Molecular analysis of an extracellular protease gene from *Vibrio parahaemolyticus*

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The structural gene *prtVp* encoding the extracellular protease of *Vibrio parahaemolyticus* strain 93 was cloned in *Escherichia coli* and sequenced. The cloned DNA fragment contained a 1761 bp ORF encoding a 587 amino acid protein. The deduced polypeptide is composed of a 25 amino acid signal peptide and a 562 amino acid extracellular polypeptide with a calculated molecular mass of 63 156 Da. Protease analysis using a gelatin-containing SDS-polyacrylamide gel detected the presence of a 62 kDa protease that was present in the culture supernatant fractions of the wild-type *V. parahaemolyticus* strain and of *E. coli* bearing a pUC119 recombinant with the *prtVp* DNA insert. The protease activity was inhibited by zinc- and metal-specific inhibitors such as EDTA and 1,10-phenanthroline, which suggested that it is a metalloprotease. The deduced amino acid sequence of *PrtVp* has 32% identity with that of the collagenase of *Vibrio alginolyticus*, but has no identity with those of the bacterial proteases. A conserved zinc-binding domain was also found in *PrtVp* from homology comparison with other metalloproteases. This *PrtVp* can cause weak haemolysis on blood agar.

Keywords: cloning, sequence, *Vibrio parahaemolyticus*, protease gene

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 haemolysin (Nishibuchi *et al.*, 1992), lethal toxins (Sarkar *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) nicks 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 Z46782.

METHODS

Bacterial strains and plasmids. These are listed in Table 1. A 5·8 kbp *Hind*III DNA fragment from plasmid pVH21 was subcloned in the vector plasmid pUC119. The resulting recombinant plasmid was designated pLS101.

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 l⁻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 *Hind*III fragment carrying the *prtVp* gene, was ³²P-labelled by using a random primed DNA labelling kit (Boehringer 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/isoamyl 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 *Hind*III (BRL) and fractionated on a 1% (w/v) agarose gel. DNA fragments of 5–12 kbp were isolated by using a Jetsorb DNA extraction kit (Genomed). The DNA fragments were ligated with the T4 DNA ligase (BRL) into *Hind*III (BRL)-digested pBR322 previously dephosphorylated. Ligated pBR322 DNA mixtures were transformed into com-

petent *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 placed 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*(pLS101) (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

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype	Source or reference
<i>Escherichia coli</i> XL1-Blue	<i>recA1 endA1 gyrA46 thi hsdR17 supE44 relA1 lac F' [proAB lacI^a lacZΔM15 Tn10(tet^r)]</i>	Bullock <i>et al.</i> (1987)
<i>Vibrio parahaemolyticus</i> 93 pBR322	<i>tdh trb</i> , Kanagawa phenomenon weak positive Ap ^r Tc ^r	Clinical isolate from Taiwan Bolivar <i>et al.</i> (1977); Sutcliffe (1979)
pUC119	Ap ^r <i>lacZ</i>	Vieira & Messing (1982, 1987)
pVH21	Ap ^r Tc ^r <i>prt</i> ⁺ , 5·8 kbp <i>Hind</i> III fragment carrying <i>prtVp</i> cloned into <i>Hind</i> III site of pBR322	This study
pLS101	Ap ^r <i>prt</i> ⁺ , 5·8 kbp <i>Hind</i> III fragment of pVH21 carrying <i>prtVp</i> subcloned into <i>Hind</i> III site of pUC119	This study
pLSD1–6	Ap ^r <i>prt</i> ⁺ , exonuclease deletion derivatives of pLS101 retaining the <i>prtVp</i> gene	This study
pLSD7–24	Ap ^r <i>prt</i> , exonuclease deletion derivatives of pLS101 deleted in the <i>prtVp</i> gene	This study

(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 *Bam*HI and *Kpn*I to leave 5' and 3' overhangs, susceptible to *Exo*III. Deletions were made in the 5' end starting from pLS101 carrying the 5.8 kbp *Hind*III insert. The series of deletion derivatives was subcloned in pUC119 to produce plasmids pLSD1–pLSD24 (Table 1) and transformed into *E. coli* strain XL1-Blue as described previously by Ausubel *et al.* (1987).

Nucleotide sequence determination. Plasmid pLSD6 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 [α -³²S]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 (Intelli-Corp), 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 *Hind*III fragment in pVH21 was present in the *V. parahaemolyticus* genome (Fig. 1). Restriction analysis of the isolated plasmid revealed that pVH21 had one *Eco*RI 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

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 *Hind*III insert fragment. Lanes: 1, *Hind*III-cleaved λ DNA molecular size markers; 2, *V. parahaemolyticus* strain 93 genomic DNA digested with *Hind*III; 3, genomic DNA of *E. coli* strain XL1-Blue digested with *Hind*III; 4, *Hind*III-digested pVH21 DNA; 5, *Hind*III 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.

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. 3. Construction of a series of deletion derivatives of plasmid pVH21 using exonuclease and the protease activities of transformants bearing these derivatives. pVH21 contains the protease gene *prtVp*. The *plac* arrow indicates the direction of transcription of *prtVp*. Plasmid pLS101 contains *prtVp* under *lac* promoter control. Plasmids pLSD1–pLSD24 were created by unidirectional deletion and subcloning. The proteolytic phenotype of the *E. coli* strain XL1-Blue carrying the different plasmids is indicated and was monitored by the presence of a halo in the gelatin agar plates. Vertical arrows indicate deletion derivatives pLSD4 and pLSD5 and pLSD8–pLSD23, respectively. H and E indicate restriction sites of *HindIII* and *EcoRI*, respectively.

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*(pLSD6), 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 pLSD6. 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 63 156 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 (Häse & Finkelstein, 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 10 mM 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

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1 AGTTGAGCTAATAACTAACTTTTTGGTCTGGTGGGATAAAATTCATTTTTGAGTCACTTTTGACTCATATTGAC
76 TTATTTAGAAAAAGGTAAATTAATGTTTTTATTAATAATTATTGAATCTTCCTGTGTGGGATTGGTCCCATTAA
-35
151 TTAGAGTAATAATTCTATTTTTGGGATCGGTTTCATAAAATTTATGATACTGATTTCACTGTTTGAGCTGTAAGG
-10 SD
226 AATATAAACCCCACTCAATTTATTTTTATAATCAATGAGTGTGTTATGTCTCATATCCGTTTTTCCCGCGTCA
M S H I R F F P R H
301 TCGTTTTGGCGCTTGCTTGCATGCTAGCGAGTGTCTTAGCTTCTCTTTTGCCCAAATCAGTGTGCTGTTGCTGA
R L A L A C M L A S V S S F S↓F A Q N Q C A V A D
376 TTTACAACAATCGCGTGATTTGGCCGCTGCCGTTTCTGGCGCTGAATATGATTGCTACCACGCTTGGTCTCTGCTG
L Q Q S R D L A A A V S G A E Y D C Y H A W F S A
451 GCCTTCAGCAACGCTGAATGATATTTATAGCGAAGCAAGTTTAAGCCGCAATCAAGTCGCGTTGGATCAAGAAAT
P S A T L N D I Y S E A S L S R I Q V A L D Q E I
526 TGCACGCTATCGTGGTGAAGCAGAACAAGCTCGCGTTCTAGAAAAATCTAGGCGAATTTGTTCTGTCGCGCGTATTA
A R Y R G E A E Q A R V L E N L G E F V R A A Y Y
601 CGTTTCGTTACAACGCTGGCACTGGCACACCTGAGTTTTTCAAGCAATGAGTCAGCGTTTTGCTCAGTCAACGAA
V R Y N A G T G T P E F S E A L S Q R F A Q S T N
676 CCTGTTTCTCAACAACCCTCATGCTTTGGATCAAGGCCGTGAGCAAGTTGGCGCAATGAAGAGCCTGACTTTGAT
L F L N N P H A L D Q G R E Q V G A M K S L T L M
751 GGTTGATAACGTGAAGCAGTTGCCGCTGACCATGGATAGCATGATAGCCGCGTTGATGCACITTAACCGTGACAC
V D N V K Q L P L T M D S M I A A L M H F N R D T
826 CGCGAAAGACACCCAATGGGTTGATGGCCTGAACAACCTTGTTTCGCTCTATGGCAGGTCACGCGGCGAACGATGC
A K D T Q W V D G L N N L F R S M A G H A A N D A
901 GTTCTACCGCTACATGGCAAACAACACTCACCATATTGATACGTTAGCGCGATTTCGCTTCAGACAATGCATGGGC
F Y R Y M A N N T H H I D T L A R F A S D N A W A
976 GTTAGATACAGATGCAAACTTTATCGTTTTCAATGCATTACGTGAAACTGGCCGCTTACTTGGCAGCCAGATCA
L D T D A N F I V F N A L R E T G R L L A S P D Q
1051 AGAAACCAAACGTAAGCATTGGCTGTTATGCAGCAAGTGATGCAGCGTTATCCATTGGGCAGTGAGCACGACAA
E T K R K A L A V M Q Q V M Q R Y P L G S E H D K
1126 ACTGTGGCTTGCCGCCGTTGAGATGATGAGTTACTACGCGCCTGAGGGTTTGAATGGTCTAAACCTAGAGCAAGC
L W L A A V E M M S Y Y A P E G L N G L N L E Q A
1201 GAACGAAGATCTAGCAGCACGCGTGATGCCAAATCGTTTTGAATGTCAGGGACCTGCCATCATTGCTCTGAAGA
N E D L A A R V M P N R F E C Q G P A I I R S E D
1276 TCTCTCCGATGCTCAAGCTGCGAAAGCGTGTGAAGTGCCTGCTGCGAAAGAAGCAGACTTCCATCAAGTTGCGAA
L S D A Q A A K A C E V L A A K E A D F H Q V A N
1351 TACTGGTAATCAACAGTGGCGGATGACTTGAACGATCGTGTGAAGTCGCGGTGTTTGCAAGCAACGACAGTTA
T G N Q P V A D D L N D R V E V A V F A S N D S Y
1426 CGTTGATTACTCTTTCATTCCCTGTTTGGCAATACAACGGATAACGGTGGTCAGTACTTAGAGGGGACTCCGTCAG
V D Y S S F L F G N T T D N G G Q Y L E G T P S R
1501 AGCCGATAATACGGCGCGTTTTGTGGCTTATCGCTACGCGAATGGCGAAGATCTATCTATTTTGAACCTTGAGCA
A D N T A R F V A Y R Y A N G E D L S I L N L E H
1576 CGAGTACACTCACTACTTGGATGCACGTTTCAACCAATACGGTTCATTGAGCGACAACCTTAGCTCATGGTCATAT
E Y T H Y L D A R F N Q Y G S F S D N L A H G H I
1651 CGTTTTGGTGGCTGGAAGGCTTTGCGGAATACATGCATTACAAACAAGGTTACAAGGCCGCGATCGATCTGATCCC
V W W L E G F A E Y M H Y K Q G Y K A A I D L I P
1726 AAGTGGTAAACTGAGCCTGTCAACCGTGTGTTGACACCACATACTCGCACGATAGCAACCGCATTTACCGTTGGGG
S G K L S L S T V F D T T Y S H D S N R I Y R W G
1801 CTACTTGGCGGTTTCGCTTCATGCTAGAAAACCAACCTCAAGATGTAGAGAGCTTGTGGCGTTGTCTCGTTTCAGG
Y L A V R F M L E N H P Q D V E S L L A L S R S G
1876 TCAGTTTGCACAATGGGCGCACGAGTTTACTGTGCTTGGTCAACAATACGATGCGGAATTTGAGCGTTGGTTGGA
Q F A Q W A H E V T V L G Q Q Y D A E F E R W L D
1951 TACGTTAGAAGTGGTAGTTGAACCGGAGCAACCGAAGGACCCAGAAGAGCCAAGCAGACCACCGATCCTGAA
T L E V V V E P E Q P G T D P E E P S R P P I L K
2026 GTGCAAGTGACGGAGCTGGCGGCGAACCAGCCCTTCAACTGAGTGGCG
C K -

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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.

VPRT	321 NRFECQGPALIRSEDLSDAQAACEVLAKEADFHQV
VACOLL	QTYTCSPTIRILSQNMTQEQHAAACSKMGYEEGYFHQS 362
VPRT	ANTGNQPVADDLNRVEVAVFASNDSYVDYSSFLFGNT
VACOLL	LETGEQPVKDDHNTQLQVNI FDSSTDYGYAGPIFDIS
VPRT	TDNGGQYLEGTPSRADNTARFVAY--RYANGEDLSILN
VACOLL	TDNGGMYLEGDPSPGNIPNFIAYEASYAN-ADHFVWN
VPRT	LEHEYTHYLDARFNQYGSFSDNLAHGHIVVWLEGFAEY
VACOLL	LEHEYVHYLDGRFDLYGGFS--HPTEKIVVWSEGIAEY
VPRT	MHYKQGYKAAIDLIPSG
VACOLL	VAQENDNQAALETILDG

Fig. 5. Alignment of the deduced amino acid sequences from *V. parahaemolyticus* strain 93 PrtVp protease (VPRT) and that of the central portion of *V. alginolyticus* collagenase (VACOLL) (Takeuchi *et al.*, 1992). The conserved domain of zinc-metalloprotease is indicated by the asterisks (zinc-binding sites) and circle (active site).

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 polypeptide, 63 kDa, correlates well with the value determined by SDS-PAGE for the protease from *V.*

parahaemolyticus. Gelatin-SDS-PAGE analysis revealed the presence of multiple proteases of different sizes in the ammonium sulphate precipitate of the culture supernatant fraction of *E. coli*(pLS101) or *E. coli*(pLSD6). Protease activity was detected as one major band corresponding to a molecular mass of 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

Bacterial sp.	Amino acids of conserved domains*	Amino acid residues in appropriate sequence	Reference
<i>Bacillus thermoproteolyticus</i>	HEALTH	142-146	Titani <i>et al.</i> (1972)
<i>Vibrio proteolyticus</i>	HEVSH	343-347	David <i>et al.</i> (1992)
<i>Vibrio anguillarum</i>	HEVSH	346-350	Milton <i>et al.</i> (1992)
<i>Vibrio cholerae</i>	HEVSH	343-347	Häse & Finkelstein (1991)
<i>Vibrio alginolyticus</i>	HEYVH	477-481	Takeuchi <i>et al.</i> (1992)
<i>Vibrio parahaemolyticus</i> 93	HEYTH	435-439	This study

* 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 *pvtVp*-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 & Iglewski, 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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