Molecular analysis of an extracellular protease gene from Vibrio parahaemolyticus

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INTRODUCTION

Vibrio parahaemolyticus is an important diarrhoeal agent associated with seafood consumption in Taiwan, Japan and many coastal areas (Janda et al., 1988). A number of possible virulence factors, including haemolysins (Nishibuchi et al., 1992), lethal toxins (Sarkat et al., 1987a, b) and vascular permeability factors (Honda et al., 1976), have been demonstrated to be associated with the pathogenicity of this species. Among the haemolysins, the thermostable direct haemolysin (TDH) has been established as an important virulence factor contributing to the enteropathogenesis of V. parahaemolyticus (Nishibuchi et al., 1992). However, other studies have reported that haemolysin or TDH may not be the sole major lethal factor for mice, and that other toxic factors lethal for mice may be present in environmental nonhaemolytic V. parahaemolyticus strains (Dai et al., 1992; Yoh et al., 1992).

In addition, it has been suggested that extracellular bacterial proteases may play an important role in virulence and may serve as useful industrial enzymes (Häse & Finkelstein, 1993). Legionella pneumophila metalloprotease exhibits haemolytic and cytotoxic activities (Dowling et al., 1992). Vibrio cholerae haemagglutinin/protease (Finkelstein et al., 1992) nickes and activates the A subunit of the cholera enterotoxin (Booth et al., 1984), and the corresponding gene has been cloned and sequenced (Häse & Finkelstein, 1991). The isolation and characterization of alkaline serine exoproteases and a collagenase of Vibrio alginolyticus has been reported (Deane et al., 1986, 1989; Takeuchi et al., 1992). Vibrio proteolyticus neutral protease has industrial applications in the synthesis of dipeptides (David et al., 1992). A purified extracellular metalloprotease from Vibrio mimicus has been demonstrated to enhance vascular permeability in skin and fluid accumulation in rabbit ileal loops (Chowdhury et al., 1991a, b). An elastase from Vibrio anguillarum has been isolated and characterized, and the corresponding gene has also been cloned and sequenced (Milton et al., 1992; Norqvist et al., 1990).

Unlike other Vibrio proteases, little is known about the proteases in V. parahaemolyticus (Iuchi & Tanaka, 1982). This paper describes the cloning and sequencing of a gene encoding a metalloprotease of V. parahaemolyticus and the biochemical characterization of the partially purified protease.

**Abbreviation**: TDH, thermostable direct haemolysin.

The EMBL/GenBank accession number for the prtVp nucleotide sequence data presented in this paper is 246782.
METHODS

Bacterial strains and plasmids. These are listed in Table 1. A 5.8 kbp HindIII DNA fragment from plasmid pH21 was subcloned in the vector plasmid pUC119. The resulting recombinant plasmid was designated pSL101.

Media and growth conditions. Escherichia coli strain XL1-Blue was grown at 35 °C in LB broth (Miller, 1972). LB medium supplemented with 100 μg ampicillin ml⁻¹ (Sigma) was used for this strain when bearing a vector or recombinant plasmid. To isolate chromosomal DNA, V. parahaemolyticus was grown in tryptic soy broth (TSB; BBL Microbiology Systems) supplemented with 3% (w/v) NaCl at 35 °C. Transformed E. coli cells were selected on LB plates supplemented with ampicillin (100 μg ml⁻¹) and tetracycline (10 μg ml⁻¹) or on LB plates containing 20 g LB broth base (Gibco) and 15 g agar 1⁻¹, supplemented with 3% (w/v) NaCl at 35 °C. These were selected on LB plates supplemented with ampicillin (100 μg ml⁻¹) and tetracycline (10 μg ml⁻¹) or on LB plates containing 20 g LB broth base (Gibco) and 15 g agar 1⁻¹, supplemented with 100 μg ampicillin ml⁻¹, 0.2 mM IPTG (Sigma), and 0.004% X-Gal (Sigma).

DNA preparation and molecular biological techniques. Isolation of plasmids, restriction endonuclease digestion, agarose gel electrophoresis of DNA, and purification of DNA fragments were done as described by Ausubel et al. (1987).

Southern blot hybridization. The isolated DNA fragment, a 5.8 kbp HindIII fragment carrying the prtVp gene, was 32P-labelled by using a random primed DNA labelling kit (Böhringer Mannheim) according to the directions of the manufacturer. Alkaline Southern blot hybridization was performed as described previously by Lee & Pan (1993).

Construction of a genomic library. The following steps were performed as described in the method of Ausubel et al. (1987). Chromosomal DNA of V. parahaemolyticus was extracted by SDS/proteinase K lysis followed by hexadecyltrimethylammonium bromide treatment, two phenol/chloroform/isooxyl alcohol extractions and ethanol precipitation. The purified DNA was then suspended in small volumes of TE buffer (10 mM Tris/HCl containing 1 mM EDTA, pH 8), partially digested with HindIII (BRL) and fractionated on a 1% (w/v) agarose gel. DNA fragments of 5-12 kbp were isolated by using a Jentsorb DNA extraction kit (Genomed). The DNA fragments were ligated with the T4 DNA ligase (BRL) into HindIII (BRL)-digested pBR322 previously dephosphorylated. Ligated pBR322 DNA mixtures were transformed into competent E. coli strain XL1-Blue according to the procedure of Ausubel et al. (1987).

Assay for protease. Protease activity was detected by the hydrolysis of gelatin in solidified agarose. Cultures were plated on LB agar containing 2% (w/v) gelatin and incubated at 35 °C for 1-2 d. A clear zone around the colony was detected after the agar plate was flooded with 15% (w/v) HgCl₂ (3 g HgCl₂ in 4 ml HCl and water added for a total volume of 20 ml) (Norqvist et al., 1990). For detection of protease activity in SDS-polyacrylamide gels, 0.2% gelatin was copolymerized in the 10% (w/v) polyacrylamide matrix. The gel was soaked in 2.5% (w/v) Triton X-100 for 1 h at 25 °C, incubated at 37 °C for 4 h in 0.1 M glycine (pH 8.3), and then fixed and stained with 0.27% Coomassie brilliant blue (Bio-Rad, R-250) in methanol/acetic acid/water (50:10:40, by vol.). Finally, the gel was destained with 7% (v/v) acetic acid/7% (v/v) methanol, and the clear zones in the SDS-gelatin-polyacrylamide gel were detected (Heussen & Dowdle, 1980).

Preparation of the protease. The V. parahaemolyticus strains were grown in 100 ml TSB containing 3% (w/v) NaCl at 35 °C for 18 h. The bacterial cells were pelleted by centrifugation (Hitachi) at 6510 g for 10 min at 4 °C. The supernatant fractions were collected, and the proteins were then precipitated on ice by the addition of solid ammonium sulphate to 50% saturation. The precipitate was pelleted by centrifugation at 4 °C for 10 min at 6510 g. The pellet was resuspended in 1 ml 0.01 M potassium phosphate buffer, pH 7.0, and dialysed at 4 °C with changing of the buffer at least three times. After overnight dialysis, the final volume was about 1 ml. E. coli(pLSD101) (Table 1) was grown in LB medium. Other stages in the preparation of protease from E. coli were the same as in the steps described earlier.

Protease inhibitors. EDTA (Stratagene) was used at a concentration of 10 mM. Aprotinin (Sigma) was used in 10 and 30 μg ml⁻¹ concentrations. 1,10-Phenanthroline (Sigma), PMSF (Sigma) and pepstatin A were first dissolved in methanol and used in 1 and 10 mM concentrations. Protease activity was measured by gelatin medium assay. Crude extracellular protease (40 μl) with inhibitor was added into a hole of 4 mm diameter in gelatin medium and incubated at 37 °C for 24 h. The presence or absence of a clear zone on the gelatin medium was observed.

Haemolytic activity. Crude extracellular protease (40 μl) was added into a hole of 4 mm diameter in NAB blood medium and incubated at 37 °C for 24 h. A clear zone around the colony was detected on the gelatin medium.

Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype or phenotype</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escherichia coli XL1-Blue</td>
<td>recA1 endA1 gyrA46 thi hsdR17 supE44 relA1 lac F' [proAB lacIq lacZAM15 Tn10(ter5')]</td>
<td>Bullock et al. (1987)</td>
</tr>
<tr>
<td>V. parahaemolyticus pBR322</td>
<td>tdb trb, Kanagawa phenomenon weak positive</td>
<td>Clinical isolate from Taiwan</td>
</tr>
<tr>
<td></td>
<td>Ap' Te'</td>
<td>Bolivar et al. (1977);</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sutcliffe (1979)</td>
</tr>
<tr>
<td>pVH21</td>
<td>Ap' Te' prep*, 5.8 kbp HindIII fragment carrying prepVp cloned into HindIII site of pBR322</td>
<td>This study</td>
</tr>
<tr>
<td>pLS101</td>
<td>Ap' prep*, 5.8 kbp HindIII fragment of pH21 carrying prepVp subcloned into HindIII site of pUC119</td>
<td>This study</td>
</tr>
<tr>
<td>pLSD1-6</td>
<td>Ap' prep*, exonuclease deletion derivatives of pLS101 retaining the prepVp gene</td>
<td>This study</td>
</tr>
<tr>
<td>pLSD7-24</td>
<td>Ap' prep, exonuclease deletion derivatives of pLS101 deleted in the prepVp gene</td>
<td>This study</td>
</tr>
</tbody>
</table>

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Vibrio parahaemolyticus protease gene

(nutrient agar containing 2.5%, v/v, blood and 100 μg ampicillin ml⁻¹) supplemented with or without EDTA and incubated at 37 °C for 48 h. Haemolytic activity was measured from the diameter of the clear zone; greater than 1 and 3 mm were designated weak and strong reactions, respectively.

Exonuclease deletion to localize DNA fragments encoding protease activity. Unidirectional deletions with exonuclease III/S1 were performed by using the Erase-a-Base System (Promega) (Henikoff, 1984). Plasmid pLS101 was digested with BamHI and KpnI to leave 5' and 3' overhangs, susceptible to ExoIII. Deletions were made in the 5' end starting from pLS101 carrying the 5.8 kbp HindIII insert. The series of deletion derivatives was subcloned in pUC119 to produce plasmids pLS1-pLS124 (Table 1) and transformed into E. coli strain XL1-Blue as described previously by Ausubel et al. (1987).

Nucleotide sequence determination. Plasmid pLS6 containing the DNA insert to be sequenced was maintained in E. coli strain XL1-Blue. The complete nucleotide sequences of both strands of DNA were determined by the dideoxy chain-termination method of Sanger et al. (1977), using the Sequenase version 2.0 sequencing kit (United States Biochemical) and [α³²P]dATP (NEN) as the label. Universal or synthetic oligonucleotide primers were synthesized with a DNA synthesizer (Oligo 1000, Beckman) by the Pan Asia Hospital Supply Company in Taiwan. Double-stranded template DNA was prepared by the method of Kho & Zarbl (1992). Sequencing gels (6%, w/v, polyacrylamide) were prepared according to the method of Chang & Wu (1992). After the fractionation, the gels were dried and exposed to X-ray film (DuPont Cornex).

DNA sequence analysis. The nucleotide sequence data and deduced amino acid sequence were analysed for similarity to the published sequences in the EMBL and PIR databases, using the FASTDB and PEP/ALIGN programs from IntelliGenetics (IntelliCorp), and based on the similarity search method of Wilbur & Lipman (1983). The nucleotide sequence and amino acid sequence of PrtVp were also analysed by PC/GENE version 6.60.

RESULTS

Cloning of the prtVp gene

To clone the gene encoding an extracellular protease of V. parahaemolyticus strain 93, a genomic library was constructed in vector pBR322 in E. coli strain XL1-Blue. One protease-positive clone, carrying pVH21, was detected by screening 3000 transformants. However, the control E. coli strain XL1-Blue(pBR322) had no detectable proteolytic activity on gelatin-LB agar. Southern blot analysis verified that the 5.8 kbp HindIII fragment in pVH21 was present in the V. parahaemolyticus genome (Fig. 1). Restriction analysis of the isolated plasmid revealed that pVH21 had one EcoRI cutting site within a 5-8 kbp DNA insert. For determination of the nucleotide sequence, the 5-8 kbp DNA insert in pVH21 was subcloned into vector plasmid pUC119 yielding plasmid pLS101.

Identification of the plasmid-encoded proteins

The protease was obtained from culture supernatant fractions of V. parahaemolyticus strains and E. coli(pLS101), and concentrated by ammonium sulphate precipitation. The samples were analysed for protease activity by gelatin-SDS-PAGE. A predominant protease band (62 kDa) and two relatively weak protease bands (97 kDa, 86 kDa) from extracts of E. coli(pLS101) and V. parahaemolyticus strain 93 were visible (Fig. 2). Bands with proteolytic activity were not present in concentrates from E. coli(pUC119).Fig. 2 shows that 92, 79 and 42 kDa

Fig. 1. Southern blot analysis confirming V. parahaemolyticus strain 93 as the source of insert DNA in pVH21. (a) Agarose gel electrophoresis; (b) Southern blot hybridization with the 32P-labelled pVH21-derived HindIII insert fragment. Lanes: 1, HindIII-cleaved λ DNA molecular size markers; 2, V. parahaemolyticus strain 93 genomic DNA digested with HindIII; 3, genomic DNA of E. coli strain XL1-Blue digested with HindIII; 4, HindIII-digested pVH21 DNA; 5, HindIII insert fragment of plasmid pVH21.

Fig. 2. Detection of protease activities in culture supernatant fraction concentrates by gelatin-SDS-PAGE. Crude protease preparations from the culture supernatant fraction of V. parahaemolyticus strain 93 (lane 1), E. coli(pLS101) (lane 2) and E. coli(pUC119) (lane 3) are shown. Lane 4, protein molecular mass (kDa) markers.
Protease bands that are present in the extract of *V. parahaemolyticus* strain 93 are absent in the recombinant *E. coli*(pLS101). Three bands (62, 86 and 97 kDa) of protease activity were observed for *E. coli*(pLS6), giving an identical result to that for *E. coli*(pLS101) (data not shown).

Expression of protease in *E. coli*

Protease activity of the recombinant *E. coli*(pLS101) increased when it was grown on gelatin-LB agar containing 0.016% IPTG (data not shown). This result suggests that the inserted 5.8 kbp DNA fragment in pLS101 is oriented in the same direction with respect to the *lac* promoter for expression of protease activity in *E. coli*. The plasmid pLS101 was subjected to unidirectional deletions as described in Methods. A series of deletion fragments was subcloned in pUC119 and the *E. coli* transformants were tested for protease activity on gelatin-LB agar. The results are shown in Fig. 3.

Nucleotide sequence of *prtVp*

The DNA sequence was determined for the smallest DNA fragment which still encoded protease activity. This DNA fragment was present in plasmid pLS6. The nucleotide sequence of *prtVp* and its flanking regions is shown in Fig. 4. Analysis of the nucleotide sequence revealed the presence of one ORF, starting with ATG at nucleotide position 272 and terminating with TGA at position 2033. A putative Shine–Dalgarno sequence (262–264) was found 10 bp upstream from the ATG start codon. The predicted -10 and -35 regions of the promoter sequence are at positions 226 through 231 and 207 through 212, respectively. The ORF encodes a 587 amino acid protein. A putative signal peptide of 25 amino acids was found, which resembled a typical signal peptide sequence for secreted proteins of prokaryotic origin. A potential signal peptidase cleavage site (Ser-25-Phe-26) conforms to the -3, -1 rule (von Heijne, 1986). The mature protein has a predicted molecular mass of 63156 Da and a predicted isoelectric point of 4.53. The GC content of 48.87% is in agreement with that of *V. parahaemolyticus*, which is 38–63% (Baumann & Schubert, 1984). Hydrophobic amino acids were observed in the initial amino acid sequence in the hydropathy profile. The mean hydrophobicity of the mature protein is -4.1, suggesting that the protein is hydrophilic in nature.

Comparison of *PrtVp* with other proteases

The deduced amino acid sequence of the protease *PrtVp* was compared with sequences in the IG-Suite EMBL/GenBank/DDBJ databases. Similarity comparisons showed that the amino acid sequence of the mature protease has 32% identity with the sequence of collagenase from *V. alginolyticus* (Takeuchi et al., 1992). A central region of the deduced collagenase amino acid sequence (167 amino acids) from *V. alginolyticus* showed 44% identity. The alignment of these sequences is shown in Fig. 5. The conserved domain of zinc-metalloproteases, HEXXH (Hase & Finkelson, 1993), is also observed in the amino acid sequence of this *PrtVp* protease. The amino acids in the active site and the zinc-binding site are all conserved at position His-435–His-439. The comparison of the amino acid sequences of the conserved domain between the *V. parahaemolyticus* strain 93 *prt* gene product and other metalloproteases is shown in Table 2.

Effect of protease inhibitors on enzyme activity

Crude protease from the culture supernatant fraction of transformant *E. coli*(pLS101) was prepared. The protease activity could be inhibited by the metal chelator EDTA (10 mM) and the zinc-specific chelator 1,10-phenanthroline (10 mM). The enzyme was resistant to inhibitors of serine and cysteine proteases, such as PMSF (10 mM) and aprotinin (30 μg ml⁻¹). It was also resistant to pepstatin A (10 mM), an inhibitor of aspartate proteases. The sensitivity of the protease to metal chelators indicates that this *PrtVp* is a metalloprotease.

Haemolytic assay of the crude extracellular protease

The haemolytic activities of *E. coli*(pLS101), *E. coli*(pUC119) and *V. parahaemolyticus* strain 93 extracellular protease were examined in the absence or presence of EDTA. The crude extracellular protease of wild-type strain *V. parahaemolyticus* 93 showed clear haemolytic activity on blood agar in either the presence or absence of EDTA, whereas that of transformant *E. coli*(pLS101) was relatively weak on blood agar only in the absence of EDTA. The transformant *E. coli*(pUC119) had no detectable haemolytic activity on blood agar. These
Fig. 4. Nucleotide sequence of the *V. parahaemolyticus* protease gene and its deduced amino acid sequence. The putative Shine–Dalgarno (SD) sequence and the -10 and the -35 regions of the promoter are indicated. The arrow pointing downwards indicates a possible signal peptide cleavage site. The stop codon is indicated by a horizontal bar.
results indicate that PrtVp is able to cause haemolysis but is not the major haemolytic factor of *V. parahaemolyticus* strain 93.

**DISCUSSION**

Cloning in *E. coli* of a protease gene from *V. parahaemolyticus* has not been reported so far. In this study, the gene encoding an extracellular protease of *V. parahaemolyticus* strain 93 was successfully cloned and sequenced. A single ORF of 1761 nucleotides encodes a large polypeptide (587 amino acids) with a signal sequence of 25 amino acids. The deduced molecular mass of the mature gene encoding an extracellular protease of *V. parahaemolyticus* strain 93 was 62 kDa, with two weaker bands at 97 and 86 kDa. It is not likely that pLSD6 contains two or three distinct protease genes since the length of the nucleotide sequence of the flanking region of *prtVp* is too small to code for the 97 kDa and 86 kDa proteins. The relationship between these weaker bands and the major 62 kDa protease is still unclear and remains to be resolved.

We found no amino acid sequence similarity of the *prtVp* gene product with those of any of the published *Vibrio* proteases and other proteases such as *Bacillus* thermolysin (Titani *et al.*, 1972), *Pseudomonas* elastase (Fukushima *et al.*, 1989), *Pseudomonas* alkaline proteinase (Okuda *et al.*, 1990) and *Serratia* proteinase (Nakahama *et al.*, 1986). However, the deduced amino acid sequence shared 32% identity with that of the *V. alginolyticus* collagenase (Fig. 5). This result suggests that these *Vibrio* proteases may belong to a new family of proteases, but further work is necessary to prove this hypothesis.

PrtVp may be regarded as a zinc-metalloprotease because the primary sequence motif HEXXH was found in PrtVp (His-435, His-439 and Glu-436). In the process of catalysis, the importance of the amino acid structure of His-Glu-Xaa-Xaa-His in facilitating electron transfer with zinc was reported previously by Häse & Finkelstein (1993). The catalytic site as well as the zinc-binding site of the *Bacillus* thermoproteolyticus protease and those of other metalloproteases of the *Vibrio* species and the predicted sites of the *V. parahaemolyticus* metalloprotease are conserved. In addition, the inhibition by zinc- and metal-specific inhibitors indicates that PrtVp binds a zinc ion at this conserved zinc-binding site. Several other bacterial zinc-metalloproteases have been shown to be extracellular proteins which need a signal sequence to aid transport across the bacterial cell membranes (David *et al.*, 1992; Milton *et al.*, 1992; Nishina *et al.*, 1992; Booth *et al.*, 1983). Our protease has also been found to have a typical signal peptide.

**Table 2.** Comparison of zinc-metalloprotease motifs between the PrtVp conserved domain and conserved domains from other zinc-metalloproteases

<table>
<thead>
<tr>
<th>Bacterial sp.</th>
<th>Amino acids of conserved domains*</th>
<th>Amino acid residues in appropriate sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus thermoproteolyticus</em></td>
<td>HELTH</td>
<td>142-146</td>
<td>Titani <em>et al.</em> (1972)</td>
</tr>
<tr>
<td><em>Vibrio proteolyticus</em></td>
<td>HEVSH</td>
<td>343-347</td>
<td>David <em>et al.</em> (1992)</td>
</tr>
<tr>
<td><em>Vibrio anguillarum</em></td>
<td>HEVSH</td>
<td>346-350</td>
<td>Milton <em>et al.</em> (1992)</td>
</tr>
<tr>
<td><em>Vibrio cholerae</em></td>
<td>HEVSH</td>
<td>343-347</td>
<td>Häse &amp; Finkelstein (1991)</td>
</tr>
<tr>
<td><em>Vibrio alginolyticus</em></td>
<td>HEYVH</td>
<td>477-481</td>
<td>Takeuchi <em>et al.</em> (1992)</td>
</tr>
<tr>
<td><em>Vibrio parahaemolyticus</em></td>
<td>HEYTH</td>
<td>435-439</td>
<td>This study</td>
</tr>
</tbody>
</table>

* H, Zinc-binding sites; E, active site.
There has been limited study of the proteases of *V. parahaemolyticus*. Previously, Iuchi & Tanaka (1982) separated and characterized four extracellular proteases of *V. parahaemolyticus* by polyacrylamide slab gel electrophoresis. These proteases comprised two serine proteases and two metalloproteases as characterized by enzyme inhibitor studies. However, the authors did not determine the molecular mass of the proteases they identified. Sarkar et al. (1987b) reported that a 65 kDa protein isolated from Kanagawa-positive strains of *V. parahaemolyticus* was observed in SDS-PAGE. The authors also concluded that this 65 kDa protein may play a vital role in the pathogenesis of the disease caused by *V. parahaemolyticus* based on the mouse lethality test (Sarkar et al., 1987b). In their study, about 26% of the Kanagawa-phenomenon-positive strains isolated from clinical sources demonstrated the presence of this 65 kDa protein along with the 21 kDa TDH in SDS-PAGE, but whether this 65 kDa protein has protease activity is unknown (Sarkar et al., 1987b). Whether this 65 kDa protein is related to that characterized herein also remains to be clarified. The isolation and purification of the *prtv*/p-encoded 62 kDa protein for testing of animal lethal activity is required.

Many extracellular bacterial proteases have been suggested to play important roles in virulence. The extracellular protease of *Vibrio vulnificus* digested all haem proteins tested and elicited haem liberation from the proteins (Nishina et al., 1992). The authors suggest that the protease contributes to the efficient utilization of haem by *V. vulnificus*. Recently, Finkelstein et al. (1992) proposed that haemagglutinin/protease of *V. cholerae* may act as a detachase which allows the bacterium to detach from host cell membranes and then to disseminate to other hosts. The alkaline protease of *Pseudomonas aeruginosa* is required for the tissue destruction observed in corneal infections by this species (Howe & Igleswki, 1984). A metalloprotease of *Aeromonas salmonicida* has been shown to produce lesions upon injection into trout and has been suggested as a virulence factor (Ellis, 1991). *Vibrio anguillarum* metalloprotease (36 kDa) and a 75 kDa protease or 30 kDa protease may also work together to enhance the pathogenicity of *V. anguillarum* (Milton et al., 1992).

Our future investigations will concern mutagenesis of the protease gene of *V. parahaemolyticus* strain 93 and animal infection studies to assess the role of the metalloprotease in the virulence of *V. parahaemolyticus*.

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