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Structural Analysis of the *Actinobacillus pleuropneumoniae*-RTX-Toxin I (ApxI) Operon

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

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***Actinobacillus pleuropneumoniae*-RTX-toxin I (ApxI), an important virulence factor, is secreted by serotypes 1, 5, 9, 10, and 11 of *A. pleuropneumoniae*. However, sequences homologous to the secretion genes *apxIBD* of the ApxI operon are present in all 12 serotypes except serotype 3. The purpose of this study was to determine and compare the structures of the ApxI operons of the 12 *A. pleuropneumoniae* serotypes. We focused on the nucleotide sequence comparison of the ApxI-coding genes, the structures of the ApxI operons, and the transcription of the ApxI operons. We determined the nucleotide sequences of the toxin-encoding *apxICA* genes of serotype 9 and found that the gene for the structural toxin, *apxIA*, was almost identical to the *apxIA* gene of serotype 1. The toxin-encoding genes of the other serotypes are also similar for the main part; nevertheless, two variants were identified, one in serotypes 1, 9, and 11 and one in serotypes 5 and 10. The two *apxIA* variants differ mainly within the distal 110 nucleotides. Structural analysis demonstrated that intact ApxI operons, consisting of the four contiguous genes *apxICABD*, are present in serotypes 1, 5, 9, 10, and 11. ApxI operons with a major deletion in the *apxICA* genes are present in serotypes 2, 4, 6, 7, 8, and 12. Serotype 3 does not contain ApxI operon sequences. We found that all ApxI operons are transcriptionally active despite the partial deletion of the operon in some serotypes. The implications of these data for the expression and secretion of ApxI and the other Apx-toxins, ApxII and ApxIII, as well as for the development of a subunit vaccine against *A. pleuropneumoniae* will be discussed.**

Porcine pleuropneumonia is a highly contagious disease that causes great economic loss to the pig-farming industry worldwide. The bacterium *Actinobacillus pleuropneumoniae* is the causative agent of this disease. At least 12 serotypes of biotype I of this bacterium are currently recognized (11, 22, 26). To fight the disease, it is important to gain a better understanding of the virulence factors of *A. pleuropneumoniae* that play a role in the pathogenesis of porcine pleuropneumonia. There is good evidence that secreted proteins are important for virulence (2, 17, 23). Therefore, we focused our research on the characterization of these proteins and their genes.

Three different cytotoxic proteins are secreted by the 12 serotypes of *A. pleuropneumoniae* (17). These toxins were named cytolysin (Cly) I, II, and III, but recently the nomenclature of these proteins and their genes was standardized and now ClyI, ClyII, and ClyIII are referred to as *A. pleuropneumoniae*-RTX-toxin I (ApxI), ApxII, and ApxIII, respectively (8). ApxI is secreted by the reference strains of serotypes 1, 5, 9, 10, and 11, ApxII is secreted by the reference strains of all serotypes except serotype 10, and ApxIII is secreted by the reference strains of serotypes 2, 3, 4, 6, and 8 (17). The Apx proteins are toxic for porcine lung macrophages and/or erythrocytes (17).

Nucleotide sequence analysis of the ApxI-, ApxII-, and ApxIII-coding genes demonstrated that these toxins are closely related members of the RTX toxin family (4, 8a, 15, 27). RTX toxins are often associated with pathogenic bacteria. Well-known examples are *Pasteurella haemolytica*, *Escherichia coli*, and *Actinobacillus actinomycetemcomitans* (6, 20, 21). RTX toxins are encoded by operons that consist of four contiguous genes, *C*, *A*, *B*, and *D*. The *A* gene encodes the structural toxin that is activated by the *C* gene-encoded protein. The *B* and *D* gene-encoded proteins are involved in the secretion of the RTX toxins (9, 11).

The ApxI operons have been studied in serotypes 1, 5, and 9 (5, 8a, 27), and the ApxIII operons have been studied in serotypes 2 and 8 (15, 24). Only the ApxII operons, however, have been studied in all 12 serotypes (13). The ApxII operons were found to be present in the reference strains of all 12 serotypes except serotype 10. All ApxII operons had similar structures and consisted only of the *apxIICA* genes and a truncated *apxIIB* gene (5, 13). No serotype-specific differences were found in the *apxIICA* genes (13). The *apxIICA* genes were also found in *Actinobacillus suis*, indicating that these genes are mobile and can be transferred among species (1, 3).

The absence of the *apxIIBD* secretion genes leads to the suggestion that the ApxII operon would be complemented by the secretion genes of other RTX operons, which were located elsewhere in the genome. Indeed, we demonstrated the presence of *apxIBD* genes in serotype 9 and homologous sequences in all other serotypes except serotype 3 (13, 27). In serotype 9, the *apxIBD* genes were preceded by the genetic determinant of ApxI (27). Secretion genes that were identical to the *apxIBD* genes of serotype 9 were also found in a serotype 5 strain by Chang et al. (5). These authors assumed, however, that these genes were *apxIIBD* secretion genes which were unlinked to the *apxIICA* genes and preceded by an *apxIIA* pseudogene (5).

The finding of *apxIBD* genes in most serotypes, even in those that do not secrete ApxI protein, implies that these serotypes have ApxI operon sequences. This finding was unexpected, and it prompted us to investigate the ApxI operon structures in the reference strains of all 12 serotypes.

* Corresponding author.

This will lead to a better understanding of the genetic basis for the differences in ApxI expression between the serotypes and the functions and interactions of the three Apx operons. In addition to performing the genetic analysis, we also wanted to determine whether the ApxI proteins of serotypes 1, 5, 9, 10, and 11 differed from each other. Insight into the divergence of these proteins is important for the development of a universal subunit vaccine against *A. pleuropneumoniae*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and DNA and RNA preparations. The reference strains for the 12 serotypes of *A. pleuropneumoniae* biotype I were used as a source of genomic DNA and RNA. The reference strains for serotypes 1 to 12 were S4047, 1536, 1421, M62, K17, Femø, WF83, 405, 13261, D13039, 56153, and 8329, respectively.

High-molecular-weight DNA was isolated by proteinase K-sodium dodecyl sulfate (proteinase K-SDS) lysis, phenol-chloroform extraction, and precipitation with ethanol (25). DNA fragments were cloned with the plasmid pGEM7Zf(+) (Promega Corp., Madison, Wis.) or pKUN plasmid vectors (19) in *Escherichia coli* JM101 by standard molecular biology techniques (25). Total bacterial RNA was isolated by the hot phenol method (25). Briefly, cells from logarithmic-phase growing bacterial cultures with an optical density at 600 nm of 0.3 were collected by centrifugation, resuspended in 150 mM NaCl–50 mM Tris-HCl (pH 7.4), and directly mixed at 65°C with phenol that was saturated with 1 M Tris-HCl (pH 5.0). After 5 min of incubation, the aqueous phase, containing RNA, was reextracted at 20°C with phenol that was saturated with 1 M Tris-HCl (pH 7.4). RNA was precipitated from the aqueous phase by the addition of 2.5 volumes of ethanol, aliquoted, and stored in suspension at −20°C. RNA was precipitated by centrifugation and dissolved in TE (10 mM Tris-HCl [pH 7.4], 1 mM EDTA) containing 3,000 U of RNA-guard (Pharmacia, Uppsala, Sweden) per ml and treated with 30 U of RNase-free DNase I (Pharmacia) per ml to remove contaminating chromosomal DNA.

Southern and Northern (RNA) hybridization and radiolabeling of DNA. Restriction fragments were separated on an 0.8% agarose gel (1 μg of DNA per lane) and blotted onto GeneScreen Plus membranes (New England Nuclear Corp., Boston, Mass.). The blots were hybridized overnight at 65°C in a solution of 1.5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM Na H_2PO_4 [pH 7.4], and 1 mM EDTA)–1% SDS. The blots were washed with 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.

For Northern blots, the RNA was size separated by formaldehyde-agarose gel electrophoresis (23) or the RNA was spotted directly onto GeneScreen Plus membranes. Agarose gels were loaded with 5 μg of RNA per lane, and dot blots were loaded with 1 μg of RNA per dot. The blots were hybridized overnight at 45°C in a solution of 0.1% SDS, 40% formamide, 1 M NaCl, 50 mM NaH_2PO_4 [pH 6.8], 5× Denhardt solution (25), 10 mM EDTA, and 10% dextran sulfate. The blots were washed with a final stringency of 0.1× SSC–0.1% SDS for 60 min at 20°C.

For probes, we used DNA fragments that were purified from agarose gels by Prep-a-Gene (Bio-Rad Laboratories, Richmond, Calif.). DNA was labeled with [α-^{32}P]dCTP (Amersham Corp., Arlington Heights, Ill.) either by use of a random prime labeling kit (Boehringer GmbH, Mannheim, Germany) or by polymerase chain reaction (PCR) (10, 16). Radioactivity was detected by autoradiography with X-Omat AR film (Eastman Kodak, Rochester, N.Y.).

PCR, restriction fragment analysis, and cloning of amplified fragments. PCR and analysis of radiolabeled restriction fragments were done as described previously (13). To assure specificity to the *apxICABD* sequences, we positioned the oligonucleotides in regions of the *apxICABD* genes that were nonconserved in the *apxICABD*, *apxIICA*, and *apxIIICABD* genes. The sequences of oligonucleotides I, II, and III and their positions in the serotype 9 *apxICA* genes were as follows: oligonucleotide I, TGTAAACCTCATATGTAATG (positions −132 to −113); oligonucleotide II, TAGCCAAGC GGTTAGATCTA (positions 3798 to 3817); oligonucleotide III, CGGATGCATTTACCGGTG (positions 3475 to 3492). The sequence of oligonucleotide IV and its position in the *apxIBD* genes of serotype 9 (GenBank-EMBL accession number X61112) is AAAAAGTAAGAAAATATGGC (positions 3587 to 3606). The sequences of oligonucleotides V and VI and their positions in the insertion of the ApxI operon of serotype 7 (GenBank-EMBL accession number X73115) were as follows: oligonucleotide V, GACGGAAATGTTTT CAC (positions 156 to 172); oligonucleotide VI, CATTA CTAGTTGATGTT (positions 272 to 288).

Genomic fragments of approximately 700 bp that were amplified from serotypes 2, 4, 6, 7, 8, and 12 by use of oligonucleotides I and II were cloned into the *Sma*I and *Bam*HI sites of pGEM7Zf(+) after treatment with T4 DNA polymerase and the restriction enzyme *Bgl*II. Genomic fragments of approximately 3.95 kbp that were amplified from serotypes 5 and 10 by use of oligonucleotides I and II were subcloned into the *Xho*I and *Bam*HI sites of pGEM7Zf(+) as a 3.4-kbp *Xho*I-*Bgl*II fragment.

Nucleotide sequence analysis. We prepared progressive unidirectional deletion clones from the 7.4-kbp *Nsi*I-*Hin*dIII fragment (27) by using the Erase-a-Base system (Promega). A series of deletion clones that differed by 250 to 300 bp in length was sequenced by use of a T7 polymerase sequencing kit (Pharmacia). The SP6 and T7 promoter primers (Promega) were used for sequencing pGEM7Zf(+) clones, M13 reverse and M13 forward primers (Promega) were used for sequencing pKUN clones, and oligonucleotide VII was used for sequencing part of *apxIA* of serotypes 5 and 10. The sequence of oligonucleotide VII and its position in the *apxIA* gene of serotype 9 is GATGATGATACGTTATTG (positions 3115 to 3131). Nucleotide sequence analysis of PCR-amplified and cloned DNA was done on a mixture of 10 independent transformants to mask PCR-introduced sequence errors.

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 numbers. The GenBank-EMBL accession numbers for primary nucleotide sequence data are X73117 for the serotype 9 *apxICA* gene, X73115 for the insertion of the ApxI operon of serotype 7, and X73116 for the 3′ end of the *apxIA* gene of serotype 5.

RESULTS

Nucleotide sequence of the *apxICA* genes of *A. pleuropneumoniae* serotype 9. We previously reported the nucleotide sequence of the *apxIBD* genes of serotype 9 and the cloning of a 7.4-kbp *Nsi*I-*Hin*dIII fragment that was located immediately in front of the *apxIBD* genes and that coded for the ApxI toxin (27). We now determined the nucleotide sequence of the region that coded for ApxI. We found two

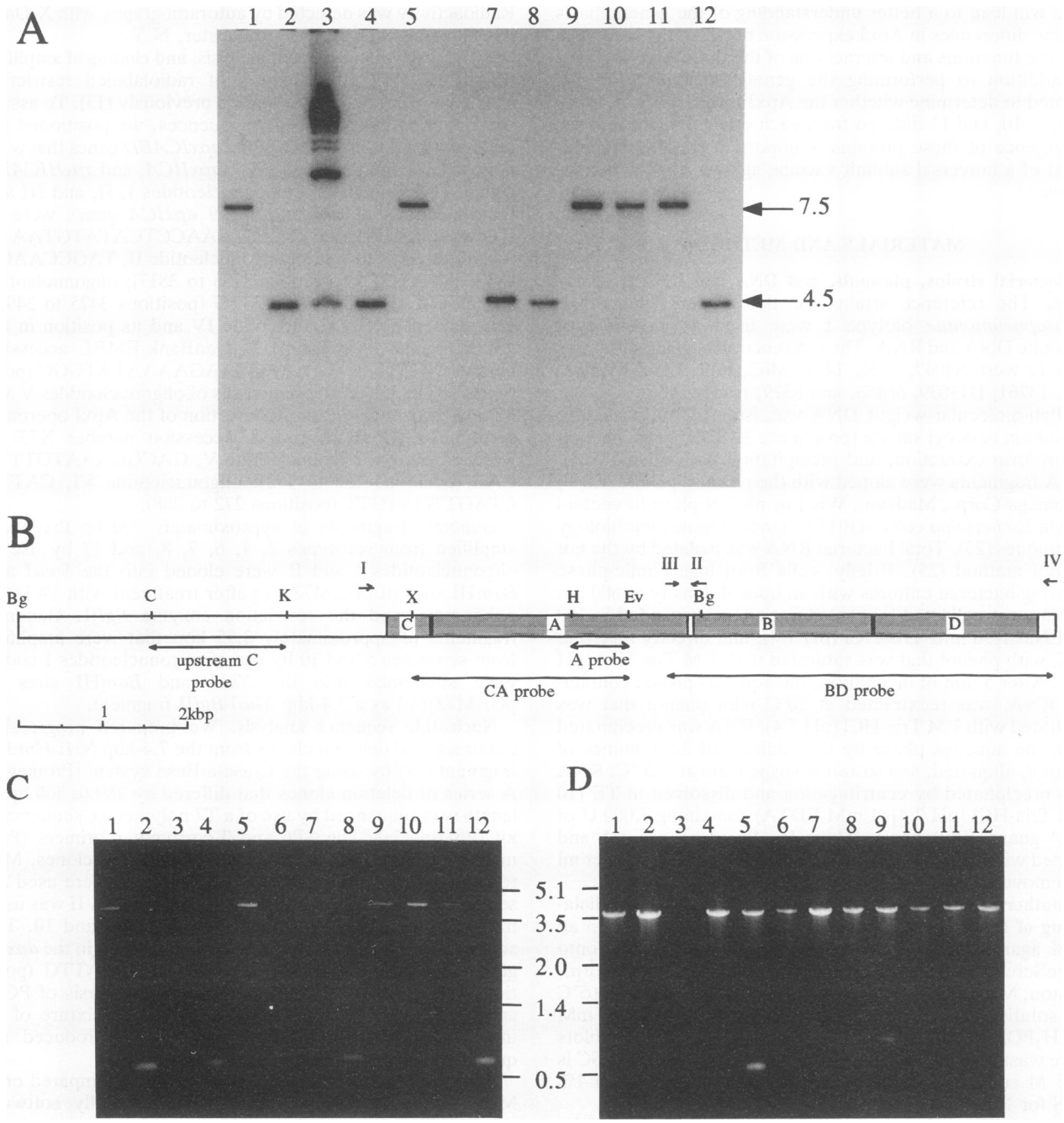

FIG. 1. (A) Autoradiogram of a Southern blot of *Bgl*II-digested genomic DNA. The 7.5-kbp fragments hybridized to the A probe and the upstream C probe. The other fragments hybridized only to the upstream C probe. Serotype numbers and molecular sizes in kilobase pairs are indicated. (B) Schematic presentation of the ApxI operon of serotype 9. The coding regions of the *apxICABD* genes are shaded. The positions of the probes, the PCR primers I to IV, and the recognition sites for the enzymes *Bgl*II (Bg), *Cla*I (C), *Eco*RV (Ev), *Hin*dIII (H), *Kpn*I (K), and *Xho*I (X) are indicated. (C and D) Ethidium bromide-stained agarose gels with PCR-amplified DNA fragments of the serotypes 1 to 12 by using oligonucleotides I and II (C) or oligonucleotides III and IV (D). Serotype numbers and molecular size markers in kilobase pairs are indicated.

major open reading frames in the sequence. The first open reading frame, from positions 1 to 516, was designated *apxIC* and coded for a protein with a molecular mass of 19.9 kDa. The second open reading frame, from positions 529 to 3594, was designated *apxIA* and coded for a protein with a molecular mass of 109.1 kDa. These data confirmed that the ApxI operon of serotype 9 consists of the four contiguous genes *apxICABD* (Fig. 1B) as was expected from ApxI expression data (27).

Comparison of the predicted amino acid sequences of the ApxIA and ApxIC proteins of serotype 9 to other *A. pleuropneumoniae* Apx proteins showed that ApxIA shared 37% of its amino acid sequence with ApxIIA of serotype 9 and 50% with ApxIIIA of serotype 8. The ApxIC protein

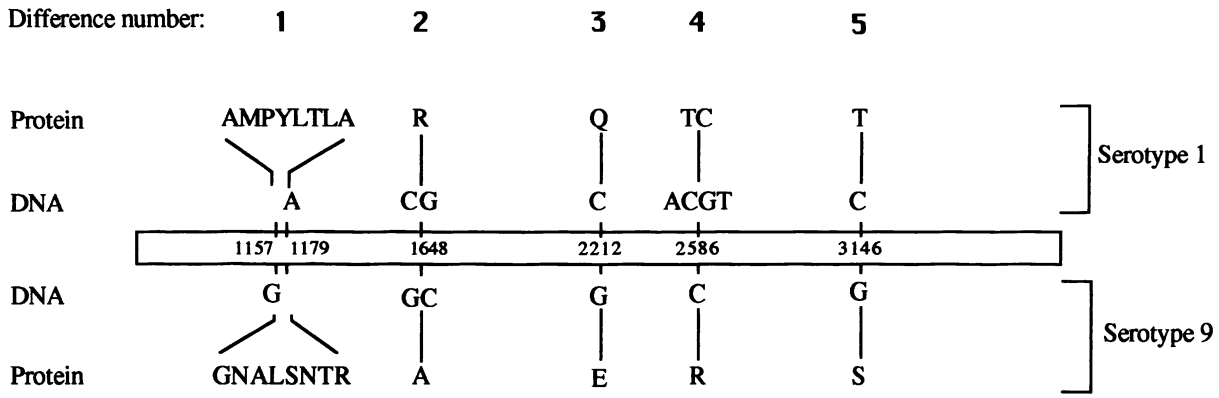

FIG. 2. Schematic presentation of the differences between the *apxIA* nucleotide sequences of serotypes 1 and 9. The bar represents the *apxIA* gene from positions 529 to 3594. The nucleotide sequence differences are numbered 1 to 5. The nucleotide differences and the encoded amino acids are indicated above the bar for serotype 1 and below the bar for serotype 9. The positions of the differences in the serotype 9 *apxICA* sequences are indicated within the bar.

shared 53% of its amino acid sequence with ApxIIC of serotype 9 and 52% with ApxIIIC of serotype 8.

Comparison of the ApxI operon structures between the serotypes. To gain insight into the overall structures of the ApxI operons of the 12 serotypes, we hybridized Southern blots of genomic *Bgl*II fragments to several fragments of the ApxI operon of serotype 9. For probes, we used a 770-bp *Hin*dIII-*Eco*RV fragment from the *apxIA* gene (A probe) and a 1,400-bp *Cla*I-*Kpn*I fragment that was located 1 kbp upstream of the *apxIC* gene (upstream C probe; Fig. 1B). Specific hybridization to *apxICA* sequences and not to *apxIICA* or *apxIIICA* sequences was assured by the low nucleotide sequence similarity of these genes (40 to 50%) and the high stringency of the hybridizations (13, 15, 27). We found that the A probe hybridized to a 7.5-kbp *Bgl*II fragment of serotypes 1, 5, 9, 10, and 11 and not to the DNA of the other serotypes. The upstream C probe, however, hybridized to the DNA of all serotypes. This probe recognized the same 7.5-kbp *Bgl*II fragment as the A probe did in serotypes 1, 5, 9, 10, and 11. In the other serotypes, a much smaller fragment of approximately 4.5 kbp was recognized. Serotype 3 contained several larger hybridizing fragments besides the 4.5-kbp fragment. This may be due to incomplete digestion of the genomic DNA or, alternatively, to the presence of multiple copies of the target sequence. The genomic DNA of serotype 6 was not included in the blot shown in Fig. 1A. In a separate experiment, we found that serotype 6 reacted in the same way as serotypes 2, 4, 7, 8, and 12. The Southern blot data demonstrated that *apxIA* sequences are present only in the serotypes that secrete the ApxI protein. Sequences located as close as 1 kbp upstream from the *apxIC* gene are present in all serotypes.

We further analyzed the ApxI operons by PCR. Two pairs of oligonucleotides were designed, namely, oligonucleotides I and II to amplify the *apxICA* genes and oligonucleotides III and IV to amplify the *apxIBD* genes (Fig. 1B). PCR amplification with oligonucleotides I and II on genomic DNA from serotypes 1, 5, 9, 10, and 11 resulted in a fragment of approximately 3.95 kbp. Amplification with the same oligonucleotides on genomic DNA from serotypes 2, 4, 6, 7, 8, and 12 resulted in a fragment of approximately 700 bp. Serotype 3 did not give an amplification product (Fig. 1C).

The amplification of a 3.95-kbp fragment from serotypes 1, 5, 9, 10, and 11 indicated the presence of complete *apxICA* genes in these serotypes. This was in agreement with the Southern hybridization data that were obtained with the A probe (Fig. 1A). The amplification of a 700-bp fragment from serotypes 2, 4, 6, 7, 8, and 12 was in agreement with the Southern hybridization data that were obtained with the upstream C probe (Fig. 1A). By PCR and Southern hybridization, we found DNA fragments that are approximately 3 kbp smaller than the corresponding fragments of the serotypes with complete *apxICA* genes. These data indicated that approximately 3 kbp was deleted from the chromosomes of serotypes 2, 4, 6, 7, 8, and 12. The deletion is located between the priming sites of oligonucleotides I and II and includes the sequences that are recognized by the A probe.

PCR amplification of genomic DNA, with oligonucleotides III and IV, resulted for all serotypes except serotype 3 in a 3.7-kbp fragment (Fig. 1D). This confirms the presence of *apxIBD* genes in all serotypes except serotype 3 as was previously suggested (13). The 3.7-kbp fragments hybridized to the BD probe, while the small bands of approximately 500 bp did not hybridize. Therefore, we considered these small bands as aspecific amplification products (data not shown). The identical sizes of the amplified fragments indicated that the *apxIBD* genes of the serotypes are intact and do not contain major insertions or deletions (Fig. 1D). The failure to amplify DNA from serotype 3 with both pairs of oligonucleotides agreed with the absence of *apxICA* and *apxIBD* sequences in this serotype (Fig. 1A) (13).

Primary structures of the *apxICA* genes of serotypes 1, 5, 9, 10, and 11. We compared the nucleotide sequences of the *apxIA* genes of serotypes 1 (8a) and 9 and found only five differences (Fig. 2), indicating that the *apxIA* genes of these two serotypes are very similar. To determine whether the *apxIA* genes of the other serotypes would be as similar, we compared the *apxICA* genes of serotypes 1, 5, 9, 10, and 11 by extensive restriction fragment analysis. For that purpose, we amplified by PCR the *apxICA* genes by use of oligonucleotides I and II (Fig. 1C). The resulting fragments of 3.95 kbp were radiolabeled and digested with the restriction enzymes *Alu*I, *Hpa*II, *Rsa*I, *Dde*I, *Sau*3AI, or *Hin*fI. These enzymes cut the *apxICA* sequence of serotype 9 at 77 positions. Major differences between the *apxICA* sequences of the other serotypes will result in different restriction sites for these enzymes. We found very similar restriction fragment patterns for the five serotypes. The patterns for serotypes 1, 9, and 11 were identical, indicating that the nucleotide sequences of the *apxIC* and *apxIA* genes of these

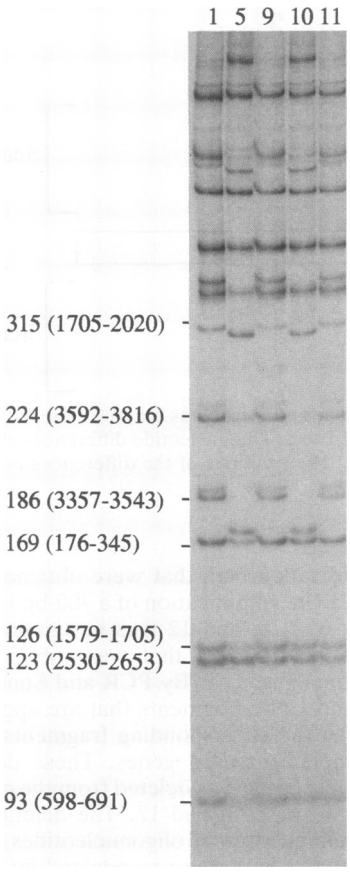

FIG. 3. Autoradiogram of *Alu*I-digested *apxICA* genes of serotypes 1, 5, 9, 10, and 11. The *apxICA* genes were PCR amplified, radiolabeled, and digested with *Alu*I. The *Alu*I fragments were separated on a 5% polyacrylamide gel. Serotype numbers are indicated at the top of the lanes. The sizes of the *Alu*I fragments in base pairs and their positions in the serotype 9 *apxICA* genes (in parentheses) are shown.

serotypes are also identical. As an example, the *Alu*I restriction patterns of serotypes 1, 5, 9, 10, and 11 are shown in Fig. 3. The restriction patterns of serotypes 5 and 10 were identical to each other but differed at several positions from the restriction patterns of serotypes 1, 9, and 11. Serotypes 5 and 10 had two extra *Alu*I sites (Fig. 3), one extra *Hpa*II site, and one extra *Hin*fI site; they were lacking two *Dde*I sites and two *Sau*3AI sites. We could not trace the exact positions of these sites in the *apxIA* genes, but five of the sites were located in the distal 500 bp of the *apxIA* gene and the other two sites were located between positions 2000 and 3000. We concluded from the restriction fragment analysis data that the *apxICA* genes of serotypes 1, 5, 9, 10, and 11 are very similar. Despite the similarity, two variants of *apxICA* genes that differ at their 3′ ends could be assigned. One variant is present in serotypes 1, 9, and 11, and the other is present in serotypes 5 and 10.

Two of the five differences that were found between the *apxIA* sequences of serotypes 1 and 9 (Fig. 2) could be checked by restriction fragment analysis. Difference 4 should result in a 126-bp *Alu*I fragment for serotype 1 and in a 123-bp fragment for serotype 9. However, we found a 123-bp *Alu*I fragment for both serotypes (Fig. 3). Difference 1 (Fig. 2) could be checked by *Afl*II digestion. The sequence of serotype 9 has the recognition sequence CTTAAG at positions 1179 to 1183, while this sequence is interrupted by an A residue in the reported sequence of serotype 1. Digestion of the PCR-amplified *apxICA* genes with *Afl*II demonstrated the presence of the *Afl*II site at positions 1179 to 1183 in both serotypes (data not shown).

Nucleotide sequences of the 3′ termini of the *apxIA* genes. We cloned the PCR products that were obtained from serotypes 5 and 10 by using oligonucleotides I and II (Fig. 1B). Since the major differences between the two *apxIA* genes are expected to be located at the distal end (see above), we sequenced the distal 420 bp of the *apxIA* gene, the *apx*IAB intergenic region, and the proximal 124 bp of the *apxIB* gene and found that serotype 5 and 10 sequences were identical but differed from the *apxIA* sequence of serotype 9 (Fig. 4A). The differences were all located within the distal 118 bp of the *apxIA* gene and in the *apxIAB* intergenic region. These nucleotide sequence data confirmed that two variants of the *apxIA* genes exist. The differences between the two *apxIA* variants are reflected in the amino acid sequences of the encoded ApxI proteins. Seventeen of the 37 distal amino acid residues differed between the two variants of the ApxIA protein (Fig. 4B).

We considered it likely that the presumed *apxIIA* pseudogene described by Chang et al. (5) and the *apxIA* gene of serotype 5 described in the present article are one and the same gene since both are located in front of the *apxIBD* genes. However, the published nucleotide sequence of the presumed *apxIIA* pseudogene differs at three sites from the distal part of the *apxIA* gene of serotype 5. One difference creates an *Eco*RI site in the presumed pseudogene, and the other two differences disrupt the open reading frame.

Characterization of the *apxICA* deletion in serotypes 2, 4, 6, 7, 8, and 12. To determine the exact position of the deletion in the *apxICA* genes, we sequenced the PCR fragments of approximately 700 bp that were obtained from serotypes 2, 4, 6, 7, 8, and 12 by use of oligonucleotides I and II (Fig. 1C). We found that the nucleotide sequences of these fragments were very similar among the serotypes. The structure of the fragment of serotype 7 is presented in Fig. 5. Starting at the priming site of oligonucleotide I, the nucleotide sequence matched the serotype 9 sequence till position −31. At that point, we found a fragment of 302 bp which did not match the *apxICA* sequences of serotype 9. This fragment consisted of 5 bp, which was followed by nine repeats of 16 bp with nucleotide sequence GATAGAAGAAATTATT and a stretch of 153 bp. Downstream from the 302 bp fragment, the nucleotide sequence again matched the *apxIA* sequence from position 3465 to the priming site of oligonucleotide II. The 153-bp sequence did not contain an open reading frame or obvious promoter-like sequences. For each of the serotypes, the same sequences were found. The only difference was the number of 16-bp repeats. Serotypes 2 and 8 had one repeat, serotype 4 had two repeats, serotype 12 had four repeats, serotype 6 had seven repeats, and serotype 7 had nine repeats.

To gain insight into the origin of the inserted sequences, we tested whether the 153-bp fragment was present elsewhere in the genomes of the 12 serotypes. The 153-bp fragment was labeled by PCR with oligonucleotides V and VI (Fig. 5) and hybridized to the blot shown in Fig. 1A. We found hybridization to the *Bgl*II fragments that also hybridized to the upstream C probe. The hybridization signals that were obtained with serotypes 5 and 10 were much weaker than the hybridization signals that were obtained with the other serotypes.

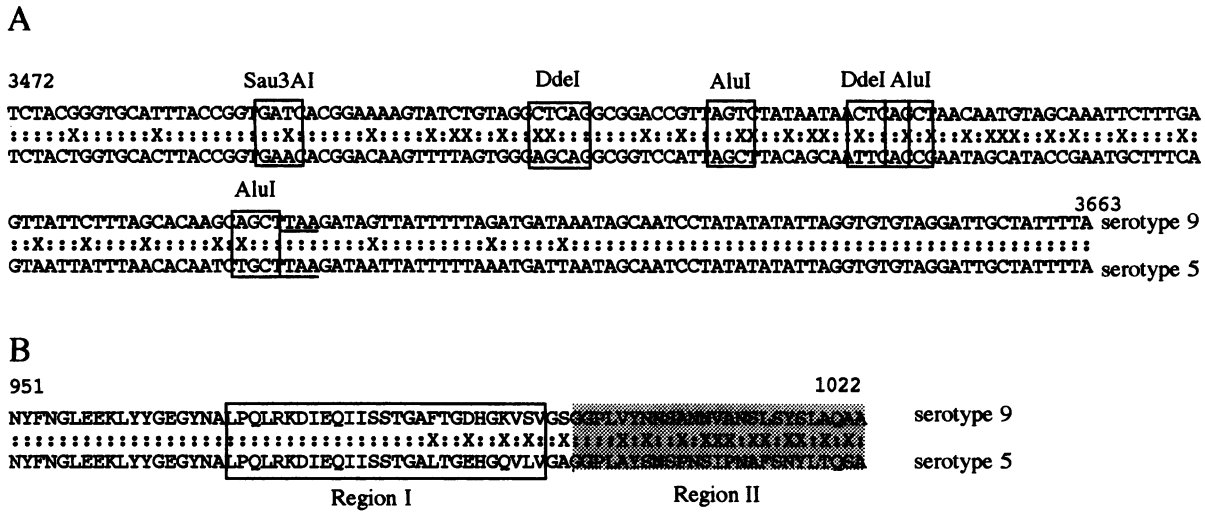

FIG. 4. (A) Alignment of the 3′ ends of the *apxIA* genes from positions 3472 to 3663 of serotypes 5 and 9. Identical nucleotides are indicated by a colon; different nucleotides are indicated by X. The recognition sites for *Sau*3AI, *Dde*I, and *Alu*I that are altered by the nucleotide sequence differences are indicated. The stop codons of the *apxIA* genes are underlined. (B) Comparison of the deduced protein sequences of the ApxIA variants from position 951 to the carboxy termini at position 1022. Identical amino acids are indicated by a colon; different amino acids are indicated by X. The potential region I (boxed) and the potential region II (shaded) are indicated.

We concluded that the ApxI operons of serotypes 2, 4, 6, 7, 8, and 12 are deleted from positions −34 to 3465. At the deletion site, sequences are present that are not related to the *apxICABD* genes but that are also present at least 1 kbp upstream of the intact ApxI operons.

Transcription of the ApxI operons. Since the ApxI operons of serotypes 2, 4, 6, 7, 8, and 12 have a major deletion, we wanted to determine whether these operons would still be functional and give rise to *apxIBD* transcripts. We tested this by hybridizing total bacterial RNA to the *apxIBD* sequences of serotype 9 (Fig. 1B, BD probe). The BD probe was amplified by PCR with oligonucleotides III and IV. RNA that hybridized to the BD probe was found in all serotypes except serotype 3 (Fig. 6A). An identical dot blot was hybridized to a 2.4-kbp *Xho*I-*Eco*RV fragment from the *apxICA* genes of serotype 9 (Fig. 1B, CA probe). As expected, we found hybridization only to the RNA from serotypes 1, 5, 9, 10, and 11. As a positive control for transcription and RNA isolation, we probed the dot blots to a 3,018-bp *Nde*I fragment of the *apxIICA* genes (apxIICA probe) of serotype 9 (Fig. 6C). Except for serotype 10, the *apxIICA* genes are present in all serotypes and have an identical structure, and ApxII is produced by all serotypes (14, 27). As expected, we found that all serotypes, except serotype 10, contained *apxIICA* transcripts. We tested whether the probes hybridized to RNA and not to contaminating chromosomal DNA. RNase treatment of the RNA preparations completely removed all hybridizing material, demonstrating that RNA and not DNA hybridized to the probes (data not shown).

DISCUSSION

In this study, we describe the nucleotide sequence of the *apxICA* genes of *A. pleuropneumoniae* serotype 9. We found that the nucleotide sequence of the *apxIA* gene of serotype 9 is almost identical to the nucleotide sequence of the *apxIA* gene of serotype 1. The published nucleotide sequences of these genes differ at only five positions (Fig. 2). At least two of these differences (differences 1 and 4) are due to cloning or sequencing artifacts. It is interesting to note that difference 4 would have resulted in a cysteine residue in the ApxIA protein of serotype 1. However, no cysteine residues have yet been found in other RTX toxins (20).

We found two variants of *apxIA* genes. One variant is present in serotypes 1, 9, and 11, and one variant is present

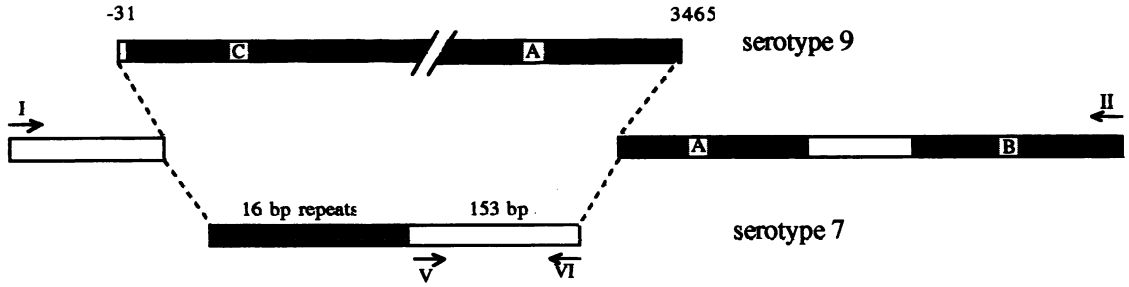

FIG. 5. Schematic presentation of a full-length and a partially deleted ApxI operon (not to scale). The operon regions that were amplified by use of oligonucleotides I and II on serotype 9 (full-length operon) or serotype 7 (partially deleted operon) are shown. The coding regions of the *apxICAB* genes and the 16-bp repeats are shaded. The positions of oligonucleotides I, II, V, and VI are indicated.

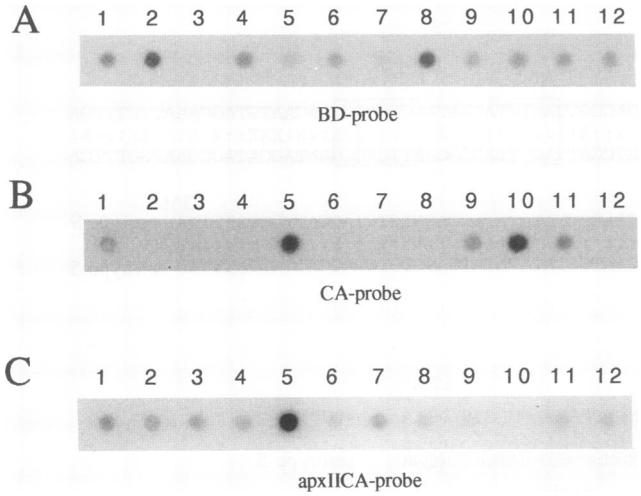

FIG. 6. Autoradiogram of Northern dot blots of RNA from serotypes 1 to 12. The blots were hybridized to the CA probe (A), the BD probe (B), and the apxIICA probe (C). Serotype numbers are indicated.

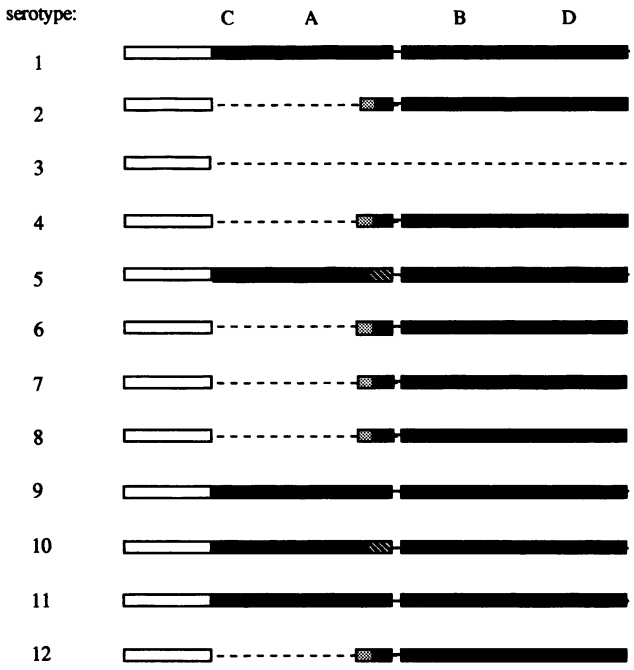

FIG. 7. Schematic overview of the ApxI operons of the reference strains of the 12 serotypes. Shaded blocks indicate the coding regions of the *apxICABD* genes, and open blocks indicate the upstream *apxIC* sequence. Stippled blocks indicate the inserted sequences in the partially deleted operons. The dashed lines indicate the absence DNA sequences. The hatched distal ends of the *apxIA* genes of serotypes 5 and 10 indicate the regions that differ most from the *apxIA* genes of serotypes 1, 9, and 11.

in serotypes 5 and 10. The hybridization data obtained with the 153-bp probe indicated that the variants also differ in the nucleotide sequences upstream of the ApxI operon. The 3′ ends of the *apxIA* genes of serotypes 5 and 10 are almost identical to the presumed *apxIIA* pseudogene described by Chang et al. (5). We have no evidence for the presence of an *apxIIA* pseudogene in the reference strain of serotype 5. It is most probable that the *apxIIA* pseudogene and the *apxIA* gene described in this article are one and the same gene. The nucleotide sequence differences may be due to cloning and/or sequencing artifacts or to the different serotype 5 strains that were used.

The two *apxIA* variants code for two different ApxIA proteins that differ mainly at their carboxy termini. Since it is well established that the carboxy termini of RTX toxins are essential for secretion, the two ApxIA variants may have different characteristics with respect to the secretion by the ApxIB and ApxID proteins. Despite extensive research, mainly on Hly RTX toxin of *E. coli*, there is no clear consensus what structural features define the secretion signal (10, 18, 28). Nevertheless, several regions and single amino acids that are important for secretion have been assigned (18, 28). For example, Stanley et al. assigned two regions within the carboxy terminus of Hly with structural features that are important for secretion. Region I consists of a potential amphiphilic helix, and region II is weakly hydrophobic, with a hydroxylated tail (28). The corresponding region I of ApxIA, as assigned by Stanley et al., and the corresponding region II of the ApxIA variants are indicated in Fig. 4B. Although 4 of the 17 differences are located in region I and 13 differences are located in region II, they probably do not affect the structural features of these regions, and therefore it might be that the ApxIA variants are functionally equal with respect to secretion. In region I, two of the differences, F to L and D to E at positions 986 and 989, are conservative. A mutation similar to the K-to-Q difference at position 992 with little effect on secretion was introduced by Kenny et al. in HlyA of *E. coli* (18). The S-to-L difference at position 994 may alter the amphipathic character but is located at the border of region I. Although the numerous differences in region II may alter the secondary structure of the protein, none of the differences strongly influences the weakly hydrophobic character and the hydroxylated tail of this region. Finally, the T and S residues at positions 1019 and 1021 of the serotype 5 ApxIA variant are also found in the hydroxylated tail of HlyA of *E. coli*. Apart from the secretion characteristics, the differences between the ApxIA variants may have an impact on their biological activity or on the immune response of the host to the toxins. Therefore, the existence of two ApxI variants should be considered in the development of universal subunit vaccines.

The organization of the ApxI operons, shown in Fig. 7, corresponds to the secretion pattern of ApxI by the serotypes (17). The serotypes having an intact ApxI operon secrete the ApxI protein, while the serotypes that do not have the operon (serotype 3) or have a partially deleted operon do not secrete ApxI. The deleted sequences of the *apxICA* genes are replaced by other sequences of which the 153-bp fragment originates from a location upstream of the intact ApxI operons. The origin of the 16-bp repeats is unknown. The inserted sequences do not point out a mechanism for the deletion and insertion events. In contrast to what has been suggested for the ApxII operons (1, 13), we have no evidence that mobile genetic elements played a role in the partial deletion of the ApxI operons.

The deletion of the *apxICA* genes apparently does not prevent transcription of the *apxIBD* genes since comparable levels of *apxIBD* mRNA are present in all serotypes that carry these genes. It is likely that the ApxIB and ApxID secretion proteins encoded by these mRNAs mediate the transport of RTX toxins other than ApxI, for example,

ApxII. Since all serotypes except serotype 10 contain ApxII operons that do not code for their own secretion proteins, it is likely that the ApxI secretion proteins secrete ApxI and ApxII. Thus, for serotypes that do not produce ApxI, the secretion of ApxII may be the sole function of the ApxI secretion proteins. The view that the ApxI operon complements the ApxII operon is supported by the work of Inzana et al., who generated a mutant of serotype 5 that, in contrast to the parent strain, did not secrete ApxI and ApxII (12). Genetic analysis of this mutant revealed that the ApxII operon was unaltered but that parts of the *apxIBD* genes were deleted (unpublished results).

Serotype 3 does not carry the *apxIBD* genes, and secretion of ApxII from the cells of this serotype should be mediated by another set of secretion proteins. Serotype 3 contains the secretion genes of the ApxIII operon (14), and we assume that in this serotype, the ApxIII secretion genes complement the ApxII operon. The ApxIII secretion genes are also found in serotypes 2, 4, 6, and 8, resulting in the interesting situation that in these serotypes two sets of secretion genes are present.

Characterization of the ApxI operons of the reference strains of *A. pleuropneumoniae* indicated that the three Apx operons interact with each other. For a complete understanding of the interactions, it will also be necessary to characterize the ApxIII operons in detail and to study genetic complementations in *A. pleuropneumoniae* itself. Insight into the interactions of the Apx operons may aid in understanding the role of each Apx toxin in the colonization of the host by *A. pleuropneumoniae*. After this article was submitted for publication, Frey et al. (7) published the structures of the ApxI operons of the 12 *A. pleuropneumoniae* serotypes. Their findings on the ApxI operon structures and *apxIA* polymorphisms agree with the data presented here.

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