INFECTION AND IMMUNITY, Nov. 1996, p. 4495–4500 Vol. 64, No. 11
0019-9567/96/$04.00+0

Cloning and Genetic Analysis of the *Vibrio cholerae* Aminopeptidase Gene

CLAUDIA TOMA* AND YASUKO HONMA

Department of Bacteriology, Faculty of Medicine, University of the Ryukyus, Nishihara, Okinawa 903-01, Japan

Received 20 May 1996/Returned for modification 25 July 1996/Accepted 15 August 1996

The structural gene for the *Vibrio cholerae* leucine aminopeptidase (*lap*) was cloned and sequenced. The cloned DNA fragment contained a 1,503-bp open reading frame potentially encoding a 501-amino-acid polypeptide with a calculated molecular mass of 54,442 Da. The deduced amino acid sequence of the entire protein showed high homology with the sequence of *Vibrio proteolyticus* leucine aminopeptidase. The residues potentially involved in binding the zinc ions were completely conserved in the *V. cholerae* aminopeptidase as well as in the *V. proteolyticus* aminopeptidase. The recombinant protein was partially purified and characterized. The molecular mass was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to be 34 kDa, suggesting a processing of the protein to acquire the mature form. The protease showed maximum activity at pH 9.0 and was thermostable at 70°C. The substrate leucyl-*p*-nitroanilide was cleaved by the protease, and its activity was inhibited by EDTA and bestatin. These results suggested that the protein was a leucine aminopeptidase. The PCR analysis of *lap* gene distribution showed that it was widely distributed among the *V. cholerae* strains. It was not present in the other species examined.

Extracellular proteases are thought to play an important role in the pathogenesis of diarrhea caused by *Vibrio cholerae* (6, 11, 14). In addition, protease-deficient mutants of *V. cholerae* are reported to be less virulent (23). Detailed study of pathogenic organisms has either demonstrated or suggested the important role extracellular proteases play in virulence (13).

Proteases are enzymes that catalyze the hydrolysis of peptide bonds in proteins or peptides. They are present in all living organisms, in which they display a variety of physiological functions. Microbial proteases are predominantly extracellular and can be classified into four groups on the basis of the essential catalytic residue at their active site. These groups are serine proteases, cysteine proteases, aspartate proteases, and the metalloproteases. The aminopeptidases (APs) form a group of zinc-dependent metalloexopeptidases that catalyze the removal of amino acids from the N terminus of a protein. These enzymes are of biological and medical importance because of their key role in protein degradation and in the metabolism of biologically active peptides. Leucine APs (LAPs), as their name implies, preferably cleave leucyl substrates, although substantial rates of enzymatic cleavage with most amino-terminal amino acids are observed (4).

Only one *V. cholerae* extracellular protease gene, the soluble hemagglutinin/protease (*hap*) (12), has been cloned and sequenced to date. However, the biochemical characterization of extracellular proteases from *V. cholerae* suggests the existence of more than one type of protease (24, 32). Furthermore, *hap*-deficient mutants have shown residual proteolytic activity that was not inhibited by anti-hemagglutinin/protease (anti-HA/P)-specific antiserum (12).

HA/P nicks the cholera toxin (3) and digests proteins that may participate in host defense against cholera, such as mucin, fibronectin, lactoferrin, and secretory immunoglubulin A (25). Although HA/P is a very attractive virulence factor, an isogenic strain of *V. cholerae* specifically mutated in the *hap* gene was no less virulent in infant rabbits than was the parent strain (10).

ToxR, a DNA-binding protein, regulates the expression of cholera toxin, toxin-coregulated pilus, and outer membrane proteins (OmpT and OmpU) in response to several environmental signals, of which osmolarity and the presence of amino acids seem to be the most important (17). There is little doubt that *V. cholerae* is exposed to amino acids that are released through the action of its own proteases on the intestinal mucus. Nevertheless, no studies have addressed this point.

In this study, the gene encoding an AP of *V. cholerae* was cloned, sequenced, and genetically analyzed. The product of this gene was partially purified and characterized.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *V. cholerae* O 1 serovar Inaba, biotype El Tor, strain N86 was used to search for a new protease. For general molecular cloning, *Escherichia coli* XL-1 Blue (Stratagene, La Jolla, Calif.) was used. Plasmids pBR 322 and pBluescript II KS$^+$ (Stratagene) were used as cloning vectors. Cells were cultivated in Luria-Bertani (LB) medium (1% tryptone [Difco Laboratories, Detroit, Mich.], 0.5% yeast extract [Difco], 0.5% NaCl [Wako Pure Chemical Co., Osaka, Japan]) or on an LB agar plate containing 100 μg of ampicillin per ml. LB agar containing 3% of skim milk was used to select the proteolytic clones.

DNA manipulation. Plasmid DNA was isolated by the method of Birnboim and Doly (2) or, for DNA sequencing, by the alkaline lysis method using a Qiagen Plasmid Kit (Funakoshi, Tokyo, Japan). Restriction enzyme digestion, ligation, gel electrophoresis, and transformation were carried out as described by Bagdasarian et al. (1).

Cloning and DNA sequencing. A DNA library for *V. cholerae* N86 was kindly provided by K. Yamamoto (Research Institute for Microbial Diseases, Osaka, Japan). *Sau*3AI-digested *V. cholerae* DNA fragments, 7 to 8 kb long, were ligated to the *Bam*HI site in pBR 322 and then introduced into *E. coli* DH10B (31).

Suitable restriction fragments were obtained and subcloned into pBluescript II KS$^+$. Nucleotide sequence analysis was performed in both directions on overlapping DNA fragments. Sequencing was done by the dideoxynucleotide triphosphate chain termination method of Sanger et al. (22) with the Dye Deoxy Terminator Cycle Sequence Kit and an ABI 373 A DNA sequencer (Applied Biosystems, Inc., Foster, Calif.). Two 20-bp synthetic oligonucleotide primers, 5′GCTTGGCGTGTAGGCCCTGA3′ (primer np1) and 5′TCAGGGCCTACA CGCCAAGC3′ (primer np2), were used to provide the nucleotide sequence information between the *Xho*I and *Eco*RV sites.

Protease activity. Protease activity was detected by using a single-diffusion technique in agar gel containing skim milk as a substrate (11). Sample solution (20 μl) was added to wells 3 mm in diameter, and the plates were incubated overnight at 37°C.

* Corresponding author. Mailing address: Department of Bacteriology, Faculty of Medicine, University of the Ryukyus, Nishihara, Okinawa 903-01, Japan. Phone: 81 98 895 3331. Fax: 81 98 895 2951. Electronic mail address: k950417@med.u-ryukyu.ac.jp.

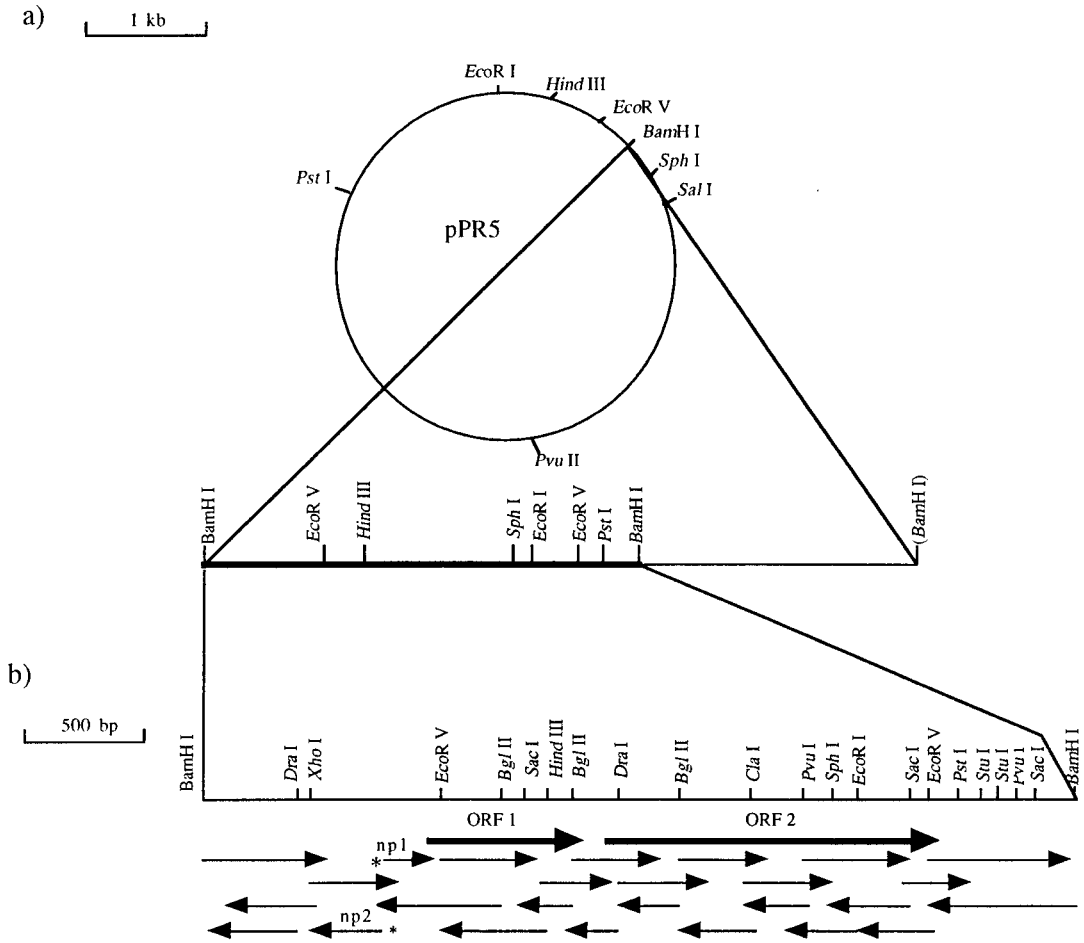

FIG. 1. (a) Physical map of the original plasmid pBR 322 containing the *lap* gene of *V. cholerae* (pPR5). The thick solid line denotes the smallest fragment that showed protease activity. (b) Restriction map and sequencing strategy for the 3.6-kb DNA fragment inserted in plasmid pPRB9 that contains the *lap* gene. Arrows indicate the directions of sequencing from the subclones. Asterisks indicate sequencing with synthetic oligonucleotide primers (np1 and np2). The two ORFs (ORF1 and ORF2) are indicated.

Estimation of recombinant LAP molecular mass. A modified conventional polyacrylamide gel electrophoresis (PAGE) (8) was performed with a slab gel. After protein separation, the gel was cut into fractions of 5 mm and the proteins were eluted with 20 mM Tris-HCl buffer (pH 7.6). The eluted fractions were examined for their proteolytic activity in skim milk agar plates. The fraction showing protease activity was analyzed by sodium dodecyl sulfate (SDS)-PAGE to estimate the protein molecular mass.

Protease purification. HA/P was purified from *V. cholerae* non-O1 strain 93Ag13 as described previously (25).

Recombinant LAP was partially purified from *E. coli* XL-1 carrying plasmid pPRB9 that harbored the *lap* gene. Bacteria were precultured in LB medium-ampicillin with shaking at 37°C for 6 h. The precultured bacteria were inoculated into 500 ml of LB medium-ampicillin in a 3-liter Erlenmeyer flask and incubated for 20 h with shaking at 37°C. The cell-free culture supernatant was salted out with 60% saturated ammonium sulfate. After centrifugation, the pellet was resuspended in 20 ml of 20 mM Tris-HCl buffer (pH 7.6) and dialyzed against the same buffer. The dialyzed material was fractionated successively by Sephadex G-100, Sepharose CL-4B, and Sephacryl S-200 chromatographies. After each gel filtration chromatography, fractions with protease activity were pooled and concentrated for the next column. Tris-HCl (20 mM; pH 7.6) was used as the elution buffer.

Antiserum preparation. Rabbits were immunized with 50 μg of either LAP or HA/P every 2 weeks. One milliliter of antigen emulsified with an equal volume of Freund's complete adjuvant was injected subcutaneously into multisites. For the boosting injection, 50 μg of protease and Freund's incomplete adjuvant was used.

Electrophoresis and Western blotting (immunoblotting). SDS-PAGE and immunoblotting were carried out by the method of Laemmli (16) and Towbin et al. (26), respectively. A prestained molecular marker (New England Biolabs, Beverly, Mass.) was used as a standard.

Leucyl-*p*-nitroanilide assay. The AP activity was measured by a leucyl-*p*-nitroanilide assay, as described by Prescott et al. (21) with some modifications. The increase in A_{405} by liberation of *p*-nitroaniline was measured. Eighty microliters of the sample was added to 2 ml of substrate (0.2 mM), and the increase in absorbance at 25°C in 1 min was observed. EDTA (10 mM) or bestatin (100 μM) was preincubated for 30 min at 37°C with an equal volume of enzyme solution to study their effect on activity. The effect of pH on leucyl-*p*-nitroanilide hydrolysis was determined with the following buffers: 100 mM citric acid (pH 3.0 to 6.0), 100 mM Tris-HCl (pH 7.0), 20 mM Tricine (pH 8.0), and glycine-NaOH (pH 9.0 and 10.0). To examine protease heat stability, protease was preincubated at 70, 80, and 90°C for 30 min, and the hydrolytic activity was compared with that of the control.

PCR conditions. A single colony was picked and resuspended in 1 ml of distilled water, boiled for 10 min, and used as template DNA. PCR cycle times were as follows: 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min (for 25 cycles). The primers used to amplify the protease coding region were 5′CGGC AGCGTTGAGCGCCAAT3′ (primer np3; residues 1700 to 1719), and 5′GCATCATCATCCGCGCCGGG3′ (primer np4; residues 2314 to 2333). The PCR product was electrophoresed in a 1.2% agarose gel with ϕX174-*Hae*III digest as a standard molecular mass marker.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession number D84215.

RESULTS

Cloning of the LAP gene (*lap*). The DNA library of *V. cholerae* O1 was introduced into *E. coli* DH10B. Transformants were grown on skim milk-LB agar plates containing ampicillin

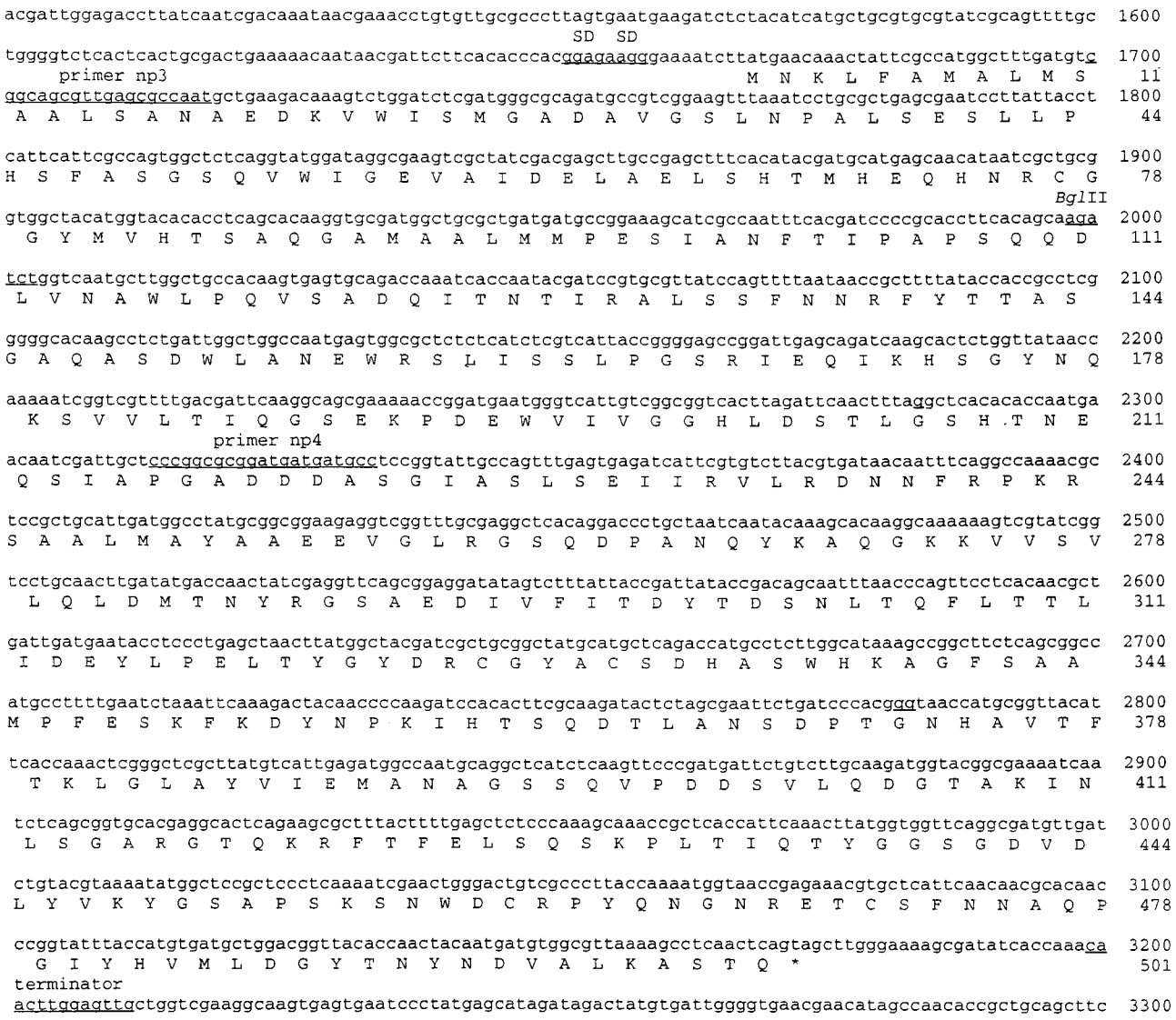

FIG. 2. Nucleotide sequence of the ORF2 (*lap*) gene and predicted amino acid sequence. The sequence of ORF1 was omitted from the figure. Putative Shine-Dalgarno (SD) sequences (GGAG-AAGG) are underlined and labelled. The primer annealing sites for the PCR are underlined and labelled. The *Bgl*II site within the PCR fragment is indicated. The putative termination consensus is also underlined. The asterisk indicates the stop codon.

and incubated at 37°C for 48 h. About 10,000 clones were screened; 6 of these clones showed a proteolytic halo around the colony. One clone showing a clear halo was selected for further investigation (pPR5).

A restriction map of pPR5 (Fig. 1) showed differences from that previously reported for the *hap* gene by Häse et al. (12). Deletion derivatives of the original recombinant plasmid (pPR5) were constructed to locate the minimum protease gene region. The 3,587-bp *Bam*HI fragment (Fig. 1) was reinserted into the *Bam*HI site of pBluescript II KS$^+$ and transformed into *E. coli* XL-1. Colonies harboring the plasmid with the *Bam*HI fragment insertion (pPRB9) showed proteolytic halos. Plasmid pPRB9 was selected for further analysis (Fig. 1).

DNA sequencing and predictive amino acid sequence. The dideoxy chain termination method of Sanger et al. (22) was employed to determine the DNA sequence of the 3,587-bp *Bam*HI fragment present on plasmid pBluescript II KS$^+$. On the basis of deletion analysis, it was determined that this was the smallest clone that still produced proteolytic activity.

Appropriate restriction fragments were introduced into the pBluescript II KS$^+$ plasmid for single-strand sequencing (Fig. 1). The sequencing of the 3,587-bp *Bam*HI fragment revealed the presence of two open reading frames (ORFs) as indicated in Fig. 1. ORF1 (residues 873 to 1632) and ORF2 (residues 1669 to 3172) were located in different reading frames. ORF1 is discussed in Discussion, and ORF2 is discussed in the rest of the paper.

The analysis of the deduced amino acid sequence of the 1,504-bp ORF2 provided convincing evidence that this was the structural gene of the secreted protease. The coding region was followed by a stem-loop structure which could function as a terminator sequence (Fig. 2). ORF2 may encode a protein composed of 501 residues with a calculated molecular mass of 54,442 Da. The N-terminal region of the predicted polypeptide revealed a potential signal sequence of 18 amino acids, ending in Ala-Asn-Ala. The alanine residues found at positions −3 and −1 (relative to the putative signal sequence cleavage site) were consistent with the "(−3, −1)" rule of signal sequence

```
LAP MNKLFAMALMSAALSANAEDKVWISMGADAVGSLNPALSESLLPHSFASGSQVWIGEVAIDELAELSHTMHEQHNR   76
AP                 AYA-------I----NQTVMKSGA--I--N-V--S----V-Q-NVAQ------N---E---

LAP CGGYMVHTSAQGAMAALMMPESIANFTIPAPSQQDLVNAWLPQVSADQITNTIRALSSFNNRFYTTASGAQASDWL  152
AP  -------P---S----SA--TTL-S-VM-PIT--AT-T------D-S---G--SS-E--T------T--------I

LAP ANEWRSLISSLPGSRIEQIKHSGYNQKSVVLTIQGSEKPDEWVIVGGHLDSTLGSHTNEQSIAPGADDDASGIASL  228
AP  -S--QA-SA---NASVK-VS----------M--T---A----IVI-------I--------V------------AV

LAP SEIIRVLRDNNFRPKRSAALMAYAAEEVGLRGSQDPANQYKAQGKKVVSVLQLDMTNYRGSAEDIVFITDYTDSNL  304
AP  T-V----SE---Q----I-F---------------L-----SE--N---A--------K---Q-V----------F

LAP TQFLTTLIDEYLPELTYGYDRCGYACSDHASWHKAGFSAAMPFESKFKDYNPKIHTSQDTLANSDPTGNGAVTFTK  380
AP  --Y--Q-M-----S----F-T------------N--YP---------N----R---T-----------SH-KK--Q

LAP LGLAYVIEMANAGSSQVPDDSVLQDGTAKINLSGARGTQKRFTFELSQSKPLTIQTYGGSGDVDLYVKYGSAPSKS  457
AP  -----A---GS-TGDTPTPGNQ-E--VPVTD---S--SNVWY----ETQ-N-Q-T-S--Y--L-----F--KA--Q

LAP NWDCRPYQNGNRETCSFNNAQPGIYHVMLDGYTNYNDVALKASTQ                                  501
AP  -------LS--N-V-T----S--T-S---T--S--SGAS-----F
```

FIG. 3. Amino acid sequence comparison of *V. cholerae* LAP and *V. proteolyticus* AP, starting from the putative signal peptide cleavage site. Residues identical in the two proteins are indicated by a dash. Residues potentially involved in binding the zinc ions are indicated by bold letters. Residues involved in forming the hydrophobic specificity pocket are double underlined. The residues (−3 to −1) from the putative cleavage site of the signal peptide are single underlined.

(29) (Fig. 3). Homology searches comparing the sequence of ORF2 with other protein sequences revealed that ORF2 is highly homologous to the *Vibrio proteolyticus* AP (28) (Fig. 3), with 67% identity and 80% homology found over the entire sequence. Amino acid residues potentially involved in binding the zinc ions were conserved in the two proteins (5, 20) (Fig. 3). These results suggest that ORF2 codes for the *V. cholerae* LAP. Considerable homology in the C-terminal region (amino acids 390 to 498) was also found with the extracellular proteases of other species: *V. cholerae* HA/P (63%) (12), *V. proteolyticus* neutral protease (60%) (7), *Vibrio alginolyticus* alkaline serine protease A (67%) (9), and *Vibrio anguillarum* metalloprotease (63%) (18).

Recombinant LAP characterization. SDS-PAGE of the crude LAP showed a 42-kDa band with proteolytic activity (data not shown). However, during the purification steps, the protein was processed to a 34-kDa form (Fig. 4). No cross-reactivity between HA/P and LAP was observed in Western blots (Fig. 4).

The substrate leucyl-*p*-nitroanilide was cleaved by LAP. EDTA and bestatin (an AP inhibitor) completely inhibited LAP activity. Protease activity was unaffected by heating at 70°C, was partially inactivated at 80°C (40% of the activity of the unheated control), and completely inactivated by heating at 90°C. The optimal pH for expressing protease activity was pH 9 (Fig. 5).

Screening of *lap* gene distribution by PCR. To detect the *lap* gene, a PCR with a set of primers (np3 and np4) was performed. The strains examined from our laboratory stock were 35 *V. cholerae* O1 strains, 35 *V. cholerae* non-O1 strains, 5 *Vibrio parahaemolyticus* strains, 3 *V. alginolyticus* strains, 13 *Aeromonas* sp. strains, and 5 *E. coli* strains. The expected product of 633 bp (Fig. 6) was obtained with 100% (35 of 35)

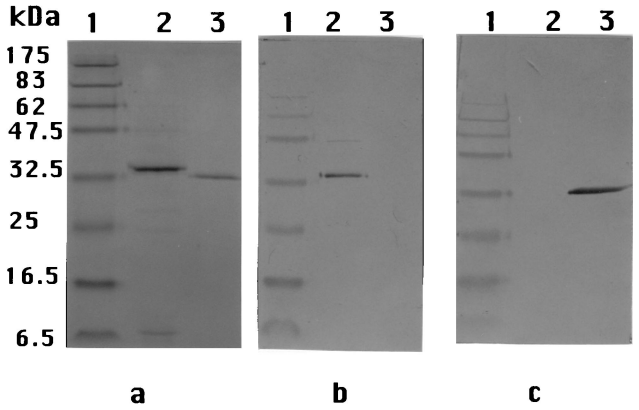

FIG. 4. (a) Coomassie brilliant blue-stained SDS–12% PAGE gel; (b) Western blot using anti-LAP antisera; (c) Western blot using anti-HA/P antisera. Lanes: 1, molecular mass markers; 2, recombinant LAP; 3, purified HA/P.

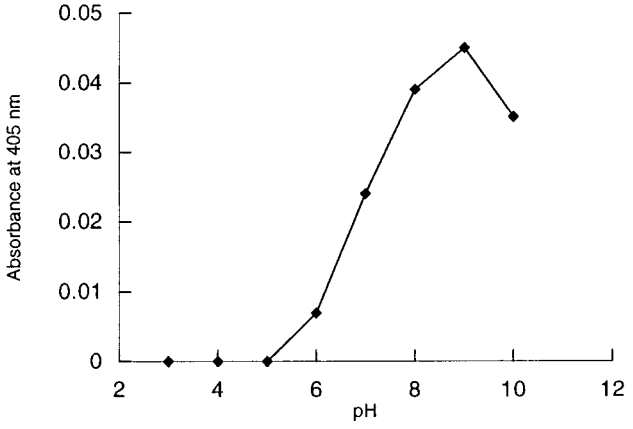

FIG. 5. Effect of pH on leucyl-*p*-nitroanilide hydrolysis by LAP. The buffers used were 100 mM citric acid (pH 3.0 to 6.0), 100 mM Tris-HCl (pH 7.0), 20 mM Tricine (pH 8.0), and glycine-NaOH (pH 9.0 and 10.0).

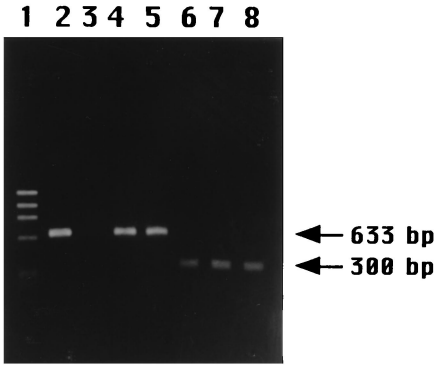

FIG. 6. Analysis of *lap* gene distribution by PCR. The primers used were np3 and np4; annealing sites are shown in Fig. 2. Lanes: 1, molecular mass markers (ϕX174-*Hae*III digest); 2, *E. coli* XL-1 harboring plasmid pPRB9; 3, *E. coli* XL-1 (negative control); 4, *V. cholerae* non-O1 strain 93Ag13; 5, *V. cholerae* O1 strain N86; 6 to 8, *Bgl*II digest of PCR product (*E. coli* XL-1 harboring the plasmid pPRB9 [lane 6], *V. cholerae* non-O1 strain 93Ag13 [lane 7], and *V. cholerae* O1 strain N86 [lane 8]).

of the *V. cholerae* O1 strains and 92% (32 of 35) of the *V. cholerae* non-O1 strains. Strains of other species were all negative. The PCR product was digested with *Bgl*II, and fragments of about 300 bp were obtained (Fig. 6), confirming that the correct region was amplified.

DISCUSSION

This report describes the cloning, sequence analysis, expression, and partial characterization of an AP gene for *V. cholerae* (*lap*). The nucleotide sequence revealed an ORF (ORF2) of 1,503 nucleotides, which may encode a polypeptide of 501 amino acids (Fig. 1). The calculated molecular mass of the protein is 54,442 Da. This is larger than the molecular mass (34 kDa) of the purified recombinant *lap* product. Another ORF (ORF1) was found upstream of the *lap* gene which may encode a protein with a predicted molecular mass of 27,396 Da. ORF1 seems to play a role in the expression of LAP recombinant protein in *E. coli* since its deletion produced clones with no proteolytic activity.

The deduced amino acid sequence of the entire LAP shows high homology (80%) with the sequence of *V. proteolyticus* (previously classified as *Aeromonas proteolyticus*) AP (28) (Fig. 3). The crystal structure study of AP reveals that the active site consists of the metal binding site and a well-defined hydrophobic specificity pocket (5). The amino acids involved in binding the zinc ions and in forming the hydrophobic pocket are conserved in both proteins (Fig. 3). The *V. proteolyticus* AP gene encodes a protein with a predicted molecular mass of 54,197 Da. However, the purified protein has a molecular mass of 30 kDa (28). The one previously reported *V. cholerae* protease *hap* gene consists of 1,827 nucleotides, with the molecular mass of the encoded protein calculated to be 69,300 Da (12). This is larger than the molecular mass of the purified HA/P (32 kDa). When HA/P is purified in the presence of protease inhibitors, a larger (approximately 45-kDa) form of HA/P is isolated (12). We also observed the presence of a 42-kDa protease during the purification of LAP. These observations suggest that proteases may undergo several stages of processing, including cleavage of the signal peptide and a further processing of the N terminus and/or C terminus, to form the mature protein. In the case of HA/P, the processing of the C-terminal region has been reported (19), but for LAP, the N-terminal amino acid sequence of the isolated protein has yet to be determined. However, it is interesting to note that the deduced C-terminal amino acid sequence of LAP exhibits some similarity to the C-terminal sequences of proteases of other *Vibrio* species. The absence of cross-reactivity in Western blots with anti-HA/P and anti-LAP antisera (Fig. 4) suggests that the C-terminal region was processed from purified proteins. The possible removal of an approximately 9-kDa polypeptide from the carboxy terminus of other proteases has been reported (18). This could explain the decrease in molecular mass of LAP, from 42 to 34 kDa observed during purification. There is evidence that the C-terminal regions of proteins secreted from gram-negative bacteria play a critical role in protein export through the outer membrane (7). Although not definitively shown here, it is plausible that the C-terminal region of LAP may also be important in secretion.

Characterization of the recombinant LAP supported the genetic analysis data. *V. cholerae* LAP was inhibited by EDTA (a metalloprotease inhibitor) and bestatin (an AP inhibitor). Moreover, leucyl-*p*-nitroanilide was cleaved by LAP, revealing a substrate specificity similar to that of AP (30). As with AP, LAP was stable in response to heat treatment. Uraleva and Gulida (27) reported a thermostable protease for *V. cholerae* that correlated with pathogenicity. Nevertheless, no other study has been done on this type of protease. The PCR analysis of *lap* gene distribution using primers corresponding to the N-terminal region showed that the gene is widely distributed among the *V. cholerae* strains examined. No positive strain was found in other species or genera. The G+C content of *lap* (47.78%) also corresponded to that observed in *V. cholerae* chromosomal DNA (47 to 49%).

HA/P is reported to cleave Arg at position 192 from the N terminus of the *E. coli* heat-labile enterotoxin (15) and between Ser-420 and Ser-421 of the human lactoferrin (25). However, the amino acid specificity of *V. cholerae* endopeptidase (HA/P) has yet to be studied in detail.

Complementary specificities between endopeptidases and exopeptidases of the same cellular origin have been reported (30). Hydrolysis of a peptide chain by an endopeptidase thus may liberate a fragment having an N-terminal residue that is susceptible to an exopeptidase. It would be very interesting to examine whether such complementary specificities occur between HA/P and LAP.

During HA/P purification, AP activity (measured by leucyl-*p*-nitroanilide hydrolysis) was observed in the crude preparations. This activity was not present in the purified HA/P (data not shown). These results suggest that both proteases are present in the *V. cholerae* culture supernatant. *V. cholerae* AP might not have been found, even now, in spite of its wide distribution among *V. cholerae* strains because its molecular mass is very similar to that of HA/P. Milton et al. (18) suggested a two-component protease system working to enhance the pathogenicity of *V. anguillarum*. Therefore, the role of proteases in the virulence mechanism of *V. cholerae*, if any, may not involve just one protease. Moreover, LAP may also play an important role in releasing the free amino acids needed for the regulation of expression of other virulence factors (17).

ACKNOWLEDGMENTS

We thank Masaaki Iwanaga for helpful discussions and for critically reading the manuscript and Koichiro Yamamoto for providing the *V. cholerae* DNA library.

REFERENCES

1. **Bagdasarian, M., and M. M. Bagdasarian.** 1994. Gene cloning and expression, p. 409–412. *In* P. Gerhardt, R. G. E. Murray, W. A. Wood, and N. R. Krieg, (ed.), Methods for general and molecular bacteriology. American

Society for Microbiology, Washington, D.C.
2. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1523.
3. **Booth, B. A., M. Boesman-Finkelstein, and R. A. Finkelstein.** 1984. *Vibrio cholerae* hemagglutinin/protease nicks cholera enterotoxin. Infect. Immun. **45:**558–560.
4. **Burley, S. K., P. R. David, A. Taylor, and W. N. Lipscomb.** 1990. Molecular structure of leucine aminopeptidase at 2.7-Å resolution. Proc. Natl. Acad. Sci. USA **87:**6878–6882.
5. **Chevrier, B., C. Schalk, H. D'Orchymont, J.-M. Rondeau, D. Moras, and C. Tarnus.** 1994. Crystal structure of *Aeromonas proteolytica* aminopeptidase: a prototypical member of the cocatalytic enzyme family. Structure **2:**283–291.
6. **Crowther, R. S., N. W. Roomi, R. E. F. Fahim, and J. F. Forstner.** 1987. *Vibrio cholerae* metalloproteinase degrades intestinal mucin and facilitates enterotoxin-induced secretion from rat intestine. Biochim. Biophys. Acta **924:**393–402.
7. **David, V. A., A. H. Deutch, A. Sloma, D. Pawlyk, A. Abdul, and D. R. Durham.** 1992. Cloning, sequencing and expression of the gene encoding the extracellular neutral protease, vibriolysin, of *Vibrio proteolyticus*. Gene **112:** 107–112.
8. **Davis, B. J.** 1964. Disc electrophoresis. II. Method and application to human serum proteins. Ann. N. Y. Acad. Sci. **121:**404–427.
9. **Deane, S. M., F. T. Robb, S. M. Robb, and D. R. Woods.** 1989. Nucleotide sequence of the *Vibrio alginolyticus* calcium-dependent, detergent-resistant alkaline serine exoprotease A. Gene **76:**281–288.
10. **Finkelstein, R. A., M. Boesman-Finkelstein, Y. Chang, and C. Häse.** 1992. *Vibrio cholerae* hemagglutinin/protease, colonial variation, virulence, and detachment. Infect. Immun. **60:**472–478.
11. **Finkelstein, R. A., M. Boesman-Finkelstein, and P. Holt.** 1983. *Vibrio cholerae* hemagglutinin/lectin/protease hydrolyzes fibronectin and ovomucin: F. M. Burnet revisited. Proc. Natl. Acad. Sci. USA **80:**1092–1095.
12. **Häse, C. C., and R. A. Finkelstein.** 1991. Cloning and nucleotide sequence of the *Vibrio cholerae* hemagglutinin/protease (HA/protease) gene and construction of an HA/protease-negative strain. J. Bacteriol. **173:**3311–3317.
13. **Häse, C. C., and R. A. Finkelstein.** 1993. Bacterial extracellular zinc-containing metalloproteases. Microbiol. Rev. **57:**823–837.
14. **Ichinose, Y., M. Ehara, T. Honda, and T. Miwatani.** 1994. The effect on enterotoxicity of protease purified from *Vibrio cholerae* O1. FEMS Microbiol. Lett. **115:**265–272.
15. **Ichinose, Y., T. Tsuji, M. Ehara, A. Miyama, and T. Naito.** 1992. The protease from *Vibrio cholerae* nicks arginine at position 192 from the N-terminus of the heat-labile enterotoxin A subunit from enterotoxigenic *Escherichia coli*. Eur. J. Epidemiol. **8:**743–747.
16. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
17. **Miller, V. L., and J. L. Mekalanos.** 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. J. Bacteriol. **170:**2575–2583.
18. **Milton, D. L., A. Norqvist, and H. Wolf-Watz.** 1992. Cloning of a metalloprotease gene involved in the virulence mechanism of *Vibrio anguillarum*. J. Bacteriol. **174:**7235–7244.
19. **Naka, A., K. Yamamoto., T. Miwatani, and T. Honda.** 1992. Characterization of two forms of hemagglutinin/protease produced by *Vibrio cholerae* non-O1. FEMS Microbiol. Lett. **98:**197–200.
20. **Prescott, J. M., F. W. Wagner, B. Holmquist, and B. L. Vallee.** 1985. Spectral and kinetic studies of metal-substitute *Aeromonas* aminopeptidase: nonidentical, interacting metal-binding sites. Biochemistry **24:**5350–5356.
21. **Prescott, J. M., and S. H. Wilkes.** 1976. *Aeromonas* aminopeptidase. Methods Enzymol. **45:**530–543.
22. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
23. **Schneider, D. R., and C. D. Parker.** 1978. Isolation and characterization of protease-deficient mutants of *Vibrio cholerae*. J. Infect. Dis. **138:**143–151.
24. **Schneider, D. R., S. P. Sigel, and C. D. Parker.** 1981. Characterization of *Vibrio cholerae* protease activities with peptide digest analysis. J. Clin. Microbiol. **13:**80–84.
25. **Toma, C., Y. Honma, and M. Iwanaga.** 1996. Effect of *Vibrio cholerae* non-O1 protease on lysozyme, lactoferrin and secretory immunoglobulin A. FEMS Microbiol. Lett. **135:**143–147.
26. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and source applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
27. **Uraleva, V. S., and M. M. Gulida.** 1988. Characterization of the protease activity of *Vibrio cholerae* strains and mutants with different biological properties. Zh. Mikrobiol. Epidemiol. Immunobiol. **9:**7–10.
28. **Van Heeke, G., S. Denslow, J. R. Watkins, K. J. Wilson, and F. W. Wagner.** 1992. Cloning and nucleotide sequence of *Vibrio proteolyticus* aminopeptidase gene. Biochim. Biophys. Acta **1131:**337–340.
29. **Von Heijne, G.** 1986. A new method for predicting signal sequence cleavage sites. Nucleic Acids Res. **14:**4683–4690.
30. **Wagner, F. W., S. H. Wilkes, and J. M. Prescott.** 1972. Specificity of *Aeromonas* aminopeptidase toward amino acid amides and dipeptides. J. Biol. Chem. **247:**1208–1210.
31. **Yamamoto, K., Y. Ichinose, H. Shinagawa, K. Makino, A. Nakata, M. Iwanaga, T. Honda, and T. Miwatani.** 1990. Two-step processing for activation of the cytolysin/hemolysin of *Vibrio cholerae* O1 biotype El Tor: nucleotide sequence of the structural gene (*hlyA*) and characterization of the processed products. Infect. Immun. **58:**4106–4116.
32. **Young, D. B., and D. A. Broadbent.** 1982. Biochemical characterization of extracellular proteases from *Vibrio cholerae*. Infect. Immun. **37:**875–883.

Editor: A. O'Brien