Full virulence of *Actinobacillus pleuropneumoniae* serotype 1 requires both ApxI and ApxII

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ABSTRACT

Most serotypes of *Actinobacillus pleuropneumoniae* produce *in vivo* more than one toxin. To determine the surplus value of the production of more than one toxin in the development of disease, we tested the pathogenicity *in vivo* of isogenic strains of *A. pleuropneumoniae* serotype 1 that are mutated in the toxin genes *apxIA* and/or *apxIIA* or in the toxin transport genes *apxIBD*. Bacteria mutated in both *apxIA* and *apxIIA* or in *apxIBD* were not able to induce pathological lesions, indicating that ApxI and ApxII are essential for the pathogenesis of pleuropneumonia. Infection with isogenic strains lacking either ApxI or ApxII did not consistently lead to pleuropneumonia in contrast to infection with the parent strain S4074. ApxII seemed at least as potent as ApxI for the development of clinical and pathological symptoms. Only one out of four pigs inoculated with an ApxII mutant strain developed mild pneumonia whereas two out of three pigs inoculated with an ApxI mutant strain developed more severe lesions. These results indicate that both ApxI and ApxII of *A. pleuropneumoniae* serotype 1 are necessary for full virulence.
INTRODUCTION

*Actinobacillus pleuropneumoniae* causes porcine pleuropneumonia, a disease which occurs world-wide and affects growing pigs of all ages. The pathology of the disease is characterised by a fibrinous pleuritis and a fibrino-haemorrhagic necrotizing pneumonia with focal pulmonary vascular thrombosis (Nicolet, 1992). Several virulence factors of *A. pleuropneumoniae* have been described that enable the bacterium to survive *in vivo* (Haesebrouck et al., 1997). Major virulence factors are the capsule, the lipopolysaccharide (LPS) and the toxins. The capsule has been shown to protect against killing by antibody and complement and against phagocytosis by polymorphonuclear leucocytes (PMNs) (Inzana et al., 1988; Ward and Inzana, 1994). Smooth lipopolysaccharide (LPS) has been shown to play a role in adherence of *A. pleuropneumoniae* to lung and tracheal frozen sections (Paradis et al., 1994). *A. pleuropneumoniae* produces four different toxins belonging to the family of RTX toxins, named ApxI, ApxII, ApxIII and ApxIV (Kamp et al., 1991; Frey et al., 1993; Jansen et al., 1995; Schaller et al., 1999). In general, RTX toxins are encoded by operons that consist of four contiguous genes C, A, B, and D (Welch, 1991; Fath and Kolter, 1993). Genes C and A are required for the production of active toxin protein and genes B and D are required for the secretion of the active toxin. ApxI, ApxII and ApxIII are expressed *in vitro* and *in vivo* and are in various degrees cytotoxic for lung macrophages, PMN, lung epithelial cells and endothelial cells (Van Leengoed et al., 1989; Serebrin et al., 1991; Dom et al., 1994; Van de Kerkhof et al., 1996). ApxI and ApxII are also haemolytic. ApxIV is expressed *in vivo* only and when expressed in *Escherichia coli* it is weakly haemolytic (Schaller et al., 1999). ApxI, ApxII and ApxIII are essential for the development of clinical disease and the typical lung lesions because mutants that produce none of these three toxins are non-pathogenic (Tascon et al., 1994; Inzana, 1991; Anderson et al., 1991; Reimer et al., 1995).

All *A. pleuropneumoniae* serotypes contain *apxIVA* (Schaller et al., 1999). Serotypes 7, 10 and 12 produce one additional Apx toxin while serotypes 1, 2, 3, 4, 5, 6, 8, 9 and 11 even produce two extra Apx toxins. Since disease and typical lung lesions produced by the different serotypes of *A. pleuropneumoniae* are very similar, the question arises what the surplus value is of the production of more than one toxin. Therefore, we tested the pathogenicity *in vivo* of isogenic strains of *A. pleuropneumoniae* serotype 1 that are mutated in the toxin genes *apxA* and/or *apxB* or in the transport genes *apxD* (Jansen et al., 1995) to determine the contribution of ApxI and ApxII in the development of disease. The results show that the presence of at least ApxI or ApxII is necessary for the development of pneumonia but that the
combination of ApxI and ApxII enhances the virulence of *A. pleuropneumoniae* serotype 1.

**MATERIAL AND METHODS**

**Bacterial strains and growth conditions** *A. pleuropneumoniae* reference strain S4074 (serotype 1) contains genes *apxIA*, *apxIIA* and *apxIVA* encoding ApxI, ApxII and ApxIV and was used to generate toxin mutants by targeted mutagenesis (Jansen *et al.*, 1995). In mutant strain 1 (S4074 Δ*apxIA*) the *apxIA* gene was inactivated, this knockout mutant secretes *in vitro* ApxII only. In mutant strain 14 (S4074 Δ*apxIIA*) the *apxIIA* gene was inactivated, this knockout mutant secretes *in vitro* ApxI only. In mutant strain 21 (S4074 Δ*apxIA* Δ*apxIIA*) both the *apxIA* and *apxIIA* genes were inactivated. This double knockout mutant secretes no Apx toxins *in vitro*. In mutant strain 6 (S4074 Δ*apxIBD*) the *apxIBD* genes were inactivated and this knockout mutant also secretes no Apx toxins *in vitro*.

For preparation of the inocula, *A. pleuropneumoniae* strains were cultured on sheep blood agar plates (SB) supplemented with 0.1% β-nicotinamide adenine dinucleotide (NAD, Calbiochem, La Jolla, USA), for 24 h at 37°C. Fifty colonies were suspended in 100 µl of Eagle’s minimal essential medium (EMEM, Gibco BRL, Paisley, UK), plated on SB+NAD and incubated for 6 h at 37°C. Each plate was then rinsed with 5 ml EMEM and suspensions were stored overnight at 4°C. To determine the number of colony forming units (CFU) of the bacterial suspensions, tenfold dilutions were plated on SB+NAD and incubated at 37°C. After 18 h, the CFU were counted, and inocula were prepared from the bacterial suspensions stored at 4°C overnight by dilution with phosphate-buffered saline solution (PBS; 0.123 M NaCl, 0.01 M Na₂HPO₄, 0.0032 M KH₂PO₄; pH 7.2) to approximately 200 CFU/ml. After inoculation of the pigs, the number of CFU was confirmed by plating 100 µl of the inoculum on SB+NAD. The average inoculum contained 640 CFU. Bacteria isolated from tissue were characterised on the basis of haemolytic activity on SB+NAD.

**Infection experiment** The experiment was performed in two similar, consecutive trials in specific pathogen free pigs from the ID-Lelystad breeding herd free of *A. pleuropneumoniae*. Per trial, ten pigs were randomly allocated to five groups of two pigs. The pigs of each group were housed in sterile stainless steel isolators. In the first trial pigs were about four weeks of age and in the second trial they were about eight weeks of age. Pigs were delivered to the experimental facilities and allowed to acclimate for four days before they were infected. For endobronchial infection, pigs were anaesthetised with a combination of azaperone
APX AND PATHOGENESIS OF *A. PLEUROPNEUMONIAE*

(Stresnil; Jansen Pharmaceutica B.V., Tilburg, The Netherlands) and ketamine hydrochloride (ketamine; Kombivet B.V., Etten-Leur, The Netherlands). Inoculation was performed as previously described (Van Leengoed and Kamp, 1989). Briefly, a catheter with an outer diameter of 2.2 mm was advanced through the trachea deep into the bronchi and 5 ml of bacterial suspension was slowly administered. A total of four pigs per strain was inoculated (divided over the two trials) with approximately 1,000 CFU of the parent strain or of one of the four mutants strains. An inoculation dose of 1,000 CFU of *A. pleuropneumoniae* serotype 9 was sufficient to induce lesions in all animals (Van Leengoed and Kamp, 1989). In the group inoculated with S4074 ΔapxIA only three pigs were infected due to technical problems during inoculation. Pigs were monitored clinically for two days after inoculation. At 0, 6, 12, 24, 36 and 48 hours post infection (hpi) rectal temperatures were measured and the pigs were inspected for clinical symptoms as depression, laboured breathing, coughing, or nasal discharge. All animal experiments were approved by the ethical committee of ID-Lelystad.

**Clinical pathology** To assess the induction and development of disease by the different *A. pleuropneumoniae* strains in the period after inoculation, blood samples were taken at 0, 6, 12, 24, 36 and 48 hpi. White blood cells (WBC) were counted in all blood samples with a Sysmex microcell counter. Serum levels of interleukin (IL) 6, IL 1 and tumour necrosis factor (TNFα) were determined by bioassays at 0 and 12 hpi. Serum IL 6 was measured with a bioassay using murine B9 cells as described by Helle et al. (1988) with slight modifications. Briefly, B9 cells were grown until confluence was reached in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) with 5% heat inactivated foetal bovine serum and sodium penicillin and streptomycin sulphate in presence of 50 U/ml human recombinant IL 6 (CLB Amsterdam, NL, nr. M1449). Cells were washed once with IL 6 free DMEM and suspended at $10^5$ cells/ml in DMEM. Serum samples were titrated in threefold and 50 µl was added to 50 µl of B9 cells in flat bottom wells. Cell proliferation was measured 72 hours after incubation by tetrazolium dye reduction (MTT assay). Results were related to a standard curve generated by dilutions in threefold of human recombinant IL 6 and expressed as units/ml. A slight background reaction was seen before infection and this background level was subtracted from the measured value at 12 hpi. An increase in IL 6 level was regarded as marked when values exceeded the mean of all pigs at 0 hpi by more than two times the standard deviation. An increase was regarded as slightly when values exceeded the mean by more than one standard deviation. Serum IL 1 was determined by using the cell proliferating capacity of IL 1 on the cloned murine T cell line D10 according to Helle et al. (1988) with the modification that proliferation was tested.
by the colorimetric MTT assay. Activity of TNFα in serum samples was determined using WEHI 164 cells, a TNF-cytotoxic cell line, according to the modified procedures of Espevik and Nissen-Meyer (1986).

Pathology Forty-eight hpi pigs were anaesthetised by intravenous injection of pentobarbital and exsanguinated. The lungs were excised and the presence of pleuritis, type of lung changes and the size of lung lesions were recorded. To avoid bias, personnel responsible for clinical inspection and pathological examinations were not informed of the groups to which the animals or tissues belonged. For bacteriological examination, tissue was sampled from the caudal lobe of the right and left lung and from the tracheobronchial lymph node. For histological examination specimens were taken from both distal caudal lung lobes and the tracheobronchial lymph node in cases with no macroscopical lesions. If lesions were present, tissue specimens were taken from the centre and the periphery of altered lung tissue. Specimens for histological examination were fixed in ten percent neutral-buffered formaline for at least 48 hours. Formaline fixed lung tissue was embedded in paraffin and sectioned at 3–5 µm and stained with hematoxylin and eosin. Immunohistological examination of lung tissue for in situ localisation of A. pleuropneumoniae was selectively done on lungs from pigs with lung lesions and on lungs from pigs that were cultured positive for A. pleuropneumoniae. Immunohistology was done by an indirect immunoperoxidase technique. Tissue sections from paraffin embedded tissue were deparaffinised, rehydrated and washed in PBS. After inactivating endogenous peroxidase (30 min in 3% H₂O₂), slides were incubated overnight at 4°C with a hyperimmune rabbit serum raised against A. pleuropneumoniae serotype 11 (dilution 1:10000) in a moist chamber. The used serum was shown to cross-react with serotype 1 (Kamp et al., 1987). To identify antibodies bound to bacteria, slides were incubated with biotin labelled goat anti rabbit immunesera (DAKO, Hamburg, Germany). Bound secondary antibodies were visualised by adding peroxidase-conjugated streptavidin (DAKO) followed by enzymehistochemical staining with 3,3’-diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, USA) and H₂O₂, which results in a brown staining of specific structures. Slides were counterstained with Mayer’s hematoxylin.

Statistical analysis Results of the different toxin mutants, expressed as the number of animals with pneumonic lesions after infection, were compared with results of the parent strain in this study and in identical previous studies with four animals per trial using Fishers exact test.
RESULTS

**Parent strain S4074** Results of clinical signs, WBC count and IL 6 levels after endobronchial infection of pigs with *A. pleuropneumoniae* serotype 1 parent strain S4074 or deletion mutants are summarised in Table 1. The frequency and severity of pleuropneumonia after endobronchial infection with *A. pleuropneumoniae* serotype 1 parent strain S4074 or deletion mutants are summarised in Table 2.

Clinically, all four pigs inoculated with the parent strain showed moderate to severe symptoms typical of an infection with *A. pleuropneumoniae* with laboured breathing and/or coughing at 18 and 24 hours post infection (hpi). Two pigs died about 36 hpi and showed large lesion volumes in the lungs (255 and 277 cm³, Table 2). Clinical signs started in three pigs from 12 hpi on, accompanied by fever of > 40°C. At 18 hpi all pigs had developed fever. The mean rectal body temperature over all time points after infection was highest in this group compared to the other groups and fever was observed in 9 out of 18 observations (Table 1). The number of WBC was increased from 12 hpi on in all four pigs and stayed at the same level or increased further until the end of the experiment. All pigs had increased IL 6 levels at 12 hpi (Table 1), however no correlation was found between levels of IL 6 at this time point and severity of pleuropneumonia. Three of the four pigs had marked elevated IL 6 levels, ranging between 601 and 86,305 U/ml and one pig, which died early, had only slightly elevated IL 6 levels of 70 U/ml (Table 1). TNFα and IL 1 were detected in none of the sera at 12 hpi. All four pigs had a moderate to severe fibrinous, necro-haemorrhagic pneumonia with fibrinous pleurisy in one side of the lung (Table 2). Lesions extended over parts of the caudal lung lobe or in two cases over the whole right lung. From lung lesions of all pigs and from tracheobronchial lymph nodes of two pigs, strongly haemolytic *A. pleuropneumoniae* were isolated (Table 2). The isolated phenotype was the same as the phenotype used for inoculation and was characterised on the basis of haemolytic activity on SB+NAD.

**S4074 ΔapxA** In contrast to the pigs inoculated with the parent strain, none of the pigs inoculated with mutant strain S4074 ΔapxA died before the end of the experimental period. All three pigs showed at 12 or 18 hpi mild clinical depression and one pig showed respiratory distress at 24 and 48 hpi. Two pigs displayed fever at 24 hpi, which started in one pig already at 12 hpi. Both pigs with fever had an increased WBC count from 6 hpi on and had marked elevated serum IL 6 levels at 12 hpi (Table 1). TNFα and IL 1 were detected in none of the sera at 12 hpi. In each of the two trials, one pig had a typical unilateral necro-haemorrhagic
### TABLE 1 Clinical signs, WBC count and IL 6 levels after endobronchial infection with *A. pleuropneumoniae* serotype 1 parent strain S4074 or deletion mutants

<table>
<thead>
<tr>
<th><em>A. pleuropneumoniae</em> strain</th>
<th>Clinical scores post infection</th>
<th>Observations &gt;40°C/total observations post infection</th>
<th>Mean increase WBC of all time points post infection (%)</th>
<th>Serum IL 6 at 12 hpi</th>
<th>Marked increase in number of pigs</th>
<th>Slight increase in number of pigs</th>
</tr>
</thead>
<tbody>
<tr>
<td>S4074</td>
<td></td>
<td>6.75 (1.50)</td>
<td>9/18</td>
<td>21963 (42895)</td>
<td>3/4</td>
<td>1/4</td>
</tr>
<tr>
<td>S4074 ΔapxIA</td>
<td></td>
<td>2.67 (2.08)</td>
<td>5/15</td>
<td>132 (128)</td>
<td>2/3</td>
<td>0/3</td>
</tr>
<tr>
<td>S4074 ΔapxIIA</td>
<td></td>
<td>0.25 (0.50)</td>
<td>1/20</td>
<td>37 (40)</td>
<td>0/3</td>
<td>1/3</td>
</tr>
<tr>
<td>S4074 ΔapxIA ΔapxIIA</td>
<td></td>
<td>0.75 (0.96)</td>
<td>0/20</td>
<td>259 (478)</td>
<td>1/4</td>
<td>1/4</td>
</tr>
<tr>
<td>S4074 ΔapxIBD</td>
<td></td>
<td>0.50 (1.00)</td>
<td>0/20</td>
<td>235 (371)</td>
<td>1/4</td>
<td>2/4</td>
</tr>
</tbody>
</table>

1 Sum of clinical scores of all observations post infection per pig, per observation a score from 0 to 3 was used, 0 = no clinical signs, 1 = depression, shivering, 2 = symptoms of score 1 plus laboured breathing and score 3 = symptoms of score 2 plus coughing or nasal discharge

2 Marked increase > 113 U/ml; slight increase > 32 U/ml < 113 U/ml

### TABLE 2 Frequency and severity of pleuropneumonia after endobronchial infection with *A. pleuropneumoniae* serotype 1 parent strain S4074 or deletion mutants, summarising results

<table>
<thead>
<tr>
<th><em>A. pleuropneumoniae</em> strain</th>
<th>Intact apxA genes</th>
<th>Number of pigs with pneumonic lesions</th>
<th>Volumes of lung lesions in cm³</th>
<th>Number of pigs from which the inoculation strain was reisolated</th>
</tr>
</thead>
<tbody>
<tr>
<td>S4074</td>
<td>apxI, apxII, apxIV</td>
<td>4/4</td>
<td>7, 73, 255, 277</td>
<td>4/4</td>
</tr>
<tr>
<td>S4074 ΔapxIA</td>
<td>apxII, apxIV</td>
<td>2/3</td>
<td>25, 91</td>
<td>2/3</td>
</tr>
<tr>
<td>S4074 ΔapxIIA</td>
<td>apxI, apxIV</td>
<td>1/4</td>
<td>14</td>
<td>1/4</td>
</tr>
<tr>
<td>S4074 ΔapxIA ΔapxIIA</td>
<td>apxIV</td>
<td>0/4</td>
<td>0</td>
<td>0/4</td>
</tr>
<tr>
<td>S4074 ΔapxIBD</td>
<td>apxI, apxII, apxIV</td>
<td>0/4</td>
<td>0</td>
<td>0/4</td>
</tr>
</tbody>
</table>

1 Not accompanied with pleuritis
pneumonia with fibrinous pleuritis with an affected lung volume of 25 or 91 cm³ (Table 2). The presence of pleuropneumonia correlated with fever and increased serum IL 6 levels. From lung lesions only, weakly haemolytic *A. pleuropneumoniae* were isolated (Table 2). The isolated phenotype was the same as the phenotype used for inoculation. The increases in WBC count after infection in the pneumonic pigs were comparable to the increases in WBC count in the group infected with the parent strain, suggesting a correlation between leucocytosis and the presence of pneumonia. These results show that infection with *A. pleuropneumoniae* S4074 in the absence of ApxI does not consistently result in pleuropneumonia as is seen with the parent strain, although this was not significant (*P > 0.05*). In cases with pleuropneumonia, the clinical course is similar to that observed after infection with the parent strain.

**S4074 ΔapxI A** All pigs inoculated with mutant strain S4074 ΔapxI A survived until the end of the experimental period. One animal showed mild signs of depression and fever at 24 hpi. WBC count was increased in two pigs at 6 hpi and WBC count peaked at 24–48 hpi in the pig that showed depression. One pig had slightly elevated serum IL 6 levels (Table 1), indicating the presence of an inflammatory response in the lungs of this pig after inoculation. In one pig IL 6 could not be determined because of haemolysis. TNFα and IL 1 were detected in none of the sera at 12 hpi. Only one out of the four pigs had a small focal pneumonia with a lesion volume of 14 cm³ (Table 2). In contrast to the lesions induced by the parent strain or the mutant strain S4074 ΔapxIA this was not accompanied by pleuritis. From the lung lesion only, strongly haemolytic *A. pleuropneumoniae* were isolated (Table 2). The isolated phenotype was the same as the phenotype used for inoculation. The pig with pathological lung alterations did not show an increase of IL 6 serum titer 12 hpi. This is probably due to a delayed induction of inflammation, which is also expressed by a delayed strong increase of WBC at 24–48 hpi in this pig. These results show that infection with *A. pleuropneumoniae* S4074 in the absence of ApxII does not consistently result in pneumonia as is seen with the parent strain (*P < 0.05*). In the case with pneumonia, the clinical course was delayed compared to that observed after infection with the parent strain.

**S4074 ΔapxI A ΔapxIIA** All pigs inoculated with mutant strain S4074 ΔapxI A ΔapxIIA survived until the end of the experimental period. Two pigs displayed mild signs of depression at 18 hpi and two pigs showed an increase of WBC count at 6 and 12 hpi, but none of the pigs developed fever (Table 1) or pneumonia. One of the pigs with clinical symptoms had a marked elevated serum IL 6 level at 12 hpi and the other pig had slightly elevated IL 6 serum
titres (Table 1). The increases in serum IL 6 levels and WBC count also express an ApxI and ApxII independent clinical response to the inoculation. TNFα and IL 1 were detected in none of the sera at 12 hpi. None of the pigs showed gross pathological lesions in contrast to the parent strain \( (P < 0.05) \) and no \textit{A. pleuropneumoniae} with the inoculation phenotype were isolated (Table 2). These results demonstrate the importance of ApxI and ApxII for the induction of the typical lung lesions. The findings show that although ApxI and ApxII were not present and no lesions were evoked, a clinical reaction occurred.

**S4074 ΔapxIBD** To confirm the results obtained with mutant strain S4074 ΔapxIA ΔapxIIA, the pathogenicity of strain S4074 ΔapxIBD was tested. The absence of ApxI and ApxII due to the lack of the toxin secretion genes also resulted in a lack of pleuropneumonia and death in contrast to the parent strain \( (P < 0.05) \), thereby confirming the conclusion that actively secreted ApxI and ApxII are essential for the pathogenesis of pleuropneumonia. In the group inoculated with mutant strain S4074 ΔapxIBD, one pig showed mild signs of depression at 18 and 24 hpi, but none of the pigs displayed fever at any time point (Table 1). The pig with clinical symptoms had an increased WBC count at 18 hpi and a marked elevated serum IL 6 level at 12 hpi, two other pigs had slightly elevated IL 6 levels at 12 hpi (Table 1). This also indicates an ApxI and ApxII independent clinical response. TNFα and IL 1 were detected in none of the sera at 12 hpi. None of the pigs showed gross pathological lesions and no \textit{A. pleuropneumoniae} were isolated (Table 2).

**Histopathology and immunohistology** Typical lesions with central necrosis of lung tissue surrounded by a dense layer of streaming cells, fibrin extravasation in interalveolar septae and thrombosis of blood vessels were observed histologically in all pigs infected with the parent strain. Similar pathological features as central necrosis surrounded by a demarcation zone were found in all pigs with pneumonia inoculated with mutant strain S4074 ΔapxIIA (Fig. 1A) or S4074 ΔapxIA. The presence of lesions correlated with the isolation of \textit{A. pleuropneumoniae}. Immunohistological examination of lung tissue was selectively done on lungs from pigs with lung lesions and on lungs from pigs that were cultured positive for \textit{A. pleuropneumoniae}. Bacteria were detected immunohistologically in macrophages and in large numbers around the edges of the necrotic areas adjacent to the alveolar epithelium (Fig. 1B) in all pigs with (pleuro)-pneumonia. Additionally, bacteria were found in smaller numbers outside necrotic areas adhering to alveolar epithelium (Fig. 1C) or to bronchiolar epithelium and in tracheal secretion. Histopathological changes were detected in none of the
pigs without gross pathological lesions and no *A. pleuropneumoniae* were isolated from pigs without gross pathological lesions.

**DISCUSSION**

In this study we determined the contribution of two Apx toxins of *A. pleuropneumoniae* serotype 1 in the induction of clinical symptoms and pneumonic lesions by using isogenic mutants in which one or two of the Apx toxin genes or the secretion genes had been inactivated. The used infection model is well established and results in lesions in all pigs when they are inoculated with approximately 1,000 CFU of *A. pleuropneumoniae* strain 13261 of serotype 9 (Van Leengoed and Kamp, 1989). This model allows the use of small numbers of animals to detect differences between strains. Strain S4074 used in this study, was in previous experiments as virulent as strain 13261 and consistently induced comparable lesions (unpublished results). In this study, all four animals inoculated with parent strain S4074 developed typical lung lesions and moderate to severe clinical symptoms.

The consistent induction of pathological lesions depended on the presence of both ApxI and ApxII. Bacteria mutated in both *apxIA* and *apxIIA* and bacteria mutated in genes *apxIBD*
required for toxin secretion were not able to induce lesions. This indicates that actively secreted ApxI and ApxII are necessary for the development of lesions in line with previous observations (Tascon et al., 1994; Reimer et al., 1995). Although no pathological lesions were found in pigs inoculated with mutant strains unable to produce or secrete Apx toxins, the majority of these pigs showed increased numbers of WBC or elevated serum IL 6 levels. Several pigs also displayed mild clinical symptoms in the period after inoculation. The induction of IL 6 production and the mild clinical symptoms in these pigs, could indicate the presence of an infection and may have been caused by the release of other compounds like LPS or ApxIV. Because a saline control was not included, a reaction due to the inoculation procedure cannot be excluded. Export of ApxII in *A. pleuropneumoniae* serotype 1 is dependent on ApxIB and ApxID. Proteins involved in the export of ApxIV have not been identified yet. If export of ApxIV depends on ApxIB and ApxID, it is not likely that ApxIV is the cause of increased IL 6 production and mild clinical symptoms because pigs infected with strain S4074 ΔapxIBD also showed these reactions.

To determine the contribution of two Apx toxins of *A. pleuropneumoniae* serotype 1 in the pathogenesis, isogenic mutants were used in which the toxin genes *apxIA* or *apxIIA* were inactivated. The presence of either ApxI or ApxII in *A. pleuropneumoniae* serotype 1 appeared not to be sufficient to consistently induce pathological lesions. Bacteria that were mutated in either *apxIA* or *apxIIA* induced less severe lesions and/or in fewer pigs than the parent strain. Histologically no differences were detected between lesions caused by the ApxI or ApxII mutants and lesions caused by the parent strain. Mutants of serotype 1 and 5, devoid of ApxI but still producing ApxII also caused typical severe clinical disease (Tascon et al., 1994; Reimer et al., 1995). However, *apxIIA* deletion mutants were not included in those studies. In our hands both ApxI and ApxII appear to be required for full virulence.

Our results indicate that ApxII is at least as potent as ApxI for the development of clinical and pathological symptoms. Only one out of four pigs inoculated with mutant strain S4074 Δ*apxIIA* developed mild pneumonia whereas two out of three pigs inoculated with mutant strain S4074 Δ*apxIA* developed more severe lesions. These results are in contrast to other studies which indicated a lower toxicity or a smaller contribution to virulence for ApxII than for ApxI and ApxIII among all serotypes (Kamp et al., 1991; Kamp et al., 1997). The reduced toxicity in *in vivo* experiments of purified recombinant ApxII compared to ApxI and ApxIII (Kamp et al., 1997), should be viewed critically since the large amounts of toxins used in that study do not reflect the natural situation. Bacteria in close contact to cells can directly target the toxin to these cells whereas instillation of recombinant toxin in the lung can result in a
more diffuse delivery. A large portion of inactive toxin was present in the ApxII preparations which might reduce the overall toxicity by competition (Kamp et al., 1997). A demarcation zone typical for A. pleuropneumoniae infections was not found after inoculation with purified toxins (Kamp et al., 1997), and is probably not dependent on both toxins but related to live bacteria and a longer lasting host defence reaction. Little is known about the production level in vivo of the different toxins.

The recently characterised ApxIV is expressed in vivo only by all serotypes of biovar 1 (Schaller et al., 1999), including reference strain S4074 used in this study. The contribution of ApxIV to the development of clinical and/or pathological symptoms remains to be elucidated. Although we did not test it, it is very likely that ApxIV is produced in vivo by the parent strain and the mutant strains devoid of ApxI and/or ApxII. If this assumption is true, ApxIV alone is not able to induce clinical pathology. Active ApxIV requires the presence of an additional gene, ORF1, which is located immediately upstream of apxIVA (Schaller et al., 1999). Whether export of ApxIV depends on ApxIB and ApxID is unknown.

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CHAPTER 2


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