Characterization of apxlIVA, a new RTX determinant of Actinobacillus pleuropneumoniae

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A fourth type of RTX determinant was identified in Actinobacillus pleuropneumoniae and was designated apxlIVA. When expressed in Escherichia coli, recombinant ApxlIVA showed a weak haemolytic activity and co-haemolytic synergy with the sphingomyelinase (beta-toxin) of Staphylococcus aureus. These activities required the presence of an additional gene, ORF1, that is located immediately upstream of apxlIVA. The apxlIVA gene product could not be detected in A. pleuropneumoniae cultures grown under various conditions in vitro; however, pigs experimentally infected with A. pleuropneumoniae serotypes 1, 5 and 7 started to produce antibodies that reacted with recombinant ApxlIVA 14 d post-infection, indicating that apxlIVA is expressed in vivo. In addition, sera from pigs naturally and experimentally infected with any of the serotypes all reacted with recombinant ApxlIVA. The apxlIVA gene from the serotype 1 A. pleuropneumoniae type strain Shope 4074 encodes a protein with a predicted molecular mass of 202 kDa which has typical features of RTX proteins including hydrophobic domains in the N-terminal half and 24 glycine-rich nonapeptides in the C-terminal half that bind Ca²⁺. The glycine-rich nonapeptides are arranged in a modular structure and there is some variability in the number of modules in the ApxlIVA proteins of different serotypes of A. pleuropneumoniae. The deduced amino acid sequences of the ApxlIVA proteins have significant similarity with the Neisseria meningitidis iron-regulated RTX proteins FrpA and FrpC, and to a much lesser extent with other RTX proteins. The apxlIVA gene could be detected in all A. pleuropneumoniae serotypes and seems to be species-specific. Although the precise role of this new RTX determinant in pathogenesis of porcine pleuropneumonia needs to be determined, apxlIVA is the first in vivo induced toxin gene that has been described in A. pleuropneumoniae.

Keywords: RTX toxin, porcine pleuropneumonia, serology, recombinant protein

INTRODUCTION

Actinobacillus pleuropneumoniae, the causative agent of porcine pleuropneumonia, is an important pathogen of swine. Twelve serotypes have been described which variously express three different proteinaceous cytotoxins belonging to the RTX toxin family: ApxI, ApxII and ApxIII (Frey et al., 1993, 1995). There are many lines of evidence that suggest that these toxins are important determinants of virulence (Frey, 1995; Reimer et al., 1995; Rycroft et al., 1991; Tascon et al., 1994; Kamp et al., 1997). ApxI, a strongly haemolytic and cytotoxic protein of 105 kDa, is secreted by the most virulent serotypes, 1, 5, 9, 10 and 11 (Beck et al., 1994). ApxII, a weakly haemolytic and moderately cytotoxic 105 kDa protein, is produced by all serotypes except for serotype

Abbreviation: DIG, digoxigenin-11-dUTP.
The GenBank accession numbers for the apxlIVA gene and 1304 bp of flanking sequences present in plasmid pJFFROK7, for the apxlIVA locus (pJFFROKS), and for the vector pETHS-1 are AF021919, AF030511 and AF012911, respectively.
10 (Chang et al., 1989; Kamp et al., 1991) whereas ApxIII, a protein of 120 kDa, is secreted by serotypes 2, 3, 4, 6 and 8. ApxIII differs from the other two toxins in that it does not possess any haemolytic activity, but it is strongly cytotoxic (Kamp et al., 1991). Co-haemolytic activity, known as the CAMP (Christie-Atkins-Munch-Petersen) effect, is associated with all three Apx toxins (Kamp et al., 1991; Macdonald & Rycroft, 1992; Frey et al., 1994; Jansen et al., 1995). The presence of particular Apx toxins is inherent to given serotypes of *A. pleuropneumoniae* and corresponds with the degree of virulence (Frey, 1995). With the exception of ApxII, which lacks the secretion genes, the operons of the Apx toxins are composed of four genes, the activator gene C, the structural toxin gene A and the secretion genes B and D. Homologues of ApxIA and ApxIIA have also been found in less virulent species, including *Actinobacillus suis* (ApxIVA, ApxIVA) (Burrows & Lo, 1992; Van Ostaaijen et al., 1997), *Actinobacillus lignieresii*, *Actinobacillus equuli* and *Actinobacillus rossii* (J. Frey unpublished results).

The RTX family of cytotoxins includes related pore-forming protein toxins found in many Gram-negative pathogens. They all have tandem repeated glycine-rich nonapeptides with the consensus sequence Leu/Ile/Phe-NAD medium was supplemented with either 0.1 mM FeCl₃ (iron-replete) or with 0.1 mM EDDA (iron-depleted); with 20 mM MgCl₂ (Mg²⁺-replete) or with 20 mM CaCl₂ (Ca²⁺-replete) or with 1 mM EGTA (Ca²⁺-depleted). Expression of ApxIVA was analysed from culture supernatants and cells from both exponential-phase and stationary-phase cultures. In addition, the expression of ApxIVA was assayed in media supplemented with 5 mM MnCl₂, 5 mM ZnCl₂, 10 mM KCl or 10 mM LiCl. The expression of ApxIVA was also measured under anaerobic conditions. For these experiments, *A. pleuropneumoniae* cultures were grown aerobically on Columbia agar (BBL) supplemented with 10 μg β-NAD ml⁻¹ (Sigma). For the ApxIVA expression assays, Columbia broth/β-NAD medium was supplemented with either 0.1 mM FeCl₃ (iron-replete) or with 0.1 mM EDDA (ethylenediamine di(o-hydroxyphenylacetic acid)) (iron-depleted); with 20 mM MgCl₂ (Mg²⁺-replete) or with 20 mM CaCl₂ (Ca²⁺-replete) or with 1 mM EGTA (Ca²⁺-depleted). Expression of ApxIVA was analysed from culture supernatants and cells from both exponential-phase and stationary-phase cultures. In addition, the expression of ApxIVA was assayed in media supplemented with 5 mM MnCl₂, 5 mM ZnCl₂, 10 mM KCl or 10 mM LiCl. The expression of ApxIVA was also measured under anaerobic conditions. For these experiments, *A. pleuropneumoniae* cultures were grown aerobically to an A₅₉₀ of 0.5, then incubated overnight in a nitrogen atmosphere. Cultures grown to mid-exponential phase (A₅₉₀ of 0.5) at 37°C then shifted to 42°C for 2 h were also assessed for ApxIVA expression. In addition, *A. pleuropneumoniae* serotype 1 strain Shope 4074₄ and serotype 3 strain HVI114 grown on chocolate blood agar, trypticase soy agar and PPLO broth (Difco) were assayed for ApxIVA. For these experiments, cells were collected from solid media in sterile 140 mM NaCl and suspended at a concentration of 10⁸ cells ml⁻¹. *Southern blotting.* Chromosomal DNAs were extracted by a guanidine thiocyanate method (Pitcher et al., 1989). ClaI-digested DNAs were analysed for the presence of RTX toxin genes and for the production of labelled gene probes.

**METHODS**

**Bacterial strains, vectors and growth conditions.** The *A. pleuropneumoniae* type and reference strains used in this study (Table 1) have been described elsewhere (Frey & Nicolet, 1990). *A. pleuropneumoniae* HVI14 is a virulent serotype 3 field strain that was isolated from a pig with severe pleuropneumonia. HVI14 shows the typical serotype 3 pattern of apx toxins (i.e. apxIICA and apxIIACABD) (Beck et al., 1994). *A. lignieresii* ATCC 49236, *A. equuli* ATCC 19392, *A. rossii* ATCC 27072, *A. suis* ATCC 15538 and serotype 1 *P. haemolytica* ATCC 14003 (Table 1) were purchased from the American Type Culture Collection, Manassas, VA, USA. *Actinobacillus minor* PNDN33, *Actinobacillus porcinus* NM319 and *Haemophilus sp.* 'minor group' strain 202 (Moller et al., 1996) were from the collection of S. Rosendal, University of Guelph, Ontario, Canada. The *E. coli* K-12 hosts XL-1 Blue (Stratagene) and HMS174(DE3), and *E. coli* B strain BL21(DE3) (Novagen) were used for cloning. Plasmid pETHis-1 was constructed by annealing a 90 bp oligonucleotide encoding a multiple cloning site and a carboxy-terminal histidine decamer tail with NdeI/ClaI-digested pET14b (Novagen). It allows the expression of fusion proteins with a N-terminal histidine hexamer and/or a C-terminal histidine decamer. *E. coli* was grown at 37°C for the production of Luria–Bertani broth or on Luria–Bertani broth supplemented when necessary with ampicillin (50 μg ml⁻¹) for selection and maintenance of recombinant plasmids, and additionally with 125 μM X-Gal for white-blue selection with pBluescript II SK(+) (Stratagene). The CAMP test for co-haemolysis (Christie et al., 1944) was performed on trypticase soy agar medium (BBL Microbiology Systems) containing 5% sheep blood and 10 mM CaCl₂ using a beta-haemolytic *Staphylococcus aureus* strain for the production of the diffusion zone of sphingomyelinase (beta-toxin) as described previously (Frey et al., 1994). In order to visualize the direct haemolysis of recombinant *E. coli* strains producing active apxIVA, the cells were removed with a spatula from the blood agar plate after growth.

**In vitro expression of apxIVA.** The presence of ApxIVA in both cells and in 10× concentrated supernatants was evaluated on immuno blots (see below). *Actinobacillus* and *Pasteurella* species were grown in Columbia broth or on Columbia agar (BBL) supplemented with 10 μg β-NAD ml⁻¹ (Sigma). For the ApxIVA expression assays, Columbia broth/β-NAD medium was supplemented with either 0.1 mM FeCl₃ (iron-replete) or with 0.1 mM EDDA (ethylenediamine di(o-hydroxyphenylacetic acid)) (iron-depleted); with 20 mM MgCl₂ (Mg²⁺-replete) or with 20 mM CaCl₂ (Ca²⁺-replete) or with 1 mM EGTA (Ca²⁺-depleted). Expression of ApxIVA was analysed from culture supernatants and cells from both exponential-phase and stationary-phase cultures. In addition, the expression of ApxIVA was assayed in media supplemented with 5 mM MnCl₂, 5 mM ZnCl₂, 10 mM KCl or 10 mM LiCl. The expression of ApxIVA was also measured under anaerobic conditions. For these experiments, *A. pleuropneumoniae* cultures were grown aerobically to an A₅₉₀ of 0.5, then incubated overnight in a nitrogen atmosphere. Cultures grown to mid-exponential phase (A₅₉₀ of 0.5) at 37°C then shifted to 42°C for 2 h were also assessed for ApxIVA expression. In addition, *A. pleuropneumoniae* serotype 1 strain Shope 4074₄ and serotype 3 strain HVI114 grown on chocolate blood agar, trypticase soy agar and PPLO broth (Difco) were assayed for ApxIVA. For these experiments, cells were collected from solid media in sterile 140 mM NaCl and suspended at a concentration of 10⁸ cells ml⁻¹. *Southern blotting.* Chromosomal DNAs were extracted by a guanidine thiocyanate method (Pitcher et al., 1989). ClaI-digested DNAs were analysed for the presence of RTX toxin genes and for the production of labelled gene probes.

**Cloning of apxIVA loci.** Plasmid DNAs were isolated by an alkaline lysis method (Ausubel et al., 1990). Partial gene libraries were prepared by cloning gel-purified ClaI fragments in pBluescript II SK(−) using conventional techniques (Ausubel et al., 1990). Recombinant plasmids were screened by colony blot assay (Ausubel et al., 1990) using the probe for the 3' end of apxIVA as described below (Fig. 1).

**PCR and production of labelled gene probes.** PCR was carried out with a DNA thermal cycler (GeneAmp 9600; Perkin Elmer Cetus) in 50 μl reaction mixes containing 10 mM Tris/HCl pH 8.3, 1.5 mM MgCl₂, 50 mM KCl, 0.005%
Table 1. Hybridization of apxIVA probes and PCR amplifications of apxIVA in different A. pleuropneumoniae strains and related species

<table>
<thead>
<tr>
<th>Species</th>
<th>Serotype/group</th>
<th>Strain</th>
<th>Hybridization</th>
<th>PCR amplification*</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>apxIVA 5' part</td>
<td>apxIVA central part</td>
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<tr>
<td>A. pleuropneumoniae</td>
<td>1†</td>
<td>Shope 4074T</td>
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<tr>
<td>A. pleuropneumoniae</td>
<td>2†</td>
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<td>A. porcinus</td>
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<tr>
<td>Haemophilus sp.</td>
<td>&quot;Minor group&quot;</td>
<td>202</td>
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<td>P. haemolytica</td>
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<td>Serotype 1</td>
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* Amplification with primer pairs APX4UP-L/APX4UP-R, APX4DWN-L/APXIVA-1R, and APXIVA-1L/APXIVA-1R.
† Reference strain.
† Weak hybridization signal.

Tween 20, 170 µM of each dNTP, 0.25 µM forward and reverse primers, 0.5 units Taq polymerase and 5 mg template DNA. The DNAs were amplified for 35 cycles (30 s denaturation at 94 °C, 30 s at the optimal annealing temperature of the primers (Table 2), and 30 s extension at 72 °C). When DNA fragments were produced by PCR for subsequent cloning and expression, the elongation steps were increased to 2 min at 72 °C, and Pfu polymerase with proofreading capacity was used instead of Taq polymerase. In addition, an extension step of 7 min at 72 °C was added at the end of the last cycle in order to ensure full-length synthesis of the different fragments.

DIG-labelled probes for the 5' terminal and the 3' terminal regions of apxIVA (Fig. 1) were made by PCR amplification using genomic DNA of the serotype 1 strain Shope 4074T and the oligonucleotide primer pairs APPIV1L-APPIV1R and APXIVA-1L/APXIVA-1R, respectively. Amplification reactions were carried out as described above except that the PCR mixtures were supplemented with 50 µM DIG. A probe for the central part of apxIVA was obtained by amplifying the 2.2 kb BamHI-Nrnl fragment of plasmid pJFFROK5 cloned in pBluescript II SK(−) using primers complementary to the T2 and T3 promoter sequences in the vector. The hybridization signals were visualized using a chemiluminescent DIG-detection kit according to the manufacturer's instructions (Boehringer Mannheim).

Nucleotide sequencing and analysis. The nucleotide sequences of apxIVA var1 and apxIVA var3 were determined with an Applied Biosystems DNA Sequenator AB373 using the Taq Dye Deoxy Terminator Cycle kit (Applied Biosystems/Perkin Elmer) and oligonucleotide primers complementary to the T3 and T7 promoters flanking the cloning site of pBluescript II SK(−). Deletion subclones for sequence analysis were obtained by exonuclease III digestion with the 'double-stranded' Nested Deletion Kit (Pharmacia Biotech). The complementary strand was sequenced using internal primers which were deduced from the sequence of the first strand. The nucleotide and deduced amino acid sequences were analysed using the PSORT programs (Bairoch et al., 1995) and PSORT (Nakai & Kanehisa, 1991). Comparisons with sequences in the GenBank/EMBL and NBRF databases were made using the BLAST programs (Altschul et al., 1990).

Production of poly-histidine-tailed fusion proteins. In order to construct plasmids encoding poly-histidine-tailed ApxIVA fusion proteins, we amplified various segments of apxIVA var1 and apxIVA var3 and full-length apxIVA var1 from genomic DNA of strains Shope 4074T and HV114, respectively, using the primers which contained restriction enzyme recognition sites (Table 2). The PCR products were purified from agarose gels and subsequently digested with Ndel and BamHI, or Ndel and EcoRI for cloning into pETHS-1 or pBluescript II SK(−) (Stratagene). The constructs were analysed by restriction
Table 2. Oligonucleotide primers

<table>
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<tr>
<th>Primer name</th>
<th>Oligonucleotide sequence 5'-3'</th>
<th>Position†</th>
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<td>APXIVA-1L</td>
<td>TGGCACCTGACCGGTCTATGAT</td>
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<td>APXIVA-1R</td>
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<td>6459-6442</td>
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<td>APX4UP-L</td>
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<td>539-556</td>
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<tr>
<td>APX4UP-R</td>
<td>CCCCTCGATTTTTCGCGGGGGGG</td>
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<tr>
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<td>6504-6488</td>
<td>54</td>
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</tbody>
</table>

* Lower case letters indicate the nucleotides that were added in order to create restriction enzyme recognition sites (underlined) for cloning.
† Nucleotide positions in AFO21919 (start of apxlVA is at 1132).
‡ Annealing temperature in °C.

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Fig. 1. Physical and genetic maps of plasmids pJFFROK5 and pJFFROK7 containing apxlVA<sub>var3</sub> and apxlVA<sub>var1</sub>, respectively. Open boxes with arrowheads indicate ORFs; putative rho-independent transcription termination signals are indicated by hairpins; the locations of putative promoter sequences are indicated by filled triangles; the promoter of the vector pBluescript II SK(-) is shown as an open triangle; filled arrows below the maps indicate the positions of the oligonucleotide primer sequences used for the production of DNA probes or for the detection of the apxlVA gene in relation to the insert in pJFFROK7; and at the bottom of the figure the locations of the DNA probes in relation to the map of pJFFROK7 are indicated.

enzyme digestion and partial DNA sequencing, then introduced by transformation into E. coli HMS174(DE3) or E. coli BL21(DE3) for expression of the fusion proteins. Unless marked specifically, vector pETHIS-1 added poly-histidine tags to both N'- and C'-termini of the peptides, which facilitated efficient purification by Ni<sup>2+</sup> chelate affinity
apxIVA, a new RTX determinant

Fig. 2. Predicted structures of ApxIVA\textsubscript{var1} and ApxIVA\textsubscript{var3}. The upper part of the figure shows the general structures: the hydrophobic domains (hatched region) and potential acylation sites (marked GK). The iterated glycine-rich nonapeptides are indicated by filled triangles and the open triangles show the positions of the DNA-polymerase family 2 signature sequences. The boxes drawn with dashed lines show the locations of the poly-histidine-tailed fusion proteins and the filled arrows indicate the positions of the oligonucleotide primers that were used for their construction. The lower part of the figure shows the details of C-terminal regions of ApxIVA\textsubscript{var1} and ApxIVA\textsubscript{var3}. The boxes 1A–1E, 2A, 2B, 3A and 3B represent the three different repeated modules which build up the C-terminal half of ApxIVA. The site of the deletion of a short peptide of 9 aa within module 1C of ApxIVA\textsubscript{var3}, as compared to ApxIVA\textsubscript{var1}, is indicated by dotted lines.

chromatography under strong denaturation conditions, which were necessary to keep these peptides soluble during the process.

Full-length poly-histidine-tailed ApxIVA was expressed from plasmid pJFFapxIVA1His2. This plasmid was constructed by amplifying a PCR fragment with primers APX4115-L/ASCH3 from genomic DNA of strain Sype 4074T and cloning this PCR fragment into the NdeI and EcoRI sites of vector PETHIS-1. The plasmid encoding the poly-histidine-tailed N-terminal half of ApxIVA was constructed using the primers APX4IIL3-L/APX4IIL3B-R (Table 2) and genomic DNA of strain Sype 4074T as template. The resultant plasmid was named pJFFapxIVA1NaHis1 and expressed the 96 kDa N-terminal half of ApxIVA, designated ApxIVAN', in E. coli BL21(DE3) (Fig. 2). Plasmid pJFFapxIVA3CHis encoding the poly-histidine-tailed C-terminal half of ApxIVA was constructed using the primers APXIVAHi51-L/APXIVAHi51-R (Table 2) and genomic DNA of strain HV114. The peptide produced from this construct was designated ApxIVAC' (80 kDa).

Full-length C-terminally His-tailed ApxIVA (named ApxIVA-10xHis) was obtained from plasmid pJFFapxIVA1HisC', which was constructed by PCR amplification of the 5' half of apxIVA using pJFFROK7 as template and oligonucleotide primers APXIVAHi5C'-R and APXIVAORFl-L (Table 2), subsequent digestion of the PCR fragment with XbaI and KpnI and replacement with the corresponding XbaI-KpnI fragment in plasmid pJFFapxIVA1His2. Plasmid pJFFORFlapxIVA1His2, which allows the co-expression of ORFl and ApxIVA-10xHis, was constructed by PCR amplification of ORFl and the 5' half of apxIVA using pJFFROK7 as template and oligonucleotide primers APXIVAORFl-L and APXIVAORFl-R, subsequent digestion of the PCR fragment with XbaI and KpnI and replacement with the corresponding XbaI-KpnI fragment in plasmid pJFFapxIVA1His2. Finally, the plasmid for the expression of ORFl alone as a control was obtained by deleting a 4382 bp EcoRV fragment in pJFFORFlapxIVA1His2. This deletion removed most of the apxIVA gene but left ORFl intact. The plasmid constructions were verified by DNA sequence analysis using apxIVA internal primers and primers matching the sequences flanking the multiple cloning site of PETHIS-1.

The production of the various ApxIVA fusion proteins was induced by the addition of 1 mM IPTG at mid-exponential phase and incubation for a further 2.5 h. Following induction, the fusion proteins were purified from cell extracts dissolved with 6 M guanidine hydrochloride using Ni\textsuperscript{2+} chelate affinity chromatography (Qiagen) according to the manufacturer's instructions. The bound fusion proteins were eluted by slowly decreasing the pH from 8.0 to 5.0 with 50 mM potassium phosphate buffer, 300 mM NaCl, 6 M guanidine hydrochloride. Following elution at pH 5.0, the fusion proteins were dialysed against 50 mM phosphate buffer, 300 mM NaCl, pH 8.0.

Production of antiserum and immunoblotting. Monospecific polyclonal antisera directed against the poly-histidine-tailed fusion proteins ApxIVA, ApxIVA' and ApxIVAC', were obtained by immunization of rabbits with 100 \mu g of the
purified recombinant proteins mixed 1:1 with complete Freund's adjuvant (Difco) followed by a booster immunization 3 weeks later containing 100 μg protein and incomplete Freund's adjuvant. Sera were collected 10 d after the second immunization. Hyperimmune rabbit sera directed against ApxI purified from serotype 10 reference strain 13039, ApxII purified from serotype 3 reference strain S1421, were prepared as described previously (Frey & Nicolet, 1991). Convalescent field sera from A. pleuropneumoniae serotype 1 infected pigs were obtained from R. Nielsen, Copenhagen, Denmark (Frey & Nicolet, 1991). Sera from pigs experimentally infected with the different A. pleuropneumoniae serotype reference strains were obtained from R. Nielsen, Copenhagen, Denmark (Frey & Nicolet, 1991).

Purified proteins, total cell preparations, or culture supernatants were mixed with an equal volume of SDS sample buffer (62.2 mM Tris/HCl pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.005% bromophenol blue) and boiled for 10 min. Proteins were separated by electrophoresis on 10% or on 5–15% gradient SDS-polyacrylamide gels and immunoblot analysis was performed as described by Ausubel et al. (1990). Pig sera were used at a dilution of 1:100 and rabbit sera at a dilution of 1:1000. Bound antibodies were visualized by using phosphate-labelled goat antibodies directed against pig IgG or rabbit IgG, respectively (Kirkegaard Perry).

**Ca**²⁺-binding assay. Proteins were separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes as described above. The membranes were then soaked in calcium-binding buffer (60 mM KCl, 5 mM MgCl₂, 10 mM imidazole hydrochloride, pH 7.2) for 10 min (Levitsky et al., 1994). Following washing, the membranes were incubated in binding buffer supplemented with 10 μCi ml⁻¹ of ⁴⁵Ca²⁺ [0.02 mM MgCl₂ (0.74 MBq μl⁻¹) of ⁴⁵CaCl₂; Amersham] for 20 min, then rinsed twice with deionized water for 5 min, dried at room temperature and visualized by autoradiography.

**RESULTS**

Detection and cloning of the apxIVA determinant

Limited nucleotide sequence analysis of the region downstream of the A. pleuropneumoniae CM5 lacZ gene revealed an ORF containing several glycine-rich nonapeptide sequences which shared homology with the C-termini of the frpA and frpB genes of N. meningitidis (Anderson & Maclnnes, 1997). The presence of this fourth RTX determinant, named apxIVA, was confirmed by hybridizing ClaI-digested genomic DNA of the serotype 1 A. pleuropneumoniae strain Shope 4074 with broad-range probes designed for screening for RTX genes (Kuhnert et al., 1997), which showed an 8 kb ClaI fragment hybridized to the frpA gene probe in addition to the predicted fragments that hybridized to the apxIA and apxIIA gene probes. In serotype 3 strain HV114, a 7 kb ClaI fragment hybridized to the frpA probe. These same ClaI fragments also hybridized to a probe made by PCR amplification of a 440 bp fragment located downstream of the lacZ gene of strain Shope 4074 using oligonucleotide primer pair APXIVA-1L/APXIVA-1R (Fig. 1). The 8 kb ClaI fragment of serotype 1 strain Shope 4074 was cloned into vector pBluescript II SK(−), resulting in plasmid pJFFROK7. Similarly, a 7 kb ClaI fragment of a virulent serotype 3 strain HV114 was cloned into pBluescript II SK(−), resulting in plasmid pJFFROK5 (Fig. 1).

**Nucleotide sequence analysis of the apxIVA loci**

Plasmid pJFFROK7 contains an ORF starting with an ATG start codon that encodes a protein of 1805 aa with a predicted molecular mass of 20130.4 Da and a theoretical pl of 4.81. This ORF is preceded 8 bp upstream of the putative start codon by sequences (GAGAGA) that share homology with the consensus sequence for a ribosome-binding site (RBS). The predicted amino acids of this ORF have significant sequence similarity with the iron-regulated RTX proteins of N. meningitidis, FrpA and FrpC (Thompson et al., 1993, 1994). The central 300 amino acids of the peptide showed highest similarity to FrpA and FrpC, with 42% identical and 50% identical plus similar amino acid residues. In addition, sequence similarity was found with all other known members of the RTX toxin family. In keeping with conventions (Frey et al., 1993), we designated this RTX determinant apxIVA_var1 and its gene product ApxIVA_var1 (Fig. 1). The apxIVA_var1 gene is preceded immediately upstream by a 474 bp ORF (ORF1) which encodes a protein of 157 amino acids with a calculated molecular mass of 18804.4 Da and a theoretical pl of 6.15 (Fig. 1). ORF1 is preceded by a putative RBS 7 bp upstream of an ATG codon. ORF1 has no similarity to any reported protein. Upstream of ORF1, there is a 105 bp segment containing several sequences with homology to canonical −35 and −10 promoter sequences. This 105 bp segment is preceded by a stem–loop structure (ΔG = 15 kcal), which could be a rho-independent transcription termination signal (Fig. 1).

Upstream of this stem–loop structure we identified an ORF with significant similarity to the C-terminal end of the E. coli methionine-rich protein MRP (mrp gene product). A putative rho-independent terminator (ΔG = 24 kcal) is also present between the 3′ ends of apxIVA_var1 and lacZ (Fig. 1).

The nucleotide sequence of the insert in pJFFROK7 containing apxIVA_var1 (serotype 3) is virtually identical to the corresponding segment in pJFFROK7. The only significant difference is a 825 bp deletion in the 3′ end of apxIVA_var1 (Fig. 1). The predicted molecular mass of ApxIVA_var3, which contains 1522 aa residues, is 170157 Da and the protein has a theoretical pl of 4.93.

**Structural features of ApxIVA_var1 and ApxIVA_var3**

The predicted amino acid sequences of the first three quarters of ApxIVA_var1 and ApxIVA_var3 are identical except for 11 aa. Both ApxIVA_var1 and ApxIVA_var3 have strongly hydrophobic domains stretching from aa 100 to aa 600 (Fig. 2). Putative lysine acylation sites (GK) resembling those found in the HlyA of E. coli (Stanley et al., 1994) are present (aa 642–643 and aa 853–854).

The C-terminal part of ApxIVA_var1 contains 24 glycine-rich nonapeptides with the consensus sequence L/V-X-G-X-G-N/D-D-X (Felmlee et al., 1985). These
apxIVA, a new RTX determinant

Fig. 3. Haemolytic and co-haemolytic activity of ApxIVA on trypticase soy agar medium containing 5% washed sheep blood and 10 mM CaCl₂. A, Vertical streak of Staphylococcus aureus; B, diffusion zone of sphingomyelinase (β-toxin) secreted by S. aureus. Horizontal streaks: E. coli HMS174(DE3) harbouring on expression vector pETHIS-1 the genes apxIVA-10xHis (1), ORF1 plus apxIVA-10xHis (2), and ORF1 (3). The zone of bacterial growth with the bacteria present (C) and the zone where the grown bacteria were removed (D) are shown.

Expression and functional analysis of recombinant ApxIVA

Recombinant, poly-histidine-tailed ApxIVA or partial peptides ApxIVAN' and ApxIVAC' were obtained from E. coli cells harbouring the expression vector pETHIS-1 containing the entire apxIVA gene or parts thereof and were purified as described in Methods. Purified histidine-tailed ApxIVA was used for immunization of rabbits in order to obtain anti-ApxIVA antiserum. The partial peptides ApxIVAN' and ApxIVAC' were obtained at significantly higher yields than the full-sized ApxIVA. Purified ApxIVAN' was quite stable in solution when kept at 4 °C or frozen at −20 °C but full-sized ApxIVA and ApxIVAC' showed some degradation, which resulted in additional minor bands on immunoblots (see Figs 4, 5 and 6).

Weak haemolysis and a co-haemolytic effect, known as the CAMP effect (Christie et al., 1944), were seen on blood agar plates when C-terminally histidine-tailed ApxIVA-10xHis together with ORF1 (Fig. 1) was produced in E. coli (Fig. 3). The CAMP effect did not show a classical arrow-shaped appearance as produced by Streptococcus agalactiae (Christie et al., 1944) but was round-shaped as were other RTX toxins (Frey et al., 1994; Jansen et al., 1995). Haemolysis was only seen when the cells were removed from the blood agar plate and showed no diffusion zone (Fig. 3). No haemolysis or co-haemolytic (CAMP) effect was seen when ApxIVA-10xHis was produced in the absence of ORF1 or when ORF1-encoded peptide was produced alone. The haemolysis and the co-haemolytic activities were the same on both sheep and swine erythrocytes. Purified ApxIVA-10xHis (produced in the presence of ORF1) did not retain haemolysis or co-haemolytic activity, probably due to protein denaturation by the harsh purification conditions used (requiring 6 M guanidine hydrochloride).

In blotting experiments we found that ⁴⁵Ca²⁺ bound recombinant ApxIVAC' peptide but not the ApxIVAN', confirming the Ca²⁺-binding of the part of the protein containing the glycine-rich repeats (Fig. 4). In a control strip from the same blot, the presence of both peptides (the N-terminal hydrophobic part and the C-terminal part with the glycine-rich repeats) could be demonstrated on the basis of their reaction with anti-ApxIVA antibodies (Fig. 4).
Expression of ApxIVA in *A. pleuropneumoniae* and antigenic specificity

Polyclonal anti-ApxIVA antibodies bound strongly to recombinant ApxIVA. However, no reaction with anti-ApxIVA was observed when total cells or with culture supernatants of any of the 12 serotype reference strains grown in PPLO medium with 5% horse serum were tested by immunoblotting (data not shown). In an attempt to induce *apxIVA* expression *in vitro*, *A. pleuropneumoniae* Shope 4074 and *A. pleuropneumoniae* HV114 were grown under a wide range of culture conditions (as described in Methods) and analysed by immunoblotting. No protein bands reacting with polyclonal anti-ApxIVA antibodies were detected when total cells or culture supernatant of *A. pleuropneumoniae* grown under any of culture conditions was analysed. However, sera from pigs which were experimentally infected with *A. pleuropneumoniae* serotypes 1, 5 and 7 reacted with recombinant ApxIVA as well as with the N-terminal and C-terminal halves of ApxIVA (Fig. 5). Whereas the sera taken 14 d post-infection reacted weakly (reactions for certain sera are hardly visible on immunoblots), sera taken 120 d post-infection showed strong reactions to ApxIVA and its N' and C'-halves. The peptides ApxIVAN' and ApxIVAC' showed similar reactions to full-length ApxIVA on immunoblots. Sera taken before infection at day 0 showed no reactions at all to ApxIVA (Fig. 5). These results demonstrate that infection of pigs with *A. pleuropneumoniae* induces antibodies against ApxIVA. Sera from pigs experimentally infected with each of the 12 serotypes of *A. pleuropneumoniae* strongly reacted with recombinant ApxIVAN' and ApxIVAC' (Fig. 6) showing that ApxIVA is produced by all 12 serotypes during infection.

Sera from pigs naturally infected with *A. pleuropneumoniae* serotype 1 or serotype 2 also reacted strongly with recombinant ApxIVAN' and ApxIVAC' whereas sera from *A. pleuropneumoniae*-negative pigs did not react with any of these antigens, nor did a strongly positive serum from an *A. suis*-infected pig (Fig. 6).

No cross-reaction of anti-ApxIVA was detected with purified ApxI, ApxII or ApxIII (Fig. 7a). Conversely, antisera to ApxI, ApxII and ApxIII reacted with purified ApxI, ApxII and ApxIII, respectively, but did not bind ApxIVA (Fig. 7b, c, d), showing that anti-ApxIVA antibodies were specific. Similarly, serum from a pig that was experimentally infected with *A. suis* (known to produce ApxI and ApxII) reacted strongly with ApxI and ApxII but not with ApxIII or ApxIVA (Fig. 7e).

Presence of *apxIVA* in *A. pleuropneumoniae* reference strains and related species

The presence of *apxIVA* or homologous sequences in the different serotype reference strains of *A. pleuropneumoniae* as well as in related species used in this study was analysed by Southern blot hybridization (Table 1). *ClaI*-digested genomic DNA was hybridized with three DIG-labelled probes representing the 5' terminal, the central and the 3' terminal part of *apxIVA* (Fig. 1). The probe for the
**Fig. 6.** Serological reactions of recombinant ApxlVA fusion proteins with sera from pigs infected with *A. pleuropneumoniae*. Immunoblots containing ApxlVAN’ and ApxlVAC’ were incubated with convalescent serum from pigs experimentally infected with *A. pleuropneumoniae* serotype reference strains (lanes 1–12), with field sera from naturally infected pigs with *A. pleuropneumoniae* serotype 1 (lanes 88–1368/3, 88–1209/14, 88–1209/58, 88–1356/675, 88–1209/35) or with serotype 2 (lanes 1925 Bre, 1926 Bre, 1930 Bre, 1935 Bre, 1765 Jen). As controls, immunoblots were reacted with sera from healthy pigs (lanes Ko91-446-Ko91-447 and Kel. 1-Kel. 4), with a strong *A. suis*-positive pig serum (lane *A. suis*), and with rabbit serum against purified recombinant ApxlVA (lane Ctr). The sizes of molecular mass markers (Std) are indicated in kDa.

**Fig. 7.** Antigenic specificity of ApxlVA. (a) Immunoblot containing purified recombinant ApxlVA (lane ApxlVA), total culture antigens of *A. pleuropneumoniae* serotype 1 strain 4074T grown in PPLO broth (lane sero 1), total culture antigen of serotype 2 strain S1536 (lane sero 2), purified Apxl (lane Apxl), purified Apxll (lane Apxll) and purified Apxlll (lane Apxlll) incubated with polyclonal anti-ApxlVA antibody. Note that in order to verify the absence of serological cross-reactions, five times more Apxl, Apxll and Apxlll proteins than ApxlVA was used. (b–e) Immunoblots containing: Apxl and ApxlVA incubated with polyclonal anti-Apxl antibody (b); Apxll and ApxlVA incubated with polyclonal anti-Apxll antibody (c); Apxlll and ApxlVA incubated with polyclonal anti-Apxlll antibody (d); and Apxl, Apxll, Apxll and ApxlVA incubated with serum from an *A. suis*-infected pig (e). The sizes of prestained molecular mass markers (Std) are indicated in kDa.
central part of apxIVA hybridized to a ClaI fragment ranging between 6 and 10 kb in all serotypes. Serotypes 7, 8 and 10 showed a second band of approximately 5 kb, indicating the presence of a ClaI site in apxIVA in these strains. A 4 kb fragment of A. lignieresii also hybridized weakly to this probe, whereas none of the other species tested showed any reaction. The probe made to the 5' part of apxIVA hybridized to the same ClaI fragments of all serotype reference strains except for serotypes 7, 8 and 10, where it only reacted with the 5 kb fragment (Table 1). The probe made to the 3' sequence of apxIVA only reacted with DNA of A. pleuropneumoniae strains (Table 1).

Using primer pair APX4UP-L/APX4UP-R matching the 5' part of apxIVA (Fig. 1), a 36 kb fragment was amplified by PCR from all A. pleuropneumoniae type and reference strains. When the same strains were analysed with the primer pair APX4DW-L/APXIVA-1R, fragments of 16, 20, 24 or 28 kb were amplified. PCR amplification with the primer pair APXIVA-1L/APXIVA-1R gave rise to a 440 bp fragment in all A. pleuropneumoniae reference strains and in HV114. In addition, amplification of the 440 bp fragment was also obtained when 97 field strains of A. pleuropneumoniae representing all serotypes and isolated from several different countries worldwide were tested using primers APXIVA-1L and APXIVA-1R (data not shown). No product was detected following PCR amplification of DNAs from any of the other species examined, including A. lignieresii, using any of the primer pairs (Table 1).

DISCUSSION

The gene encoding ApxIVA, a new RTX determinant of A. pleuropneumoniae, was cloned and characterized from the A. pleuropneumoniae serotype 1 strain Shope 4074' and from the serotype 3 field strain HV114. In both strains, the apxIVA genes are preceded by, and may be co-transcribed with, ORF1. ORF1 is flanked upstream by the mrp gene locus; downstream of apxIVA there is a lacZ gene which is divergently transcribed (Fig. 1). When apxIVA is expressed together with ORF1 on an expression vector in E. coli, it confers weak haemolytic activity and a co-haemolytic (CAMP) effect. In this respect ApxIVA most closely resembles ApxII (Frey, 1995). The fact that the haemolytic activity of the recombinant ApxIVA-10xHis is only visible directly below the growing cells and that the CAMP effect occurs only in a relatively narrow diffusion zone around the growing cells may be due to the lack of active secretion of ApxIVA in E. coli, to the presence of additional His residues, to the low diffusion capacity of this large protein, or some combination of these factors. When ORF1 or apxIVA are expressed individually in E. coli, neither CAMP nor direct haemolytic effects are detected. These effects are therefore not due to activation of cryptic E. coli host-specific functions.

The predicted protein sequence of ApxIVA shows significant similarity to the N. meningitidis iron-regulated RTX exoproteins FrpA and FrpC (Thompson et al., 1994). Like FrpA and FrpC, the operons encoding ApxIVA contain an ORF (ORF1) upstream of the apxIVA gene which is similar in size to the ORF of unknown function found upstream of frpA and frpC, but shares no significant homology with it. The ORF1 gene upstream of apxIVA has no similarity with any known protein, including hlyC gene homologues that are generally found upstream of the structural (A) genes of RTX toxins (Welch, 1991; Welch et al., 1995). ORF1, however, seems to be involved in activation of ApxIVA and is required for the observed haemolytic and co-haemolytic (CAMP) phenotypes. It must be noted that ApxIVA contains two canonical lysine acylation sites (Fig. 2) resembling those which are the targets of HlyC-mediated activation of HlyA in E. coli (Stanley et al., 1994).

ApxIVA has structural characteristic features of RTX proteins, including N-terminal hydrophobic domains, signature sequences for potential acylation sites and repeated glycine-rich nonapeptide repeats, DNA polymerase 2 signature sequences, whose role is unknown, are also found as repeats in this region and form together with nonapeptide repeats a modular structure (Fig. 2). Sequencing and PCR studies suggested that deletions of certain copies of the modules must have occurred in apxIVA genes of serotype 3 (Fig. 2) and other serotypes. However, ApxIVA from serotypes 1 and 3 had the same haemolytic and co-haemolytic activities and were immunologically indistinguishable.

Using a sensitive immunoblot assay with monospecific polyclonal anti-ApxIVA antibodies, we could not detect production of ApxIVA in A. pleuropneumoniae grown in different media, or under a wide variety of different culture conditions in either exponential phase or stationary phase. The culture conditions and media supplements chosen included those known to be involved in regulation of gene expression (e.g. iron-depleted, Ca²⁺-replete, oxygen-limited and high temperature). However, regulation of in vivo expressed genes (Finlay & Falkow, 1989; Heithoff et al., 1997) is so far unknown in A. pleuropneumoniae.

Infection of pigs with A. pleuropneumoniae induced antibodies against ApxIVA, indicating that the apxIVA gene is expressed during infection, in contrast to in vitro conditions. Convalescent sera from pigs which were naturally or experimentally infected with all of the different A. pleuropneumoniae serotypes reacted strongly in immunoblots with recombinant ApxIVA. Sera from healthy pigs as well as a positive A. suis swine antiserum did not react with ApxIVA although the latter reacted strongly with the exotoxins ApxI and ApxII, which are secreted by both A. pleuropneumoniae and A. suis. These results are consistent with the genetic findings that A. suis is devoid of ApxIVA and provide further evidence that ApxIVA is specific to the species A.
pleuropneumoniae. The fact that A. suis does not produce ApxIVA might in part explain why A. suis is less virulent than A. pleuropneumoniae despite its ability to express ApxI and ApxII. No serological cross-reactions could be detected between recombinant ApxIVA and the other RTX toxins of A. pleuropneumoniae (Fig. 5). Similarly, there was no reaction between antibodies to the ApxIVA recombinant protein and ApxI, ApxII or ApxIII. These results indicated that ApxIVA is antigenically specific and make this protein a promising candidate for serodiagnosis of A. pleuropneumoniae infection.

Homologues of apxIVA could be detected by both PCR amplification and Southern blotting in the reference strains of all A. pleuropneumoniae serotypes but were absent in the closely related Actinobacillus spp. and P. haemolytica serotype I. The only exception was A. lignieresi, in which some hybridization to the 5' half of apxIVA could be detected by Southern blotting. It is not known whether this homologous region is a pseudogene or part of yet another RTX protein. The 3' half of apxIVA, however, seems to be very specific to the species A. pleuropneumoniae as shown both by DNA-DNA hybridization and by PCR analysis using the primer pair APXIVA-1L/APXIVA-1R (Table 2). The apxIVA gene may therefore be a useful target for rapid, species-specific genetic detection and identification of the species A. pleuropneumoniae.

In summary, we have cloned and characterized a new RTX determinant encoding a putative protein toxin, ApxIVA, in A. pleuropneumoniae that is expressed in vivo but not in vitro. To our knowledge this is the first gene described in A. pleuropneumoniae which is expressed only in vivo. Sequences with homology to apxIVA_var1 are present in all A. pleuropneumoniae serotypes, but there appears to be some heterogeneity in number of nonapeptide repeats. The similarity of ApxIVA to other known RTX toxins, its strong immunogenicity in infected pigs and the haemolytic activity of recombinant ApxIVA suggests that ApxIVA may play a role in the pathogenesis of A. pleuropneumoniae.

ACKNOWLEDGEMENTS

We are grateful to Yvonne Schlatter and Margrit Krawinkler for technical assistance; to Serge Lariviére, Montréal, Canada, and Ragnhild Nielsen, Copenhagen, Denmark for their gifts of sera; and to Jos Cox, Geneva, Switzerland, for help with Ca++-binding experiments. This work was supported by grant no. 3100.39123.93 of the Swiss National Science Foundation and the Natural Sciences and Engineering Research Council of Canada.

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Received 21 December 1998; revised 14 April 1999; accepted 4 May 1999.