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Purification, Characterization, and Primary Structure of *Clostridium perfringens* Lambda-Toxin, a Thermolysin-Like Metalloprotease

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The lambda-toxin of *Clostridium perfringens* type B NCIB10691 was purified by ammonium sulfate precipitation, followed by size exclusion, anion-exchange, and hydrophobic interaction chromatography. The purified toxin had an apparent molecular mass of 36 kDa, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The toxin possessed casein-hydrolyzing activity, which was inhibited specifically by metal chelators, indicating that the toxin is a metalloprotease. The gene encoding the lambda-toxin (*lam*), which was shown by Southern analysis to be located on a 70-kb plasmid, was cloned into *Escherichia coli* cells. Nucleotide and N-terminal amino acid sequencing revealed that the *lam* gene encodes a 553-amino-acid protein, which is processed into a mature form, the molecular mass of which was calculated to be 35,722 Da. The deduced amino acid sequence of the mature enzyme contains an HEXXH motif characteristic of zinc metalloproteases and is homologous to other known enzymes belonging to the thermolysin family. The purified toxin degraded various biologically important substances, such as collagen, fibronectin, fibrinogen, immunoglobulin A, and the complement C3 component. It caused an increase in vascular permeability and hemorrhagic edema on injection into the dorsal skin of mice. These results suggest that the toxin contributes to the pathogenesis of histolytic infection by lambda-toxin-producing *C. perfringens*.

Clostridium perfringens is an anaerobic gram-positive rod, which is widely distributed in nature and lives in the large intestines of man and other animals. It produces a variety of toxins and hydrolytic enzymes, some of which have been characterized well both biochemically and genetically (10, 29). However, others, including the lambda-toxin, remain poorly understood. This toxin has not been purified yet, and the only information available is that it is encoded by a plasmid (5) and that a partially purified lambda-toxin preparation can hydrolyze casein, gelatin, and hemoglobin (3). The concerted action of various toxins and hydrolytic enzymes seems to be the basis of the pathogenesis of clostridial infection, and the lambda-toxin seems to be one such virulence factor. Therefore, identification and characterization of the toxin would provide useful information for understanding the molecular mechanism underlying the disease process of clostridial infection.

The lambda-toxin is produced by most type B and E strains and some type D strains of *C. perfringens*, which cause enteritis and enterotoxemia in domestic animals (3, 10, 29). We found that the culture supernatant of a lambda-toxin-producing type B strain showed high levels of caseinolytic activity, which was inhibited by metal-chelating agents (unpublished data). This led us to suspect that the toxin is a zinc metalloprotease and contributes to the pathogenicity by degrading certain protein components of host cells, such as well-defined bacterial zinc metalloproteases, the neurotoxins of *Clostridium botulinum* and *Clostridium tetani* (24, 34, 40), clostridial collagenases (20, 42), and *Pseudomonas aeruginosa* elastase (26). Alternatively, the toxin may contribute to the pathogenesis by activating other potent toxins, such as the epsilon- and iota-toxins, produced by these type strains.

In order to elucidate the possible role of the lambda-toxin in the pathogenicity of *C. perfringens*, we attempted to purify it and to determine its biochemical properties. We also attempted to clone and sequence the lambda-toxin gene (*lam*) to gain insight into its primary structure and also to determine the extent of its pathogenic role by searching for sequence similarity with other known metalloproteases. Finally, we examined the in vivo effect of the toxin by injecting purified lambda-toxin into the dorsal skin of mice. This paper presents evidence that the lambda-toxin is a zinc metalloprotease belonging to the thermolysin family and that it functions as a virulence factor causing an increase in vascular permeability and hemorrhagic edema.

MATERIALS AND METHODS

Bacterial strains, plasmid vectors, and culture conditions. *C. perfringens* type B NCIB10691 used in this study was a gift from S. Nakamura, Department of Bacteriology, School of Medicine, Kanazawa University, Kanazawa, Japan. The medium used for its growth was Gifu anaerobic medium broth (GAM broth; Nissui Pharmaceutical Co., Tokyo, Japan), which was chilled in ice water and covered with paraffin oil after autoclaving to maintain anaerobic conditions (13). A preculture grown at 37°C overnight was diluted 100-fold with 1 liter of fresh GAM broth and then grown at 37°C for 6 h. *C. perfringens* 13 (18) and pJIR418 (35) were used as the host strain and plasmid vector, respectively, for the transformation of *C. perfringens*. The selection and culture of strain 13 transformants were performed as described previously (19). *Escherichia coli* JM109 (41) was used as the host strain for plasmid pUC18 (41) and derivatives. It was grown in LB broth (Gibco BRL Ltd., Paisley, United Kingdom) containing 50 μg of ampicillin per ml with shaking at 37°C.

Lambda-toxin purification. One-liter volumes of cultures were chilled in ice water, and approximately 700-ml amounts of cultures were siphoned into centrifuge tubes to avoid contamination by the overlaid paraffin oil. The cultures were centrifuged at 15,000 × *g* for 20 min at 4°C. A solution of 1 M $CaCl_2$ was added to 600 ml of the culture supernatant to a final concentration of 5 mM. All purification steps were carried out at 0 to 4°C. Ammonium sulfate was added to the culture supernatant to give 60% saturation, and the precipitate was collected by centrifugation at 15,000 × *g* for 30 min. The pellet was dissolved in approximately 16 ml of TSC buffer (50 mM Tris-HCl, 50 mM NaCl, 5 mM $CaCl_2$ [pH 7.5]) and then centrifuged at 28,000 × *g* for 30 min to remove undissolved materials. The supernatant was applied to a Sephacryl S-100 column (2.2 by 77 cm; Pharmacia LKB Biotechnology, Uppsala, Sweden). Proteins were eluted with the same buffer at a flow rate of 80 ml/h. The caseinase which eluted the first

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peak of activity was collected. This fraction (46.5 ml) was applied to a DEAE Sephadex A-25 column (1.6 by 23 cm; Pharmacia) preequilibrated with TSC buffer. After the column had been washed with 200 ml of the buffer, proteins were eluted with a 240-ml linear gradient of 0.05 to 0.6 M NaCl in TC buffer (50 mM Tris-HCl, 5 mM $CaCl_2$ [pH 7.5]). Lambda-toxin activity was eluted in the range of 0.25 to 0.6 M NaCl. Ammonium sulfate was added to 60% saturation. After centrifugation as described above, the precipitate was dissolved in 5 ml of TSC buffer and then applied to a Sephadex G-100 column (2.2 by 95 cm; Pharmacia). Proteins were eluted with the same buffer. The lambda-toxin fraction eluted as a single peak was collected. Ammonium sulfate was added to the fraction (72 ml) to give 25% saturation. The sample was subjected to hydrophobic interaction chromatography on a TSK gel ether-Toyopearl column (1.4 by 8 cm; Tosoh, Co., Tokyo, Japan), which was preequilibrated with TC buffer containing ammonium sulfate (25% saturation). After the column had been washed with 50 ml of the same buffer, proteins were eluted first with 90 ml of the TC buffer containing ammonium sulfate (15% saturation) and then with 130 ml of a linear gradient of ammonium sulfate, from 15 to 0% saturation, in TC buffer. The lambda-toxin fraction (30 ml) eluted as the last peak was collected and used as the purified toxin without further treatment. The purified toxin was stored at −80°C.

Enzyme assays and protein determination. The protease activity of the lambda-toxin was semiquantitatively assayed for the detection of the enzyme-containing fraction as follows. A 10 μl-fraction was added to a well in skim milk agar (1% agar, 1% skim milk, 1 mM $CaCl_2$, 100 mM imidazole buffer [pH 6.8]). After incubation at 37°C for 1 h, the diameter of the transparent zone formed around the well was measured. Proteolytic activity was quantitatively determined by using azocasein (Sigma Chemical Co., St. Louis, Mo.) as described by Kessler et al. (14), except that TC buffer was used as the incubation buffer. One unit of activity was defined as the amount of enzyme that caused an increase in A_{400} of 1/min under the assay conditions. Elastolytic activity was determined by using elastin-Congo red (Sigma) as described by Bjorn et al. (4) except that the pH of the reaction buffer was adjusted to 7.5. Protein concentrations were determined by using the Pierce bicinchoninic acid protein assay reagent (36) with bovine serum albumin as a standard.

Effects of inhibitors, metal ions, temperature, and pH on enzyme activity. The ability of the following proteinase inhibitors to inhibit the casein-hydrolyzing activity of the purified lambda-toxin was assessed: (4-amidinophenyl)methanesulfonyl fluoride (APMSF), 3,4-dichloroisocoumarine (3,4-DCI), ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), phosphoramidon, bestatin, and *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E64) (all from Sigma); leupeptin A, pepstatin, 1,10-phenanthroline, EDTA, dithiothreitol, and $HgCl_2$ (all from Wako Pure Chemical Industries, Ltd., Osaka, Japan); phenylmethylsulfonyl fluoride (PMSF) (Calbiochem Co., La Jolla, Calif.); and iodoacetic acid (Seikagaku Co., Tokyo, Japan). The toxin was preincubated with an inhibitor at room temperature for 10 min before residual casein-hydrolyzing activity was determined. The effect of pH was examined under the standard assay conditions, except for the buffer. The buffers used were 50 mM bis-Tris-HCl (pH 5 to 7) containing 5 mM $CaCl_2$ and 50 mM Tris-HCl (pH 7 to 9) containing 5 mM $CaCl_2$. Heat stability was examined by preincubating the toxin in TC buffer at various temperatures for 30 min prior to determination of caseinase activity.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (17) on a slab gel comprising a stacking 3% acrylamide gel over a 12.5% resolving gel. The samples were heated in sample buffer consisting of 62.5 mM Tris-HCl (pH 6.8), 1% 2-mercaptoethanol, 1% SDS, 10% glycerol, and 0.001% bromophenol blue at 100°C for 3 min. Electrophoresis was performed at 100 V until the dye front reached the bottom of the gel. The gel was stained with 0.2% Coomassie brilliant blue R by the conventional method. Pharmacia low-molecular-mass (94, 67, 43, 30, 20.1, and 14.4 kDa) and high-molecular-mass (212, 170, 116, 76, and 53 kDa) standards were used as markers.

N-terminal sequencing of purified lambda-toxin. Approximately 20 μg of the purified enzyme was applied to an SDS-PAGE gel. After electrophoresis, the protein was electrophoretically transferred to a polyvinylidene difluoride membrane (Trans-Blot transfer medium; Bio-Rad Laboratories, Richmond, Calif.) and stained as described previously (42). The area containing the band was cut out and subjected to N-terminal amino sequence analysis with a protein sequencer (model 473A; Applied Biosystems, Foster City, Calif.).

Ability of lambda-toxin to cleave host cellular proteins. The ability of the lambda-toxin to cleave host cellular proteins was assessed by using the following proteins: human immunoglobulin G (IgG; Caltag Laboratories, South San Francisco, Calif.), human IgA (Caltag), porcine skin collagen (type I; Wako), bovine lens capsule collagen (type IV; Wako), porcine placenta collagen (type V; Wako), human fibrinogen (Servio Laboratory, Gennevilliers, France), human fibronectin (Chemicon International, Inc., Temecula, Calif.), human complement C3 component (Morinaga Institute of Biological Science, Yokohama, Japan), and human α_2-macroglobulin (Athens Research and Technology, Inc., Athens, Ga.). The proteins (16 to 60 μg) were incubated with purified lambda-toxin (2 μg) at 37°C in 90 μl of TC buffer containing 85 mM NaCl for 2 h. Samples were heated at 100°C for 3 min with the sample buffer, and then the cleavage products were resolved by SDS-PAGE as described above.

In vivo study. Purified lambda-toxin was concentrated by ammonium sulfate (60% saturation) precipitation. The precipitate was dissolved in TC buffer containing 85 mM NaCl and dialyzed against 1 liter of the same buffer. The concentrated lambda-toxin was diluted to various concentrations with 0.15 M NaCl, and a 50-μl sample of each dilution was injected into the dorsal skin of a male ddY mouse (6 to 8 weeks old and 30 to 35 g in weight) which had previously been injected intravenously with 0.3 ml of 0.5% Evans blue in 0.15 M NaCl. At the indicated time postinjection, the mouse was sacrificed, the dorsal skin was flayed, and then the diameter of the blue spot caused by extravasation of Evans blue was determined.

Cloning of *lam* gene. Plasmid DNA was isolated from *C. perfringens* NCIB10691 as described by Roberts et al. (28) and then purified by centrifugation on a cesium chloride density gradient (31). Plasmid DNA was partially digested with *Hin*cII and then ligated into the *Hin*cII site of pUC18. *E. coli* JM109 was transformed with the ligation mixture. Transformants were replica plated onto LB agar containing 50 μg of ampicillin per ml and 1% skim milk, and then the plate was screened for colonies producing clear zones of hydrolysis. Casein-hydrolyzing transformants were disrupted with a French press, and the lysates were subjected to the skim milk agar plate test to verify the caseinase phenotype. Other subclones were constructed from the original plasmid (pFJ1) conferring casein-hydrolyzing activity by similar procedures.

DNA sequencing and sequence analysis. DNA sequencing was performed by the dideoxy chain termination method of Sanger et al. (32), with Sequenase (United States Biochemical, Cleveland, Ohio) and α-^{35}S-dATP (>1,000 Ci/mmol; Amersham Japan Inc., Tokyo, Japan). Universal and reverse primers were used to obtain the initial sequences within the inserts, and then specific primers for sequences within the inserts were prepared with a DNA synthesizer (model 381; Applied Biosystems).

The deduced amino acid sequence was analyzed for similarity to sequences in protein databases PIR and SwissProt by using the BLAST algorithm (2). To test the statistical significance of similarity between two protein sequences, a Monte-Carlo test was performed by using the Lipman-Pearson alignment algorithm with Dayhoff similarity scoring (27) as described previously (20).

Mutagenesis and DNA manipulations. To determine which was the initiation codon of the *lam* gene of three possible codons, the initiation codon, ATG, at nucleotide 196 (see Fig. 3) was converted to a termination codon, TAG, as follows. A 2.3-kb *Hin*dIII fragment containing the *lam* gene was cloned into pBluescript II SK(+), and then site-directed mutagenesis was carried out with a synthetic oligonucleotide, 5′-ATGGGGTGATTTTTAGAAAAAAATATTA-ATATC-3′, as described by Kunkel (16). The base substitution was confirmed by nucleotide sequencing. The 2.3-kb *Hin*dIII fragments containing the wild-type and mutagenized *lam* genes were ligated into pJIR418, and the resulting plasmids were named pJIRlam and pJIRlamM, respectively. They were introduced into *C. perfringens* 13 by a modification of the method of Allen and Blaschek (1). All recombinant DNA procedures were carried out as described by Sambrook et al. (31).

Southern hybridization and pulsed-field electrophoresis. To prepare a DNA probe, plasmid DNA of pUC18 containing a 0.55-kb *Eco*RI-*Pst*I fragment was completely digested with *Eco*RI and *Pst*I, and then the 0.55-kb insert was isolated, purified, and labeled with digoxigenin-11-dUTP as described previously (42). Plasmid DNAs from the NCIB strain were digested with *Eco*RI, *Hin*cII, or *Eco*RI and *Pst*I at 37°C overnight. The digests (1 μg each) were electrophoresed, hybridized, and detected as described elsewhere (20). The plasmid DNAs from the NCIB strain were also separated by counter-clamped homogeneous electric field gel electrophoresis (7). We ramped the pulse time from 10 to 30 s in the course of 12 h. λ Concatemers were used as size markers.

Nucleotide sequence accession number. The sequence data presented here will appear in the GenBank, EMBL, and DDBJ databases under accession no. D45904.

RESULTS

Purification of lambda-toxin. Because of the ability of strain NCIB10691 to produce a high level of casein-hydrolyzing activity of the lambda-toxin in GAM broth, we chose it for subsequent studies on purification of the toxin. When we grew the organism in the broth, the highest level of casein-hydrolyzing activity was produced in the late log phase. The specific activity of total lambda-toxin in the culture supernatant, as determined as azocasein hydrolysis, was 0.013 U/mg of protein (Table 1). Casein-hydrolyzing activity of the lambda-toxin decreased on dialysis against 50 mM Tris-HCl (pH 7.5), while virtually none was lost on dialysis against the buffer containing 5 mM $CaCl_2$. Therefore, the toxin was stabilized by the addition of $CaCl_2$ prior to ammonium sulfate precipitation, and 5 mM $CaCl_2$-containing buffers were used for all purification steps. Each of the five purification steps in our protocol yielded increasingly purer lambda-toxin (Table 1 and Fig. 1). The purification and recovery of the lambda-toxin were estimated to be 87-fold and

TABLE 1. Purification of lambda-toxin

Step	Vol (ml)	Protein[a] (mg)	Activity[b] (U)	Sp act (U/mg)	Purification[c] (fold)	Recovery[d] (%)
Culture supernatant	600.0	12,600	165	0.0131		
Ammonium sulfate (60%)	16.3	300	171	0.570	1	100
Sephacryl S-100	46.5	58.9	178	3.02	5.3	104
DEAE Sephadex A-25	182.0	15.5	114	7.35	13	64.0
Sephadex G-100	76.5	4.97	108	21.7	38	63.1
Ether-Toyopearl	30.2	1.05	52.2	49.7	87	30.5

[a] Determined by using the Pierce bicinchoninic acid protein assay reagent. The results are the averages of three determinations; values did not vary by more than 10%.
[b] Deteremained by using azocasein as described in Materials and Methods. The results are the averages of three determinations; values did not vary by more than 10%.
[c] Ratio of each fraction to the ammonium sulfate fraction value.
[d] Percentage of the ammonium sulfate fraction value.

30.5%, respectively, when the ammonium sulfate fraction was regarded as the starting material (Table 1). The yields of the caseinase activity in the ammonium sulfate precipitate and Sephacryl S-100 fraction were slightly higher than that in the starting culture supernatant. Bidwell (3) described that dialysis of the ammonium sulfate fraction against water resulted in an increase in lambda-toxin activity. Such an increase in enzyme activity at an early purification stage could be due to the removal of an inhibitor.

Properties of lambda-toxin. The lambda-toxin migrated as a single band corresponding to an estimated molecular mass of 36 kDa on SDS-PAGE under reducing conditions. The molecular mass of the enzyme, as determined by gel filtration on Sephacryl S-100 calibrated with standard proteins, was in the range of 32 to 36 kDa. These results indicate that the purified lambda-toxin is in a monomeric form with an apparent molecular mass of 36 kDa. Enzyme activity was detectable over a broad range of pH 5 to 8, and the pH for optimum activity was

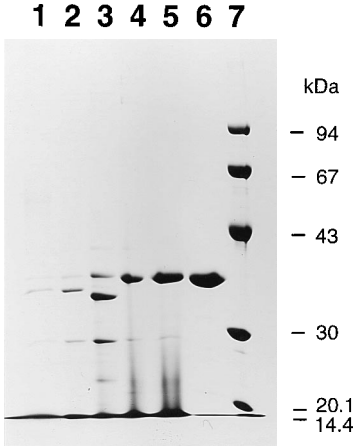

FIG. 1. SDS-PAGE of samples from all purification steps. Samples were denatured by being heated at 100°C for 3 min with the sample buffer and then electrophoresed on a 12.5% polyacrylamide gel. Lanes: 1, culture supernatant; 2, 60% $(NH_4)_2SO_4$ fraction; 3, Sephacryl S-100 fraction; 4, DEAE Sephadex A-25 fraction; 5; Sephadex G-100 fraction; 6, ether-Toyopearl fraction; 7, molecular size markers. Lane 1 contained 15 μl of the sample, and lanes 2 to 6 contained 20 μg of protein. The numbers on the right are the sizes (in kilodaltons) of the markers.

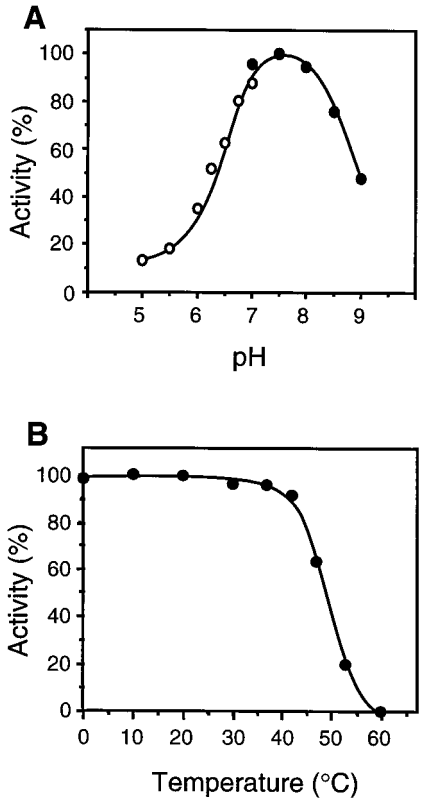

FIG. 2. pH profile and thermostability of purified lambda-toxin. (A) The effect of pH was examined in 50 mM Tris-HCl–5 mM $CaCl_2$ (●) (pH 7.0 to 9.0) or 50 mM bis-Tris-HCl–5 mM $CaCl_2$ (○) (pH 5.0 to 7.0). (B) The enzyme was incubated in 50 mM Tris-HCl (pH 7.5)–5 mM $CaCl_2$ at the indicated temperature for 30 min prior to determination of the caseinase activity. Each reaction was performed with 0.35 μg of purified lambda-toxin.

7.5 (Fig. 2A). The enzyme activity was unstable above 42°C, as shown in Fig. 2B.

Effects of enzyme inhibitors and metal ions. A number of potential inhibitors were incubated with purified lambda-toxin to determine their influence on its azocaseinase activity (Table 2). From the observation that the stability of the lambda-toxin increased in the presence of Ca^{2+}, we expected the enzyme to be sensitive to chelating agents. The results here confirmed this expectation, as both the metal chelators EDTA and EGTA and the zinc-specific chelator 1,10-phenanthroline inhibited azocaseinase activity. Azocaseinase activity was also inhibited by phosphoramidon, an inhibitor of thermolysin and thermolysin-related metalloproteases (39). Serine or cysteine protease inhibitors, such as PMSF, APMSF, 3,4-DCI, leupeptin, E-64, dithiothreitol, and $HgCl_2$, were virtually ineffective in blocking the enzymatic reaction. Iodoacetic acid inhibited the activity, suggesting that thiol groups may have a conformational role. Azocaseinase was also resistant to bestatin, an inhibitor of aminopeptidases, and to pepstatin, an inhibitor of aspartate proteases. After complete inactivation of the enzyme with 2 mM 1,10-phenanthroline, it could be reactivated 57, 62, and 15% by the addition of Zn^{2+}, Co^{2+}, and Mn^{2+}, respectively, to a final concentration of 2.5 mM but could not be reactivated by the addition of Mg^{2+} and Ca^{2+} at the same concentration (data not shown). The enzyme inactivated by treatment with 5 mM EDTA was reactivated 20% by dialysis against 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM Zn^{2+} and 5 mM Ca^{2+}, while no activity was restored by dialysis against the

TABLE 2. Effects of various inhibitory substances on proteolyic activity of purified lambda-toxin

Inhibitory substance	Concn (mM)	% Inhibition[a]
Serine proteases		
PMSF	5	2
APMSF	0.2	9
3,4-DCI	1.0	2
Leupeptin	0.2	13
Metalloproteases		
Phosphoramidon	0.5	74
1,10-Phenanthroline	2	97
EDTA	5	99
EGTA	2	100
Thiol protease		
E64	0.8	3
Dithiothreitol	2	20
Iodoacetic acid	0.02	100
$HgCl_2$	0.5	13
Others		
Bestatin	0.15	10
Pepstatin A	0.07	0

[a] Lambda-toxin (0.35 μg) was incubated with the compounds at the indicated concentrations for 10 min at room temperature prior to the addition of azocasein. The values, the averages for triplicate assays, did not vary by more than 15%.

buffer containing each divalent cation alone (data not shown). These results suggest that the enzyme requires both Zn^{2+}, possibly as a prosthetic metal ion, and Ca^{2+}, probably as a stabilizer.

Cloning of *lam* gene. Plasmid DNAs from the NCIB strain were partially digested with *Hin*cII, ligated into pUC18, and then introduced into *E. coli* JM109. One clone, which produced a distinctive transparent zone with an outer translucent halo on a skim milk agar plate, contained an 8.4-kb plasmid (pUC18 plus a 5.8-kb DNA insert). The 5.8-kb insert consisted of 3.95- 1.75- and 0.1-kb *Hin*cII fragments. When each fragment was subcloned into pUC18, caseinase activity was exhibited by a transformant with the 3.95-kb fragment-containing plasmid (pFJ2). To localize the *lam* gene within the 3.95-kb fragment, pFJ2 was digested with *Eco*RI, *Hin*cII, and *Pst*I and then subcloned into pUC18. A subclone harboring pUC18 with the 1.95-kb *Eco*RI-*Hin*cII fragment exhibited caseinase activity. This fragment was assumed to contain at least the main, if not the whole, region of the *lam* gene. Therefore, we determined its nucleotide sequence.

Analysis of nucleotide sequence and open reading frame. The sequences aligned by their overlaps to form a single contig revealed the presence of a reading frame encoding a polypeptide of 534 amino acids. Since the N-terminal portion of this frame was truncated at the left *Eco*RI end, about 250 nucleotides upstream of the *Eco*RI site of the 1.95-kb *Eco*RI-*Hin*cII fragment was also sequenced (Fig. 3). An open reading frame starting at nucleotide 196 encodes a protein consisting of 553 amino acid residues. The N-terminal amino acid sequence of the purified lambda-toxin is the same as that predicted from the nucleotide sequence starting at nucleotide 901, as shown in Fig. 3. The molecular mass predicted from this reading frame is 35,272 Da, which is in good agreement with that determined by SDS-PAGE for the purified lambda-toxin (36 kDa).

There are three possible initiation codons at nucleotides 196, 289, and 355, all of which are preceded by a consensus ribosome binding sequence. The ATG codon at nucleotide 196 is located at the beginning of a sequence which resembles that of a typical signal peptide (two positively charged amino acids followed by a run of hydrophobic residues), while the sequences starting at the other two ATG codons each lack a typical signal peptide sequence. Therefore, the former seems to be the initiation codon. To confirm this, we replaced this initiation codon with a termination codon by site-directed mutagenesis and examined its effect on the production of the active enzyme. A transformant of *C. perfringens* 13 with pJIRlam containing the wild-type *lam* gene produced high levels of caseinolytic activity, while one with pJIRlamM containing a mutant *lam* gene did not (data not shown). Therefore, the codon at nucleotide 196 is likely to be the main translational initiation site. The N-terminal amino acid of the mature enzyme is separated by a long sequence (approximately 200 amino acid residues) from the putative signal peptide, indicating that the lambda-toxin is secreted as a proenzyme and thereafter processed to the mature enzyme.

The deduced amino acid sequence contains not only the motif HEXXH and but also the consensus sequence around the three zinc-binding sites dictated homologous to that of the thermolysin family (12). Thus, the lambda-toxin seems to belong to the thermolysin family. We searched for proteins with amino acid sequences similar to that of the lambda-toxin, using the BLAST algorithm to align all protein sequences deposited in the databases (20). This search identified the metalloproteases belonging to the thermolysin family, all of which showed high degrees of similarity (Poisson *P* value, $<1e-6$).

Location of *lam* gene. Plasmid DNAs from the NCIB strain were digested with *Eco*RI, *Hin*cII, or both *Eco*RI and *Pst*I and then separated on a 0.8% agarose gel. To confirm that the cloned *lam* gene was derived from the plasmids of the NCIB strain, Southern hybridization was performed with a *lam* gene probe. For the *Eco*RI, *Hin*cII, and *Eco*RI plus *Pst*I digests, the hybridized bands were 1.4, 3.95, and 0.55 kb long, respectively (Fig. 4), these sizes being in good agreement with those calculated from the restriction map of pFJ2. This indicates that in the NCIB strain the *lam* gene is located on a plasmid. For the undigested plasmid DNAs, the hybridized bands were longer than the 23.1-kb λ *Hin*dIII fragment (Fig. 4, lane 5). To accurately determine the size of a *lam* gene-containing plasmid, we carried out Southern hybridization after pulsed-field electrophoresis. Of the plasmids of various sizes (3 to >100 kb), one large plasmid was hybridized with the *lam* gene probe. The length of this plasmid was estimated to be 70 kb, as determined by pulsed-field electrophoresis with λ ladder molecular standards (data not shown). *Apa*I cleaved the chromosomal DNA of the NCIB strain at nine sites, while it did not cleave the hybridized DNA (data not shown), ruling out the possibility that chromosomal-borne *lam* might have contaminated our plasmid preparation.

Ability of lambda-toxin to degrade host cellular proteins. Included in the thermolysin family are metalloproteases of pathogenic bacteria, which have been suggested to play roles in their virulence (9). The lambda-toxin is expected to contribute to the pathogenicity of toxin-producing *C. perfringens*. We evaluated the ability of the purified toxin to cleave a variety of biologically important substances, such as immunoglobulins, the complement C3 component, fibrinogen, various collagens, and fibronectin, by analyzing incubation mixtures on an SDS-polyacrylamide gel. The purified lambda-toxin degraded all substrates but IgG, as shown in Fig. 5. The toxin selectively cleaved the heavy chain of IgA (Fig. 5, lane 1) and the α-chain of the human complement C3 component (Fig. 5, lane 6). All α-, β- and γ-chains of fibrinogen were cleaved by the toxin (Fig. 5, lane 8).

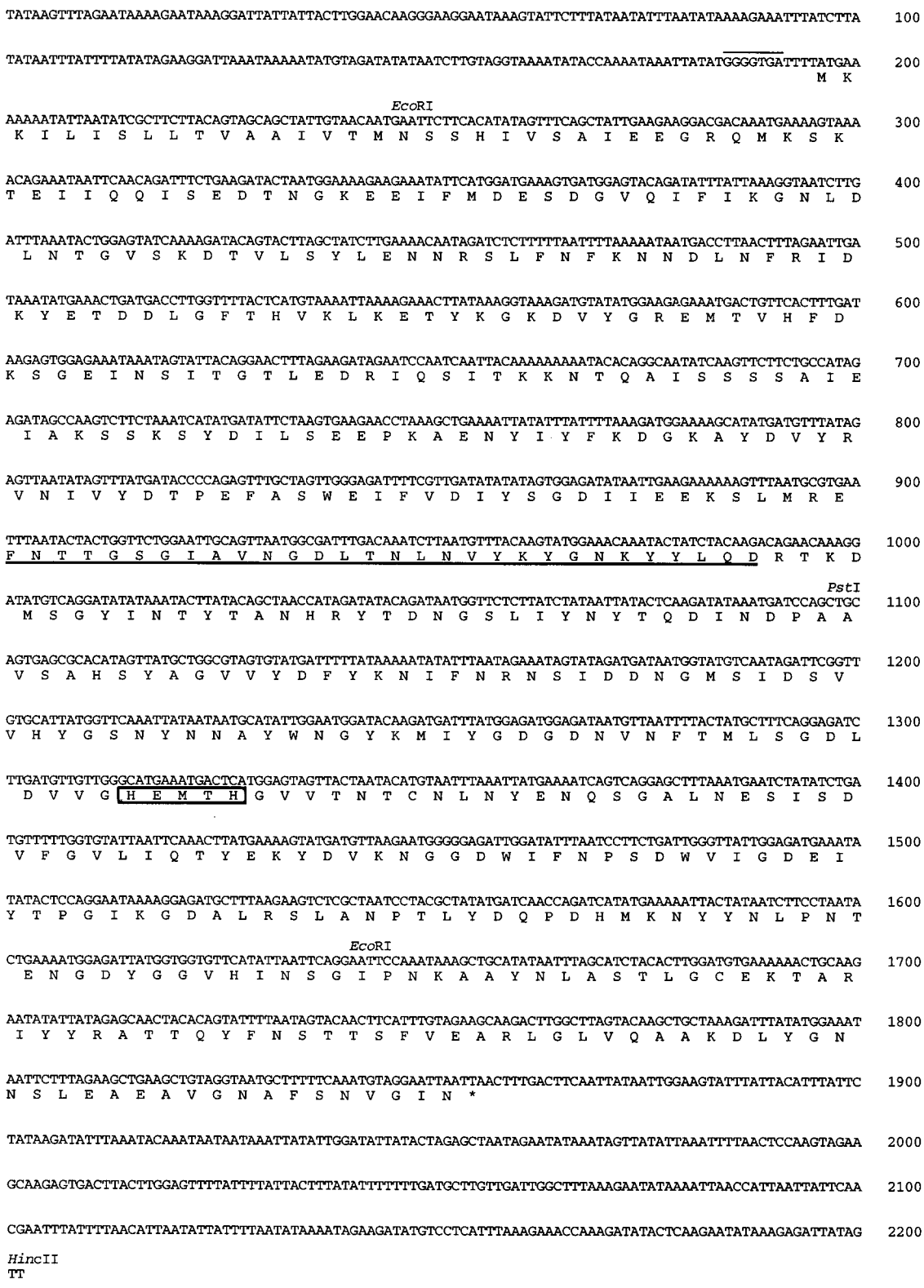

FIG. 3. Nucleotide sequence of *lam* gene. The deduced amino acid sequence is shown below the DNA sequence. The potential ribosome binding site is overlined. The N-terminal amino acid sequence determined for the purified lambda-toxin is underlined. The HEXXH motif is boxed. Some of the restriction sites are also shown.

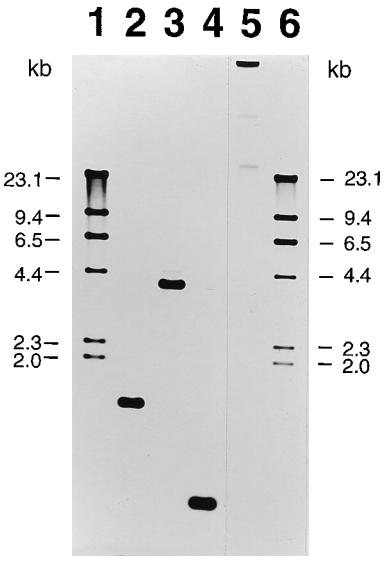

FIG. 4. Southern analysis of plasmid DNAs from *C. perfringens* NCIB10691. Bands separated on a 0.8% agarose gel were transferred onto a nylon membrane and then hybridized with a *lam* gene probe (0.55-kb *Eco*RI-*Pst*I fragment). Lanes: 1 and 6, λ *Hin*dIII size markers; 2, *Eco*RI digest; 3, *Hin*cII digest; 4, *Eco*RI and *Pst*I digest; 5, plasmid DNAs without digestion. Each lane contained 1 μg of plasmid DNA. The numbers on the left and right are the sizes (in kilobases) of the markers.

The substrate repertoire of the lambda-toxin described above is similar to that of *P. aeruginosa* elastase (9, 25), which also belongs to the thermolysin family and is homologous to the lambda-toxin. To determine whether the lambda-toxin degrades elastin, elastin-Congo red was incubated with the purified toxin. It showed a ΔA_{495} of 0.001/μg/h, which was far less than the reported value, 0.207/μg/h, for the *P. aeruginosa* elastase standard (33). Thus, elastin is resistant to the lambda-toxin.

In vivo effects of lambda-toxin. The results of an in vitro experiment indicate that the lambda-toxin is capable of degrading components of connective tissue as well as ones of the host defense system. To assess its effect on connective tissue, purified lambda-toxin was injected intradermally into a mouse. At 2 h postinjection, a bloody edematous lesion was observed in the skin area injected with 10 μg of the toxin. The edema formation seems to be due to an increase in vascular permeability. This was verified by the observation that the toxin rendered the injected site permeable to circulating Evans blue (Fig. 6). The permeability-enhancing effect was dose dependent in the range of 0.1 to 3 μg (data not shown). The time course of the permeability-enhancing effect was examined by measuring the diameter of the blue spot at various times after injection of 3 μg of the toxin. The blue spot was visible at 2 min postinjection and reached a plateau at 10 min postinjection.

DISCUSSION

Characterization of the lambda-toxin and its encoding gene reported here show that it is a zinc metalloprotease. The predicted amino acid sequence of the lambda-toxin is homologous to those of *Bacillus thermoproteolyticus* thermolysin (11) and *P. aeruginosa* elastase (PAE [38]), whose tertiary structures have been refined by X-ray crystallography. The degrees of identity and similarity on alignment of the lambda-toxin and thermolysin are 45.8 and 75.4%, respectively, while those of the lambda-toxin and PAE are 26.7 and 45.7%, respectively. Thus, the lambda-toxin seems to be evolutionarily closer to thermolysin than to PAE. However, the lambda-toxin seems to be functionally more related to PAE than to thermolysin, since their substrate repertoires are similar. The difference in reactivities toward substrates between PAE and thermolysin has been suggested to be due to the more open substrate-binding cleft in PAE (38). This raises the question of whether this is also the case for the lambda-toxin. Another important question is what causes the difference in elastolytic activity between the lambda-toxin and PAE. A crystallographic study of the toxin would answer these questions and provide useful information with respect to the structure-function relationship of prokaryotic zinc metalloproteases.

The only plasmid-encoded bacterial protease so far reported is the surface protease of *Yersinia pestis*, a virulence factor of the organism (37), and probably a lethal factor of *Bacillus anthracis*, which has a metalloprotease consensus sequence (15).

The *lam* gene is located on a plasmid with a 70-kb nucleotide length in strain NCIB 10691, while it was reported to be on a 2.1-MDa (3.2-kb) plasmid in strain ATTC 3626B (5). Thus, the

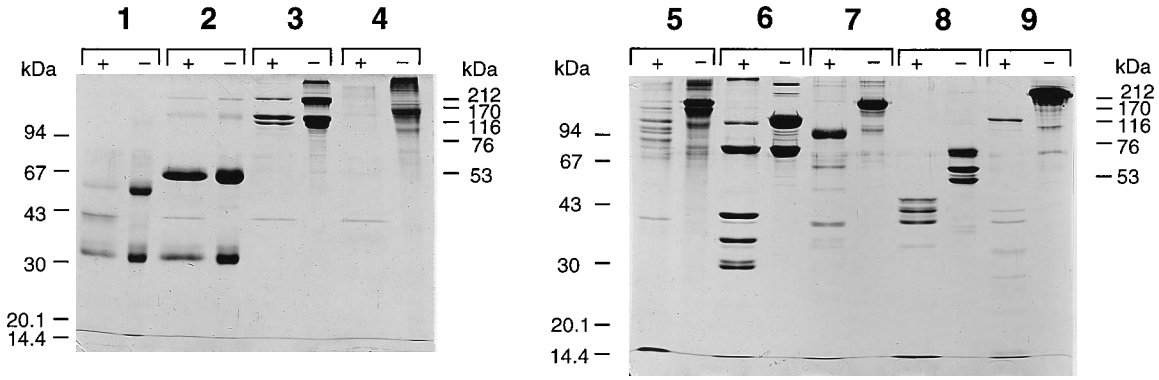

FIG. 5. Cleavage of host cellular proteins by lambda-toxin. Various proteins (16 to 60 μg) were incubated with the purified lambda-toxin (2 μg) in 90 μl of TC buffer containing 85 mM NaCl at 37°C for 2 h. Samples were heated at 100°C for 3 min with 1% 2-mercaptoethanol in the sample buffer, and then the aliquots were electrophoresed on an SDS–12.5% polyacrylamide gel. Protein bands were visualized by staining with Coomassie brilliant blue R. Lanes labeled − and + represent proteins incubated without and with the toxin, respectively. Lanes: 1, IgA (5 μg); 2, IgG (5 μg); 3, collagen type I (10 μg); 4, collagen type IV (10 μg); 5, collagen type V (10 μg); 6, complement C3 component (10 μg); 7, α_2-macroglobulin (5 μg); 8, fibrinogen (10 μg); 9, fibronectin (3 μg). The numbers on the left and right are the sizes of low- and high-molecular-mass standards, respectively.

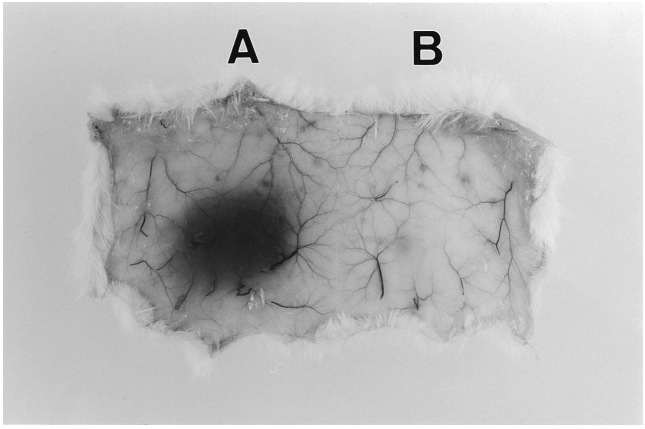

FIG. 6. Permeability-enhancing activity of purified lambda-toxin (3 μg) without treatment (A) heated at 100°C for 5 min (B). The toxin was injected into the dorsal skin of a mouse, and then Evans blue dye was injected intravenously. The mouse was sacrificed at 2 h postinjection.

lam gene is probably located on different plasmids in the two strains. How plasmids bearing the *lam* gene prevail in *C. perfringens* is of interest and would be revealed by a comparative study of *lam* genes and plasmids in lambda-toxin-producing strains. The *lam* gene seems to have evolved similarly to the chromosomal genes of *C. perfringens*, since the GC content of the *lam* gene is 30 mol%, which is close to that of the chromosomal DNA. It seems unlikely that *C. perfringens* Lam^+ strains acquired plasmids bearing the *lam* gene very recently.

An effect on the vascular permeability similar to that of the lambda-toxin described here has been demonstrated for the epsilon-toxin (6). The beta-toxin has also been shown to elicit dermonecrosis on intradermal injection into a guinea pig, even at a dose as low as 2 ng (30). Since type B strains can produce both the beta- and epsilon-toxins, it cannot be ruled out that our purified lambda-toxin sample contains these toxins in trace amounts and thus that the permeability enhancement is due to their activities. To disprove this, the lambda-toxin was purified from a culture of *C. perfringens* 13 carrying pJIRlam, which does not produce the beta- or epsilon-toxin, by the same procedure as that described above. The toxin from the recombinant strain increased vascular permeability in a fashion similar to that of the wild-type strain (data not shown). Thus, the toxin has a permeability-enhancing effect, as do many microbial proteases (8, 23). The metalloprotease of *Vibrio vulnificus* exhibits the effect through activation of the Hageman factor and prekallikrein (22, 23). The dose dependency and time course of the permeability-enhancing reaction of the lambda-toxin are similar to those of *V. vulnificus* protease (21). Therefore, it seems likely that the same mechanism underlies the effect of the lambda-toxin.

The present work dealt with the skin edematous lesion caused by the lambda-toxin to simply assess its in vivo effect. Its ability to degrade various components of connective tissue and of the host defense mechanism suggests that it may exhibit detrimental effects on many tissues other than blood vessels and that it is a potent virulence factor of *C. perfringens* involved in invasion and tissue destruction. One approach for determining the extent of the pathogenic role of the lambda-toxin, which we are currently undertaking, is to compare the pathogenicities of the wild-type and lambda-toxin mutant strains.

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