***Actinobacillus pleuropneumoniae* RTX-toxins: uniform designation of haemolysins, cytotoxins, pleurotoxin and their genes**

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The three different pore-forming RTX-toxins of *Actinobacillus pleuropneumoniae* are reviewed, and new and uniform designations for these toxins and their genes are proposed. The designation ApxI (for *Actinobacillus pleuropneumoniae* RTX-toxin I) is proposed for the RTX-toxin produced by the reference strains for serotypes 1, 5a, 5b, 9, 10 and 11, which was previously named haemolysin I (HlyI) or cytotoxin I (ClyI). This protein is strongly haemolytic and shows strong cytotoxic activity towards pig alveolar macrophages and neutrophils; it has an apparent molecular mass in the range 105 to 110 kDa. The genes of the *apxI* operon will have the designations *apxIC*, *apxIA*, *apxIB*, and *apxID* for the activator, the structural gene and the two secretion genes respectively. The designation ApxII is proposed for the RTX-toxin which is produced by all serotype reference strains except serotype 10 and which was previously named App, HlyII, ClyII or Cyt. This protein is weakly haemolytic and moderately cytotoxic and has an apparent molecular mass between 103 and 105 kDa. The genes of the *apxII* operon will have the designations *apxIIC* for the activator gene and *apxIIA* for the structural toxin gene. In the *apxII* operon, no genes for secretion proteins have been found. Secretion of ApxII seems to occur via the products of the secretion genes *apxIB* and *apxID* of the *apxI* operon. The designation ApxIII is proposed for the non-haemolytic RTX-toxin of the reference strains for serotypes 2, 3, 4, 6 and 8, which was previously named cytotoxin III (ClyIII), pleurotoxin (Ptx), or macrophage toxin (Mat). This protein is strongly cytotoxic and has an apparent molecular mass of 120 kDa. The genes of the *apxIII* operon have the designations *apxIIIC*, *apxIIIA*, *apxIIIB* and *apxIIID* for the activator gene, the structural gene and the two secretion genes respectively.

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Introduction

Actinobacillus pleuropneumoniae is a Gram-negative bacterium belonging to the family *Pasteurellaceae*. It is the aetiological agent of swine pleuropneumonia, a severe disease causing large economic losses in industrialized swine production (Shope, 1964; Nicolet, 1992). Among the twelve different serotypes, three different exotoxins which belong to the group of pore-forming RTX-toxins (Welch, 1991) have been detected (Frey & Nicolet, 1988*a*, 1990; Frey *et al.*, 1991*b*, 1992; Chang *et al.*, 1989; Rycroft *et al.*, 1991*a*; Kamp *et al.*, 1991; Smits *et al.*, 1991; Lalonde *et al.*, 1989; Jansen *et al.*, 1993, 1992*b*; Macdonald & Rycroft, 1992; van den Bosch, 1991; Gygi *et al.*, 1990). Two of these RTX-toxins possess both haemolytic and cytotoxic activity (Frey & Nicolet, 1988*a*; Rosendal *et al.*, 1988; Kamp *et al.*, 1991) while the third has cytotoxic, but not haemolytic activity (Rycroft *et al.*, 1991*a*; Kamp *et al.*, 1991). The RTX-toxins have molecular masses of 100–120 kDa and can be distinguished serologically by polyclonal and monoclonal antibodies (Frey *et al.*, 1992; Kamp *et al.*, 1991; Rycroft *et al.*, 1991*a*; Kamp & van Leengoed, 1989). Reference and field strains of several serotypes produce two different RTX-toxins (Frey *et al.*, 1992; Kamp *et al.*, 1991; Rycroft *et al.*, 1991*a*; Jansen *et al.*, 1992*a*, *b*, 1993), a situation which is rather unusual among RTX-toxin-producing bacteria and which created confusions in the serological distinction of different RTX-proteins. All RTX-toxins of *A. pleuropneumoniae* have been shown to be major immunogenic factors in experimentally and

naturally infected pigs (Devenish *et al.*, 1990*a*, *b*; Ma & Inzana, 1990; Frey & Nicolet, 1991; Rycroft *et al.*, 1991*a*) and seem to be important virulence factors (Inzana *et al.*, 1991; Kamp *et al.*, 1991; Stine *et al.*, 1991; Rycroft *et al.*, 1991*a*, *b*; Udeze & Kadis, 1992; Devenish *et al.*, 1992; Dom *et al.*, 1992). They have also been shown to be involved in the induction of protective immunity and are suggested to be important components in vaccines against swine pleuropneumonia (Bosse *et al.*, 1992; Bhatia *et al.*, 1991; Inzana *et al.*, 1991; Rycroft *et al.*, 1991*a*; Rossi Campos *et al.*, 1992; van den Bosch *et al.*, 1992; van den Bosch, 1991; Devenish *et al.*, 1990*a*; Fedorka Cray *et al.*, 1990).

In general, the operons encoding RTX-toxins consist of four contiguous genes in the order C A B D. The A gene encodes the structural toxin protein, the C gene encodes an activator protein which is involved in fatty acylation in the conversion of the protoxin to active toxin, and the B and D genes encode membrane-associated proteins for secretion of the toxins (Issartel *et al.*, 1991; Welch, 1991; Felmlee *et al.*, 1985). While this is also true for several RTX-operons of *A. pleuropneumoniae* (Gygi *et al.*, 1992; Jansen *et al.*, 1993, 1992*b*), partial RTX-operons containing two genes have been found in *A. pleuropneumoniae* and *Actinobacillus suis* (Burrows & Lo, 1992; Frey *et al.*, 1993; Chang *et al.*, 1991; Jansen *et al.*, 1992*a*, *b*; Smits *et al.*, 1991). The presence of different combinations of the three RTX-toxins in strains from the same species originally led to considerable confusion, probably because of the very similar molecular masses of these toxins. Due to the

Table 1. New designations and synonyms used for the various RTX-toxins found in *A. pleuropneumoniae*

New designation				Synonyms*					
Name of toxin	Protein/ gene symbols	Name of toxin	Protein/ gene symbols	Name of toxin	Protein/ gene FCR symbols	Name of toxin	Protein/ gene symbols	Name of toxin	Protein/ gene symbols
RTX-toxin I	ApxI apxIC apxIA apxIB apxID	Haemolysin I	HlyI ^e hlyIC ^{f,g} hlyIA ^{f,g} hlyIB ^g hlyID ^g	Cytolysin I	ClyI ^k clyIC ⁱ clyIA ⁱ clyIB ⁱ clyID ⁱ		appB ^d appD ^d		
RTX-toxin II	ApxII apxIIC apxIIA	Haemolysin II	HlyII ^e hlyIIC ^f hlyIIA ^f	Cytolysin II	ClyII ^k clyIIC ⁱ clyIIA ⁱ	Haemolysin	App ^c appC ^c appA ^c	Cytolysin	Cyt ^a cytC ^a cytA ^a
RTX-toxin III	ApxIII apxIIIC apxIIIA apxIIIB apxIIID	Pleurotoxin	Ptx ^m ptxA ^l	Cytolysin III	ClyIII ^k clyIIIC ^{h,j} clyIIIA ^{h,j} clyIIIB ^h clyIIID ^h			Macrophage- toxin	Mat ^b

* References: *a*, Anderson *et al.* (1991); *b*, van den Bosch *et al.* (1992); *c*, Chang *et al.* (1989); *d*, Chang *et al.* (1991); *e*, Frey & Nicolet (1988*a*); *f*, Frey *et al.* (1992); *g*, Gygi *et al.* (1992); *h*, Jansen *et al.* (1992*a*); *i*, Jansen *et al.* (1992*b*); *j*, Jansen *et al.* (1993); *k*, Kamp *et al.* (1991); *l*, Macdonald & Rycroft (1992); *m*, Rycroft *et al.* (1991*a*).

intense research efforts on *A. pleuropneumoniae* RTX toxins which have been made simultaneously by various groups during the last few years, several different toxin names and gene designations have been used. A combination of the results from the molecular characterization at the genetic level and serological and toxicity studies has resulted in the present understanding of RTX-toxins and genes in *A. pleuropneumoniae* strains. The researchers working on *A. pleuropneumoniae* RTX toxins therefore made the effort to agree upon a common, uniform nomenclature for these toxins which was proposed at the Fallen Leaf Lake Conference 1992 on 'Bacterial Virulence Mechanisms'. These new designations and their synonyms are listed in Table 1 and are briefly described below. They are abbreviated by Apx, standing for *Actinobacillus pleuropneumoniae* RTX-toxins. ApxI, ApxII and ApxIII will be used according to numbering used in previously published work (Jansen *et al.*, 1992a; Smits *et al.*, 1991; Kamp *et al.*, 1991; Frey & Nicolet, 1988a, 1990). For RTX toxins from other species the same strategy for abbreviations could be used.

ApxI (previously HlyI or ClyI)

A. pleuropneumoniae-RTX-toxin I (ApxI) was initially described as the strongly haemolytic haemolysin I (HlyI), purified from serotype 1 type strain 4074 as an active protein with an apparent molecular mass of 105 kDa (Frey & Nicolet, 1988a, b). ApxI was also found to be cytotoxic for alveolar macrophages and neutrophils and therefore it was also named cytotoxin I (ClyI) (Kamp *et al.*, 1991). ApxI was found to be present in the reference strains of serotypes 1, 5a, 5b, 9, 10 and 11 (Frey & Nicolet, 1990; Frey *et al.*, 1992; Kamp *et al.*, 1991). In these reference strains the operon of ApxI contains four genes: *apxIC*, the activator gene; *apxIA*, the structural toxin gene; and *apxIB* and *apxID*, the secretion genes (Table 2). These genes were previously named *hlyIC*, *hlyIA*, *hlyIB* and *hlyID* respectively in serotype 1 (Gygi *et al.*, 1990, 1992; Frey *et al.*, 1993) or *clyIC*, *clyIA*, *clyIB* and *clyID* in serotype 9 (Jansen *et al.*, 1992a, b; Smits *et al.*, 1991). The *apxIA* genes of serotypes 1, 9 and 11 are similar whereas they show allelic variation in serotypes 5a, 5b and 10 (Jansen *et al.*, 1992b; Frey *et al.*, 1993). The genes *apxIB* and *apxID* correspond to the genes *appB* and *appD*, which were initially believed to be the secretion genes of ApxII (initially named App) (Chang *et al.*, 1991). The *apxI* operon is transcribed into two RNA species: a major one of 3.5 kb which is Ca^{2+} inducible and contains *apxIC* and *apxIA*, and a minor transcript of 7.5 kb which is not induced by Ca^{2+} and contains the whole operon *apxICABD* (Gygi *et al.*, 1992). The reference strains of serotypes 2, 4, 6, 7, 8 and

12 do not possess *apxIC* and *apxIA* and therefore these serotypes do not produce ApxI. Instead these serotypes possess a truncated *apxI* operon containing *apxIB* and *apxID* and the C-terminal end of *apxIA* (Frey *et al.*, 1993; Jansen *et al.*, 1992b). The serotype 3 reference strain lacks the entire *apxI* operon (Frey *et al.*, 1993; Jansen *et al.*, 1992b). The structural gene *apxIA* has been sequenced and was shown to be similar to the *Escherichia coli* haemolysin gene *hlyA* and to a lesser extent to *Pasteurella haemolytica* leukotoxin gene *lktA* (Frey *et al.*, 1991b, 1993). *apxIA* encodes the protein ApxIA, which has a molecular mass of 110.2 kDa as deduced from the DNA sequence. It contains 13 glycine-rich repeats and binds $^{45}\text{Ca}^{2+}$ (Frey *et al.*, 1991b). This binding is required for haemolytic activity and binding to neutrophils (van Leengoed & Dickerson, 1992; Devenish & Rosendal, 1991).

ApxII (previously HlyII, ClyII, App or Cyt)

ApxII was initially described as the weakly haemolytic toxin HlyII (haemolysin II) with an apparent molecular mass of 105 kDa isolated from serotype 2 reference strain S1536 (Frey & Nicolet, 1988a, 1990; Frey *et al.*, 1991a). ApxII was identified as a 103 kDa protein by monoclonal antibodies in the reference strains of all serotypes except 10 (Kamp *et al.*, 1991). Besides its haemolytic activity, ApxII was also found to be cytotoxic for alveolar macrophages and neutrophils and was designated cytotoxin II (ClyII) (Kamp *et al.*, 1991). The genes encoding the activator and the structural protein of ApxII were first cloned from serotype 5 and sequenced (Chang *et al.*, 1989) and designated *appC* and *appA* respectively. These sequences appeared to be nearly identical to the *apxIICA* (*clyIICA*) genes of serotype 9 (Smits *et al.*, 1991). Using PCR amplification, gene expression and monoclonal and polyclonal antibodies, the AppA protein was shown to be identical to ApxIIA and to be produced by all serotypes except 10 (Frey *et al.*, 1992; Kamp *et al.*, 1991; Jansen *et al.*, 1992a; Smits *et al.*, 1991). *apxIIA* is present in all serotype reference strains except 10 and shows no significant allelic variations (Frey *et al.*, 1993; Jansen *et al.*, 1992a). The *apxII* operon was shown to contain only the activator gene *apxIIC* and the gene for the structural protein *apxIIA*, but not the genes for the secretion proteins B and D (Jansen *et al.*, 1992a; Frey *et al.*, 1993; Chang *et al.*, 1989; Smits *et al.*, 1991) (Table 2). The secretion genes were probably deleted, since only short segments of B-like genes were found downstream of *apxIIA* (Chang *et al.*, 1989; Smits *et al.*, 1991). Secretion of the ApxIIA protein seems to occur by *trans*-complementation with *apxIB* and *apxID* gene products (Frey *et al.*, 1993; Jansen *et al.*, 1992a). DNA sequence

Table 2. Presence of the different *apx* genes in the reference strains for the different serotypes of *A. pleuropneumoniae*

Serotype	<i>apxI</i> operon				<i>apxII</i> operon		<i>apxIII</i> operon			
1	<i>apxIC</i>	<i>apxIA</i>	<i>apxIB</i>	<i>apxID</i>	<i>apxIIC</i>	<i>apxIIA</i>				
2			<i>apxIB</i>	<i>apxID</i>	<i>apxIIC</i>	<i>apxIIA</i>	<i>apxIIIC</i>	<i>apxIIIA</i>	<i>apxIIIB</i>	<i>apxIIID</i>
3					<i>apxIIC</i>	<i>apxIIA</i>	<i>apxIIIC</i>	<i>apxIIIA</i>	<i>apxIIIB</i>	<i>apxIIID</i>
4			<i>apxIB</i>	<i>apxID</i>	<i>apxIIC</i>	<i>apxIIA</i>	<i>apxIIIC</i>	<i>apxIIIA</i>	<i>apxIIIB</i>	<i>apxIIID</i>
5a	<i>apxIC</i>	<i>apxIA</i>	<i>apxIB</i>	<i>apxID</i>	<i>apxIIC</i>	<i>apxIIA</i>				
5b	<i>apxIC</i>	<i>apxIA</i>	<i>apxIB</i>	<i>apxID</i>	<i>apxIIC</i>	<i>apxIIA</i>				
6			<i>apxIB</i>	<i>apxID</i>	<i>apxIIC</i>	<i>apxIIA</i>	<i>apxIIIC</i>	<i>apxIIIA</i>	<i>apxIIIB</i>	<i>apxIIID</i>
7			<i>apxIB</i>	<i>apxID</i>	<i>apxIIC</i>	<i>apxIIA</i>				
8			<i>apxIB</i>	<i>apxID</i>	<i>apxIIC</i>	<i>apxIIA</i>	<i>apxIIIC</i>	<i>apxIIIA</i>	<i>apxIIIB</i>	<i>apxIIID</i>
9	<i>apxIC</i>	<i>apxIA</i>	<i>apxIB</i>	<i>apxID</i>	<i>apxIIC</i>	<i>apxIIA</i>				
10	<i>apxIC</i>	<i>apxIA</i>	<i>apxIB</i>	<i>apxID</i>						
11	<i>apxIC</i>	<i>apxIA</i>	<i>apxIB</i>	<i>apxID</i>	<i>apxIIC</i>	<i>apxIIA</i>				
12			<i>apxIB</i>	<i>apxID</i>	<i>apxIIC</i>	<i>apxIIA</i>				

Data were compiled from: Jansen *et al.* (1992a, b); Frey *et al.* (1992, 1993); Gygi *et al.* (1992); Smits *et al.* (1991); Chang *et al.* (1989, 1991). See text for details.

analysis of *apxIIA* reveals that the ApxIIA protein has a calculated molecular mass of 102.5 kDa and contains eight glycine-rich repeats (Chang *et al.*, 1989; Smits *et al.*, 1991). ApxIIA resembles the *P. haemolytica* leukotoxin LktA, and to a lesser extent ApxIA and the *E. coli* haemolysin HlyA (Smits *et al.*, 1991; Chang *et al.*, 1989). *apxIIC* and *apxIIA* were also cloned from serotype 7 and were named *cytC* and *cytA* respectively (Anderson *et al.*, 1991; Rossi Campos *et al.*, 1992) and were shown by DNA sequence analysis to be identical with the *apxIICA* sequences of serotype 5 and 9 (Chang *et al.*, 1989; Smits *et al.*, 1991).

ApxIII (previously Ptx, ClyIII, Mat)

The third RTX-toxin of *A. pleuropneumoniae*, ApxIII, is a protein with an apparent molecular mass of 120 kDa. ApxIII is present in the reference strains of serotypes 2, 3, 4, 6 and 8 and was initially named cytolysin III (ClyIII) (Kamp *et al.*, 1991; Jansen *et al.*, 1992b, 1993), or pleurotoxin (Ptx) (Rycroft *et al.*, 1991a; Macdonald & Rycroft, 1992), or macrophage toxin (Mat) (van den Bosch *et al.*, 1992; van den Bosch, 1991). ApxIII has no haemolytic activity but shows strong cytotoxic activity towards alveolar macrophages and neutrophils (Kamp *et al.*, 1991; Rycroft *et al.*, 1991a; Macdonald & Rycroft, 1992; van den Bosch, 1991). The gene *apxIIIA* for the structural protein ApxIIIA was cloned and expressed in *E. coli* and designated *ptxA*, or *clyIIIA* (Macdonald & Rycroft, 1992; Jansen *et al.*, 1992b). It was found to be present in the reference strains of serotypes 2, 3, 4, 6 and 8 (Macdonald & Rycroft, 1992; Jansen *et al.*, 1992b) (Table 2). In all these serotypes a complete operon consisting of the activator gene *apxIIIC*, the structural gene for the toxin *apxIIIA*, and two secretion genes

apxIIIB and *apxIIID* were found (Table 2) and previously named *clyIIIC*, *clyIIIA* or *ptxA*, *clyIIIB* and *clyIIID* respectively (Jansen *et al.*, 1992b; Macdonald & Rycroft, 1992). The DNA sequence of *apxIIIC* and *apxIIIA* from the serotype 8 reference strain has been established (Jansen *et al.*, 1993). The derived amino acid sequence for ApxIIIA shows the typical features of RTX-toxins, with the three hydrophobic domains in the N-terminal half of the protein and 13 glycine-rich repeats in the C-terminal part of the protein (Jansen *et al.*, 1993). The calculated molecular mass of ApxIIIA is 112.8 kDa (Jansen *et al.*, 1993).

Concluding remarks

Detailed genetic and biochemical data are now available on *A. pleuropneumoniae* RTX-toxins. Their exact functions and target cells still remain to be established. It is therefore most important that all research groups working on these toxins use the uniform designations ApxI, ApxII and ApxIII proposed here, in order to avoid confusion and to ensure rapid research progress in the field.

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