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Identification and Characterization of a Zinc Metalloprotease Associated with Invasion by the Fish Pathogen *Vibrio anguillarum*

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An invasiveness-defective mutant of the fish-pathogenic bacterium *Vibrio anguillarum* was isolated. Compared with the wild type, this mutant had a 1,000-fold higher 50% lethal dose after immersion infection of rainbow trout, *Oncorhynchus mykiss*, while after intraperitoneal infection, the mutant had only a 10-fold higher 50% lethal dose. In addition, the mutant showed a lower level of protease activity. Two forms of the protease (Pa and Pb) were found after sodium dodecyl sulfate-polyacrylamide gel electrophoresis of nonheated samples. Pa was found predominantly in protease preparations of the wild type, while Pb was the predominant form in the mutant. Conversion of Pb to Pa was observed in protease preparations after incubation at 4°C. Characterization of the protease showed that it was an elastolytic enzyme which required Zn^{2+} for activity and Ca^{2+} for stability. The molecular mass of the protease was 36 kilodaltons. N-terminal amino acid sequence analysis of the protease of *V. anguillarum* revealed homology to the elastase of *Pseudomonas aeruginosa* and the protease of *Legionella pneumophila*.

Vibrio anguillarum is an important infectious agent, causing vibriosis in fish. The mode of entrance of the pathogen is not known. The first step of infection probably requires attachment of the bacterium to the host and then penetration of the epithelial cell layers, since the later stages of infection involve hemorrhagic septicemia. The pathogen can easily be isolated in relatively large numbers from the kidneys of moribund fish. The only well-characterized virulence property of *V. anguillarum* is its ability to sequester iron from high-affinity iron-binding proteins of the host via a plasmid-encoded mechanism (1, 5). Siderophore mutants which do not sequester iron from the host have been shown to be avirulent. However, experimental fish infections with a wild type and a mutant showed that the inability of the mutant to sequester iron could be complemented in vivo by using the siderophore of the wild type (20). Thus, the iron-sequestering mechanism of *V. anguillarum* is important to the pathogenic process.

Besides the iron uptake system, little is known about other virulence mechanisms of *V. anguillarum*. However, extracellular compounds such as a hemolysin and a protease have been implicated in virulence (9, 15). Intraperitoneal injections of the protease of *V. anguillarum* into goldfish have been shown to be lethal (9). Although this suggests a role in virulence, no conclusive data, such as from mutant studies, have been presented. Proteases have also been suggested to be associated with the virulence of other pathogens (3, 11, 19). The best-studied system in this respect is the zinc metalloprotease of *Pseudomonas aeruginosa* (14). Mutants defective in this enzyme have reduced ability to cause infections. However, the role of the elastase in virulence has not been established.

In general, the molecular mechanisms of bacterial invasion are poorly understood. The use of fish as a model system to study invasion of bacterial pathogens has advantages, since the fish can be infected both intraperitoneally (i.p.) and by immersion. In addition, fish can be used to screen for a large number of mutants (17). As a result, the fish model system facilitates isolation of mutants that are defective in host invasion but are still virulent after i.p. infection.

In this study, we applied this strategy and showed that invasive mutants can be isolated. A mutant was isolated that was, compared with the wild type, restricted in the ability to infect rainbow trout, *Oncorhynchus mykiss*, by immersion but was almost as virulent as the wild type following i.p. infection. The mutant exhibited a lowered level of expression of an extracellular 36-kilodalton (kDa) zinc metalloprotease which shows N-terminal amino acid sequence homology to the elastase of *P. aeruginosa*. On the basis of these results, we suggest that the extracellular zinc metalloprotease plays an important role in promoting invasion.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *V. anguillarum* NB10, serotype O1, is an isolate of this laboratory from the Gulf of Bothnia outside the Norrby Laboratory, Umeå, Sweden. Rifampin-resistant mutants were isolated by spreading 10^{10} cells of *V. anguillarum* NB10 on Trypticase soy agar (TSA; BBL Microbiology Systems) containing 200 μg of rifampin per ml. Colonies appearing at a frequency of 10^{-8} were picked and streaked on TSA-rifampin plates before being frozen at −70°C.

The media used in this study were nutrient broth (NB; Difco Laboratories and Oxoid Ltd.), Trypticase soy broth (BBL), brain heart infusion broth (Difco), MOPS (morpholinepropanesulfonic acid) minimal medium (16), thiosulfate-citrate-bile-sucrose agar (Difco), and TSA. All media were supplemented with NaCl to a final concentration of 2%.

Preparation of the protease. Bacteria were grown in NB (Difco) at 18°C for 48 h. The bacterial suspension was centrifuged for 15 min at 13,000 × *g*, and the supernatant was filtered through a 0.22-μm (pore size) membrane filter (Millipore Corp.). Protein in the supernatant was precipitated overnight upon addition of 60% ammonium sulfate and collected by centrifugation at 16,000 × *g* for 60 min. The pellet was suspended in 50 mM phosphate-buffered saline

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(pH 7.4) and frozen in aliquots at −70°C (crude preparation). When further purification of the protease was required, it was applied to a Sephacryl S-200 (Pharmacia) gel filtration column equilibrated with 50 mM ammonium hydrogen carbonate and then eluted with the same buffer at 4°C. The elution rate was 8 ml/h, and the fraction volume was 4 ml. Elution profiles were recorded at 280 nm, and the protease-containing fractions were immediately lyophilized.

SDS-PAGE and analytical gel filtration analysis. Protease preparations were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (12). Electrophoresis was performed at 175 V for 60 min. Gels were fixed in 40% methanol–10% acetic acid, stained in 0.2% Coomassie brilliant blue in 40% methanol–10% acetic acid, and destained in 40% methanol–10% acetic acid.

Analytical gel filtration using high-pressure liquid chromatography was performed on a Superose-12 (Pharmacia) column. The column buffer contained 50 mM Tris (pH 8.0), 0.1 M NaCl, and 0.1% SDS. The flow rate was 0.25 ml/min.

Determination of proteolytic activity and protein content. Detection of proteolytic activity on solid media was performed by patching the bacteria on TSA plates containing 1% gelatin. After incubation at 18°C for 24 h, the plates were flooded with 12.5% $HgCl_2$ in 1 M HCl. A transparent zone around the colony indicated proteolytic activity. TSA plates containing 1% skim milk or 0.3% elastin were also used, and a transparent zone around the colony indicated proteolytic activity. Proteolytic activity was also determined by an X-ray film method as follows. X-ray film having a gelatin support (we found DuPont Cronex 4 to be suitable) was exposed to light before use. When culture supernatants were to be tested, 4 μl of supernatant was spotted on the film and incubated for 1 h at 37°C. After incubation, the film was flushed with water. A clear zone showing degradation of the gelatin indicated activity. When enzymatic activity in SDS-polyacrylamide gels was tested, SDS was removed from the gel by soaking the gel at room temperature in 2.5% Triton X-100 for 30 min and repeating this once with a fresh Triton X-100 solution. The gel was then put on the X-ray film for 1 h at 37°C, whereafter it was flushed with water.

Caseinolytic activity was determined as described by Sakai (19). Elastolytic activity was estimated by the method described by Bjorn et al. using elastin-Congo red (2), and activities are given as units of porcine pancreas type 1 elastase (Sigma Chemical Co.) per milligram of protein.

Protein content was estimated by the method of Lowry et al. (13) with bovine serum albumin as the standard.

Protease inhibition studies. Protease was incubated with inhibitor for 60 min in phosphate-buffered saline at 37°C and then assayed by the X-ray film method. The inhibitors used were 1,10-phenanthroline (OPA), EDTA, ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), phenylmethylsulfonyl fluoride, *N*-α-L-rhamnopyranosyloxy-(hydroxyphosphinyl)-L-leucyl-L-tryptophan (phosphoramidone), and benzamidine hydrochloride.

Experimental fish infections. Rainbow trout, *O. mykiss*, in the size range of 10 to 15 g were infected i.p. or by immersion as previously described (17). The 50% lethal doses (LD_{50}) were calculated as described by Reed and Muench (18).

Gel scanning analysis. A computerized video densitometer (Bio-Rad Laboratories 620) was used for gel scanning analysis of polyacrylamide gels.

N-terminal amino acid sequence analysis. The N-terminal amino acid sequences of peptides blotted from SDS-polyacrylamide gels to Immobilon were determined by using an Applied Biosystems 470A gas-liquid-phase sequencer with on-line detection of phenylthiohydantoin derivatives. Twenty cycles were run, and the amino acid residues were identified by comparison to a β-lactoglobulin standard.

TABLE 1. Infection of fish i.p. or by immersion with NB11 and NB10 mixed at different ratios

Infection method and NB11/NB10 ratio[a]	% of bacteria isolated that were NB11[b]
Immersion	
10^4	95
10^3	<1
10^2	<1
10^1	<1
i.p.	
10^3	90
10^2	16
10^1	12
10^0	<1

[a] The numbers of NB10 cells used were 10^3/ml in the immersion experiment and 10 in the i.p. experiment.

[b] Kidney samples were withdrawn from moribund fish and plated on thiosulfate-citrate-bile-sucrose agar. Colonies growing on thiosulfate-citrate-bile-sucrose agar were patched on TSA-rifampin plates, and the percentages of isolated bacteria that were NB11 were estimated.

RESULTS

Isolation of an invasiveness-defective mutant of *V. anguillarum*. Six spontaneously rifampin-resistant mutants of *V. anguillarum* were screened for the inability to cause immersion infections in rainbow trout, *O. mykiss*. Survival rates in brackish water of the wild type (NB10) and all tested mutants were equal during the infections (data not shown). The degree of virulence was scored by comparing the LD_{50}s of the mutants and the wild type. One mutants (NB11) of six tested had an LD_{50} of 10^6/ml, compared with 10^3/ml for NB10. When this mutant was tested by i.p. infection, only a 10-fold increase in the LD_{50} was observed (10^2 bacteria for NB11 compared with 10 bacteria for NB10), indicating that this mutant was defective in the ability to invade a host.

In addition, experiments were performed in which NB10 and NB11 were mixed at different ratios before infection of fish. After both i.p. and immersion infections, the kidneys of moribund fish were analyzed for the presence of the two strains. For immersion infection, isolation of the mutant from moribund fish required infection with a 10^4 excess of NB11 bacteria. However, after i.p. infection with an NB11/NB10 ratio of only 10, 12% of the bacteria isolated from moribund fish were NB11 (Table 1). These results confirmed our LD_{50} results, which strongly suggested that the mutant was affected in its invasive capability.

Comparisons of hemolytic and proteolytic activities. To test whether the mutant was affected in its hemolytic or proteolytic activity, equal numbers of NB10 and NB11 cells were spotted on blood agar plates and TSA plates containing 1% gelatin. NB11 was as hemolytic as NB10, showing no difference in the size of the hemolytic zone. However, when the proteolytic activities of the two strains were tested on gelatin plates, a marked difference in the cleared zones around the colonies was observed, indicating that NB11 had a reduced ability to produce gelatinolytic activity (Fig. 1). The specificities of the proteolytic activities of NB10 and NB11 were also tested by spotting them on TSA plates containing either casein or elastin. Both strains were able to degrade casein and elastin, but the mutant again showed a reduced clear zone.

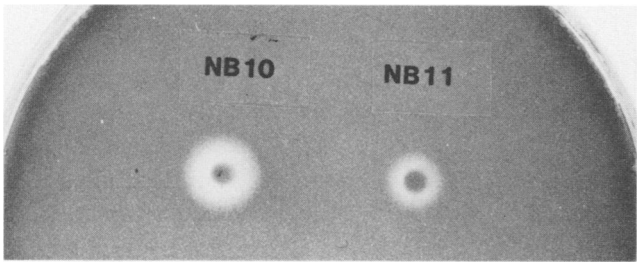

FIG. 1. Secreted proteolytic activities of NB10 and NB11 after growth on TSA with 1% gelatin.

Growth conditions for protease production. It has previously been shown that the protease of *V. anguillarum* is secreted from the cells (9). However, to our knowledge, no suitable liquid media for the production of the protease have been reported. Strain NB10 was grown to the stationary phase in five different liquid media; NB from Difco, NB from Oxoid, BHI, MOPS minimal medium, and Trypticase soy broth. When the supernatants were tested for proteolytic activity by the X-ray film method, it was found that secretion of the protease was medium dependent. NB from Difco was the only medium that gave detectable proteolytic activity in the culture supernatant. After 24 h of incubation, the culture contained approximately 10^9 cells per ml and protease activity was detectable. If the bacteria were incubated for 48 h, higher yields of the protease were obtained although the number of cells had not increased (data not shown).

Characterization of the protease. The protease was precipitated from the culture supernatant by addition of ammonium sulfate and further purified by gel filtration chromatography. Inamura et al. have previously shown that the protease of *V. anguillarum* is inhibited by EDTA (9), suggesting that it is a metalloprotease. Our results confirm that removal of metal ions by EDTA and OPA and the more specific removal of Ca^{2+} by EGTA inhibits the protease (Table 2). Moreover, the protease was also inhibited by the competitive metalloprotease inhibitor phosphoramidone but not by serine protease inhibitors (Table 2).

The metal ion requirement of the protease was tested, and it was found that Zn^{2+} ions restored activity but Ca^{2+} or Mg^{2+} did not (Table 2). The protease was further characterized by SDS-PAGE. When lyophilized samples were reconstituted in SDS-PAGE sample buffer with no heating of the samples before application to the gel, two predominant protein bands, Pa and Pb, were found. When the samples were heated to 95°C for 5 min before application, only one predominant band, Pc, was detected (Fig. 2). The apparent molecular masses of Pa and Pb, compared to molecular mass standard proteins, were greatly dependent on the concentration of the acrylamide in the gel. When acrylamide concentrations between 6 and 17% were used, a gradual decrease in the apparent molecular masses of Pa and Pb was observed. In 6% gels, Pa was apparently 94 kDa and Pb was 82 kDa. However, in 17% gels, both Pa and Pb appeared as smaller proteins with apparent molecular masses of 43 kDa for Pa and 40 kDa for Pb. The apparent molecular mass of Pc was always 36 kDa.

TABLE 2. Inhibition and metal ion requirements of the protease

Addition (concn)	Activity[a]
None	+
OPA (10 mM)	−
EGTA (10 mM)	−
EDTA (10 mM)	−
Phenylmethylsulfonylfluoride (1 mM)	+
Benzamidine (2 mg/ml)	+
Phosphoramidone (1 μg/ml)	−
OPA (10 mM) + Zn^{2+} (20 mM)	+
OPA (10 mM) + Ca^{2+} (20 mM)	−
OPA (10 mM) + Mg^{2+} (20 mM)	−

[a] Activity was scored by the X-ray film method. Requirements for metal ions were tested after incubation for 60 min with OPA, followed by addition of the ions. The protein content of each sample was approximately 1.5 μmol.

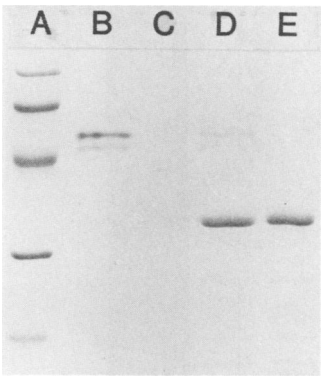

FIG. 2. SDS-(12%) PAGE of protease preparations of *V. anguillarum* NB10. Lanes: A, molecular mass standards (from the top, 94, 67, 43, 30, and 20 kDa) heated to 95°C; B, OPA-treated protease; C, EGTA-treated protease; D, OPA-EGTA-treated protease; E, protease heated to 95°C for 5 min.

The electrophoretic behaviors of the Pa and Pb forms of the protease were also studied after treatment with metal chelators. With OPA, no effect was found. However, after treatment of the protease with EDTA or EGTA, no protein bands were detected in the SDS-polyacrylamide gel (Fig. 2). This suggested the possibility that a metal ion other than Zn^{2+} stabilized the protease, and when this ion was removed by the chelator, the protease was rapidly degraded. Since the Ca^{2+}-specific chelator EGTA gave this result, the protease may be stabilized by Ca^{2+}. This was further examined by inhibiting the protease first with OPA, whereafter EGTA was added. As a result of this treatment, the 36-kDa band appeared with concomitant loss of Pa and Pb. Analytical gel filtration in the presence of SDS also gave a molecular mass of 36 kDa both before and after treatment with OPA and OPA plus EGTA.

Both Pa and Pb were proteolytically active (Fig. 3), and the OPA-treated samples were also active after SDS-PAGE. In samples treated with both OPA and EGTA, in which a protein band was found in the Pc position, no proteolytic activity was detected. However, in these samples, low residual activities were found at the positions of Pa and Pb.

Comparison of the proteases of NB10 and NB11. The reduction of lysis zones on the different substrate plates indicated that the mutant, NB11, was defective either in the specific activity of the protease or in the level of expression or secretion of the protease. Specific activity measurements showed that the protease activities of NB10 and NB11 were the same when both casein (61 U/mg of protein for NB10 and 64 U/mg of protein for NB11) and elastin (15 U/mg of protein for NB10 and 14 U/mg of protein for NB11) were used as substrates. Another observation made during this work was that Pb was much more abundant than Pa in protease preparations of the mutant. However, in NB10, Pa was the

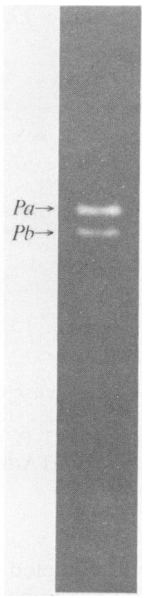

FIG. 3. Proteolytic activity of *V. anguillarum* protease after SDS-12% PAGE. Activity in the gel was scored by the X-ray film method after the gel was soaked twice for 30 min each time in 2.5% Triton X-100.

most abundant band after SDS-PAGE. When crude preparations of the protease from NB10 were kept or dialyzed at 4°C overnight, only Pa was observed after SDS-PAGE. An explanation of this observation may be that Pb had been proteolytically degraded in these preparations. To study this in more detail, crude preparations of the proteases from NB10 and NB11 were incubated at 4°C overnight. In the preparation from NB10, Pb disappeared. However, in the preparation from NB11 consisting mostly of Pb, a decrease in the amount of Pb was followed by a concomitant increase in the amount of Pa, indicating that Pb was converted to Pa (Fig. 4). The kinetics of this process was monitored in a crude preparation of the wild type by incubation at 4°C. Samples were removed at regular time intervals, whereafter they were subjected to SDS-PAGE. Gel scanning analysis revealed that the amount of Pa increased with a concomitant decrease of Pb over time. At each time point, the amount of Pa and Pb accounted for approximately 80% of the total protein while the remaining 20%, consisting of proteins with lower molecular masses, remained constant. Thus, it seems likely that Pa originated from Pb. Interestingly, the gel filtrated preparations showed no conversion of Pb to Pa.

N-terminal amino acid sequence analysis. The N-terminal amino acid sequence of the 36-kDa form of the protease was determined. When comparing the first 20 amino acids of the protease by computer analysis with PC Gene programs for comparisons of amino acid sequence similarities, a high degree of homology with the elastase of *V. vulnificus* (11) and also considerable homology with the *P. aeruginosa*

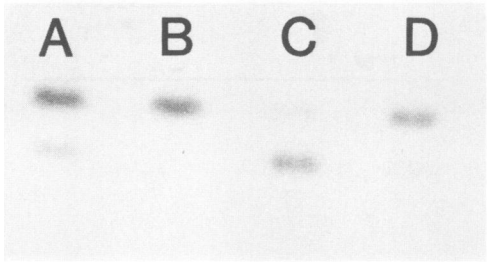

FIG. 4. Conversion of Pb to Pa in ammonium sulfate precipitates of culture supernatants of NB10 and NB11. SDS-(7.5%) PAGE stained with Coomassie brilliant blue. Lanes: A, NB10 not incubated overnight; B, NB10 incubated at 4°C overnight; C, NB11 not incubated overnight; D, NB11 incubated at 4°C overnight. Only part of the gel with Pa and Pb is shown.

elastase (8) were found. In addition, a small but significant homology with the protease of *Legionella pneumophila* (3) was observed (Fig. 5).

DISCUSSION

Invasive microorganisms must be able to penetrate body surfaces before they can cause systemic infections. This process is, to a large extent, unknown. Use of bacterial mutants is one way to study this mechanism. A good model system which allows rapid, inexpensive, and extensive screening is required to find such mutants. We chose the pathogen *V. anguillarum* and a naturally occurring host, *O. mykiss*, as a model since they fulfill these requirements (17).

V. anguillarum infects fish through water and causes an invasive disease. Thus, the pathogen must be able to penetrate the epithelial cell barriers. An expected phenotype of a mutant defective in invasion could be that it is avirulent when fish are infected by immersion but virulent after i.p. infection. However, the virulence determinants involved in invasion may have several roles in pathogenesis, i.e., adherence, invasion, and subsequent promotion of bacterial proliferation. Therefore, mutations of structural genes that encode invasive properties may be difficult to identify, while regulatory mutations causing decreased ability to invade could be easier to isolate. Hence, in a first attempt to characterize invasive properties of *V. anguillarum*, we chose to isolate regulatory mutations rather than structural gene mutations.

It has been found that spontaneous mutants of *Yersinia pseudotuberculosis* that are resistant to high concentrations of rifampin are avirulent at a high frequency (4). Such mutants are affected in the beta subunit of RNA polymerase, and thus an avirulent phenotype must be associated with a regulatory disorder caused by the altered RNA polymerase. Hence, we isolated a number of spontaneously rifampin-resistant mutants of *V. anguillarum* NB10 and screened for avirulence by infecting rainbow trout, *O. mykiss*. Compared with the wild type, one mutant, NB11, was clearly defective in invasiveness. No difference in the hemolysin production

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V. vulnificus:   Ala-Gln-Ala-Asn-Gly-Thr-Gly-Pro-Gly-Gly-Asn-Ser-Lys-Thr-Gly-Arg-Tyr-Glu-Phe-Gly
                  *       *       *  *  *  *  *  *  *       *  *  *       *     :  *
V. anguillarum:  Ala-Glu-Ala-Thr-Gly-Thr-Gly-Pro-Gly-Gly-Asn-Gln-Lys-Thr-Gly-Phe-Tyr-Gln-Tyr-Gly
                  *  *  *       *     *                                    *
P. aeruginosa:   Ala-Glu-Ala-Gly-Gly-Pro-Gly-Gly-Asn-Gln-Lys-Ile-Gly-Lys-Tyr-Thr-Tyr-Gly-Ser-Asp
                     *          *     *                                    :
L. pneumophila:  Ala-Lys-Gly-Met-Gly-Phe-Gly-Gly-Asn-Ala-Lys-Ile-Gly-Glu-Tyr-Gln-Phe-Gly-Lys-Asp
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FIG. 5. Comparison of N-terminal amino acid sequences of *V. anguillarum*, *V. vulnificus*, *P. aeruginosa*, and *L. pneumophila*. Asterisks indicate identity, and colons signify conservation of side group, size, or charge.

was found. We were also unable to detect any differences in lipopolysaccharide structures, membrane protein profiles, or total proteins as determined by two-dimensional SDS-PAGE (data not shown). The only previously well-characterized virulence property of *V. anguillarum* is the plasmid-encoded ability to sequester iron. Mutants defective in this system have been shown to be avirulent even after i.p. infections (6). We conclude that NB11 is not affected in its iron uptake system, since the mutant was virulent after i.p. infections. Thus, since the only difference detected between the wild type and the mutant was that the mutant showed reduced proteolytic activity, we suggest that the protease is important for invasion.

The *V. anguillarum* protease is an elastolytic metalloprotease dependent on Zn^{2+} for its activity and Ca^{2+} for its stability. In SDS-PAGE, an untreated or OPA-treated enzyme appeared as two bands, Pa and Pb, which both showed activity even after the samples had been treated with OPA before separation on SDS-PAGE. Interestingly, when activity was inhibited by OPA and then Ca^{2+} was removed by EGTA, the protease of *V. anguillarum* appeared as one band (Pc) in SDS-PAGE having a molecular mass of 36 kDa. Since no activity could be detected in the Pc band after OPA-EGTA treatment, we conclude that removal of Ca^{2+} from the protease leads to denaturation. Thus, in its active form, the protease is stabilized by Ca^{2+}. The N-terminal amino acid sequence analysis of the Pc form of the protein gave one single sequence, and no other polypeptides could be detected in SDS-polyacrylamide gels after the samples were heated. We therefore suggest that the protease consists of a single polypeptide with a molecular mass of 36 kDa. The Pb form of the protease was found to be converted to the Pa form. The mutant, NB11, showed a higher amount of Pb than did the wild type. This suggests that there was a correlation between the lowered proteolytic activity and the relative increase in the amounts of the Pb form of the protease, possibly indicating that Pb is an inactive precursor of the protease, similar to what has been described for *P. aeruginosa* (7). The observation that the conversion was found only in crude preparations of the protease indicates that a low-molecular-mass compound is involved in this process.

The elastase of *P. aeruginosa* has been implicated in virulence as a factor promoting spread of infection in the burned-mouse model (14). Recently, it has been shown by Black et al. (3) that the metalloprotease of *L. pneumophila* shows a high degree of homology to the *Pseudomonas* elastase, and cumulative results have indicated a role for the *Legionella* protease in virulence. The role of an elastolytic protease in the virulence of the halophilic bacterium *V. vulnificus*, which occasionally causes rapidly developing would infections and septicemia in humans, has also been suggested (11). Therefore, our observation that the elastase of *V. anguillarum* shows homology to the *Pseudomonas* elastase, the *Legionella* protease-cytotoxin, and the elastase of *V. vulnificus* is of considerable interest. These results show that there may be a family of related metalloproteases of different bacterial genera which seems to be involved in pathogenesis.

Although much effort has been expended in showing that these species are dependent on proteases for virulence, definitive evidence has not been established. Use of the model system for infection presented here makes it possible to distinguish between mutants defective in the different stages of the pathogenetic process and will facilitate further studies concerning the actual role of proteases in the process of virulence. The present study has focused on invasion of fish by *V. anguillarum* and the correlation between virulence and production of a secreted protease. The rifampin-resistant mutant used in this study is not affected in the structural gene of the protease, but is likely to be regulatorily disordered because of an altered RNA polymerase which also could have caused a pleiotropic phenotype, as has been shown in *Escherichia coli* (10). Thus, we cannot exclude the possibility that the three different phenotypes of the mutant, i.e., reduced ability to invade fish, a lowered level of protease activity, and affected conversion of the two different forms of the protease, are independent events. However, the lowered protease activity and the affected conversion are most probably linked. Since coupling of reduced invasion capability and a lowered protease level is possible, we suggest that the protease is involved in invasion. To investigate our hypothesis further, we have initiated cloning of the structural gene for the protease for mutant studies.

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