INFECTION AND IMMUNITY, Mar. 1993, p. 947–954
0019-9567/93/030947-08$02.00/0
Copyright © 1993, American Society for Microbiology
Vol. 61, No. 3

Cloning and Characterization of the *Actinobacillus pleuropneumoniae*-RTX-Toxin III (ApxIII) Gene

RUUD JANSEN,[1] JAN BRIAIRE,[1] ELBARTE M. KAMP,[2] ARNO L. J. GIELKENS,[1] AND MARI A. SMITS[1]*

Departments of Molecular Biology[1] and Bacteriology,[2] DLO-Central Veterinary Institute, Postbox 65, 8200 AB Lelystad, The Netherlands

Received 14 July 1992/Accepted 30 November 1992

To study the role of *Actinobacillus pleuropneumoniae*-RTX-toxin III (ApxIII) in the pathogenesis of porcine pleuropneumonia, we cloned and characterized the gene encoding this toxin. For that purpose, we screened an expression library of genomic DNA of serotype 8 with an ApxIII-specific monoclonal antibody and isolated a 425-bp fragment of an immunoreactive clone. Using this fragment as a probe, we identified and cloned an overlapping chromosomal *NsiI* restriction fragment of 5.0 kbp. *Escherichia coli* cells that contained this fragment produced a protein similar to ApxIII. Like ApxIII, the protein had a molecular mass of approximately 120 kDa, was recognized by an ApxIII-specific antibody, killed porcine lung macrophages, and was not lytic for sheep erythrocytes. We concluded from these data that the 5.0-kbp *NsiI* fragment contained the ApxIII-coding gene. Nucleotide sequence analysis of the 5.0-kbp *NsiI* fragment revealed the presence of two genes, *apxIIIC* and *apxIIIA*. These genes coded for proteins ApxIIIC and ApxIIIA, respectively, which were 53 and 50% identical to the prototypic RTX proteins HlyC and HlyA of *E. coli*. We assumed that the *apxIIIA* gene coded for the structural RTX toxin and that the *apxIIIC* gene coded for its activator. In addition, we found that ApxIII could be secreted from *E. coli* by the heterologous RTX transporter proteins HlyB and HlyD. The deduced amino acid sequence of ApxIIIA was 50% identical to that of ApxIA and 41% identical to that of ApxIIA. We concluded that, beside ApxI and ApxII, ApxIII is the third RTX toxin produced by *A. pleuropneumoniae*.

Porcine pleuropneumonia is a highly contagious disease that causes serious economic losses in the pig farming industry worldwide. The disease is caused by *Actinobacillus pleuropneumoniae*, a gram-negative bacterium that colonizes the lung tissues (23, 29). In pigs, pneumonic lesions similar to the lesions induced by *A. pleuropneumoniae* can be induced with a sterile culture supernatant of *A. pleuropneumoniae* (1, 25). Therefore, exotoxins have been implicated as important virulence factors.

In an earlier study, we identified three different toxin proteins in the culture supernatants of reference strains of the 12 *A. pleuropneumoniae* serotypes (16a). For several reasons, we named these toxins cytolysins, but several different names were used by other research groups; these included Hly (9, 10), AppA (5), and pleurotoxin (26). Recently, most groups agreed to use a standardized nomenclature (8). According to this nomenclature, the proteins were named *A. pleuropneumoniae*-RTX-toxin I (ApxI), *A. pleuropneumoniae*-RTX-toxin II (ApxII), and *A. pleuropneumoniae*-RTX-toxin III (ApxIII). These three toxins were not secreted by all 12 *A. pleuropneumoniae* serotypes. ApxI, a protein of 105 kDa, was secreted by serotypes 1, 5, 9, 10, and 11, ApxII, a protein of 103 kDa, was secreted by all serotypes except for 10, and ApxIII, a protein of 120 kDa, was secreted by serotypes 2, 3, 4, 6, and 8 (16a).

ApxI and ApxII are both RTX toxins, like the well-characterized alpha hemolysin (Hly) of *Escherichia coli*. In general, RTX toxins are encoded by an operon consisting of four genes, *C*, *A*, *B*, and *D* (32). The *A* gene encodes the structural toxin, which is activated by an acylation reaction that is mediated by the protein encoded by the *C* gene (12). The *B* and *D* genes encode proteins that are involved in the secretion of the toxin. At the amino acid sequence level, ApxIA and ApxIIA of serotype 9 share 37% identical amino acids. Despite this low amino acid identity, ApxI and ApxII do share several properties. ApxI and ApxII have similar biological activities; both toxins lyse erythrocytes and kill porcine lung macrophages (16a) and require Ca^{2+} for these activities (33). Furthermore, ApxI and ApxII seem to be immunologically related, since at least one monoclonal antibody (MAb) cross-reacts with the two proteins (16a), although a specific polyclonal antiserum for ApxII has also been reported (10). ApxIII is the least well-characterized cytolysin of *A. pleuropneumoniae*. ApxIII differs from ApxI and ApxII in several aspects. Immunologically, ApxIII seems to be unrelated to ApxI and ApxII. An antiserum raised against a serotype 3 mutant that solely secretes ApxIII does not cross-react with ApxII (26). In addition, MAbs raised against ApxI or ApxII do not cross-react with ApxIII, and an MAb raised against ApxIII does not cross-react with ApxI or ApxII (16a). ApxIII also differs from ApxI and ApxII in its biological activity. ApxIII kills porcine lung macrophages but does not lyse porcine erythrocytes, while ApxI and ApxII affect both cell types (33).

Nothing is known yet about the molecular structure and mode of action of ApxIII. To gain insight into these matters, we have cloned and characterized the genetic determinant that encodes ApxIII of the reference strain of serotype 8.

MATERIALS AND METHODS

Bacterial strains, genomic DNA, and DNA manipulations. We used reference strain 405 of *A. pleuropneumoniae* serotype 8 as a source of genomic DNA. *E. coli* JM101 and LE392 were used as hosts for cloning vectors pUEX2 (4) and

* Corresponding author.

pGEM7Zf(+) (Promega Corp., Madison, Wis.). Manipulations of DNA and isolation of high-molecular-weight DNA by proteinase K-sodium dodecyl sulfate (SDS) lysis, phenol-chloroform extractions, and ethanol precipitation were done as described by Sambrook et al. (28).

Construction and screening of a DNA library. We made a DNA library of *A. pleuropneumoniae* serotype 8 in expression vector pUEX2 by cloning genomic DNA that had been partially digested with *Sau*3AI to an average size of 1,000 bp. We optimized the size of the library by preventing circularization of the *Sal*I-digested vector and the *Sau*3AI fragments. Therefore, the *Sal*I ends of the vector were partially filled in with dTTP and dCTP and the *Sau*3AI ends of the genomic fragments were partially filled in with dATP and dGTP by use of the Klenow fragment of DNA polymerase I (34). We transformed *E. coli* LE392 by electroporation with a Gene pulser (Bio-Rad, Richmond, Calif.). The recombinant clones were grown overnight at 30°C to prevent expression of the fusion proteins, replica plated onto nitrocellulose filters, and incubated for 2 h at 42°C, allowing expression of the fusion proteins (4). The bacteria were lysed by transferring the filters to paper that had been soaked in 5% SDS. The bacterial proteins were electroblotted onto the filters for 30 min at a constant current of 400 mA in transfer buffer (2.93 g of glycine, 5.81 g of Tris, 0.375 g of SDS, and 200 ml of methanol per liter) by use of a BioRad semidry transfer cell. Residual bacterial debris and DNA were removed from the filters by washing the filters once in wash buffer (0.9 g of NaCl per ml, 10 mM Tris-HCl [pH 7.4], 0.05% Tween 20) and by incubating the filters for 15 min at 20°C in wash buffer supplemented with 100 μg of DNase I per ml. We detected immunoreactive proteins with MAb CVI-Apcly-2.2 (16a) and rabbit anti-mouse immunoglobulin G–alkaline phosphatase conjugate (Zymed Laboratories Inc., San Francisco, Calif.) as described by Sambrook et al. (28).

Southern blot analysis. Restriction fragments of genomic DNA were separated by agarose gel electrophoresis (1 μg of DNA per lane) and blotted onto GeneScreen plus membranes (New England Nuclear Corp., Boston, Mass.). We hybridized the blots overnight at 65°C in a solution of 1.5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4 [pH 7.4], and 1 mM EDTA)–1% SDS. DNA probes were labeled with [α-^{32}P]dCTP (Amersham Corp., Arlington Heights, Ill.) by use of a random primer labeling kit (Boehringer GmbH, Mannheim, Germany). The blots were washed at a final stringency of 0.2× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate)–1% SDS for 15 min at 65°C. Radioactivity was detected by autoradiography with X-Omat AR film (Eastman Kodak, Rochester, N.Y.) and intensifying screens.

Expression and analysis of recombinant proteins. Recombinant proteins were produced in *E. coli* LE392 harboring pGEM7Zf(+) as a carrier of the recombinant genes. For secretion studies, we cotransformed these *E. coli* cells with pLG575, a plasmid that contained the *hlyB* and *hlyD* genes of *E. coli* (11). Transformants were grown in Luria-Bertani medium containing 50 μg of ampicillin per ml and/or 25 μg of chloramphenicol per ml and supplemented with 10% fetal calf serum to stabilize the cytolytic activity of ApxIII. The cultures were inoculated from an overnight culture and grown for 4 h at 37°C to an optical density at 600 nm of 0.3 to 0.4. During growth of the cultures, we added fresh antibiotics every 2 hours, since we noticed that the bacteria lost the plasmids when the antibiotic pressure dropped.

To concentrate the proteins of the culture supernatant, we precipitated the proteins with 3 volumes of acetone and redissolved the pellet in 0.1 volume of SDS sample buffer (28). Bacterial cells were lysed in SDS sample buffer after one freeze-thaw step. We separated the proteins on an SDS-polyacrylamide gel (4% stacking gel and 7% separating gel) and transferred the proteins to GeneScreen membranes by electroblotting. Immunoreactive proteins were visualized as described above. We measured the cytotoxic activity of culture supernatants towards porcine lung macrophages and the hemolytic activity towards sheep erythrocytes as described by Kamp and van Leengoed (17). In short, serial twofold dilutions were made of the supernatants and incubated with fresh porcine lung macrophages or sheep erythrocytes. The hemolysin and cytolysin titers were expressed as the reciprocal of the highest dilution showing at least 50% hemolysis of erythrocytes or 50% killing of macrophages.

PCR and construction of deletion clones. We constructed deletion clones by use of polymerase chain reaction (PCR)-amplified DNA. The PCR was essentially done as described previously (16). Plasmid DNA (10 ng) was used as a template, and the PCR was run for only 15 cycles to prevent the excessive accumulation of PCR-induced nucleotide sequence mistakes in the amplified DNA. The PCR primers that we used were the T7 promoter primer (Promega) and an oligonucleotide with the nucleotide sequence 5′ CTATAT AATTAACGGTTCTT 3′ (positions −42 to −23; Fig. 1B). The amplified DNA was purified from an agarose gel with Prep-a-Gene (Bio-Rad) and used for the construction of pApxIIICA−42 and pApxIIICA+142 from parent plasmid pApxIIICA (Fig. 1B). For the generation of pClyIIICA−42, we replaced the DNA fragment from the 5′ *Nsi*I site to the *Kpn*I site at position 837 with the blunt-ended and *Kpn*I-digested PCR product (Fig. 1B). For the generation of pClyIIICA+142, we replaced the same fragment with the *Bcl*I and *Kpn*I-digested PCR product. The plasmids were constructed by a three-part ligation with the following DNA fragments: *Sma*I-*Nsi*I-digested pGEM7Zf(+) vector DNA, the 2,952-bp *Kpn*I-*Nsi*I fragment of pApxIIICA, and the 880-bp blunt-ended and *Kpn*I-digested PCR product for pApxIIICA−42 or the 695-bp *Bcl*I-*Kpn*I-digested PCR product for pApxIIICA+142.

Nucleotide sequence analysis. We prepared progressive unidirectional deletion clones by use of the Erase-a-Base system (Promega) and sequenced a series of deletion clones that differed 250 to 300 bp in length. For sequencing, we used the T7 polymerase sequencing kit (Pharmacia) and the SP6 and T7 promoter primers. Nucleotide sequences were analyzed and compared on a Macintosh computer by use of the MacMolly software package (Soft Gene GmbH, Berlin, Germany).

Nucleotide sequence accession number. The GenBank-EMBL accession number for the primary nucleotide sequence data is X68815.

RESULTS

Immunoscreening of the expression library. We constructed an expression library of genomic DNA of serotype 8 and screened 90,000 recombinant clones with the ApxIII-specific MAb CVI-Apcly-2.2. We found three clones that produced an immunoreactive fusion protein. Two clones, 3 and 5, contained inserts of 425 bp, and one clone, clone 7, contained an insert of approximately 1,000 bp. We compared the inserts by Southern hybridization with the 425-bp insert of clone 3 as a probe. All three clones showed an equally strong hybridization signal, indicating that the three clones shared overlapping sequences (data not shown).

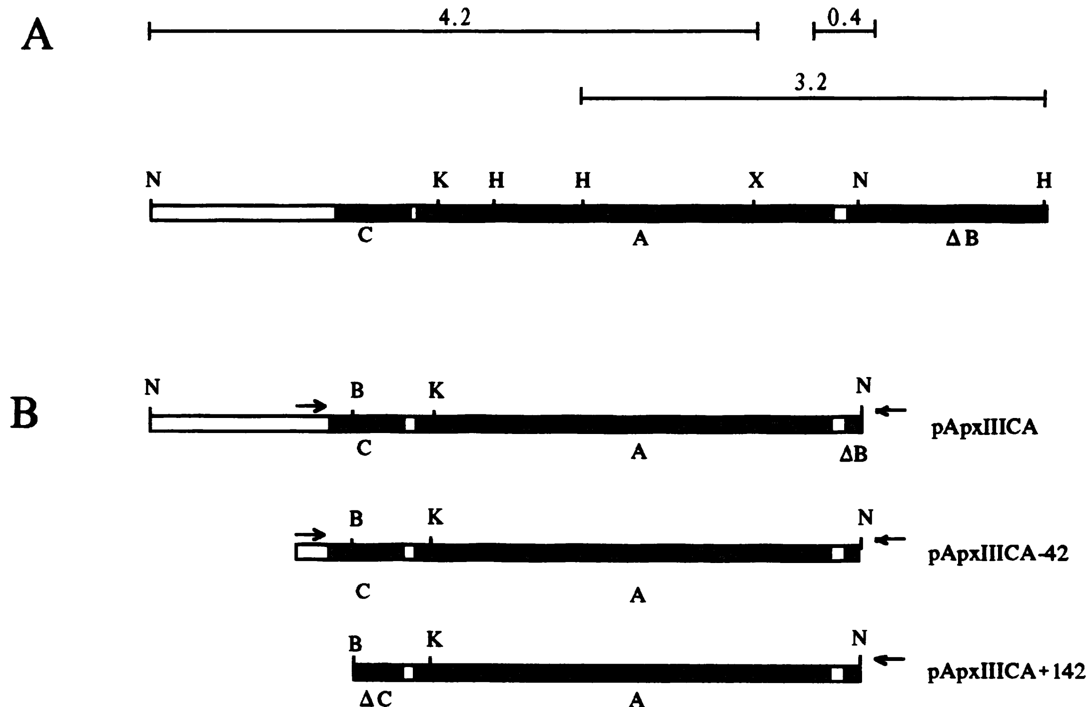

FIG. 1. Schematic presentation of the ApxIII genomic clones and constructs. (A) The bar represents a genomic fragment of 6.2 kbp. The sizes and positions of the 4.2-kbp *Nsi*I-*Xba*I fragment, the 3.2-kbp *Hin*dIII fragment, and the 425-bp clone 3 fragment are indicated by the solid lines. Restriction sites for *Hin*dIII (H), *Xba*I (X), *Nsi*I (N), and *Kpn*I (K) are indicated. Hatched areas show the positions of the open reading frames of *apxIIIC* (C), *apxIIIA* (A), and the proximal part of *apxIIIB* (ΔB). (B) Inserts of plasmids pApxIIICA, pApxIIICA−42, and pApxIIICA+142 used for expression studies. The *Kpn*I (K), *Nsi*I (N), and *Bcl*I (B) restriction sites and the positions of the PCR primers are indicated.

To determine the nucleotide sequence of the clone 3 insert, we excised the insert from plasmid pUEX2 with the restriction enzymes *Pst*I and *Bam*HI and subcloned it into the *Nsi*I and *Bam*HI sites of vector pGEM7Zf(+). We compared the nucleotide sequence with sequences in the GenBank-EMBL DNA sequence data base and found that 170 nucleotides at the 3′ end resembled a part of the RTX Hly operon of *E. coli* (6). The homologous region encompassed the transcription termination signal located between the *hlyA* and *hlyB* genes and the 5′ terminus of the *hlyB* gene. This finding indicated that the clone 3 fragment formed part of an RTX operon. The 255 nucleotides at the 5′ end of the fragment contained an incomplete open reading frame of 83 codons. Because of its location, we expected that this open reading frame formed part of an RTX *A* gene, although neither the nucleotide sequence nor the derived amino acid sequence showed similarity to any RTX sequence. We postulated that the insert of clone 3 contained the distal 83 codons of an RTX *A* gene, which we designated *apxIIIA*, a transcription termination signal, and the proximal 35 codons of an RTX *B* gene, which we designated Δ*apxIIIB*.

Because MAb CVI-Apcly-2.2 recognized the β-galactosidase fusion protein of clone 3, we concluded that the epitope for this MAb was located within the distal 83 amino acids of the postulated *apxIIIA* gene product. The nucleotide sequence of the clone 3 insert is included in Fig. 2 and comprises nucleotides 3433 to 3858.

Cloning and expression of the complete *apxIIIA* gene. We expected to find the remaining part of the *apxIIIA* gene upstream of the clone 3 insert. By Southern blot analysis, we identified two restriction fragments in this part of the serotype 8 genome (data not shown). These were a 3.2-kbp *Hin*dIII fragment, which included the 425-bp insert of clone 3 and approximately 1,700 bp upstream and 1,100 bp downstream of this insert, and a 4.2-kbp *Nsi*I-*Xba*I fragment, which overlapped the *Hin*dIII fragment at its 5′ end by 1,200 bp (Fig. 1A). Together, the *Hin*dIII and *Nsi*I-*Xba*I fragments encompassed approximately 6.2 kbp of DNA, of which 4.7 kbp was located upstream and 1.1 kbp was located downstream of the clone 3 insert. The two genomic fragments were isolated from limited libraries that were made in pGEM7Zf(+) by use of size-selected *Hin*dIII or *Nsi*I-*Xba*I genomic DNA fragments.

We constructed from these genomic restriction fragments a 5.0-kbp *Nsi*I fragment, which was expected to contain the complete *apxIIIA* gene and, if present, the *apxIIIC* gene. The *Nsi*I fragment was constructed by the addition of an 800-bp *Xba*I-*Nsi*I fragment, derived from the 3.2-kbp *Hin*dIII fragment, to the 4.2-kbp *Nsi*I-*Xba*I fragment (Fig. 1A). This *Nsi*I fragment was ligated in the sense orientation downstream of the *lac* promoter of pGEM7Zf(+) to generate pApxIIICA (Fig. 1B).

We transformed *E. coli* LE392 with pApxIIICA and analyzed the proteins present in the culture medium and the lysate of these cells by immunoblotting with MAb CVI-Apcly-2.2 (Fig. 3). The cell lysate of *E. coli* LE392(pApxIIICA) (lane 4) contained a 120-kDa immunoreactive protein that comigrated with ApxIII of *A. pleuropneumoniae* (lane 3). Neither the culture medium of *E. coli* LE392(pApxIIICA) (lane 5) nor the cell lysate and culture medium of control *E. coli* LE392 (lanes 1 and 2, respectively) contained immunoreactive proteins. We concluded that the 5.0-kbp *Nsi*I frag-

```
-190 GTAGATATTCTTTTAATATCAAACAACTATTGTTATTTGTCTGAGTGTAGATATGTAGCATTGTGTATTTCTTTATTTACAACTCTAATCTTAATCTAAA
                                                                                                  M  S  Y  K
- 90 AAGATTTCTATATTTTCTTTGTAAGAAATTTTGTTAAAATCCGACTAACTATATAATTAACGGTTCTTAAAGTGGATAAATAATAAAATTATGAGTTATA
       N  V  K  N  L  T  D  D  F  T  T  L  G  H  I  A  W  L  W  A  N  S  P  L  H  K  E  W  S  I  S  L  F
11   AAAATGTTAAAAATTTAACAGATGATTTTACAACTTTAGGGCATATCGCTTGGTTGTGGGCTAATTCTCCGTTACATAAGGAGTGGTCTATCTCTTTGTT
      T  K  N  I  L  P  A  I  Q  H  D  Q  Y  I  L  L  M  R  D  E  F  P  V  A  F  C  S  W  A  N  L  T  L
111  TACTAAGAATATTTTGCCAGCCATTCAACATGATCAATATATTTTACTTATGCGAGATGAGTTCCCTGTAGCGTTTTGTAGTTGGGCAAATTTAACGTTA
      T  N  E  V  K  Y  V  R  D  V  T  S  L  T  F  E  D  W  N  S  G  E  R  K  W  L  I  D  W  I  A  P  F  G
211  ACTAATGAAGTGAAGTATGTACGTGATGTGACGTCATTGACTTTTGAAGATTGGAATTCAGGAGAACGAAAATGGTTGATCGACTGGATTGCGCCATTTG
      D  N  N  T  L  Y  R  Y  M  R  K  K  F  P  N  E  V  F  R  A  I  R  V  Y  P  G  S  T  E  A  K  I  I
311  GGGATAACAATACGCTTTATAGATATATGCGTAAAAAATTTCCTAATGAAGTATTCCGGGCCATTCGAGTATATCCTGGTTCTACAGAAGCGAAAATCAT
      H  V  Q  G  G  Q  I  N  K  F  T  A  K  K  L  I  Q  Q  Y  Q  E  E  L  I  Q  V  L  N  N  H  K  K  I
411  TCATGTTCAAGGAGGACAAATTAATAAATTTACAGCTAAAAAATTAATACAACAATATCAGGAAGAACTTATTCAAGTTCTTAACAATCACAAAAAAATT
      V  R  G        M  S  T  W  S  S  M  L  A  D  L  K  K  R  A  E  E  A  K  R  Q  A  K  K  G  Y  D  V  T
511  GTAAGAGGATAAAATATGAGTACTTGGTCAAGCATGTTAGCCGACTTAAAAAAACGGGCTGAAGAAGCCAAAAGACAAGCCAAAAAAGGCTACGATGTAA
      K  N  G  L  Q  Y  G  V  S  Q  A  K  L  Q  A  L  A  A  G  K  A  V  Q  K  Y  G  N  K  L  V  L  V  I
611  CTAAAAATGGTTTGCAATATGGGGTGAGTCAAGCAAAATTACAAGCATTAGCAGCTGGTAAAGCCGTTCAAAAGTACGGTAATAAATTAGTTTTAGTTAT
      P  K  E  Y  D  G  S  V  G  N  G  F  F  D  L  V  K  A  A  E  E  L  G  I  Q  V  K  Y  V  N  R  N  E
711  TCCAAAAGAGTATGACGGAAGTGTTGGTAACGGTTTCTTTGATTTAGTAAAAGCAGCTGAGGAATTAGGCATTCAAGTTAAATATGTTAACCGTAATGAA
      L  E  V  A  H  K  S  L  G  T  A  D  Q  F  L  G  L  T  E  R  G  L  T  L  F  A  P  Q  L  D  Q  F  L  Q
811  TTGGAAGTTGCCCATAAAAGTTTAGGTACGGCAGACCAATTCTTGGGTTTAACAGAACGTGGACTTACTTTATTTGCACCGCAACTAGATCAGTTCTTAC
      K  H  S  K  I  S  N  V  V  G  S  S  T  G  D  A  V  S  K  L  A  K  S  Q  T  I  I  S  G  I  Q  S  V
911  AAAAACATTCAAAAATTTCTAACGTAGTGGGCAGTTCTACTGGTGATGCAGTAAGTAAACTTGCTAAGAGTCAAACTATTATTTCAGGAATTCAATCTGT
      L  G  T  V  L  A  G  I  N  L  N  E  A  I  I  S  G  G  S  E  L  E  L  A  E  A  G  V  S  L  A  S  E
1011 ATTAGGTACTGTATTAGCAGGTATTAATCTTAATGAAGCTATTATTAGTGGCGGTTCAGAGCTCGAATTAGCTGAAGCTGGTGTTTCTTTAGCCTCTGAG
      L  V  S  N  I  A  K  G  T  T  T  I  D  A  F  T  T  Q  I  Q  N  F  G  K  L  V  E  N  A  K  G  L  G  G
1111 CTCGTTAGTAATATTGCTAAAGGTACAACAACAATAGATGCTTTCACTACACAAATCCAGAACTTTGGGAAATTAGTGGAAAATGCTAAAGGGTTAGGTG
      V  G  R  Q  L  Q  N  I  S  G  S  A  L  S  K  T  G  L  G  L  D  I  I  S  S  L  L  S  G  V  T  A  S
1211 GTGTTGGCCGCCAATTACAGAATATTTCAGGTTCTGCATTAAGCAAAACTGGATTAGGTTTGGATATTATCTCAAGCTTACTTTCAGGAGTAACTGCAAG
      F  A  L  A  N  K  N  A  S  T  S  T  K  V  A  A  G  F  E  L  S  N  Q  V  I  G  G  I  T  K  A  V  S
1311 TTTTGCTTTAGCGAATAAGAATGCTTCAACAAGCACTAAAGTTGCTGCTGGCTTTGAACTCTCAAATCAAGTAATTGGTGGTATTACGAAAGCAGTATCA
      S  Y  I  L  A  Q  R  L  A  A  G  L  S  T  T  G  P  A  A  A  L  I  A  S  S  I  S  L  A  I  S  P  L  A
1411 AGCTATATTCTTGCACAGCGTTTAGCTGCTGGTTTATCAACGACAGGTCCTGCTGCAGCACTAATTGCGTCTAGTATTTCTTTAGCAATCAGTCCATTGG
      F  L  R  V  A  D  N  F  N  R  S  K  E  I  G  E  F  A  E  R  F  K  K  L  G  Y  D  G  D  K  L  L  S
1511 CGTTTTTACGTGTAGCTGATAATTTTAATCGTTCTAAAGAAATTGGCGAATTTGCTGAACGTTTCAAAAAATTGGGCTATGACGGCGATAAACTACTTTC
      E  F  Y  H  E  A  G  T  I  D  A  S  I  T  T  I  S  T  A  L  S  A  I  A  A  G  T  A  A  A  S  A  G
1611 AGAGTTTTATCACGAAGCTGGTACTATTGATGCCTCAATTACTACAATTAGTACAGCACTTTCTGCTATCGCAGCTGGAACGGCCGCCGCGAGTGCAGGT
      A  L  V  G  A  P  I  T  L  L  V  T  G  I  T  G  L  I  S  G  I  L  E  F  S  K  Q  P  M  L  D  H  V  A
1711 GCATTAGTTGGCGCACCAATTACTTTGTTGGTTACTGGTATCACAGGATTAATTTCTGGTATTTTAGAGTTCTCTAAACAACCAATGTTAGATCATGTTG
      S  K  I  G  N  K  I  D  E  W  E  K  K  Y  G  K  N  Y  F  E  N  G  Y  D  A  R  H  K  A  F  L  E  D
1811 CATCGAAAATTGGTAACAAAATTGACGAATGGGAGAAAAAATACGGTAAAAATTACTTCGAGAATGGCTATGATGCTCGTCATAAAGCTTTCTTAGAAGA
      S  F  S  L  L  S  S  F  N  K  Q  Y  E  T  E  R  A  V  L  I  T  Q  Q  R  W  D  E  Y  I  G  E  L  A
1911 TTCATTCTCATTATTGTCTAGTTTTAATAAACAATATGAAACTGAAAGAGCTGTTTTAATTACACAACAACGTTGGGATGAATATATTGGCGAACTTGCG
      G  I  T  G  K  G  D  K  L  S  S  G  K  A  Y  V  D  Y  F  Q  E  G  K  L  L  E  K  K  P  D  D  F  S  K
2011 GGTATTACTGGCAAAGGTGACAAACTCTCTAGTGGTAAGGCGTATGTAGATTACTTTCAAGAAGGTAAATTATTAGAGAAAAAACCTGATGACTTTAGCA
      V  V  F  D  P  T  K  G  E  I  D  I  S  N  S  Q  T  S  T  L  L  K  F  V  T  P  L  L  T  P  G  T  E
2111 AAGTAGTTTTCGATCCAACTAAGGGCGAAATTGATATTTCAAATAGCCAAACGTCAACGTTGTTAAAATTTGTTACGCCATTATTAACACCAGGTACAGA
      S  R  E  R  T  Q  T  G  K  Y  E  Y  I  T  K  L  V  V  K  G  K  D  K  W  V  V  N  G  V  K  D  K  G
2211 GTCACGTGAAAGAACTCAAACAGGTAAATATGAATATATCACGAAGTTAGTTGTAAAAGGTAAAGATAAATGGGTTGTTAATGGCGTTAAAGATAAAGGT
      A  V  Y  D  Y  T  N  L  I  Q  H  A  H  I  S  S  S  V  A  R  G  E  E  Y  R  E  V  R  L  V  S  H  L  G
2311 GCCGTTTATGATTATACTAATTTAATTCAACATGCTCATATTAGTTCATCAGTAGCACGTGGTGAAGAATACCGTGAAGTTCGTTTGGTATCTCATCTAG
      N  G  N  D  K  V  F  L  A  A  G  S  A  E  I  H  A  G  E  G  H  D  V  V  Y  Y  D  K  T  D  T  G  L
2411 GCAATGGTAATGACAAAGTGTTCTTAGCTGCGGGTTCCGCAGAAATTCACGCTGGTGAAGGTCATGATGTGGTTTATTATGATAAAACCGATACAGGTCT
      L  V  I  D  G  T  K  A  T  E  Q  G  R  Y  S  V  T  R  E  L  S  G  A  T  K  I  L  R  E  V  I  K  N
2511 TTTAGTAATTGATGGAACCAAAGCGACTGAACAAGGGCGTTATTCTGTTACGCGCGAATTGAGTGGTGCTACAAAAATCCTGAGAGAAGTAATAAAAAAT
      Q  K  S  A  V  G  K  R  E  E  T  L  E  Y  R  D  Y  E  L  T  Q  S  G  N  S  N  L  K  A  H  D  E  L  H
2611 CAAAAATCTGCTGTTGGTAAACGTGAAGAAACCTTGGAATATCGTGATTATGAATTAACGCAATCAGGTAATAGTAACCTAAAAGCACATGATGAATTAC
      S  V  E  E  I  I  G  S  N  Q  R  D  E  F  K  G  S  K  F  R  D  I  F  H  G  A  D  G  D  D  L  L  N
2711 ATTCAGTAGAAGAAATTATTGGAAGTAATCAGAGAGACGAATTTAAAGGTAGTAAATTCAGAGATATTTTCCATGGTGCCGATGGTGATGATCTATTAAA
       G  N  D  G  D  D  I  L  Y  G  D  K  G  N  D  E  L  R  G  D  N  G  N  D  Q  L  Y  G  G  E  G  N  D
2811 TGGTAATGATGGGGATGATATTCTATACGGTGATAAAGGTAACGATGAGTTAAGAGGTGATAATGGTAACGACCAACTTTATGGTGGTGAAGGTAATGAC
      K  L  L  G  G  N  G  N  N  Y  L  S  G  G  D  G  N  D  E  L  Q  V  L  G  N  G  F  N  V  L  R  G  G  K
2911 AAACTATTAGGAGGTAATGGCAATAATTACCTCAGTGGTGGTGATGGCAATGATGAGCTTCAAGTCTTAGGCAATGGTTTTAATGTGCTTCGTGGCGGTA
       G  D  D  K  L  Y  G  S  S  G  S  D  L  L  D  G  G  E  G  N  D  Y  L  E  G  G  D  G  S  D  F  Y  V
3011 AAGGCGATGATAAACTTTATGGTAGCTCAGGTTCTGATTTACTTGATGGTGGAGAAGGTAATGATTATCTAGAAGGAGGCGATGGTAGCGATTTTTATGT
      Y  R  S  T  S  G  N  H  T  I  Y  D  Q  G  K  S  S  D  L  D  K  L  Y  L  S  D  F  S  F  D  R  L  L
3111 TTATCGTTCCACTTCAGGTAATCATACTATTTATGATCAAGGTAAATCTAGTGATTTAGATAAACTATATTTGTCTGATTTTTCCTTCGATCGTCTTCTT
      V  E  K  V  D  D  N  L  V  L  R  S  N  E  S  S  H  N  N  G  V  L  T  I  K  D  W  F  K  E  G  N  K  Y
3211 GTTGAGAAAGTTGATGATAACCTTGTACTTAGAAGTAATGAAAGTAGTCATAATAATGGAGTACTCACAATCAAAGACTGGTTTAAAGAAGGGAATAAAT
      N  H  K  I  E  Q  I  V  D  K  N  G  R  K  L  T  A  E  N  L  G  T  Y  F  K  N  A  P  K  A  D  N  L
3311 ATAACCATAAAATTGAACAAATTGTTGATAAAAATGGTAGAAAATTGACAGCAGAGAATTTAGGAACTTATTTCAAAAATGCTCCAAAAGCTGACAATTT
      L  N  Y  A  T  K  E  D  Q  N  E  S  N  L  S  S  L  K  T  E  L  S  K  I  I  T  N  A  G  N  F  G  V
3411 GCTTAATTATGCAACTAAAGAAGATCAGAATGAAAGCAATTTATCTTCACTTAAAACTGAATTAAGTAAAATTATTACTAATGCAGGTAATTTTGGTGTG
      A  K  Q  G  N  T  G  I  N  T  A  A  L  N  N  E  V  N  K  I  I  S  S  A  N  T  F  A  T  S  Q  L  G  G
3511 GCAAAACAAGGTAATACTGGAATCAATACAGCTGCCTTGAACAATGAAGTGAATAAAATCATTTCTTCTGCTAATACCTTTGCTACTTCACAATTGGGTG
      S  G  M  G  T  L  P  S  T  N  V  N  S  M  M  L  G  N  L  A  R  A  A                    >>>>>>>>>>>
3611 GCTCAGGGATGGGAACATTACCATCAACGAATGTAAATTCAATGATGCTAGGTAACCTAGCTAGAGCAGCTTAATCATCTGCAATAATCAATAGCAATCC
     >>           <<<<<<<<<<<<<                      M  P  F  N  E  K  I  D  Y  G  L  H  A  L  V  I  L  A  Q  Y
3711 TATGGCTATTCTAGGATTGCTATTTTATTTATGGAGTCACAAATGCCTTTTAACGAAAAAATAGATTACGGATTACATGCATTGGTAATTCTCGCGCAAT
      H  N  V  A  V  N  P  E  E  V  K  H  K  F  D
3811 ATCACAATGTTGCCGTAAACCCTGAAGAGGTAAAACATAAATTTGATC
```

FIG. 2. Nucleotide sequence of the major part of the pApxIIICA insert from positions −190 to 3858, relative to the putative start site of *apxIIIC*, and the deduced amino acid sequences encoded by the *apxIIIC*, *apxIIIA*, and Δ*apxIIIB* open reading frames. In the nucleotide sequence, putative ribosome binding sites are underlined, start codons are in boldface type, and the potential transcription termination signal is indicated by <<< >>>. In the amino acid sequence of the ApxIIIA protein, the glycine-rich repeats with the consensus sequence I/L-X-G-G-X-G-N/D-D-X are underlined.

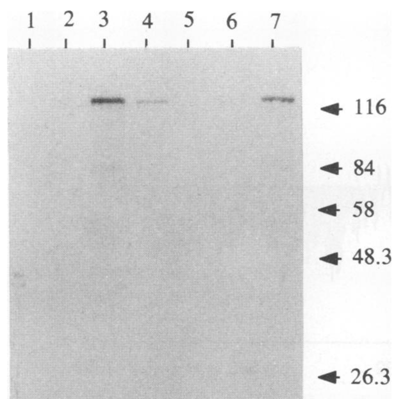

FIG. 3. Immunoblot of ApxIII proteins. Lysates and culture media of *E. coli* LE392 and culture media of *A. pleuropneumoniae* serotype 8 were separated by polyacrylamide gel electrophoresis and analyzed for proteins immunoreactive with MAb CVI-Apcly-2.2. Lanes: 1 and 2, lysate and culture medium, respectively, of *E. coli* LE392(pLG575); 3, culture medium of *A. pleuropneumoniae* serotype 8; 4 and 5, lysate and culture medium, respectively, of *E. coli* LE392(pApxIIICA); 6 and 7, lysate and culture medium, respectively, of *E. coli* LE392(pApxIIICA, pLG575). The positions of molecular weight markers are indicated (in thousands).

ment contained the genetic information for ApxIII. To confirm that ApxIII is an RTX toxin, we studied whether ApxIII could be secreted from *E. coli* by heterologous RTX secretion proteins. Therefore, we co-transformed *E. coli* LE392(pApxIIICA) with pLG575, which carries the *E. coli* RTX *hlyB* and *hlyD* genes. The 120-kDa ApxIII protein was now secreted into the culture medium, while no immunoreactive protein was detectable in the cell lysate (lanes 6 and 7). These results confirmed the RTX character of the ApxIII protein. To study the biological activity of the 120-kDa ApxIII protein produced by *E. coli* LE392(pApxIIICA, pLG575), we measured its potential to kill porcine lung macrophages and to hemolyse sheep erythrocytes. The culture medium of *E. coli* LE392(pApxIIICA, pLG575) killed porcine lung macrophages but did not lyse sheep erythrocytes. In a typical experiment, we found a cytolytic titer of 2,048 and a hemolytic titer of 0, titers that are comparable to the hemolytic and cytolytic titers of the culture medium of *A. pleuropneumoniae* serotype 8. We also tested the hemolytic activity of *E. coli* LE392(pApxIIICA, pLG575) on blood agar plates and, as expected, found no hemolytic zones around the colonies after overnight growth. We concluded that the in vitro cytolytic activities of the proteins encoded by pApxIIICA and of the ApxIII protein of *A. pleuropneumoniae* were similar.

Nucleotide sequence of the ApxIII determinant. We determined the nucleotide sequence of the major part of the pApxIIICA insert by sequencing a selection of progressive unidirectional deletion clones that were prepared from the 3.2-kbp *Hin*dIII fragment and the 4.2-kbp *Nsi*I-*Xba*I fragment (Fig. 1A). We found two intact open reading frames; the first, from nucleotide positions 1 to 519, was designated *apxIIIC*, and the second, from nucleotide positions 526 to 3681, was designated *apxIIIA*. A third open reading frame, designated Δ*apxIIIB*, started at position 3753 but had no termination codon within the sequenced area. Figure 2 shows the nucleotide sequence, and a schematic presentation of the genes is given in Fig. 1A. The proteins encoded by *apxIIIC* and *apxIIIA* were homologous to the RTX proteins HlyC and HlyA of *E. coli*. The amino acid sequences of ApxIIIC and HlyC were 53% identical, and those of ApxIIIA and HlyA were 50% identical. It is noteworthy that the putative ribosome binding sites for ApxIIIC and HlyC were at an unusually long distance, 15 bp, from the ATG codons.

The open reading frame of the ApxIIIC protein was expected to start at position 1 because of the homology of ApxIIIC to HlyC. However, two potential ATG translation initiation codons could be assigned for the *apxIIIC* gene, one at position 1 and one at position 160. Even though no putative ribosome binding site could be assigned in front of the second ATG codon, we checked whether the translation of the *apxIIIC* gene was initiated from position 1. Therefore, we constructed two deletion derivatives of pApxIIICA and tested their ability to produce cytotoxic activity, assuming that the intact ApxIIIC protein is required for the activation of the ApxIIIA protein. In the first deletion derivative, pApxIIICA−42, we deleted the region from the *Nsi*I site upstream of the *apxIIIC* gene to position −42, leaving both putative *apxIIIC* initiation codons present (Fig. 1B). In the second, pApxIIICA+142, we deleted the region from the *Nsi*I site upstream of the *apxIIIC* gene to position 142, leaving only the putative initiation codon at position 160 present. The proteins encoded by pApxIIICA, pApxIII CA−42, and pApxIIICA+142 were expressed in *E. coli* LE392(pLG575). All three clones produced equal amounts of immunoreactive ApxIII, as determined by dot blotting with serial dilutions of the culture media of the clones (data not shown). However, the ApxIII proteins produced by *E. coli* LE392(pApxIIICA, pLG575) and *E. coli* LE392(pApx IIICA−42, pLG575) were cytotoxic for porcine lung macrophages, while the ApxIII protein produced by *E. coli* LE392(pApxIIICA+142, pLG575) was not cytotoxic for porcine lung macrophages. We concluded that the ApxIIIA protein secreted by *E. coli* LE392(pApxIIICA+142, pLG575) was not activated and hence that the translation of the *apxIIIC* gene was initiated from the ATG codon at position 1. We constructed pApxIIICA−42 and pApxIII CA+142 by using PCR-amplified DNA (see Materials and Methods). Since PCR may introduce errors in the DNA, we analyzed three independent clones for each construct. Within each group, no differences were found in the amounts and cytotoxic activities of the ApxIII proteins.

Comparison of ApxIII with other RTX toxins. We compared the amino acid sequences of ApxIIIC and ApxIIIA with those of the corresponding proteins of the RTX Hly operon of *E. coli* and the RTX ApxI and ApxII operons of *A. pleuropneumoniae* serotype 9. The amino acid sequence alignments, based on amino acid identity and allowing for a minimal addition of gaps, revealed that ApxIIIC showed 53% identity to HlyC, 50% identity to ApxIC, and 52% identity to ApxIIC. ApxIIIA showed 50% identity to HlyA, 50% identity to ApxIA, and 41% identity to ApxIIA. Despite the low percentages of identity of the primary amino acid sequences of these proteins, they showed almost identical hydropathy patterns. The hydropathy pattern of ApxIIIA is presented in Fig. 4A. In this pattern, most prominent are three hydrophobic domains, designated I, II, and III. These domains are believed to be involved in pore formation by the cytotoxins (21, 22, 24). The most conserved amino acid sequences of the HlyA, ApxIA, ApxIIA, and ApxIIIA proteins were located in this hydrophobic region. A sequence alignment of this region of HlyA, ApxIA, ApxIIA, and ApxIIIA is presented in Fig. 4B. Also shown are the positions of two peptides, pep1 (LSTSAAAAGL) and pep2

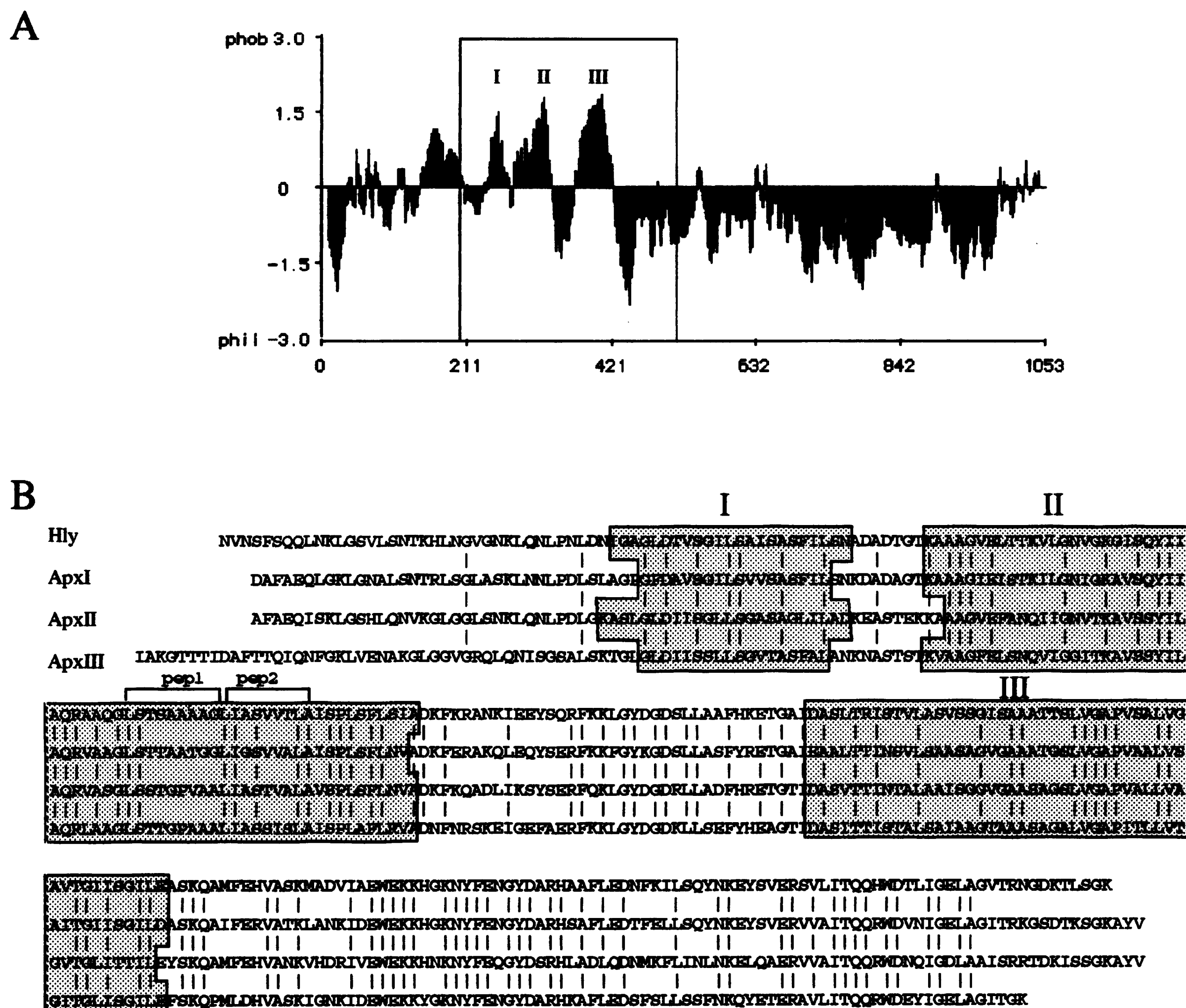

FIG. 4. Hydrophobic domains of HlyA, ApxIA, ApxIIA, and ApxIIIA. (A) Hydropathy plot of ApxIIIA. Positive values on the vertical axis are hydrophobic (phob), and negative values are hydrophylic (phil). The horizontal axis represents the amino acid sequence of ApxIIIA. The boxed region represents amino acids 200 to 500, which were used for the comparison shown in panel B. The hydrophobic domains are designated I, II, and III. (B) Sequence alignment of amino acids 200 to 500 of the hydrophobic domains of HlyA, ApxIA, ApxIIA, and ApxIIIA. Identical amino acids in all four proteins are indicated by vertical lines. The boxed regions represent hydrophobic domains I, II, and III indicated in panel A. Also indicated are the positions of pep1 (LSTSAAAAGL) and pep2 (IASVVTLA) of plasmid-encoded *E. coli* HlyA.

(IASVVTLA) of plasmid-encoded HlyA of *E. coli*, that were demonstrated to have pore-forming properties (24).

We found 13 glycine-rich repeats near the carboxy terminus of ApxIIIA with the consensus sequence L/I-X-G-G-X-G-N/D-D-X (31). The positions of the glycine-rich repeats are indicated in the ApxIIIA sequence in Fig. 2. The repeat clusters of ApxIIIA, ApxIA, and HlyA were similar in structure and consisted of 13 repeats, which were interrupted between repeats 9 and 10 by 12 amino acids (HlyA) or 10 amino acids (ApxIIIA and ApxIA). The repeat cluster of ApxIIA differed from the others; it consisted of eight repeats, which were not interrupted (5, 30). Repeats 6, 7, 9, and 10 of ApxIIIA, ApxIA, and HlyA fully matched the consensus sequence L/I-X-G-G-X-G-N/D-D-X. The other repeats contained one or more amino acids in at least one of the proteins that did not match those in the consensus sequence. Figure 5 shows the alignment of the repeat clusters of HlyA, ApxIA, and ApxIIIA. It was remarkable that amino acids that did not match those in the consensus sequence often were conserved among the three proteins. For example, amino acids 1, 3, 4, 6, 8, and 9 of repeat 2 were conserved among the three proteins, while only amino acids 3 and 8 matched corresponding amino acids in the consensus sequence. Similar conserved amino acids that did not match those in the consensus sequence were found in repeats 3, 4, 5, 8, and 11.

DISCUSSION

We cloned and characterized the *apxIIIC* and *apxIIIA* genes and the proximal part of the *apxIIIB* gene of *A. pleuropneumoniae* serotype 8. The *apxIIIC* and *apxIIIA* genes encode RTX toxin ApxIIIA and its activator protein ApxIIIC. From the presented data it is clear that ApxIII is a member of the RTX toxin family. Thus, besides ApxI and

```
Hly     LIGTTRADK FFGSKFADI FHGADGDDH IEGNDGNDR LYGDKGNDT LSGGNGDDQ LYGGDGNDK LIGGAGNNY
ApxI    IIGSNRKDK FFGSRFTDI FHGAKGDDE IYGNDGHDI LYGDDGNDV IHGGDGNDH LVGGNGNDR LIGGKGNNF
ApxIII  IIGSNQRDE FKGSKFRDI FHGADGDDL LNGNDGDDI LYGDKGNDE LRGDNGNDQ LYGGEGNDK LLGGNGNNY
            1         2         3         4         5         6         7         8

        LNGGDGDDE LQVQGNSLAKNV LSGGKGNDK LYGSEGADL LDGGEGNDL LKGGYGNDI
        LNGGDGDDE LQVFEGQY--NV LLGGAGNDI LYGSDGTNL FDGGVGNDK IYGGLGKDI
        LSGGDGNDE LQVLGNGF--NV LRGGKGDDK LYGSSGSDL LDGGEGNDY LEGGDGSDF
            9                     10        11        12        13
```

FIG. 5. Comparison of the glycine-rich repeat clusters of HlyA, ApxIA, and ApxIIIA. The repeats are numbered from 1 to 13. Identical amino acids in all three proteins are indicated by vertical lines. Amino acids that match those in the consensus sequence L/I-X-G-G-X-G-N/D-D-X in all three proteins are indicated by dashes in the top line. Amino acids that do not match those in the consensus sequence are indicated by dots in the top line. Nonmatching amino acids that are identical in HlyA, ApxIA, and ApxIIIA are indicated by arrows.

ApxII, ApxIII is the third RTX toxin produced by *A. pleuropneumoniae.*

An amino acid sequence comparison showed that ApxIII is more related to ApxI (50%) than to ApxII (41%), while ApxI and ApxII are the least related members (37%) of this toxin family. ApxIII is as much related to ApxI and ApxII as to RTX toxins from other species, such as Hly of *E. coli* and the leukotoxins of *Pasteurella haemolytica* and *Actinobacillus actinomycetemcomitans* (19, 20). On the basis of their biological activities and immunological properties, we expected that ApxI and ApxII would be more related to each other than to ApxIII. Perhaps some functional domains and epitopes of ApxI and ApxII are more conserved than the overall amino acid sequences of these proteins.

We found three hydrophobic domains and a cluster of glycine-rich repeats in the amino acid sequence of ApxIIIA. These features are found in all RTX toxins and indicate that these proteins act through the same mechanism. The hydrophobic domains are thought to be involved in the formation of pores in target cell membranes. It was demonstrated that deletions in this region of *E. coli* HlyA completely abolished its pore-forming ability in an artificial bilayer (21, 22). Furthermore, two peptides, pep1 and pep2, derived from the hydrophobic domains of plasmid-encoded HlyA of *E. coli*, formed pores in an artificial bilayer, and antibodies raised against pep1 completely inhibited the lytic activity of HlyA (24). pep1 and pep2 are located in highly conserved regions of chromosomally encoded HlyA, ApxIA, ApxIIA, and ApxIIIA.

The structures of the glycine-rich repeat clusters are similar in ApxIA, ApxIIIA, and HlyA, suggesting a similar function for the repeat clusters of these three RTX toxins. For HlyA, it was demonstrated that the repeat cluster is involved in the binding of Ca^{2+}, which is required for binding of the protein to erythrocyte membranes (2, 3, 7, 21). The binding of ApxIA to the membranes of porcine erythrocytes and neutrophils is also Ca^{2+} dependent (8a, 33). It is not known whether ApxIIIA binds Ca^{2+}, but it was demonstrated that Ca^{2+} is required for the binding of ApxIIIA to porcine neutrophils (33). Therefore, we expect that ApxIIIA binds Ca^{2+} as well. Besides the glycine-rich repeat clusters that seem to be required for target cell binding, other parts of the RTX toxins may also play a role in the binding and recognition of target cells. For example, Ludwig et al. showed that deletions in the hydrophobic domains not only reduced the hemolytic activity of Hly but also altered the ability of Hly to bind to the erythrocyte membrane (21). In addition, in this study we found that ApxIA and ApxIIIA have similar repeat clusters, although they have different target cell specificities. This result suggests that other regions of the proteins are also involved in target cell binding and recognition and need to be identified in further studies. The roles of the repeat clusters and Ca^{2+} binding may be even more complex, however, in some other RTX toxins. The leukotoxin of *A. actinomycetemcomitans* has a repeat cluster similar to those in HlyA, ApxIA, and ApxIIIA, except that it is not interrupted and consists of 14 repeats (19). The effect of Ca^{2+} on the activity of leukotoxin towards a leukemia cell line seems to be adverse: Ca^{2+} protects these cells against leukotoxin activity rather than stimulating leukotoxin activity (13).

The organization of the ApxIII operon is similar to that of other RTX toxin operons. We recently cloned the remaining part of the *apxIIIB* gene and the major part of the *apxIIID* gene of the serotype 8 reference strain (14). This result indicates that the ApxIII operon of serotype 8 consists of the genes *apxIIIC*, *apxIIIA*, *apxIIIB*, and *apxIIID*. Previously, we demonstrated that the ApxII operon consists only of the *apxIICA* genes. We suggested for serotype 9 that the ApxI and ApxII operons complement each other and that the ApxIIA protein could be secreted by the proteins encoded by the *apxIB* and *apxID* genes (15, 16). Serotype 8 probably contains a complete ApxIII operon, and for this serotype, it may be that ApxIIA is secreted by the *apxIIIB*- and *apxIIID*-encoded proteins. Alternatively, serotype 8 may contain *apxIIBD* genes that are split from the *apxIICA* genes, as was found by Chang et al. for a serotype 5 strain (5a).

The cloning of the ApxIII genes completes our efforts to clone the Apx-coding genes of *A. pleuropneumoniae*, since there is strong evidence that not more than three Apx toxins are secreted by this bacterium (18). It is still not clear what role each of the Apx toxins plays in the pathogenesis of porcine pleuropneumonia. Thus far, no pathogenic strains of *A. pleuropneumoniae* that secrete all three Apx toxins have been found. The fact that some pathogenic strains secrete only one of the toxins may indicate that the toxins have similar functions. On the other hand, the toxins differ in their lytic activities and in their target cell specificities, suggesting a more specialized role for each of the toxins. An additive effect of ApxII and ApxIII on virulence was suggested by the experiments of Rycroft et al. (27). They showed that a serotype 2 mutant that only secreted ApxIII still was able to

invade the host and cause disease, but the pigs were less severely affected.

Now that we can produce biologically active ApxI and ApxII (30) and ApxIII (this work) separately in *E. coli*, free from other *A. pleuropneumoniae* components, the role of each of the Apx toxins in the pathogenesis of porcine pleuropneumonia may be resolved.

ACKNOWLEDGMENTS

We thank Johan Popma and Thea Vermeulen for culturing *A. pleuropneumoniae* and for performing the hemolysin and cytotoxin assays.

REFERENCES

1. **Bertram, T. A.** 1990. *Actinobacillus pleuropneumoniae*: molecular aspects of virulence and pulmonary injury. Can. J. Vet. Res. **54:**S53–S56.
2. **Boehm, D. F., R. A. Welch, and I. S. Snyder.** 1990. Calcium is required for binding of *Escherichia coli* hemolysin (HlyA) to erythrocyte membranes. Infect. Immun. **58:**1951–1958.
3. **Boehm, D. F., R. A. Welch, and I. S. Snyder.** 1990. Domains of *Escherichia coli* hemolysin (HlyA) involved in binding of calcium and erythrocyte membranes. Infect. Immun. **58:**1959–1964.
4. **Bressan, G. M., and K. K. Stanley.** 1987. pUEX, a bacterial expression vector related to pEX with universal host specificity. Nucleic Acids Res. **15:**10056.
5. **Chang, Y., R. Young, and D. K. Struck.** 1989. Cloning and characterization of a hemolysin gene from *Actinobacillus* (*Haemophilus*) *pleuropneumoniae*. DNA **8:**635–647.

5a. **Chang, Y., R. Young, and D. K. Struck.** 1991. The *Actinobacillus pleuropneumoniae* hemolysin determinant: unlinked *appCA* and *appBD* loci flanked by pseudogenes. J. Bacteriol. **173:**5151–5158.

6. **Felmlee, T., S. Pellett, and R. A. Welch.** 1985. Nucleotide sequence of an *Escherichia coli* chromosomal hemolysin. J. Bacteriol. **163:**94–105.
7. **Felmlee, T., and R. A. Welch.** 1988. Alterations of amino acid repeats in the *Escherichia coli* hemolysin affect cytolytic activity and secretion. Proc. Natl. Acad. Sci. USA **85:**5269–5273.
8. **Frey, J.** Personal communication.

8a. **Frey, J., R. Meier, D. Gygi, and J. Nicolet.** 1991. Nucleotide sequence of the hemolysin I gene from *Actinobacillus pleuropneumoniae*. Infect. Immun. **59:**3026–3032.

9. **Frey, J., and J. Nicolet.** 1990. Hemolysin patterns of *Actinobacillus pleuropneumoniae*. J. Clin. Microbiol. **28:**232–236.
10. **Frey, J., H. van den Bosch, R. Segers, and J. Nicolet.** 1992. Identification of a second hemolysin (HlyII) in *Actinobacillus pleuropneumoniae* serotype 1 and expression of the gene in *Escherichia coli*. Infect. Immun. **60:**1671–1676.
11. **Gygi, D., J. Nicolet, J. Frey, M. K. Cross, V. Koronakis, and C. Hughes.** 1990. Isolation of the *Actinobacillus pleuropneumoniae* hemolysin gene and the activation and secretion of the prohemolysin by the HlyC, HlyB and HlyD proteins of *Escherichia coli*. Mol. Microbiol. **4:**123–128.
12. **Issartel, J. P., V. Koronakis, and C. Hughes.** 1991. Activation of *Escherichia coli* prohaemolysin to the mature toxin by acyl carrier protein-dependent fatty acylation. Nature (London) **351:**759–761.
13. **Iwase, M., E. T. Lally, P. Berthold, H. M. Korchak, and N. S. Taichman.** 1990. Effects of cations and osmotic protectants on cytolytic activity of *Actinobacillus actinomycetemcomitans* leukotoxin. Infect. Immun. **58:**1782–1788.
14. **Jansen, R., et al.** Unpublished data.
15. **Jansen, R., J. Briaire, E. M. Kamp, and M. A. Smits.** 1991. Cloning and characterization of the *Actinobacillus pleuropneumoniae* cytolysin genes, abstr. 301, p. 53. Abstr. 72nd Annu. Meet. Conf. Res. Workers Anim. Dis.
16. **Jansen, R., J. Briaire, E. M. Kamp, and M. A. Smits.** 1992. Comparison of the cytolysin II genetic determinants of *Actinobacillus pleuropneumoniae* serotypes. Infect. Immun. **60:**630–636.

16a. **Kamp, E. M., J. K. Popma, J. Anakotta, and M. A. Smits.** 1991. Identification of hemolytic and cytotoxic proteins of *Actinobacillus pleuropneumoniae* by using monoclonal antibodies. Infect. Immun. **59:**3079–3085.

17. **Kamp, E. M., and L. A. M. G. van Leengoed.** 1990. Serotype-related differences in production and type of heat-labile hemolysin and heat-labile cytotoxin of *Actinobacillus pleuropneumoniae*. J. Clin. Microbiol. **27:**1187–1191.
18. **Kamp, E. M., T. M. M. Vermeulen, and M. A. Smits.** 1991. Production of cytolysins (Clys) by *Actinobacillus pleuropneumoniae* field isolates, abstr. 296, p. 52. Abstr. 72nd Annu. Meet. Conf. Res. Workers Anim. Dis.
19. **Kraig, E., T. Dailey, and D. Kolodrubetz.** 1990. Nucleotide sequence of the leukotoxin gene from *Actinobacillus actinomycetemcomitans*: homology to the alpha-hemolysin/leukotoxin gene family. Infect. Immun. **58:**920–929.
20. **Lo, R. Y. C., C. A. Strathdee, and P. E. Shewen.** 1987. Nucleotide sequence of the leukotoxin of *Pasteurella haemolytica* A1. Infect. Immun. **55:**1987–1996.
21. **Ludwig, A., T. Jarchau, R. Benz, and W. Goebel.** 1988. The repeat domain of *Escherichia coli* haemolysin (HlyA) is responsible for its Ca^{2+}-dependent binding to erythrocytes. Mol. Gen. Genet. **214:**553–561.
22. **Ludwig, A., A. Schmid, R. Benz, and W. Goebel.** 1991. Mutations affecting pore formation of haemolysin from *Escherichia coli*. Mol. Gen. Genet. **226:**198–208.
23. **Nicolet, J.** 1986. Haemophilus infections, p. 426. *In* A. D. Lemon, B. Straw, R. D. Glock, W. L. Mengeling, R. H. C. Penny, and E. Scholl (ed.), Diseases of swine, 6th ed. Iowa State University Press, Ames.
24. **Oropeza-Wekerle, R. L., S. Muller, J. P. Briand, R. Benz, and W. Goebel.** 1992. Haemolysin-derived synthetic peptides with pore-forming and haemolytic activity. Mol. Microbiol. **6:**115–121.
25. **Rosendal, S., J. Devenish, J. I. MacInnes, J. H. Lumsden, S. Watson, and H. Xun.** 1988. Evaluation of heat-sensitive, neutrophil-toxic and hemolytic activity of *Actinobacillus* (*Haemophilus*) *pleuropneumoniae*. Am. J. Vet. Res. **49:**1053–1058.
26. **Rycroft, A. N., D. Williams, J. M. Cullen, and J. Macdonald.** 1991. The cytotoxin of *Actinobacillus pleuropneumoniae* (pleurotoxin) is distinct from the haemolysin and is associated with a 120-kDa polypeptide. J. Gen. Microbiol. **137:**561–568.
27. **Rycroft, A. N., D. Williams, I. A. P. McCandlish, and D. J. Taylor.** 1991. Experimental reproduction of acute lesions of porcine pleuropneumonia with a haemolysin-deficient mutant of *Actinobacillus pleuropneumoniae*. Vet. Rec. **16:**441–443.
28. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
29. **Sebunya, T. N. K., and J. R. Saunders.** 1983. *Haemophilus pleuropneumoniae* infection in swine: a review. J. Am. Vet. Med. Assoc. **182:**1331–1337.
30. **Smits, M. A., J. Briaire, R. Jansen, H. Smith, E. M. Kamp, and A. L. J. Gielkens.** 1991. Cytolysins of *Actinobacillus pleuropneumoniae* serotype 9. Infect. Immun. **59:**4497–4504.
31. **Strathdee, C. A., and R. Y. C. Lo.** 1987. Extensive homology between the leukotoxin of *Pasteurella haemolytica* A1 and the alpha-hemolysin of *Escherichia coli*. Infect. Immun. **55:**3233–3236.
32. **Strathdee, C. A., and R. Y. C. Lo.** 1989. Cloning, nucleotide sequence, and characterization of genes encoding the secretion function of the *Pasteurella haemolytica* leukotoxin. J. Bacteriol. 171:916–928.
33. **Van Leengoed, L. A. M. G., and H. W. Dickerson.** 1992. Influence of calcium on secretion and activity of the cytolysins of *Actinobacillus pleuropneumoniae*. Infect. Immun. **60:**353–359.
34. **Zabarovsky, E. R., and R. L. Allikmets.** 1986. An improved technique for the efficient construction of gene libraries by partial filling-in of cohesive ends. Gene **42:**119–123.