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RTX Toxin Genotypes and Phenotypes in *Actinobacillus pleuropneumoniae* Field Strains

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Actinobacillus pleuropneumoniae **serotype reference strains and 204** ***A. pleuropneumoniae*** **field strains representing all 12 serotypes and both biovars 1 and 2, obtained from laboratories from various countries worldwide, were analyzed for the presence of the toxin genes** ***apxIC*****,** ***apxIA*****,** ***apxIB*****,** ***apxID*****,** ***apxIIC*****,** ***apxIIA*****,** ***apxIIIC*****,** ***apxIIIA*****,** ***apxIIIB*****, and** ***apxIIID*** **by DNA-DNA hybridization with specific gene probes. Expression of the toxins ApxI, ApxII, and ApxIII was assessed by immunoblot analysis with monoclonal antibodies. The results show that the patterns of** ***apx*** **genes and those of the expressed Apx toxins in biovar 1 field strains are the same as those of the genes and toxins of corresponding serotype reference strain. We found only three strains which had certain** ***apx*** **genes missing compared with the genes in their serotype reference strains. Analysis of the expression of the three toxins showed that nearly all strains expressed their** ***apx*** **genes and produced the same Apx toxins as their serotype reference strain. We found only one strain that did not produce ApxI, although it contained the** ***apxICABD*** **genes, and one strain which did not express ApxII but which contained** ***apxIICA*****. Several field strains which initially showed that their serotype did not correspond to the** ***apx*** **gene profile of the reference strain and which had an unexpected virulence for the given serotype revealed that their initial serotyping was erroneous. We show that the** ***apx*** **gene profiles are inherent to a given serotype. The method cannot differentiate between all 12 serotypes. However, it allowed us to distinguish five groups of toxin gene patterns which showed pathological, toxicological, and epidemiological significance. None of the biovar 2 strains contained** ***apxIII*** **genes. The** ***apxI*** **and** ***apxII*** **genes in the biovar 2 strains, however, were the same as those found in the serotype reference strains of biovar 1.**

Actinobacillus pleuropneumoniae, the etiological agent of porcine pleuropneumonia, can be differentiated into two biovars (biovar 1, NAD dependent; biovar 2, NAD independent) and into 12 serotypes (31). Serotyping is one of the most important and most frequently used tools in epidemiology and sanitation programs to control porcine pleuropneumonia (31). Several different serotyping methods are currently used (33). They are mainly based on the serologies of the lipopolysaccharides and capsular polysaccharides. A complete analysis of the chemical compositions of the capsular polysaccharide K antigens and lipopolysaccharide O antigens of all 12 serotypes has recently been accomplished (3, 35). The similarity or identity of K and O antigens of certain serotypes (3) explains the strong cross-reactivity among certain groups of serotypes which were described earlier (17, 27, 29, 32) and which strongly hinder the typing of field strains and the epidemiological surveillance of certain serotypes that cause severe outbreaks.

Among the 12 serotypes significant differences in virulence have been observed. It has frequently been reported that serotypes 1 and 5, and to some extent also serotypes 9 and 11, are involved in severe outbreaks with high levels of mortality and severe pulmonary lesions; the other serotypes are less virulent, cause lower levels of mortality, but are frequently found in outbreaks in many countries (4, 11, 26, 31, 36, 38). Some serotypes, in particular serotype 3, are considered to be of very low virulence and of no epidemiological importance in certain countries but seem to be epidemic in others (4, 9, 30, 31).

It is most likely that the degree of virulence of the different *A. pleuropneumoniae* serotypes is for the most part associated with the exotoxins expressed by the different strains (13, 19). Three different exotoxins, the strongly hemolytic ApxI, the weakly hemolytic ApxII, and the nonhemolytic ApxIII, which all belong to the pore-forming RTX toxins have been identified (15). Characteristically, it is known that strains belonging to the strongly hemolytic serotypes 1, 5, 9, and 11, for which it was shown that their reference strains produce ApxI, are particularly virulent in experimentally infected mice (13, 24).

Genetic analyses have been conducted and the expression of Apx toxins has been studied so far in *A. pleuropneumoniae* serotype reference strains. The genetic determinants for ApxI or ApxIII are operons consisting of the four genes *apxICABD* or *apxIIICABD*, respectively, as is characteristic for RTX toxins, where *apxIA* codes for the structural toxin, *apxIC* codes for an activation protein, and *apxIB* and *apxID* code for two proteins involved in the secretion of the toxin. The genetic determinant for ApxII consists of an operon with the genes *apxIICA* but which lacks the typical genes for secretion (Fig. 1) (6, 15, 18, 25, 39). The complete *apxI* operon is found in serotype reference strains 1, 5a, 5b, 9, 10, and 11 (Fig. 1), which are strongly hemolytic and produce ApxI. The *apxII* operon and the expression of ApxII are found in all serotypes except serotype 10, and the *apxIII* operon is found in serotypes 2, 3, 4, 6, and 8, which produce ApxIII (Fig. 1) (15). It is, however, not known whether this is representative for strains isolated from

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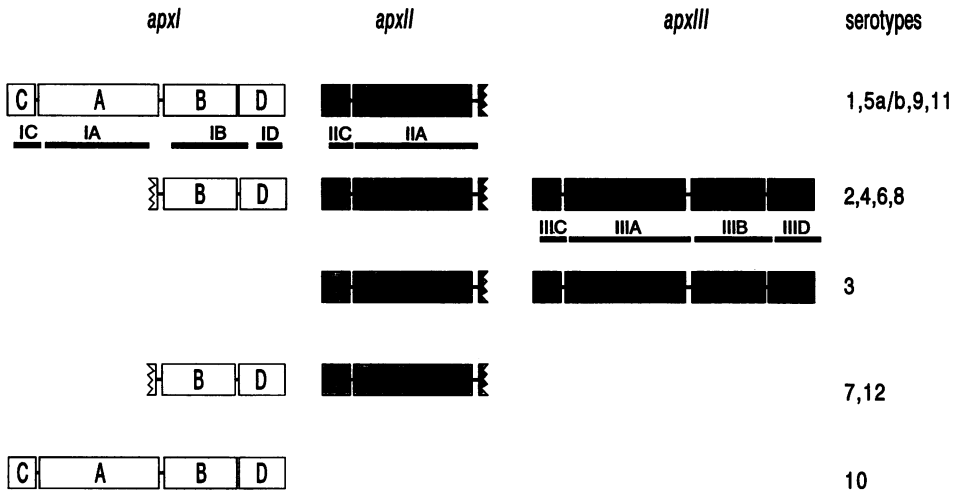

FIG. 1. Operons of the three RTX toxins ApxI, ApxII, and ApxIII in *A. pleuropneumoniae* serotype reference strains. Open boxes represent genes of the *apxI* operon, grey boxes represent genes of the *apxII* operon, and dark grey boxes represent genes of the *apxIII* operon. Broken boxes represent 3′-terminal truncated *apxIA* or 5′-terminal truncated *apxIB* genes. Solid bars represent the segments of the hybridization probes. Data for the *apx* operons were compiled from hybridization experiments in this paper and from previously published data (6–8, 14, 15, 18, 20–23).

the field. In order to get a better view of the impact of the different Apx toxins in virulence and epidemiological relevance and to determine whether the production and the gene profiles of the different Apx toxins can be correlated to a given serotype of *A. pleuropneumoniae*, we analyzed the expression and the genetic bases of ApxI, ApxII, and ApxIII in 204 field isolates of *A. pleuropneumoniae* representing all 12 serotypes and both biovars, which we received from various countries worldwide. This analysis shows that the *apx* gene profiles are stable in strains of a given serotype.

MATERIALS AND METHODS

Bacterial strains and plasmids. *A. pleuropneumoniae* reference strains of the following serotypes were used: serotype 1, 4074; serotype 2, S1536; serotype 3, S1421; serotype 4, M62; serotype 5a, K17; serotype 5b, L20; serotype 6, femϕ; serotype 7, WF83; serotype 8, 405; serotype 9, CVI 13261; serotype 10, 13039; serotype 11, 56153; serotype 12, 8329, as described previously (16). *A. pleuropneumoniae* field strains were obtained from various countries including Australia, Belgium, Brazil, Canada, the Czech Republic, Denmark, France, Italy, Japan, The Netherlands, Norway, Romania, Spain, Sweden, Switzerland, Taiwan, the United Kingdom, and the United States. They were retyped by the indirect hemagglutination test, the agar gel diffusion test (34), and the ring precipitation test (28). *A. pleuropneumoniae* strains were grown in Columbia broth (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 0.01% β-NAD (Sigma Chemicals Co.) and 25 mM $CaCl_2$ at 37°C with shaking. *Escherichia coli* XL1-Blue, *supE44 hsdR17 recA1 endA1 gyrA96 thi relA1* del[*proAB-lac*], F′[*proAB lacI*^{q} *lacZ*delM15, Tn*10* (Tc^r)] (5), was used as the host for recombinant plasmids. *E. coli* strains were grown in Luria Bertani broth (2). Culture media were supplemented with 25 μg of ampicillin per ml for the selection for pBluescriptIISK$^-$ or 25 μg of tetracycline per ml for the cultivation of strain XL1-Blue. DNA fragments for the use of gene probes were cloned on vector pBluescriptIISK$^-$ (Stratagene, La Jolla, Calif.). Plasmid pJFF750 was the source of *apxICABD* genes (18).

Hybridization DNA probes and PCR amplification. Hybridization DNA probes for the *A. pleuropneumoniae apxI* genes were isolated from plasmid pJFF750, which originated from *A. pleuropneumoniae* serotype 1 strain 4074 (18). The probe for *apxIC* was a 400-bp *Ssp*I-*Xho*I fragment, the probe for *apxIB* was a 2.1-kbp *Bgl*II-*Bgl*II fragment, and the probe for *apxID* was a 600-bp *Eco*RI-*Eco*RV fragment of pJFF750 (Fig. 1). The probe for *apxIA* was generated by PCR amplification of the 3.0-kbp coding sequence of *apxIA* with the primers HLYIA-L (5′-TGGCTAACTCTCAGCTCG-3′) and HLYIA-R (5′-ATA GACTAACGGTCCGCC-3′), corresponding to the beginning and the end of *apxIA*, respectively, and subsequent digestion with *Eco*RV to produce a 2.2-kbp fragment lacking the very 3′-terminal end of *apxIA*.

The probes for the *apxII* genes were produced by PCR amplification by using chromosomal DNA of *A. pleuropneumoniae* serotype 1 strain 4074. The probe for *apxIIA* was a 2.9-kbp PCR product amplified with the primers APP5A-LT (5′-CCCATATGGATCCGTCAAAAATCACTTTGTCAT CATT-3′) and APP5A-RT (5′-TCCGGAATTCAAGCGGCT CTAGCTAATTGA-3′) according to the beginning and the end of the *apxIIA* sequence, respectively (Fig. 1). The probe for *apxIIC* was obtained by PCR amplification of the *apxIICA* genes by using the primers APP5C-LT (5′-CGCGGATCCG TTGCCTTGTTTTCCTTCAC-3′) and APP5A-RT, corresponding to the beginning *apxIIC* and the end of the *apxIIA* sequence, respectively (7), and subsequent isolation of the 400-bp *Nde*I fragment containing *apxIIC* (Fig. 1).

The probes for the *apxIII* operon were obtained by PCR amplification of serotype 2 S1536 chromosomal DNA. Primers APXIIIC-L1 (5′-CGGGATCCGACTAACTATATAATTAA-3′) and APXIIIA-R1 (5′-CGGGATCCTAGAATAGCCAT AGG-3′), corresponding to the 5′ end of *apxIIIC* and the 3′ end of *apxIIIA*, respectively, were used to amplify a 3.8-kbp fragment containing *apxIIICA*. It was cloned into the *Bam*HI site of vector pBluescriptIISK$^-$. The probe for *apxIIIC* was obtained as a 570-bp *Bam*HI-*Asp*700 fragment; the probe for *apxIIIA* was obtained as a 3.2-kbp *Bam*HI-*Asp*700 fragment. The genes *apxIIIBD* were amplified by using the primers APXIIIBD1-L (5′-CCCAAACTAACCGTTACC-3′) and APXIIIBD1-R (5′-GCAATAACGCTCATTCCA-3′) and di-

gestion of the PCR product with the restriction enzyme *Nco*I. The probe for *apxIIIB* was the 1.7-kbp fragment, and the probe for *apxIIID* was the 1.5-kbp fragment.

As a control for the hybridization of the dot blots we used the gene encoding *16SrRNA* of *A. pleuropneumoniae* serotype 1 which was amplified by PCR by using the universal primers UNI16SRNA-L (5′-AGAGTTTGATCATGGCTCA-3′) and UNI16SRNA-R (5′-GTGTGACGGGCGGTGTGTA-3′).

Dot blot hybridizations. For dot blot hybridizations bacterial cells, at a concentration of about 10^9 cells per ml, were lysed in 10 mM Tris-HCl (pH 9)–50 mM KCl–0.01% gelatin–1.5 mM $MgCl_2$–0.1% Triton X-100–0.05 mg of proteinase K per ml–20 mM dithiothreitol–1.5 μM sodium dodecyl sulfate (SDS) for 1 h at 37°C. Proteinase K was inactivated for 10 min at 95°C. A total of 20 μl of lysate was denatured with 50 μl of 1 M NaOH for 30 min and neutralized by adding 50 μl of 1 M HCl. Dot blot hybridization was carried out with a Bio-Rad Bio-Dot apparatus on Hybond C-extra nitrocellulose filters (Amersham plc, Little Chalfont, United Kingdom) as described elsewhere (2).

DNA probes were labelled with [^{32}P]dCTP (3,000 Ci/mmol; Amersham plc) by random priming (12). Hybridizations were carried out for 18 h at 37°C in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M trisodium citrate [pH 7.0])–50% formamide–5% polyethylene glycol 6000–0.5% SDS–100 μg of denatured and sonicated salmon sperm DNA per ml. Filters were washed in 0.1% SDS–0.1× SSC at 25°C corresponding to the melting temperature of DNA-DNA duplexes with about 75% sequence identity. Autoradiography was carried out for 6 h on Fuji RX films by using an intensifying screen. As a control of the relative DNA concentrations on the dot blot, filters were washed two times for 30 min in 0.2 M NaOH, neutralized for 30 min in 0.1 M Tris HCl (pH 7.5) to remove the hybridized probes, and then rehybridized with the PCR-amplified *16SrRNA* gene probe of *A. pleuropneumoniae.*

Analysis of expression of ApxI, ApxII, and ApxIII. Expression of the toxins ApxI, ApxII, and ApxIII by the different strains was assessed by immunoblot analysis with monoclonal antibodies. Toxins in cell-free 6-h culture supernatants were concentrated 50 times by 55% saturated ammonium sulfate precipitation. Aliquots of 50 μl of concentrated supernatants, diluted 2:3 in sample buffer, were separated on SDS–9% polyacrylamide gels and were transblotted to Immobilon P membranes (Millipore). The membranes were then incubated with monoclonal antibody Int 40-4-5-1 to detect ApxI, monoclonal antibody Int 16-10-1 to detect ApxII, and monoclonal antibody Int 33-8-1-2 to detect ApxIII. Antibody binding was visualized by successive incubation with peroxidase-conjugated goat anti-mouse immunoglobulin G (heavy and light chains; Kirkegaard & Perry Laboratories, Gaithersburg, Md.), urea peroxide, and 3,3′-diaminobenzidine–4HCl.

RESULTS

The genotypic and phenotypic analyses of the three Apx toxins were performed with *A. pleuropneumoniae* serotype reference strains and 5 to 34 field isolates of each serotype by using dot blot hybridization and immunoblotting. The results are given in Table 1.

Analysis of *apx* genes in *A. pleuropneumoniae* strains. Dot blot hybridization of *A. pleuropneumoniae* field strains was carried out by using DNA probes for the individual genes *apxIC*, *apxIA*, *apxIB*, *apxID*, *apxIIC*, *apxIIA*, *apxIIIC*, *apxIIIA*, *apxIIIB*, and *apxIIID*. The probe for the *apxIA* gene was truncated at its 3′ end (Fig. 1) in order to avoid hybridization with the short 3′-end *apxIA* segment present upstream of the *apxIBD* genes of the truncated *apxI* operons in serotypes 2, 4, 6, 7, 8, and 12 (14, 15, 21). No cross-hybridization between the individual genes was detected. The gene probe for *apxIA* did not distinguish between the two variants of the *apxIA* gene (14, 21). The *apx* gene profiles of the serotype reference strains (Table 1) were as described previously (15, 22) and are shown in Fig. 1.

Among the 191 biovar 1 field strains analyzed, 188 showed the same *apx* genes as their respective serotype reference strain (Table 1). Three of them had altered gene patterns. Serotype 2 strain 19/79 from the United Kingdom lacked the *apxIIIC-ABD* genes. Serotype 6 strain 1166 originating from Denmark lacked the entire *apxII* operon and was nonhemolytic when grown on blood agar plates. Serotype 11 strain S499/92 from Switzerland lacked genes *apxICA* and was only weakly hemolytic on blood agar plates. It was difficult to serotype this strain, which showed cross-reactions with several other serotypes.

Several biovar 1 strains showed a gene pattern which did not correspond to that of the initially determined serotype. However, upon retyping of these strains we found that the initial serotype was erroneous and that the newly determined serotype corresponded in most cases to the *apx* gene pattern of the corresponding reference strain. In particular we analyzed strains originating from pleuropneumonia outbreaks, which initially were typed as serotype 3 strains. These strains had an *apx* gene pattern that was atypical for serotype 3 strains because of the presence of the *apxIB* and *apxID* genes (giving an *apx* gene profile the same as those for serotypes 2, 4, 6, and 8). The presence of *apxIB* and *apxID* in these strains was also associated with the secretion of the ApxII toxin into the growth medium at a much higher level than that by serotype 3 strains, which lack *apxIB* and *apxID* (data not shown). Retyping of these strains revealed that they were serotype 8 strains and hence had a typical *apx* gene pattern for this serotype (Fig. 1). Serotyping of these strains, however, proved to be extremely difficult because of cross-reactions between serotype 3 and 8 strains. For four field strains from various origins no obvious serotype could be determined. Their gene profile was the same as those for serotype 7 and 12 reference strains. However, data for these strains are not included in Table 1.

Biovar 2 field strains of serotypes 2 and 4 showed an *apx* gene profile different from those of biovar 1 strains with these serotypes. All strains of both serotypes were devoid of *apxIIICABD*. They contained *apxIBD* and *apxIICA* genes as their biovar 1 analogs. The gene profiles of biovar 2 strains of serotypes 7 and 9 were the same as those for biovar 1 strains with these serotypes.

Expression of ApxI, ApxII, and ApxIII. The immunoblot analysis showing the expression of ApxI, ApxII, and ApxIII toxins by specific monoclonal antibodies in the serotype reference strains reflected the presence of the corresponding *apx* genes in these strains. Four toxin-producing groups can be distinguished. Group 1, which included serotypes 1, 5a/b, 9, and 11, produced toxins ApxI and ApxII; group 2, which included serotypes 2, 3, 4, 6, and 8, produced toxins ApxII and ApxIII; group 3, which included serotypes 7 and 12, produced toxin ApxII; and group 4, which included serotype 10, produced only ApxI.

The 204 field strains of biovars 1 and 2 showed a correlation between the Apx toxins that they produced and the presence of the corresponding *apxA* gene for the toxins with the exception of only two strains, indicating that the *apx* genes are in general expressed. Serotype 2 strain 618 showed no production of ApxII (Table 1), despite the presence of the *apxIICA* genes, which were present in full length, as verified by PCR analysis with the primers corresponding to the beginning of *apxIIC* and

TABLE 1. *apx* genotypes and Apx phenotypes of *A. pleuropneumoniae* reference and field strains

Serotype	Biotype	Strain no.	Origin	Phenotype			Genotype									
				ApxI	ApxII	ApxIII	*apxIC*	*apxIA*	*apxIB*	*apxID*	*apxIIC*	*apxIIA*	*apxIIIC*	*apxIIIA*	*apxIIIB*	*apxIIID*
1	1	4074	Reference strain	+	+	−	+	+	+	+	+	+	−	−	−	−
	1	13 field strains	Four different countries	+	+	−	+	+	+	+	+	+	−	−	−	−
2	1	S1536	Reference strain	−	+	+	−	−	+	+	+	+	+	+	+	+
	1	17 field strains	Five different countries	−	+	+	−	−	+	+	+	+	+	+	+	+
	1	618	The Netherlands[a]	−	−	+	−	−	+	+	+	+	+	+	+	+
	1	19/79	United Kingdom[b]	−	+	−	−	−	+	+	+	+	−	−	−	−
3	1	S1421	Reference strain	−	+	+	−	−	−	−	+	+	+	+	+	+
	1	9 field strains	Two different countries	−	+	+	−	−	−	−	+	+	+	+	+	+
4	1	M62	Reference strain	−	+	+	−	−	+	+	+	+	+	+	+	+
	1	5 field strains	Spain	−	+	+	−	−	+	+	+	+	+	+	+	+
5a	1	K17	Reference strain	+	+	−	+	+	+	+	+	+	−	−	−	−
5b	1	L20	Reference strain	+	+	−	+	+	+	+	+	+	−	−	−	−
5	1	18 field strains	Five different countries	+	+	−	+	+	+	+	+	+	−	−	−	−
6	1	femϕ	Reference strain	−	+	+	−	−	+	+	+	+	+	+	+	+
	1	16 field strains	Three different countries	−	+	+	−	−	+	+	+	+	+	+	+	+
	1	1166	Denmark[b]	−	−	+	−	−	+	+	−	−	+	+	+	+
7	1	WF83	Reference strain	−	+	−	−	−	+	+	+	+	−	−	−	−
	1	34 field strains	Nine different countries	−	+	−	−	−	+	+	+	+	−	−	−	−
8	1	405	Reference strain	−	+	+	−	−	+	+	+	+	+	+	+	+
	1	24 field strains	Nine different countries	−	+	+	−	−	+	+	+	+	+	+	+	+
9	1	CVI13261	Reference strain	+	+	−	+	+	+	+	+	+	−	−	−	−
	1	16 field strains	Five different countries	+	+	−	+	+	+	+	+	+	−	−	−	−
10	1	13039	Reference strain	+	−	−	+	+	+	+	−	−	−	−	−	−
	1	11 field strains	Five different countries	+	−	−	+	+	+	+	−	−	−	−	−	−
	1	269	Norway[a]	−	−	−	+	+	+	+	−	−	−	−	−	−
11	1	56153	Reference strain	+	+	−	+	+	+	+	+	+	−	−	−	−
	1	8 field strains	Two different countries	+	+	−	+	+	+	+	+	+	−	−	−	−
	1	S499/92	Switzerland[b]	−	+	−	−	−	+	+	+	+	−	−	−	−
12	1	8329	Reference strain	−	+	−	−	−	+	+	+	+	−	−	−	−
	1	15 field strains	Four different countries	−	+	−	−	−	+	+	+	+	−	−	−	−
2	2	4 field strains	Switzerland	−	+	−	−	−	+	+	+	+	−	−	−	−
4	2	1 field strain	Italy	−	+	−	−	−	+	+	+	+	−	−	−	−
7	2	2 field strains	Italy	−	+	−	−	−	+	+	+	+	−	−	−	−
9	2	6 field strains	Italy	+	+	−	+	+	+	+	+	+	−	−	−	−

[a] Strains with the same Apx gene patterns as the reference strain but with toxin phenotypes different from that of the reference strain.
[b] Strains with Apx gene patterns and toxin phenotypes different from those of the reference strain.

to the end of *apxIIA* (data not shown). Serotype 10 strain 269 expressed no ApxI (Table 1), despite the presence of the full *apxI* operon. For this strain we verified the full length of the *apxICA* genes by PCR with primers corresponding to the beginning of *apxIC* and the end of *apxIA* (data not shown). These two strains therefore seem to contain punctual mutations in either the promoters or the coding sequences of *apxIICA* (strain 618) or *apxICA* (strain 269).

DISCUSSION

Our hybridization results revealed a high degree of correlation between the serotypes and the pattern of *apx* toxin genes in biovar 1 strains originating from very different geographic locations worldwide. This indicates that the *apx* operons are inherent to their serotypes. The analysis of production of the different toxin proteins ApxI, ApxII, and ApxIII revealed that in all except two strains analyzed the structural toxin genes are expressed. Since strains expressing ApxIA were strongly hemolytic and strains expressing ApxIIA but not ApxIA were weakly hemolytic, we conclude that not only the structural toxin genes *apxIA* and *apxIIA*, respectively, but also the activator genes *apxIC* and *apxIIC*, respectively, were expressed. The two strains that did not express ApxI or ApxII but that possessed *apxICA* or *apxIICA*, respectively, are thought to contain punc-

tual mutations in the promoter or the coding regions. Such strains are rare, however, and the presence of the *apx* toxin genes can be considered potential indicators of the production of the corresponding Apx toxins.

At a first glance, we observed much less correlation between the *apx* gene profiles and the serotypes which were initially given to the field strains. However, retyping of the strains revealed that their initial serotypes were erroneous. After retyping the strains used in the study, we observed a very high degree of correlation between the serotype and the *apx* gene profiles. Of particular interest were several strains that were initially believed to be serotype 3 strains and that were known to be virulent and isolated from *A. pleuropneumoniae* outbreaks. These field strains showed an *apx* profile that was different from that of the serotype 3 reference strain because of the presence of the *apxIBD* genes. In contrast to the serotype 3 reference strain (13), they also strongly secreted ApxII, which we assumed was due to the *apxIBD* gene products. Retyping of these strains revealed that they belonged to serotype 8. In this respect it must be noted that the serological differentiation between serotypes 3 and 8 is difficult because of the presence of the same O3 antigen in both of these serotypes (3, 35).

It is interesting that three strains which showed divergent *apx* gene profiles were lacking either *apxICA* or *apxIICA* or the complete *apxIII* operon, suggesting that deletions might have occurred at hot spots of recombination or via the action of insertion sequence-like elements. It has been shown previously that spontaneous deletions of approximately 8 kbp in length can occur, removing the *apxIICA* genes from serotype 7 strains, which seemed to be induced by insertion sequence-like sequences (1). It must also be noted that it was difficult to attribute unambiguously the serotype to these strains with atypical *apx* gene patterns. Unless the chemical structures of K and O antigens of such strains have been determined and their serological cross-reactions established, it is not clear whether they could represent an unknown serotype or a subtype. The same could be the case for four strains, data for which are not included in Table 1, which gave a toxin gene profile like those for serotypes 7 and 12 but which could not be serotyped.

It has been speculated before that the different Apx toxins of *A. pleuropneumoniae* are involved in virulence, in particular on the basis of the observation that the reference strains of serotypes 1, 5, 9, and 11, which are known to be involved in highly virulent outbreaks, all produced the toxin ApxI (13, 16, 24). The results of the present study support this view, since it allows direct comparison between virulence, serotype, toxin genes, and the expression of Apx toxins in field strains. In this respect it is interesting that several strains which we received and which were reported to be highly virulent but which belonged to one of the serotypes generally known for their low virulence were shown in our study to express ApxI and to contain the *apx* gene profile of the group of serotypes 1, 5, 9, and 11. Retyping of these strains then revealed the true serotypes to be 1, 5, 9, or 11.

Thirteen strains of biovar 2 belonging to serotypes 2, 4, 7, and 9 were tested. They showed no genes of the *apxIII* operon, leading to a different gene pattern for serotypes 2 and 4 compared with those of biovar 1 strains. With regard to the *apxI* and *apxII* genes, biovar 2 strains have the same patterns as their corresponding serotypes of biovar 1. The biovar 2 strains of serotypes 7 and 9 therefore show the same *apx* gene profiles as their biovar 1 analogs. It has been described previously (10) that biovar 2 strains of serotype 2 were less virulent than biovar 1 strains of the same serotype. Although we did not use the same strains in our study, the difference in virulence that we observed might be explained by the lack of ApxIII in biovar 2 strains and would support previous findings on the involvement of the ApxIII toxin in virulence (37).

In conclusion, we showed that the *apx* genes of *A. pleuropneumoniae* are inherent to a given serotype and are an indication of the degree of virulence of a given strain. Typing of *apx* genes in *A. pleuropneumoniae* by means of specific hybridizations or PCR-based methods to reveal genes *apxICA* and *apxIICA* and the *apxIII* operon or significant parts of these genes or the analysis of expression of the different Apx toxins by specific antibodies therefore represents a valuable tool complementary to the serotyping of *A. pleuropneumoniae* in diagnostics and epidemiology. In addition, the results of the study will have a large impact in the design of efficient vaccines for the prevention of porcine pleuropneumonia.

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REFERENCES

1. **Anderson, C., A. A. Potter, and G. F. Gerlach.** 1991. Isolation and molecular characterization of spontaneously occurring cytolysin-negative mutants of *Actinobacillus pleuropneumoniae* serotype 7. Infect. Immun. **59:**4110–4116.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1990. Current protocols in molecular biology. Wiley Interscience, New York.
3. **Beynon, L. M., J. C. Richards, and M. B. Perry.** 1993. Characterization of the *Actinobacillus pleuropneumoniae* serotype K11/01 capsular antigen. Eur. J. Biochem. **214:**209–214.
4. **Brandreth, S. R., and I. M. Smith.** 1987. Comparative virulence of some English strains of *Haemophilus pleuropneumoniae* serotypes 2 and 3 in the pig. Res. Vet. Sci. **42:**187–193.
5. **Bullock, W. O., J. M. Fernandez, and J. M. Short.** 1987. XL1-Blue: a high frequency efficiency plasmid transforming recA *Escherichia coli* strain with beta-galactosidase selection. BioTechniques **5:**376–378.
6. **Chang, Y. F., J. Shi, D. Ma, S. Shin, and D. H. Lein.** 1993. Molecular analysis of the *Actinobacillus pleuropneumoniae* RTX toxin-III gene cluster. DNA Cell Biol. **12:**351–362.
7. **Chang, Y. F., R. Young, and D. K. Struck.** 1989. Cloning and characterization of a hemolysin gene from *Actinobacillus* (*Haemophilus*) *pleuropneumoniae*. DNA **8:**635–647.
8. **Chang, Y. F., R. Y. Young, and D. K. Struck.** 1991. The *Actinobacillus pleuropneumoniae* hemolysin determinant: unlinked *appCA* and *appBD* loci flanked by pseudogenes. J. Bacteriol. **173:**5151–5158.
9. **Desrosiers, R., K. R. Mittal, and R. Malo.** 1984. Porcine pleuropneumonia associated with *Haemophilus pleuropneumoniae* serotype 3 in Quebec. Vet. Rec. **115:**628–629.
10. **Dom, P., and F. Haesebrouck.** 1992. Comparative virulence of NAD-dependent and NAD-independent *Actinobacillus pleuropneumoniae* strains. Zentralbl. Veterinarmed. **39:**303–306.
11. **Fales, W. H., L. G. Morehouse, K. R. Mittal, C. Bean Knudsen,**

S. L. Nelson, L. D. Kintner, J. R. Turk, M. A. Turk, T. P. Brown, and D. P. Shaw. 1989. Antimicrobial susceptibility and serotypes of *Actinobacillus* (*Haemophilus*) *pleuropneumoniae* recovered from Missouri swine. J. Vet. Diagn. Invest. **1**:16–19.

12. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132**:6–13.
13. **Frey, J.** 1994. RTX-toxins in *Actinobacillus pleuropneumoniae* and their potential role in virulence, p. 325–340. *In* C. I. Kado and J. H. Crosa (ed.), Molecular mechanisms of bacterial virulence. Kluwer Academic Publishers, Dordrecht, The Netherlands.
14. **Frey, J., M. Beck, U. Stucki, and J. Nicolet.** 1993. Analysis of hemolysin operons in *Actinobacillus pleuropneumoniae*. Gene **123**: 51–58.
15. **Frey, J., J. T. Bosse, Y. F. Chang, J. M. Cullen, B. Fenwick, G. F. Gerlach, D. Gygi, F. Haesebrouck, T. J. Inzana, R. Jansen, E. M. Kamp, J. Macdonald, J. I. MacInnes, K. R. Mittal, J. Nicolet, A. N. Rycroft, R. P. A. M. Segers, M. A. Smits, E. Stenbaek, D. K. Struck, J. F. Vandenbosch, P. J. Willson, and R. Young.** 1993. *Actinobacillus pleuropneumoniae* RTX-toxins—uniform designation of haemolysins, cytolysins, pleurotoxin and their genes. J. Gen. Microbiol. **139**:1723–1728.
16. **Frey, J., and J. Nicolet.** 1990. Hemolysin patterns of *Actinobacillus pleuropneumoniae*. J. Clin. Microbiol. **28**:232–236.
17. **Gutierrez, C. B., R. I. Tascon, J. A. Vazquez, and E. F. Rodriguez Ferri.** 1991. Cross-reactivity between *Actinobacillus pleuropneumoniae* serotypes comparing different antigens and serological tests. Res. Vet. Sci. **50**:308–310.
18. **Gygi, D., J. Nicolet, C. Hughes, and J. Frey.** 1992. Functional analysis of the Ca^{2+}-regulated hemolysin I operon of *Actinobacillus pleuropneumoniae* serotype 1. Infect. Immun. **60**:3059–3064.
19. **Inzana, T. J., J. Todd, J. N. Ma, and H. Veit.** 1991. Characterization of a nonhemolytic mutant of *Actinobacillus pleuropneumoniae* serotype 5: role of the 110 kilodalton hemolysin in virulence and immunoprotection. Microb. Pathog. **10**:281–296.
20. **Jansen, R., J. Briaire, E. M. Kamp, A. L. J. Gielkens, and M. A. Smits.** 1993. Cloning and characterization of the *Actinobacillus pleuropneumoniae* RTX-toxin III (ApxIII) gene. Infect. Immun. **61**:947–954.
21. **Jansen, R., J. Briaire, E. M. Kamp, A. L. J. Gielkens, and M. A. Smits.** 1993. Structural analysis of the *Actinobacillus pleuropneumoniae* RTX-toxin-I (ApxI) operon. Infect. Immun. **61**:3688–3695.
22. **Jansen, R., J. Briaire, E. M. Kamp, and M. A. Smits.** 1992. The cytolysin genes of *Actinobacillus pleuropneumoniae*. Proceedings of the 12th International Pig Veterinary Society, The Hague, The Netherlands **12**:197. (Abstract.)
23. **Jansen, R., J. Briaire, E. M. Kamp, and M. A. Smits.** 1992. Comparison of the cytolysin II genetic determinants of *Actinobacillus pleuropneumoniae* serotypes. Infect. Immun. **60**:630–636.
24. **Komal, J. P., and K. R. Mittal.** 1990. Grouping of *Actinobacillus pleuropneumoniae* strains of serotypes 1 through 12 on the basis of their virulence in mice. Vet. Microbiol. **25**:229–240.
25. **Macdonald, J., and A. N. Rycroft.** 1992. Molecular cloning and expression of *ptxA*, the gene encoding the 120-kilodalton cytotoxin of *Actinobacillus pleuropneumoniae* serotype 2. Infect. Immun. **60**: 2726–2732.
26. **Martineau, G. P., R. Desrosiers, R. Charette, and C. Moore.** 1984. Control measures and economical aspects of swine pleuropneumonia in Quebec, p. 97–111. *In* Proceedings of the American Association of Swine Practitioners, Kansas City, Mo.
27. **Mittal, K. R., and S. Bourdon.** 1991. Cross-reactivity and antigenic heterogeneity among *Actinobacillus pleuropneumoniae* strains of serotypes 4 and 7. J. Clin. Microbiol. **29**:1344–1347.
28. **Mittal, K. R., R. Higgins, and S. Lariviere.** 1982. Evaluation of slide agglutination and ring precipitation tests for capsular serotyping of *Haemophilus pleuropneumoniae*. J. Clin. Microbiol. **15**: 1019–1023.
29. **Mittal, K. R., R. Higgins, and S. Lariviere.** 1988. Quantitation of serotype-specific and cross-reacting group-specific antigens by coagglutination and immunodiffusion tests for differentiating *Actinobacillus* (*Haemophilus*) *pleuropneumoniae* strains belonging to cross-reacting serotypes 3, 6, and 8. J. Clin. Microbiol. **26**:985–989.
30. **Mittal, K. R., R. Higgins, S. Lariviere, and D. Leblanc.** 1984. A 2-mercaptoethanol tube agglutination test for diagnosis of *Haemophilus pleuropneumoniae* infection in pigs. Am. J. Vet. Res. **45**: 715–719.
31. **Nicolet, J.** 1992. *Actinobacillus pleuropneumoniae*, p. 401–408. *In* A. D. Leman, B. E. Straw, W. L. Mengeling, S. D'Allaire, and D. J. Taylor (ed.), Diseases of swine. Iowa State University Press, Ames.
32. **Nielsen, R.** 1985. *Haemophilus pleuropneumoniae* (*Actinobacillus pleuropneumoniae*). Serotypes 8, 3 and 6. Serological response and cross immunity in pigs. Nord. Vet. Med. **37**:217–227.
33. **Nielsen, R.** 1990. New diagnostic techniques: a review of the HAP group of bacteria. Can. J. Vet. Res. **54**(Suppl.):S68–S72.
34. **Nielsen, R., and P. J. O Connor.** 1984. Serological characterization of 8 *Haemophilus pleuropneumoniae* strains and proposal of a new serotype: serotype 8. Acta Vet. Scand. **25**:96–106.
35. **Perry, M. B., E. Altman, J. R. Brisson, L. M. Beynon, and J. C. Richards.** 1990. Structural characteristics of the antigenic capsular polysaccharides and lipopolysaccharides involved in the serological classification of *Actinobacillus* (*Haemophilus*) *pleuropneumoniae* strains. Serodiagn. Immunother. Infect. Dis. **4**:299–308.
36. **Rapp, V. J., R. F. Ross, and B. Z. Erickson.** 1985. Serotyping of *Haemophilus pleuropneumoniae* by rapid slide agglutination and indirect fluorescent antibody tests in swine. Am. J. Vet. Res. **46**:185–192.
37. **Rycroft, A. N., D. Williams, I. A. McCandlish, and D. J. Taylor.** 1991. Experimental reproduction of acute lesions of porcine pleuropneumonia with a haemolysin-deficient mutant of *Actinobacillus pleuropneumoniae*. Vet. Rec. **129**:441–443.
38. **Turk, J. R., W. H. Fales, C. W. Maddox, J. A. Ramos, J. R. Fischer, G. C. Johnson, J. M. Kreeger, M. A. Miller, L. W. Pace, S. E. Turnquist, and H. S. Gosser.** 1993. Pleuropneumonia in Missouri swine. J. Vet. Diagn. Invest. **5**:101–103.
39. **Welch, R. A.** 1991. Pore-forming cytolysins of gram-negative bacteria. Mol. Microbiol. **5**:521–528.