. Indeed, in Gram-negative bacteria including *Escherichia coli*, *Salmonella enterica* serovar Typhimurium and *Helicobacter pylori*, production of the signal substance operating the quorum-sensing system has been documented to be regulated by metabolic or environmental conditions such as growth phase, pH, and osmolarity [19,20].

However, the quorum-sensing system is not a sole regulation mechanism in *V. vulnificus* biotype 1. Jeong et al. [21] reported positive regulation of VVP production by cAMP-CRP (cAMP receptor protein) at 30 ^{o}C. We also found that the addition of 0.5 % glucose caused remarkable reduction of the extracellular protease activity when *V. vulnificus* L-180 was cultivated at 25 ^{o}C, suggesting contribution of the cAMP-CRP regulation system (data not shown).

Bacterial growth and protease production in human serum

Each bacterial strain was inoculated into heat-treated 50 % human serum and incubated at 37 ^{o}C with shaking. *V. vulnificus* L-180 showed significant growth, however, others including *V. vulnificus* E86 could not multiply even when incubated for 28 h (Fig. 4A). The cell-free serum sample was collected periodically, and dialyzed against 60 % acetone followed by 0.9 % NaCl to regenerate the proteolytic activity blocked by α-macroglobulin [22], a broad-spectrum protease inhibitor in human plasma [23]. The sample obtained at 12-h incubation showed a very low, but significant proteolytic activity, while the sample from 28-h incubation revealed much higher proteolytic activity (Fig. 4B). Since the specific antibody neutralized the activity almost perfectly, the biotype 1 strain was confirmed to produce VVP during incubation in human serum.

Heat-inactivated human serum is known to restrict bacterial growth because of the absence of essential nutrients including free iron [24,25]. However, the infection caused by *V. vulnificus* biotype 1 is often associated with patients who have diseases predisposing them to iron overload [26,27]. The inability of biotype 1 to multiply in human serum in the presence of ethylene diamine di(*o*-hydroxyphenyl acetic acid), an iron chelator used for removal of free iron, has been documented [28,29]. However, present study clearly indicated that expression of the iron-assimilating system resulting in sufficient bacterial growth was triggered in human serum if the iron chelator is absent. *V. vulnificus* biotype 1 synthesizes a phenolate-type siderophore named vulnibactin [30], which can sequester transferrin-bound iron for growth in human serum. Biosca et al. [31] have reported that a siderophore produced by biotype

2 was a hydoxamate-type, not a phenolate-type. The results in Fig.4 may suggest the inability of the hydroxamate-type siderophore to remove iron from human transferrin. Although it is essential for iron-assimilation, vulnibactin can remove iron only from transferrin digested by VVP [32]. The present study, in which VVP production was observed even at early growth phase, may support our previous conclusion. VVP-dependence of iron-assimilation in human serum was confirmed by the following experiment. When the VVP-deficient mutant [33] was inoculated into human serum, neither bacterial growth nor VVP production was observed (data not shown).

The virulent potential of *V. vulnificus* biotype 2 to mice has been reported to be equal to that of biotype1 [34]. We recently found that mouse serum, as well as rabbit serum [28], contained much free iron and allowed enough bacterial growth [29]. As shown in Fig. 5, both biotypes revealed sufficient multiplication and protease production when incubated in 50 % rabbit serum. This result suggests that *V. vulnificus* biotype 2 also cause systemic infections in individuals whose serum iron levels are extremely increased. Indeed, a few strains isolated from human clinical sources have been identified to be biotype 2 [4,7]. In contrast to *V. vulnificus*, *V. anguillarum* and *V. proteolyticus* could not secrete a protease because of negligible multiplication, indicating deficiency of the virulence determinant(s) other than the iron-assimilating system.

Mice were used for evaluation of the virulent potential of *V. vulnificus* biotype 1. Using this experimental animal, VVP-null mutants constructed by disruption of the *vvp* gene revealed full virulence [35,36]. However, these findings may not lead the conclusion that VVP is not a virulence determinant because biotype 1 administrated to mouse is possible to grow sufficiently without production of VVP.

The bacterial pathogenicity is determined by invasiveness, resistance to host defense systems, production of virulence factor(s) and others. So far, no virulence determinant specific to *V. vulnificus* biotype 1 is not known. For instance, both biotypes 1 and 2 can express capsule, which resists to the vibriocidal action of human serum, and can produce cytolytic exotoxin, which may support bacterial invasion. However, in the present study, biotype 1 showed the distinguished and excellent potential for multiplication and protease production in human serum. This may be one of the important prominences to cause drastic human diseases, such as septicemia accompanied with serious skin lesions.

Materials and Methods

Protease preparations

V. vulnificus E86 provided kindly by Dr. C. Amaro (Universidad de Valencia, Spain) was cultivated in tryptic soy broth (Difco Laboratories, Detroit, Mich., USA) containing 1.5 % NaCl at 25 ^{o}C for 24 h with shaking (120 cycles/min). After cultivation, the culture supernatant was collected by centrifugation at 7,000 x g for 40 min, and ammonium sulfate was added to 60 % saturation. The resulting precipitate was collected, dissolved in distilled water, and applied to a HiLoad 26/60 Sephacryl S-200 HR column (Amersham Biosciences, Piscataway, N.J., USA) equilibrated with 20 mM Tris-HCl buffer containing 1 mM $CaCl_2$, 0.9 % NaCl and 0.02 % NaN_3 (pH 7.4). The fractions showed the high proteolytic activity toward azocasein (Sigma-Aldrich, St. Louis, Mo., USA) were collected and dialyzed against 20 mM Tris-HCl buffer supplemented with 1 mM $CaCl_2$ (pH 7.4). This dialyzed preparation was loaded on a HiLoad 10/2 Phenyl Sepharose column (Amersham Biosciences) equilibrated with the

same buffer. The bound protease was eluted by a linear gradient with 0 to 30 % ethylene glycol in the same buffer.

The recombinant VVP was isolated from the periplasmic fraction of a transformant, *Escherichia coli* DH5α/pVVP7-1, by ammonium sulfate precipitation followed by column chromatography on a HiLoad 16/10 Phenyl Sepharose column (Amersham Biosciences) as described [37].

Bacterial growth experiments

V. vulnificus L-180 isolated from human blood [38], *V. vulnificus* E86 from a diseased eel [4], *V. anguillarum* FY-8701 from a diseased turbot, and *V. proteolyticus* ATCC 15338 were cultivated in TYE broth supplemented with 2.0 % NaCl at 25 ^{o}C for overnight. After cultivation, the bacterial cells were harvested, rinsed twice with 0.9 % NaCl, and inoculated into 2 ml of TYE broth containing 0.9 or 3.0 % NaCl at a cell-density of 1 x 10^{7} CFU/ml. Thereafter, the bacteria were cultured at 25 or 37 ^{o}C with shaking, and growth was periodically monitored by measuring absorbance at 600 nm.

For studies on bacterial growth in mammalian serum, rabbit or human serum (Chemicon, Temecula, Calif., USA) was heat-treated at 56 ^{o}C for 30 min, and the precipitate was removed by centrifugation at 12,000 x g for 30 min. The heat-treated serum was diluted two-fold with 0.9 % NaCl and sterilized by membrane filtration (pore size 0.22 μm). The bacterial cells precultured at 25 ^{o}C in TYE broth containing 2.0 % NaCl were inoculated into 2 ml of the diluted serum at a cell-density of 1 x 10^{7} CFU/ml, and incubated at 37 ^{o}C with shaking.

SDS-PAGE

To inactivate the protease, the sample was mixed with an equal volume of 20 % trichloroacetic acid, and the resulting precipitate was collected, rinsed twice with ethanol, and treated with 1 % SDS at 100 ^{o}C for 3 min in the presence or absence of 5 % 2-mercaptoethanol. Electrophoresis was performed on a PhastSystem with a PhastGel Gradient 10-15 % gel (Amersham Biosciences) according to the manufacturer's manual. After electrophoresis, the gel was stained with 0.5 % Coomassie brilliant blue R-250 and destained with 25 % ethanol-8 % acetic acid.

Assay of the proteolytic activity

The protease activity toward azocasein was assayed as described previously [38]. The sample was allowed to act at 30 ^{o}C for an appropriate period on 1.0 mg of azocasein in 0.6 ml of 50 mM Tris-HCl buffer (pH 8.0), and the reaction was stopped by addition of 1.4 ml of 5 % trichloroacetic acid. After centrifugation at 1,000 x g for 5 min, an aliquot of the supernatant was withdrawn and mixed with an equal volume of 0.5 M NaOH. Thereafter, the increase of absorbance at 440 nm was measured. One protease unit (PU) was defined as the amount of the sample hydrolyzing 1 μg of the substrate in 1 min.

The elastase activity toward elastin-Congo red was determined by the method of Kothary and Kreger [39]. The sample was allowed to act at 30 ^{o}C for 3 h on 2.0 mg of elastin-Congo red in 0.2 ml of 50 mM Tris-HCl buffer (pH 8.0), and the reaction was stopped by addition of 1.0 ml of the ice-cold buffer. Thereafter, the increase of absorbance at 495 nm was measured. One elastase unit (EU) was defined as the amount of the sample hydrolyzing 1 μg of the substrate in 1 min.

PCR for the *vvp* gene

Genomic DNA was prepared by the miniprep procedure from the bacterial cells cultivated in Luria-Bertani broth containing 1.0 % NaCl. The primers for amplification of the segment of the *vvp* gene (5'-CATGTGTTCTCTTCCAGTCAC-3', sense primer, and 5'-TCACCACGCTCTTTTCCATCG-3', antisense primer) corresponding to bases at 653 to 673 and at 1300 to 1320, respectively, were designed from the DNA sequence of the *vvp* gene of *V. vulnificus* L-180 submitted to the DDBJ, EMBL, and GeneBank databases (accession number AB084580). After denaturation at 95 ^{o}C for 2 min, the amplification reactions were performed for 30 cycles as follows; 30-sec denaturation at 95 ^{o}C, 30-sec annealing at 52 ^{o}C, and 1-min extension at 72 ^{o}C. The PCR products were elctrophoresed on a 1.2 % agarose gel and visualized by staining with ethidium bromide.

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Figure legends

Fig. 1. Agarose gel electrophoresis of PCR products obtained with primers for the *vvp* gene. Lane M, molecular size markers; lanes 1-3, *V*. *vulnificus* biotype 1 strains (L-180, 332, and E-4); lane 4, *V. vulnificus* biotype 2 strain E86.

Fig. 2. Growth of vibrios in TYE broth. The bacterial cells (2 x 10^7 CFU) inoculated into 2 ml of TYE broth containing 0.9 or 3.0 % NaCl were cultivated at 25 or 37 ^oC with shaking, and growth (A_{600}) was measured periodically.

Fig. 3. Protease production by vibrios in TYE broth. The culture supernatant was obtained at mid-logarithmic or stationary growth phase, and the proteolytic activity toward azocasein was determined. Data represent the mean ± SD of four experiments.

Fig. 4. Growth of and protease production by vibrios in human serum. (A) The bacterial cells (2 x 10^7 CFU) inoculated into 2 ml of heat-treated 50 % human serum were incubated at 37 ^oC with shaking, and growth (A_{600}) was measured periodically. (B) The cell-free serum sample was collected at the indicated times, and the activity of the extracellular protease was measured with azocasein. Data represent the mean ± SD of four experiments.

Fig. 5. Growth of and protease production by vibrios in rabbit serum. (A) The bacterial cells (2 x 10^7 CFU) inoculated into 2 ml of heat-treated 50 % rabbit serum were incubated at 37 ^oC with shaking, and growth (A_{600}) was measured periodically.

(B) The cell-free serum sample was collected at the indicated times, and the activity of the extracellular protease was measured with azocasein. Data represent the mean ± SD of four experiments.

Fig. 1

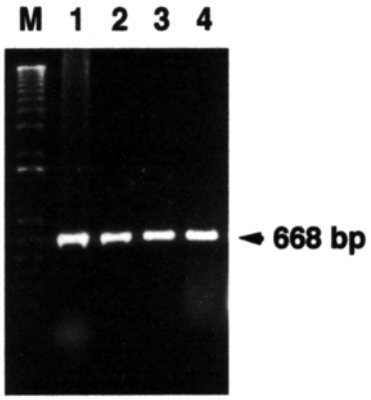

M 1 2 3 4
668 bp

Fig. 2

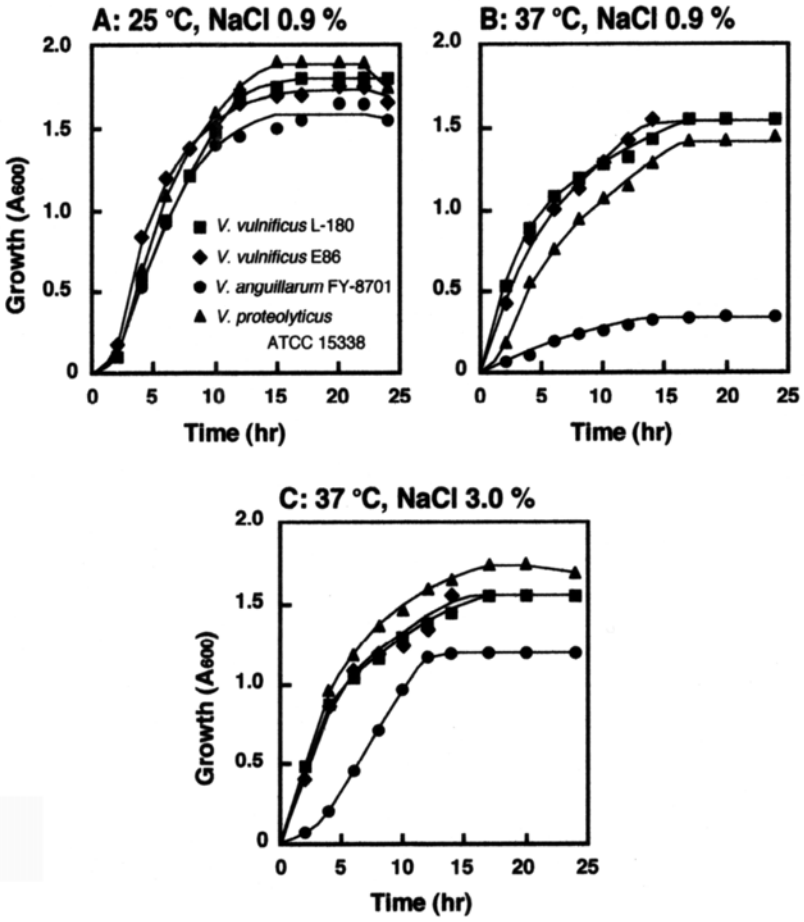

A: 25 °C, NaCl 0.9 %
B: 37 °C, NaCl 0.9 %
C: 37 °C, NaCl 3.0 %
Growth (A600)
Time (hr)
V. vulnificus L-180
V. vulnificus E86
V. anguillarum FY-8701
V. proteolyticus ATCC 15338

Fig. 3

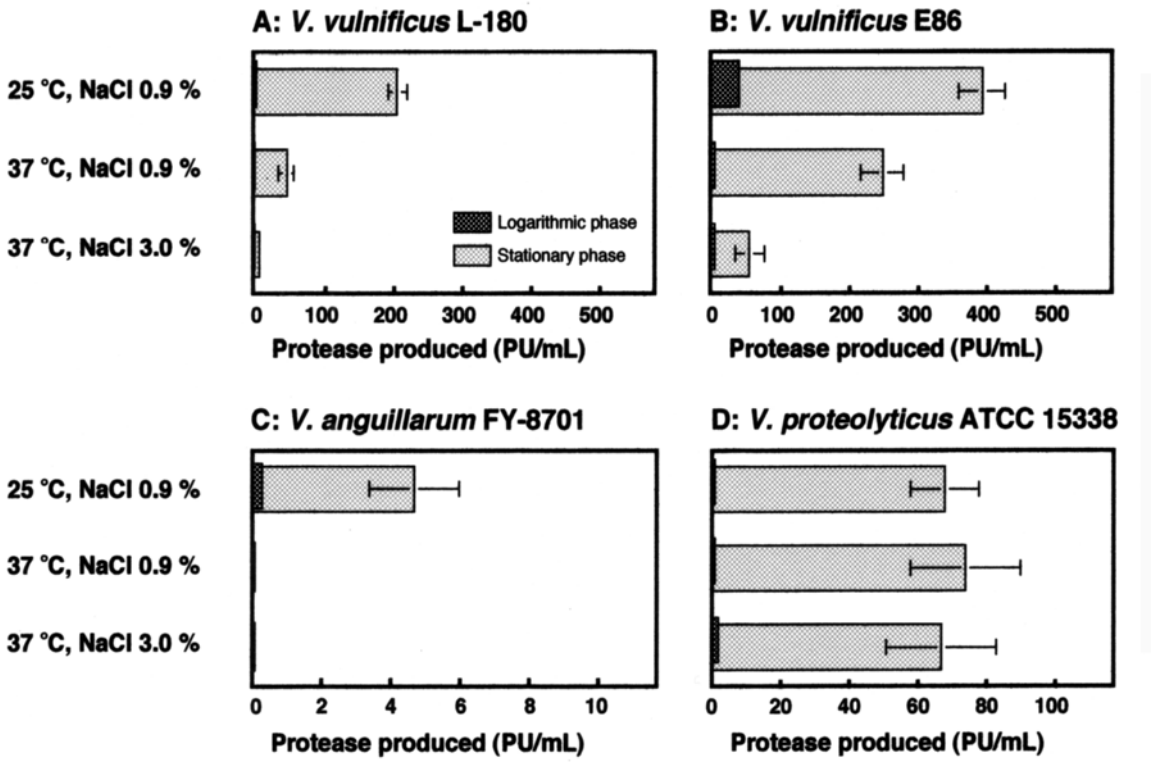

A: V. vulnificus L-180
B: V. vulnificus E86
C: V. anguillarum FY-8701
D: V. proteolyticus ATCC 15338
25 °C, NaCl 0.9 %
37 °C, NaCl 0.9 %
37 °C, NaCl 3.0 %
Logarithmic phase
Stationary phase
0 100 200 300 400 500
0 2 4 6 8 10
0 20 40 60 80 100
Protease produced (PU/mL)

Fig. 4

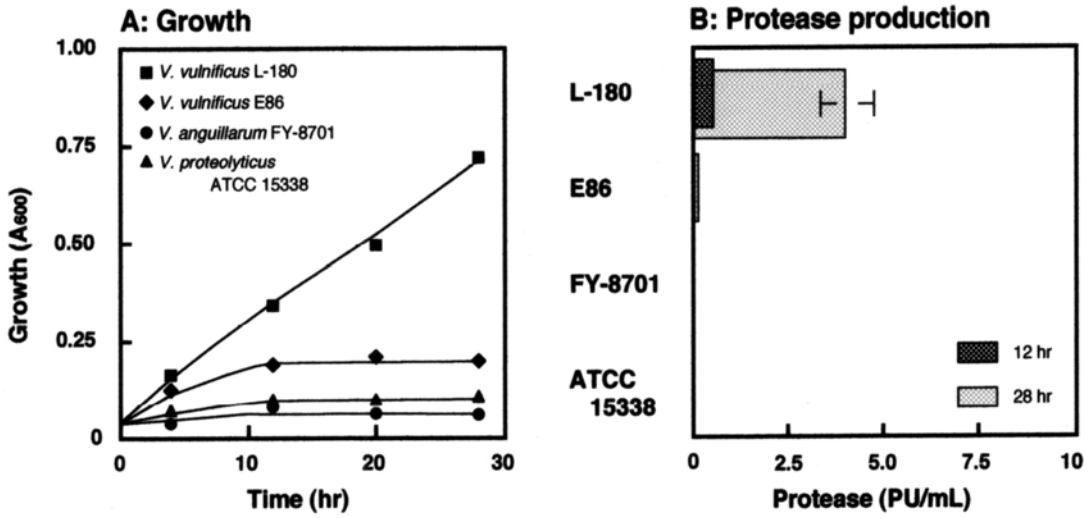
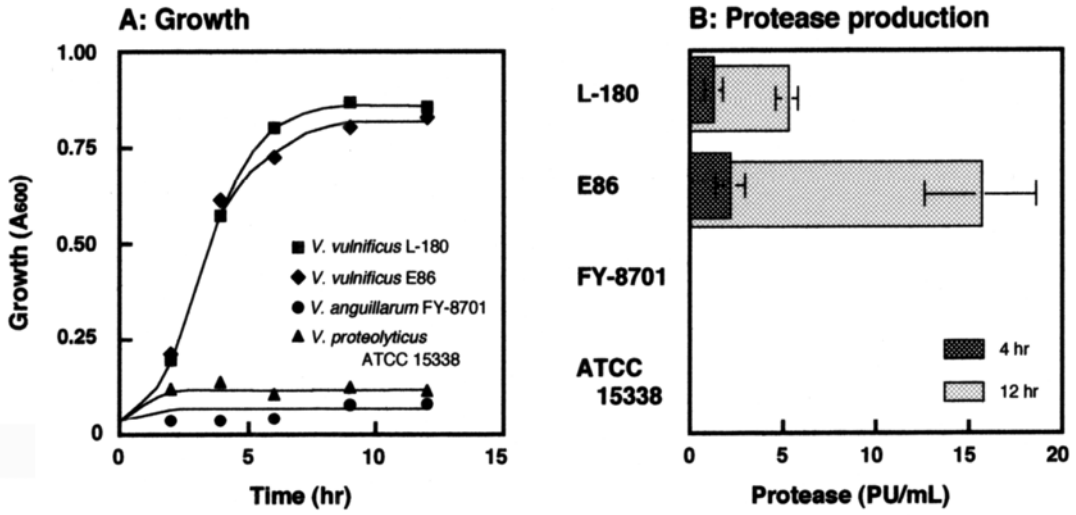

A: Growth
B: Protease production
V. vulnificus L-180
V. vulnificus E86
V. anguillarum FY-8701
V. proteolyticus ATCC 15338
Growth (A600)
1.00
0.75
0.50
0.25
0
0 10 20 30
Time (hr)
L-180
E86
FY-8701
ATCC 15338
12 hr
28 hr
0 2.5 5.0 7.5 10
Protease (PU/mL)

Fig. 5


A: Growth
1.00
0.75
0.50
0.25
0
Growth (A600)
0
5
10
15
Time (hr)
V. vulnificus L-180
V. vulnificus E86
V. anguillarum FY-8701
V. proteolyticus ATCC 15338
B: Protease production
L-180
E86
FY-8701
ATCC 15338
4 hr
12 hr
0
5
10
15
20
Protease (PU/mL)