

Characterization of *apxIVA*, 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 *apxIVA*. When expressed in *Escherichia coli*, recombinant ApxIVA 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 *apxIVA*. The *apxIVA* 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 ApxIVA 14 d post-infection, indicating that *apxIVA* is expressed *in vivo*. In addition, sera from pigs naturally and experimentally infected with any of the serotypes all reacted with recombinant ApxIVA. The *apxIVA* gene from the serotype 1 *A. pleuropneumoniae* type strain Shope 4074^T 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 ApxIVA proteins of different serotypes of *A. pleuropneumoniae*. The deduced amino acid sequences of the ApxIVA 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 *apxIVA* 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, *apxIVA* 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

Abbreviation: DIG, digoxigenin-11-dUTP.

The GenBank accession numbers for the *apxIVA*_{var1} gene and 1304 bp of flanking sequences present in plasmid pJFFROK7, for the *apxIVA*_{var3} locus (pJFFROK5), and for the vector pETHIS-1 are AF021919, AF030511 and AF012911, respectively.

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

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* (ApxIA_{var.suis}, ApxIIA_{var.suis}) (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-Xaa-Gly-Gly-Xaa-Gly-Asn/Asp-Asp-Xaa. The RTX toxin family includes the α -haemolysin of *Escherichia coli* (HlyA) (Felmlee *et al.*, 1985), the bi-functional adenylate cyclase haemolysin of *Bordetella pertussis* (CyaA) (Glaser *et al.*, 1988), the leucotoxins of *Pasteurella haemolytica* (LktA) (Lo *et al.*, 1987) and *Actinobacillus actinomycetemcomitans* (AaltA) (Lally *et al.*, 1989), and two iron-regulated exoproteins (FrpA, FrpC) of *Neisseria meningitidis* (Thompson *et al.*, 1994). Recently, sequences with similarity to the *frpA* and *frpC* genes of *N. meningitidis* were described immediately downstream of the *A. pleuropneumoniae lacZ* gene in a serotype 1 strain, CM5, indicating that a fourth type of RTX gene may be present in *A. pleuropneumoniae* (Anderson & MacInnes, 1997). In this paper we describe the complete cloning, sequencing, expression and characterization of two variants of this new RTX determinant, *apxIVA*_{var1} and *apxIVA*_{var3} from serotype 1 and 3 strains, respectively.

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* HV114 is a virulent serotype 3 field strain that was isolated from a pig with severe pleuropneumonia. HV114 shows the typical serotype 3 pattern of *apx* toxin genes (i.e. *apxIIICA* and *apxIIICABD*) (Beck *et al.*, 1994). *A. lignieresii* ATCC 49236, *A. equuli* ATCC 19392, *A. rossii* ATCC 27072, *A. suis* ATCC 15558 and serotype 1 *P. haemolytica* ATCC 14003 (Table 1) were purchased from the American Type Culture Collection, Manassas, VA, USA. *Actinobacillus minor* PNDN33, *Actinobacillus porcicus* 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 in Luria–Bertani broth or on Luria–Bertani broth agar supplemented when necessary with ampicillin (50 µg ml⁻¹) for selection and maintenance of recombinant plasmids, and additionally with 125 µM X-Gal for blue-white selection with pBluescript II SK(-). 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 immunoblots (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 A₆₅₀ of 0.5, then incubated overnight in a nitrogen atmosphere. Cultures grown to mid-exponential phase (A₆₅₀ 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 4074^T and serotype 3 strain HV114 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 10⁹ 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 with a set of digoxigenin-11-dUTP (DIG)-labelled DNA probes (Kuhnert *et al.*, 1997) in Southern blots using low-stringency conditions (Ausubel *et al.*, 1990).

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 a 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

Species	Serotype/group	Strain	Hybridization			PCR amplification*
			<i>apxIVA</i> 5' part	<i>apxIVA</i> central part	<i>apxIVA</i> 3' part	
<i>A. pleuropneumoniae</i>	1†	Shope 4074 ^T	+	+	+	+
<i>A. pleuropneumoniae</i>	2†	S1536	+	+	+	+
<i>A. pleuropneumoniae</i>	3†	S1421	+	+	+	+
<i>A. pleuropneumoniae</i>	4†	M62	+	+	+	+
<i>A. pleuropneumoniae</i>	5a†	K17	+	+	+	+
<i>A. pleuropneumoniae</i>	5b†	L20	+	+	+	+
<i>A. pleuropneumoniae</i>	6†	femø	+	+	+	+
<i>A. pleuropneumoniae</i>	7†	WF83	+	+	+	+
<i>A. pleuropneumoniae</i>	8†	405	+	+	+	+
<i>A. pleuropneumoniae</i>	9†	CVI 13261	+	+	+	+
<i>A. pleuropneumoniae</i>	10†	13039	+	+	+	+
<i>A. pleuropneumoniae</i>	11†	56153	+	+	+	+
<i>A. pleuropneumoniae</i>	12†	8392	+	+	+	+
<i>A. pleuropneumoniae</i>	3	HV114	+	+	+	+
<i>A. lignieresii</i>		ATCC49236 ^T	+‡	+‡	-	-
<i>A. equuli</i>		ATCC19392 ^T	-	-	-	-
<i>A. rossii</i>		ATCC27072 ^T	-	-	-	-
<i>A. suis</i>		ATCC15558 ^T	-	-	-	-
<i>A. minor</i>		PNDN33	-	-	-	-
<i>A. porcinus</i>		NM319	-	-	-	-
<i>Haemophilus</i> sp.	'Minor group'	202	-	-	-	-
<i>P. haemolytica</i>	Serotype 1	ATCC14003	-	-	-	-

* 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 μ M of each dNTP, 0.25 μ M forward and reverse primers, 0.5 units *Taq* polymerase and 5 ng 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 4074^T and the oligonucleotide primer pairs APPIV1-L/APPIV1-R and APXIVA-1L/APXIVA-1R, respectively. Amplification reactions were carried out as described above except that the PCR mixtures were supplemented with 50 μ M DIG. A probe for the central part of *apxIVA*_{var1} (Fig. 1) was obtained by amplifying the 2.2 kb *Bam*HI-*Nru*I fragment of plasmid pJFFROK5 cloned in pBluescript II SK(-) using primers complementary to the T7 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 PCGENE programs PROSITE (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 *Ap*xIVA fusion proteins, we amplified various segments of *apxIVA*_{var1} and *apxIVA*_{var3} and full-length *apxIVA*_{var1} from genomic DNA of strains Shope 4074^T 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 *Nde*I and *Bam*HI, or *Nde*I and *Eco*RI for cloning into pETHIS-1 or pBluescript II SK(-) (Stratagene). The constructs were analysed by restriction

Table 2. Oligonucleotide primers

Primer name	Oligonucleotide sequence 5'-3'	Position†	Temp.‡
APXIVA-1L	TGGCACTGACGGTGATGAT	6018-6036	52
APXIVA-1R	GGCCATCGACTCAACCAT	6459-6442	52
APX4UP-L	GATTCGCTGTTGGACTGC	539-556	52
APX4UP-R	CCCTTCGAATTGTTTCGC	4128-4111	52
APX4DWN-L	GCGAAACAATTGGAAGGG	4111-4128	52
APPIV1-L	GGGACAGTGGCTCAATTAAG	1895-1914	52
APPIV1-R	AGCTGTAAACTCCACCAACG	2652-2633	52
APXIVAHIS1-L	agccatATGGGGCGATTTAAATTTTCAG	3532-3551	54
APXIVAHIS1-R	tat ggaTCCTCCGTGCTTCTGAGC	6483-6466	54
APX4II3-L	cgccatatgCGCGCTATATCTGGAAT	1543-1560	54
APX4II3B-R	cgcgaaatcTTCCCTTCGAATTGTTT	4131-4114	54
APX4II5-L	cgccatATGACAAAATTAATACTATGCAAG	1132-1153	54
APXIVAHISC'-R	cgctctagaGAGCAAGCTACTTATATT	1093-1110	54
APXIVAORF1-L	CCCCTTTTCAACCTCTTTTATTT	2475-2453	54
APXIVAORF1-R	cgctctagaGTTAGTGAATTATTTTAA	601-618	54
ASCH3	cgcgaaatcAGCGACACAAGAGATAT	6504-6488	54

* 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 *apxIVA* is at 1132).

‡ Annealing temperature in °C.

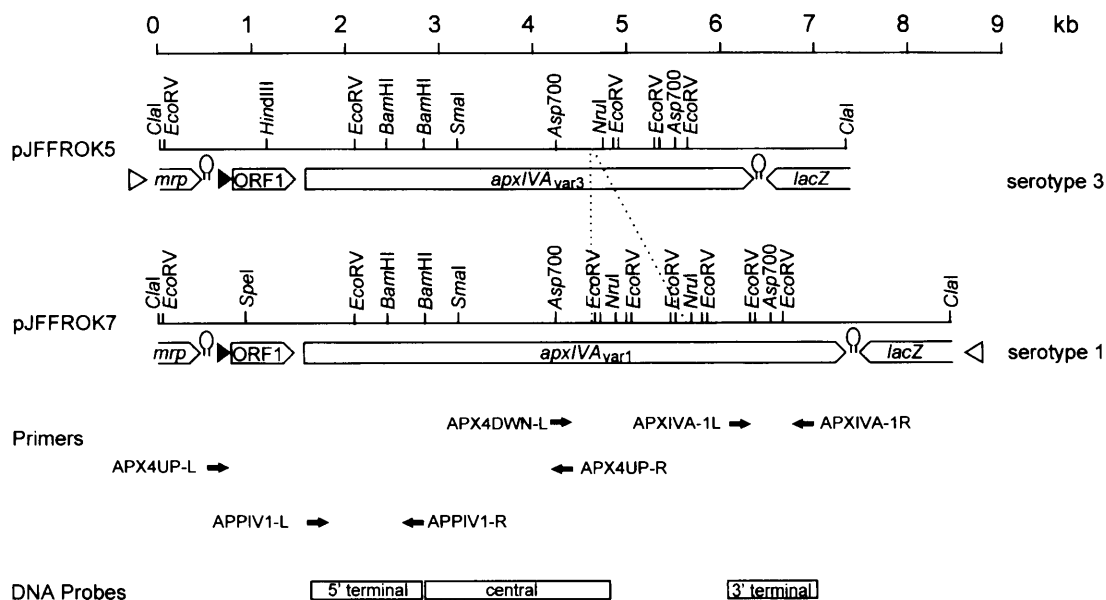


Fig. 1. Physical and genetic maps of plasmids pJFFROK5 and pJFFROK7 containing *apxIVA*_{var3} and *apxIVA*_{var1}, respectively. Open boxes with arrowheads indicate ORFs; putative *rho*-independent transcription termination signals are indicated by hairpins; the locations of putative promoter sequences are indicated by filled triangles; the *p*_{lac} 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 *apxIVA* 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 Ni²⁺ chelate affinity

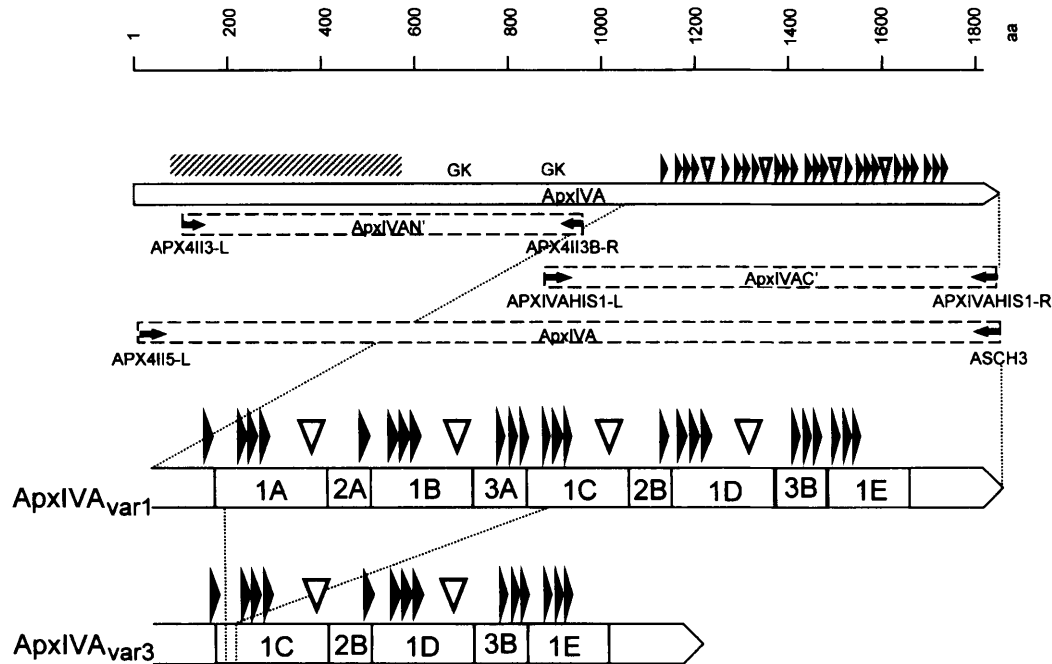


Fig. 2. Predicted structures of ApxIVA_{var1} and ApxIVA_{var3}. The upper part of the figure shows the general structures: the hydrophobic domains (hatched region) and potential acylation sites (marked GK). The iterated glycine-rich nonapeptides are indicated by filled triangles and the open triangles 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_{var1} and ApxIVA_{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_{var3}, as compared to ApxIVA_{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 APX4II5-L/ASCH3 from genomic DNA of strain Shope 4074^T and cloning this PCR fragment into the *Nde*I and *Eco*RI sites of vector pETHIS-1. The plasmid encoding the poly-histidine-tailed N-terminal half of ApxIVA was constructed using the primers APX4II3-L/APX4II3B-R (Table 2) and genomic DNA of strain Shope 4074^T as template. The resultant plasmid was named pJFFapxIVA1NaHis1 and expressed the 96 kDa N-terminal half of ApxIVA, designed 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 APXIVAHIS1-L/APXIVAHIS1-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 APXIVAHISC'-R and APXIVAORF1-L (Table 2), subsequent digestion of the PCR fragment with *Xba*I plus *Kpn*I and replacement with the corresponding *Xba*I–*Kpn*I fragment in plasmid pJFFapxIVA1His2. Plasmid pJFFORF1apxIVA1His2, which allows the co-expression of

ORF1 and ApxIVA-10xHis, was constructed by PCR amplification of ORF1 and the 5' half of *apxIVA* using pJFFROK7 as template and oligonucleotide primers APXIVAORF1-L and APXIVAORF1-R, subsequent digestion of the PCR fragment with *Xba*I and *Kpn*I and replacement with the corresponding *Xba*I–*Kpn*I fragment in plasmid pJFFapxIVA1His2. Finally, the plasmid for the expression of ORF1 alone as a control was obtained by deleting a 4382 bp *Eco*RV fragment in pJFFORF1apxIVA1His2. This deletion removed most of the *apxIVA* gene but left ORF1 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 Ni²⁺ 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 antisera and immunoblotting. Monospecific polyclonal antisera directed against the poly-histidine-tailed fusion proteins ApxIVA, ApxIVAN' and ApxIVAC', were obtained by immunization of rabbits with 100 µg 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 7 reference strain WF83, and ApxIII 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 S. Larivière, Montréal, Canada (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 phosphatase-labelled goat antibodies directed against pig IgG or rabbit IgG, respectively (Kirkegaard Perry)

Ca²⁺-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 1.0 µCi ml⁻¹ of ⁴⁵Ca²⁺ [0.02 mCi µg⁻¹ (0.74 MBq µg⁻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 & MacInnes, 1997). The presence of this fourth RTX determinant, named *apxIVA*, was confirmed by hybridizing *Clal*-digested genomic DNA of the serotype 1 *A. pleuropneumoniae* strain Shope 4074^T with broad-range probes designed for screening for RTX genes (Kuhnert *et al.*, 1997), which showed an 8 kb *Clal* 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 *Clal* fragment hybridized to the *frpA* probe. These same *Clal* 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^T using oligonucleotide primer pair APXIVA-1L/APXIVA-1R (Fig. 1). The 8 kb *Clal* fragment of serotype 1 strain Shope 4074^T was cloned into vector pBluescript II SK(-), resulting in plasmid pJFFROK7. Similarly, a 7 kb *Clal* 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 202130.4 Da and a theoretical pI 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 pI 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 pJFFROK5 containing *apxIVA*_{var3} (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*_{var3} (Fig. 1). The predicted molecular mass of ApxIVA_{var3}, which contains 1522 aa residues, is 170157 Da and the protein has a theoretical pI 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-G-X-G-N/D-D-X (Felmlee *et al.*, 1985). These

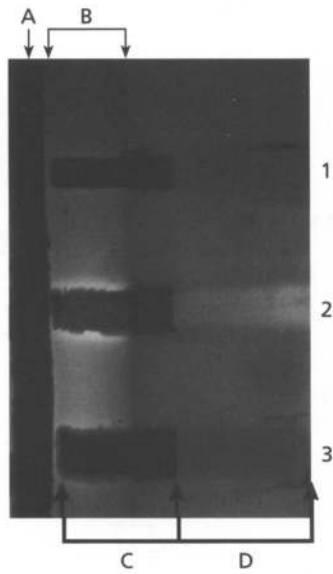


Fig. 3. Haemolytic and co-haemolytic activity of ApxIVA on trypticase soy agar medium containing 5% washed sheep blood and 10 mM CaCl₂. A, Vertical streak of *Staphylococcus aureus*; B, diffusion zone of the sphingomyelinase (β -toxin) secreted by *S. aureus*. Horizontal streaks: *E. coli* HMS174(DE3) 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.

glycine-rich nonapeptides are interspersed by four regions with homology to consensus sequences for DNA polymerase family 2 signatures, which are thought to be involved in Mg²⁺ binding (Argos, 1988). ApxIVA_{var3} contains 14 glycine-rich nonapeptides and two DNA polymerase family 2 signature sequences in the C-terminal region. The organization of this region is identical to that of ApxIVA_{var1} except that aa 1146–1428 have been deleted and a short peptide sequence (YSDSANSKK) is present in their place (Fig. 2).

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

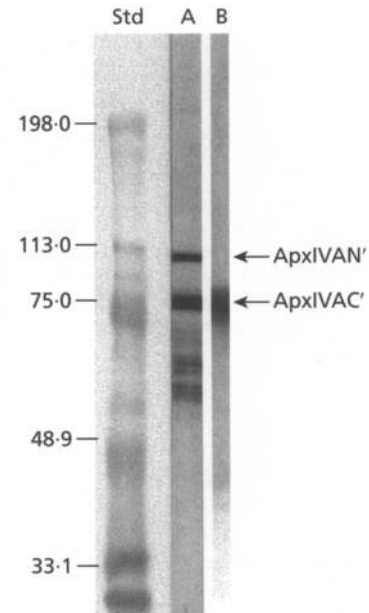


Fig. 4. Binding of ⁴⁵Ca²⁺ to ApxIVA. ApxIVAN' and ApxIVAC' were run together in the same lane, and then blotted on nitrocellulose membranes. The transferred proteins were probed with anti-ApxIVA antibodies as a control on one strip of the blot (lane A), and were reacted with ⁴⁵Ca²⁺ on a second strip of the same blot (lane B). The expected positions of ApxIVAN' and ApxIVAC' are indicated. The sizes of prestained molecular mass markers (lane Std) are shown in kDa. Note that the faint bands which are visible on the immunoblot below ApxIVAC' are degradation products of this protein.

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).

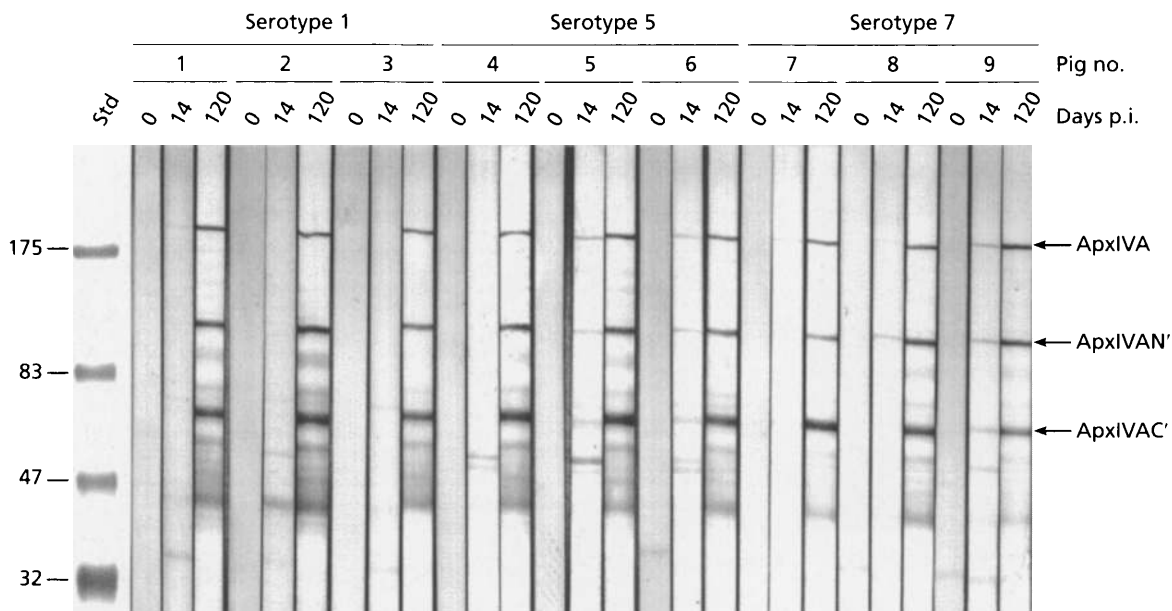


Fig. 5. Induction of antibodies directed against ApxIVA by infection of pigs with *A. pleuropneumoniae* serotypes 1, 5 and 7. Sera from three pigs per serotype were tested. Immunoblots containing recombinant ApxIVA and the peptides ApxIVAN' and ApxIVAC' (marked on the right side of the figure) were reacted with sera from pigs experimentally infected with *A. pleuropneumoniae* serotypes 1, 5 and 7, taken prior to infection at day 0, and 14 and 120 d post-infection (p.i.). The sizes of molecular mass markers (Std) are indicated in kDa.

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^T 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). *Clal*-digested genomic DNA was hybridized at high stringency with three DIG-labelled probes representing the 5' terminal, the central and the 3' terminal part of *apxIVA* (Fig. 1). The probe for the

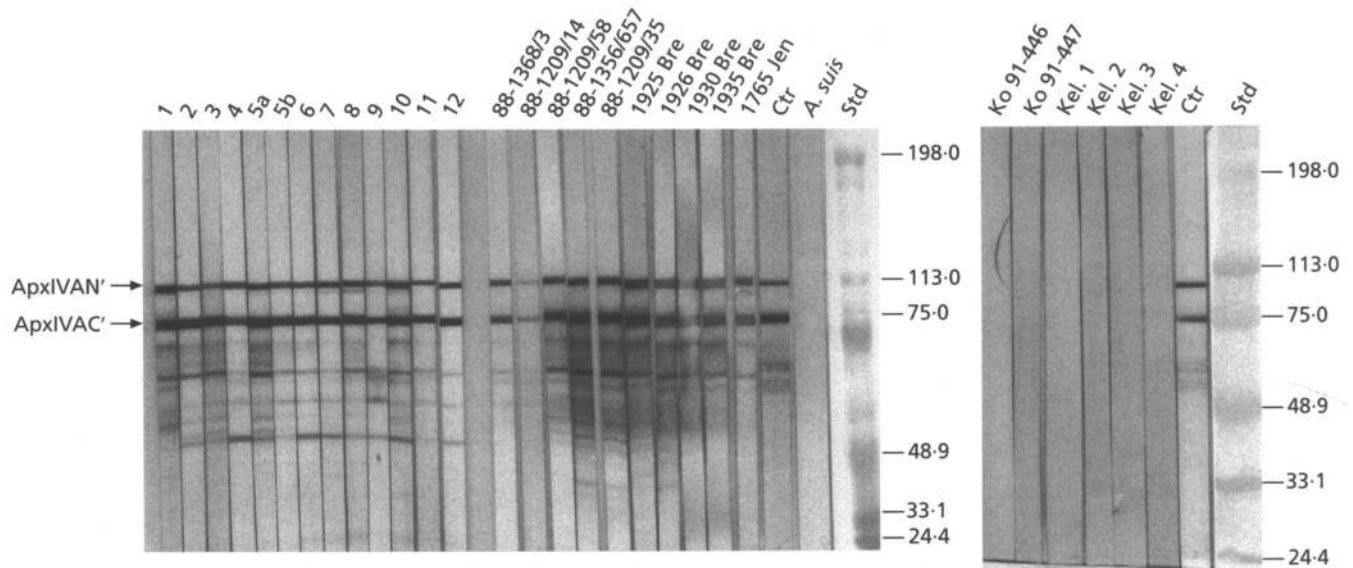


Fig. 6. Serological reactions of recombinant ApxIVA fusion proteins with sera from pigs infected with *A. pleuropneumoniae*. Immunoblots containing ApxIVAN' and ApxIVAC' were incubated with convalescent serum from pigs experimentally infected with *A. pleuropneumoniae* serotype reference strains (lanes 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 ApxIVA (lane Ctr). The sizes of molecular mass markers (Std) are indicated in kDa.

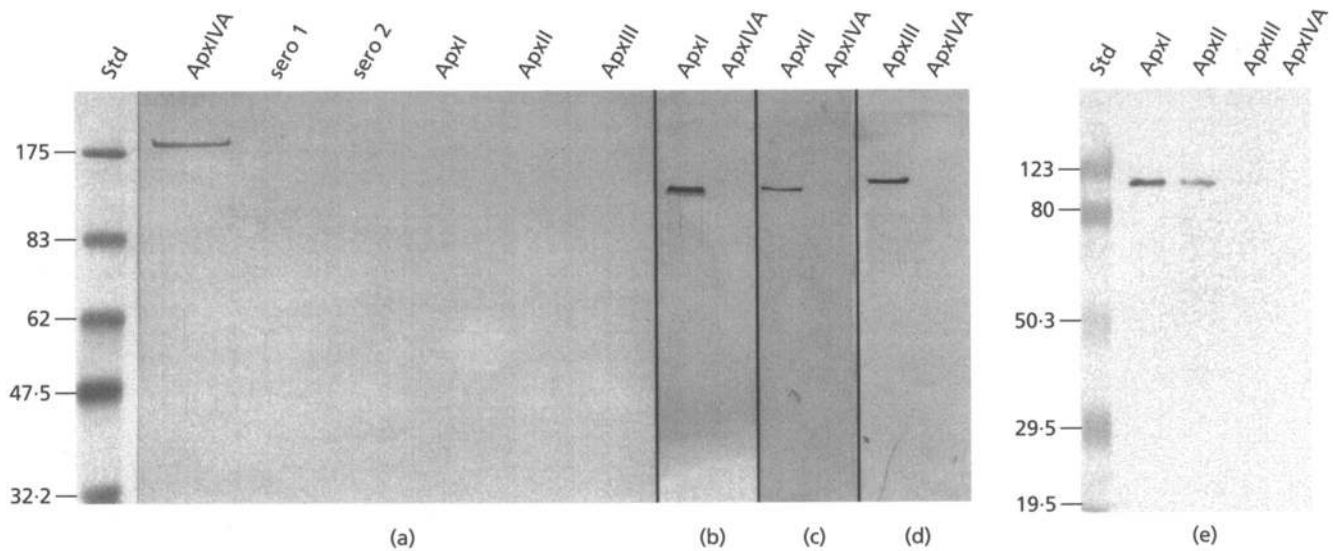


Fig. 7. Antigenic specificity of ApxIVA. (a) Immunoblot containing purified recombinant ApxIVA (lane ApxIVA), total culture antigens of *A. pleuropneumoniae* serotype 1 strain 4074^T grown in PLO broth (lane sero 1), total culture antigen of serotype 2 strain S1536 (lane sero 2), purified ApxI (lane ApxI), purified ApxII (lane ApxII) and purified ApxIII (lane ApxIII) incubated with polyclonal anti-ApxIVA antibody. Note that in order to verify the absence of serological cross-reactions, five times more ApxI, ApxII and ApxIII proteins than ApxIVA was used. (b–e) Immunoblots containing: ApxI and ApxIVA incubated with polyclonal anti-ApxI antibody (b); ApxII and ApxIVA incubated with polyclonal anti-ApxII antibody (c); ApxIII and ApxIVA incubated with polyclonal anti-ApxIII antibody (d); and ApxI, ApxII, ApxIII and ApxIVA 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 4.4 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 3.6 kb fragment was amplified by PCR from all *A. pleuropneumoniae* type- and reference strains. When the same strains were analysed with the primer pair APX4DN-L/APXIVA-1R, fragments of 1.6, 2.0, 2.4 or 2.8 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^T 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 *mvp* 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 *blyC* 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 (GK) (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 nonapeptides in the C-terminal half of the protein that are known to bind Ca²⁺ (Welch, 1991) (Figs 2 and 4). The arrangement of the C-terminal end is unusual however. In addition to the typical 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 1. The only exception was *A. lignieresii*, 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*.

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