Pathogenesis of *Actinobacillus pleuropneumoniae*: Role of toxins and fimbriae

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Pathogenese van *Actinobacillus pleuropneumoniae*: Rol van toxinen en fimbriae

(Met een samenvatting in het Nederlands)

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Chapter 1

General Introduction

Actinobacillus pleuropneumoniae is a small, Gram-negative capsulated rod with typical coccobacillary morphology (Nicolet, 1992; Shope *et al.*, 1964). *A. pleuropneumoniae* causes porcine pleuropneumonia, a disease that has serious impact on the economy in most pig-rearing countries (Nicolet, 1992). Infection of pigs with *A. pleuropneumoniae* is considered one of the most important causes of acute respiratory disease in pigs and can result in high morbidity and mortality (Cruijsen, 1995, 1996; Loeffen *et al.*, 1999; Nicolet, 1992). In addition to losses due to mortality, infection with *A. pleuropneumoniae* can be costly due to higher medication costs, reduced feed conversion, lower weaning rates, and reduced market value of animals. While individual animals can be examined for clinical symptoms and pathological alterations, infections with *A. pleuropneumoniae* at the herd level can be diagnosed by measurement of antibodies against *A. pleuropneumoniae* in for example enzyme linked immuno sorbent assays or complement fixation tests. Alternatively, bacteria can be cultured from typical lung lesions.

A. pleuropneumoniae strains can be differentiated into strains that depend on nicotinamide adenine dinucleotide (NAD) (biotype 1) and strains that are NAD-independent (biotype 2). Based on antigenic diversity of capsule and lipopolysaccharide (LPS) composition, 15 serotypes of *A. pleuropneumoniae* have been identified regardless of their NAD requirement (Blackall *et al.*, 2002; Schaller *et al.*, 2001). All serotypes are haemolytic (Taylor, 1999) and can cause severe disease and death in pigs. Subclinically infected carriers or animals recovered from the disease are believed to transmit *A. pleuropneumoniae* within and between herds (Chiers *et al.*, 2001; Møller *et al.*, 1993; Sidibe *et al.*, 1993). Direct transmission of *A. pleuropneumoniae* from pig to pig occurs (Velthuis *et al.*, 2002) and is believed to be an important infection route, since the bacterium can not survive for a long time in the environment (Nicolet, 1992).

The pathology of pleuropneumonia is characterised by a fibrinous pleuritis and a fibrino-haemorrhagic necrotising pneumonia with focal pulmonary vascular thrombosis (Nicolet, 1992). Pulmonary lesions are characterised by severe oedema, inflammation, haemorrhage and necrosis (Ajito *et al.*, 1996; Bertram, 1985; Rosendal *et al.*, 1985). Marked infiltration of polymorphonuclear leucocytes (PMNs), oedema, and fibrinous exudate are apparent in the early stages of disease (Ajito *et al.*, 1996; Bertram, 1985; Liggett *et al.*, 1987). Infection of pigs with *A. pleuropneumoniae* leads to a rapid local production of the proinflammatory cytokines tumor necrosis factor α (TNFα), interleukin (IL) 1, IL 6 and the potent PMN chemoattractant IL 8 (Baarsch *et al.*, 1995; Baarsch *et al.*, 2000; Huang *et al.*, 1999). TNFα, IL 1 and IL 8 are pivotal in the development of lung inflammation and cell infiltration.

Rapid recruitment and activation of PMNs leads to a release of toxic oxygen radicals and proteolytic enzymes (Dom *et al.*, 1992b) which can result in deleterious effects on host cells. Tissue damage and activation of neutrophils and macrophages have been attributed to toxins produced by *A. pleuropneumoniae* (Dom *et al.*, 1992a; Dom *et al.*, 1992b; Dom *et al.*, 1994a; Jansen *et al.*, 1995; Kamp *et al.*, 1997). *In vivo, A. pleuropneumoniae* can be found within alveolar and interlobular fluid and within alveolar macrophages and PMNs (Min and Chae, 1998). Epithelial cells are not invaded by *A. pleuropneumoniae* (Min and Chae, 1998). *A. pleuropneumoniae* mainly colonises the lower respiratory tract, but the bacterium can be isolated from tonsils of chronically infected animals (Møller *et al.*, 1993; Mousing *et al.*, 1990; Sidibe *et al.*, 1993). Experimental epidemiological studies showed a high correlation between infectivity of pigs and tonsils positive for *A. pleuropneumoniae* at necropsy (Velthuis *et al.*, 2002), but the role of tonsils in the pathogenesis is unclear. The disease can be reproduced in animal models that involve aerosol, intranasal or endobronchial exposure of pigs (Hensel *et al.*, 1995; Liggett *et al.*, 1987; Van Leengoed and Kamp, 1989) which mimic in various degrees the natural infection route of respiratory tract infections. Aerosol application by a nose-only exposure system and endobronchial infection are highly reproducible and well standardised.

To control *A. pleuropneumoniae* infections in pigs, several different vaccines are being used. Immunisation with killed bacteria provides at least serotype-specific protection (Nielsen, 1984; Rosendal *et al.*, 1981; Thacker and Mulks, 1988). Improvements have been made by subunit vaccines containing the RTX toxins ApxI, ApxII and ApxIII which induce good protection against clinical disease caused by any serotype (Fedorka-Cray *et al.*, 1993; Kamp *et al.*, 1992; van den Bosch *et al.*, 1992; van Leengoed, 1988). Although these vaccines reduce mortality and morbidity, they do not prevent the development of colonisation nor transmission (Beskow *et al.*, 1993; Chiers *et al.*, 1998; Hensel *et al.*, 2000; Møller *et al.*, 1993; Van Overbeke *et al.*, 2001; Velthuis *et al.*, 2002). Prevention or a sufficient reduction of colonisation may lead to eradication of *A. pleuropneumoniae* from a population. Protection against clinical disease was achieved with several of the known virulence factors but never complete. Together, more information concerning the role of virulence factors is required to improve available vaccines against *A. pleuropneumoniae*.

The molecular pathogenesis of *A. pleuropneumoniae* infections is not completely understood. Several virulence factors have been described that contribute to microbial fitness in the host and to the pathogenesis (Bosse *et al.*, 2002; Haesebrouck *et al.*, 1997) and will be discussed below. For establishment of infection, *A. pleuropneumoniae* needs to colonise and

to evade the host defence system. Therefore, we divided the virulence factors in colonisation factors and bacterial attack and defence factors. This is not a strict division since some virulence factors exhibit more than one property.

COLONISATION FACTORS

Adherence

In general, adherence of micro-organisms to epithelial cells is regarded as the initial step in the colonisation of the host and often involves a specific interaction between bacterial adhesins and complementary receptors on host cells (Hoepelman and Tuomanen, 1992; Hultgren *et al.*, 1993). By attaching to host cells, microbes are thought to avoid elimination by e.g. mucociliary clearance. The attached state possibly enhances their toxicity to the host, their resistance to deleterious agents, their ability to trap nutrients and allows the bacteria to multiply more efficiently than do unattached bacterial cells (Cundel, 1995; Svanborg *et al.*, 1996). Host or tissue specificity in adherence may be explained by the presence of specific receptors that interact with distinct adhesins present at the bacterial cell surface.

A. pleuropneumoniae adheres *in vitro* to porcine tracheal and lung cells (Belanger *et al.*, 1990; Jacques *et al.*, 1991; Paradis *et al.*, 1994; Paradis *et al.*, 1999), mucus of the respiratory tract (Belanger *et al.*, 1992; Belanger *et al.*, 1994) and to methanol-fixed alveolar epithelial cells (Haesebrouck *et al.*, 1996; Van Overbeke *et al.*, 2002). *In vivo* after intranasal inoculation, *A. pleuropneumoniae* has been shown to be associated predominantly with the cilia of terminal bronchioli and the epithelium of alveoli and only sporadically with the cilia or the epithelium of the bronchi and trachea (Dom *et al.*, 1994a), suggesting that lung cells may be a primary infection target.

Information on adhesins of *A. pleuropneumoniae* is limited. LPS has been suggested to play a role in adherence of *A. pleuropneumoniae*. LPS is a major constituent of the outer membrane of Gram-negative bacteria and consists of lipid A and a polysaccharide moiety containing O-antigen and core region (Hitchcock *et al.*, 1986). Adherence of *A. pleuropneumoniae* to tracheal and lung tissue could be inhibited with purified LPS, fractionated high-molecular-mass polysaccharide and with monoclonal antibodies directed against the O-antigen of LPS (Belanger *et al.*, 1990; Paradis *et al.*, 1994; Paradis *et al.*, 1999). Further adherence studies suggested that the core region of LPS acts as an adhesin, a function that is masked by the O-antigen (Abul-Milh *et al.*, 1999; Rioux *et al.*, 1999).

Inhibition studies of *A. pleuropneumoniae* adherence to alveolar epithelial cells indicated that both LPS and proteins are involved in adherence (Van Overbeke *et al.*, 2002). However, proteins that mediate adherence of *A. pleuropneumoniae* have not been identified so far. Many bacterial pathogens carry fimbriae that can mediate adherence of bacteria to host cells (Gilsdorf *et al.*, 1996; Hahn, 1997; Nassif *et al.*, 1994; Strom and Lory, 1993). Fimbriae or pili are filamentous polymeric structures that protrude from the bacterial cell surface (Wu and Fives-Taylor, 2001). Recently, fimbriae have been isolated from *A. pleuropneumoniae* and were found to belong to the type IV family of fimbriae (Zhang *et al.*, 2000). The role of these fimbriae in the pathogenesis of *A. pleuropneumoniae* remains to be elucidated.

Bacterial metabolism

For efficient colonisation or establishment of infection, bacteria must be able to acquire all essential nutrients. Within the host, the variety and quantity of available nutrients is restricted depending on the niche (MacFadyen and Redfield, 1996). The ability to overcome these nutritional limitations within the host may be considered a virulence trait. Iron is the most important nutrient used by bacteria since it is needed as a cofactor for a large number of metabolic enzymes. Due to complexation by the host glycoproteins transferrin and lactoferrin, iron is not readily available in the extracellular environment of the host. Many bacterial pathogens overcome this iron-restriction by expressing receptor proteins at the bacterial surface involved in direct binding of the host's transferrin or bacterial siderophores. *A. pleuropneumoniae* produces two distinct transferrin binding proteins (TbpA and TbpB) that are both required for optimal utilisation of transferrin as a sole source of iron and for virulence (Baltes *et al.*, 2002; Fuller *et al.*, 1998; Gonzalez *et al.*, 1990; Litt *et al.*, 2000; Ricard *et al.*, 1991). The restriction of the transferrin binding proteins to porcine transferrin (Niven *et al.*, 1989) may explain the host specifitiy of *A. pleuropneumoniae*. Recently, a ferric hydroxamate uptake (*fhu*) operon has been identified that putatively encodes a second iron acquisition mechanism (Mikael *et al.*, 2002). Although there are indications that FhuA is expressed *in vivo* (Mikael *et al.*, 2002), FhuA is not required for virulence of *A. pleuropneumoniae* (Baltes *et al.*, 2003). After binding at the bacterial cell surface, transport of iron across the outer membrane is enabled by a protein complex of ExbB, ExbD and TonB (Moeck and Coulton, 1998). Mutants in *exbB* or *tonB* of *A. pleuropneumoniae* are avirulent, demonstrating the essential role of this complex in iron acquisition *in vivo* (Baltes *et al.*, 2001; Fuller *et al.*, 2000).

Additional virulence genes of *A. pleuropneumoniae* have been identified with signature tagged mutagenesis (STM) and *in vivo* expression technology (IVET) (Fuller *et al.*, 1999; Fuller *et al.*, 2000). Genes identified by STM represented five broad functional classes: biosynthetic enzymes, putative colonisation factors, cellular regulation components, translation components and unknowns (Fuller *et al.*, 2000). In the IVET study, ten loci were identified that were specifically induced *in vivo* (Fuller *et al.*, 1999), but information on the correspondingly induced genes is limited. Further analysis of all loci identified with STM and IVET will likely lead to new insights into the pathogenesis of *A. pleuropneumoniae*.

BACTERIAL DEFENCE AND ATTACK FACTORS

During infection, bacteria are subject to phagocytosis by macrophages and PMNs and to killing by antibody and complement. Most pathogens have the ability to counteract these activities. *A. pleuropneumoniae* produces several factors involved in defence and attack which may have concomitant destructive effects on host tissue.

Toxins

A major factor involved in impairment of host-defence mechanisms is the production of exotoxin. Many Gram-negative bacteria that are human or animal pathogens, including *A. pleuropneumoniae*, produce extracellular pore forming toxins belonging to the RTX (repeats in toxin) family (Welch *et al.*, 1995). There are many indications that RTX toxins play a role in the evasion of the host's first line of defence, i.e. phagocytosis and killing by macrophages and neutrophils (Welch, 1991; Welch *et al.*, 1995). *A. pleuropneumoniae* produces four different toxins belonging to the RTX family, named ApxI, ApxII, ApxIII and ApxIV (Frey *et al.*, 1993; Schaller *et al.*, 1999). The toxins ApxI, ApxII and ApxIII are essential for the development of clinical disease and the typical lung lesions (Anderson *et al.*, 1991; Inzana, 1991; Kamp *et al.*, 1997; Reimer *et al.*, 1995; Tascon *et al.*, 1994). These three toxins are expressed both *in vitro* and *in vivo* and are in various degrees cytotoxic for lung macrophages, PMNs, lung epithelial cells and endothelial cells (Bendixen *et al.*, 1981; Dom *et al.*, 1992a; Dom *et al.*, 1992b, 1994b; Kamp *et al.*, 1991; Serebrin *et al.*, 1991; Tarigan *et al.*, 1994; Van de Kerkhof *et al.*, 1996; Van Leengoed and Kamp, 1989). ApxI and ApxII are also haemolytic. ApxIV in *A. pleuropneumoniae* is expressed *in vivo* only and when expressed in *Eschericha coli* it is weakly haemolytic (Schaller *et al.*, 1999). The contribution

of ApxIV to the pathogenesis is not clear since bacteria deficient in ApxI, ApxII and ApxIII but producing ApxIV are not able to cause lesions or disease (Reimer *et al.*, 1995; Tascon *et al.*, 1994).

The ability of *A. pleuropneumoniae* to produce multiple Apx toxins varies between serotypes (Table 1). The surplus value of the production of more than one toxin is unknown. It may account for the variation in virulence among *A. pleuropneumoniae* strains. ApxII appears to contribute to a lesser extent than ApxI and ApxIII to lesion formation in animal experiments with purified toxins and to *in vitro* toxicity (Kamp *et al.*, 1991; Kamp *et al.*, 1997). However, strains of serotype 7 and mutants of serotype 1 and 5 which produce only ApxII and ApxIV, are capable of causing severe disease with typical lung lesions (Reimer *et al.*, 1995; Tascon *et al.*, 1994). This prompts the question, what the relative contribution in the pathogenesis is of the different toxins in a given serotype.

Table 1 Distribution of Apx toxins in *A. pleuropneumoniae* strains of biovar 1

Serotype	Secreted Apx toxins *in vivo*			
1, 5, 9, 11	I	II		IV
10	I			IV
2, 3, 4, 6, 8		II	III	IV
7, 12		II		IV

This table was compiled using data from Frey *et al.* (1993) and Schaller *et al.* (1999).

Capsule

Many bacterial pathogens produce a capsule that protects the bacterium against the immune system of the host (Moxon and Kroll, 1990). All serotypes of *A. pleuropneumoniae* produce a capsule which mainly consists of derivatised repeating oligosaccharides (Beynon *et al.*, 1993; Perry *et al.*, 1990). Non-encapsulated mutants of *A. pleuropneumoniae* were considerably less virulent compared to their capsulated parent strains (Inzana *et al.*, 1993; Rioux *et al.*, 2000; Rosendal and MacInnes, 1990; Ward *et al.*, 1998). Capsule protects the bacterium against killing by antibody and complement by limiting the deposition of C9, a component of the membrane attack complex (Rycroft and Cullen, 1990; Ward and Inzana, 1994). High-molecular-weight surface carbohydrates present in capsule and LPS may participate in scavenging of free oxygen radicals (Bilinski, 1991). Because of the protective capacities of capsule, phagocytosis and subsequent killing of *A. pleuropneumoniae* by PMNs requires opsonisation with antibody (Cruijsen *et al.*, 1992; Inzana *et al.*, 1988).

Lipopolysaccharide

LPS, present at the bacterial surface, contributes to complement resistance by binding LPS-specific antibodies in a fashion that does not result in the formation of a functional complement membrane attack complex (Ward and Inzana, 1994). Purified LPS of *A. pleuropneumoniae* has all characteristics of endotoxin and, through activation of factor XII, initiates coagulation and fibrinolysis which ultimately results in necrosis (Fenwick and Osburn, 1986; Fenwick *et al.*, 1986; Maudsley *et al.*, 1986; Udeze *et al.*, 1987). Purified LPS is able to induce lung lesions that are similar to lesions induced by *A. pleuropneumoniae* or purified toxins although the typical haemorrhagic necrosis is missing and large amounts are required (Fenwick and Osburn, 1986; Fenwick *et al.*, 1986; Maudsley *et al.*, 1986; Udeze *et al.*, 1987). The contribution of LPS in the development of lesions is not clear. Infection with a mutant strain lacking ApxI and ApxII but with normal LPS did not result in lesions or clinical disease (Tascon *et al.*, 1994). Possibly, the inflammatory responses induced by LPS (Huang *et al.*, 1999; Lin *et al.*, 1994; Ulich *et al.*, 1991) may explain the similarity of lesions induced by purified LPS and toxins.

Urease

Urease is a potential contributor to survival in the host and is produced by virtually all strains of *A. pleuropneumoniae*. Urease hydrolyses urea to ammonia which has been shown to inhibit phagosome-lysosome fusion and to elevate intralysosomal pH in macrophages, resulting in depression of acid hydrolase (Gordon *et al.*, 1980). Urease negative mutants of *A. pleuropneumoniae* were still able to produce acute infection at a high challenge dose (10^5-10^6 CFU/ml in aerosol infection) but not at a low challenge dose (10^3 CFU/ml in aerosol infection) (Baltes *et al.*, 2001; Bosse and MacInnes, 2000). The latter could be due to the rapid clearance of urease negative mutants compared to the wild-type strain (Baltes *et al.*, 2001; Bosse and MacInnes, 2000).

Proteolytic activity

Immunoglobulin A (IgA) is a major class of immunoglobulin present in the mucosa of the healthy respiratory tract and is thought to be the most important immunoglobulin for lung defence (Lamm, 1997). IgA in respiratory secretions can bind via its carbohydrate

moieties to lectinlike bacterial adhesins, thereby blocking their epithelial colonisation. Several micro-organisms colonising mucosal surfaces, including *A. pleuropneumoniae*, produce proteases which are capable of cleaving secretory IgA antibodies (Kilian *et al.*, 1988; O'Reilly and Bhatti, 1986; Plaut, 1983) thereby possibly enhancing survival of the micro-organism. In addition to IgA, *A. pleuropneumoniae* is able to degrade IgG, porcine gelatin and haemoglobin (Negrete-Abascal *et al.*, 1994; Negrete-Abascal *et al.*, 1998). The specificity and the contribution to the pathogenesis of these proteolytic activities of *A. pleuropneumoniae* is unclear.

Superoxide dismutase

During the course of infection, many bacterial pathogens are exposed to high levels of oxygen radicals generated by inflammatory cells during oxidative burst. The capacity of superoxide dismutases (SODs) to degrade exogenous superoxide suggests a role of these enzymes in the pathogenesis. *A. pleuropneumoniae* contains a periplasmic copper-zinc superoxide dismutase (SodC) (Langford *et al.*, 1996). Although a SodC deficient mutant of *A. pleuropneumoniae* was highly sensitive to the microbial action of superoxide *in vitro*, it remained fully virulent in pigs (Sheehan *et al.*, 2000). Possibly, SodC activity is complemented by redundant oxidoreductase activities which compensate for the defect in *sodC*.

SCOPE AND OUTLINE OF THIS THESIS

As discussed above, for improvement of control strategies, more knowledge is needed about virulence factors contributing to survival of *A. pleuropneumoniae in vivo*. In this study, we investigated the relative contribution of two distinct toxins of *A. pleuropneumoniae* serotype 1 (ApxI and ApxII) in the development of lesions (Chapter 2). To study the adherence of *A. pleuropneumoniae*, we set out to design an *in vitro* adherence model using lung epithelial cells (Chapter 3). Adherence is regarded as an important first step in the colonisation and subsequent infection of the host, but little is known about factors contributing to adherence of *A. pleuropneumoniae*. In our search for possible adhesins of *A. pleuropneumoniae*, we focused on the type IV fimbriae recently identified in *A. pleuropneumoniae*. Fimbriae have been implicated in adherence of various species and purified fimbriae have been shown to confer protection. The entire type IV fimbriae operon of *A. pleuropneumoniae* serotype 1 was

cloned and characterised (Chapter 4) and the conditions driving fimbriae expression were studied (Chapter 5). The significance of these findings for the pathogenesis are discussed in Chapter 6.

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Chapter 2

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 *Eschericha 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 *apxIA* and/or *apxIIA* or in the transport genes *apxIBD* (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% ß-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 Na_2HPO_4, 0.0032 M KH_2PO_4; 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

(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 Δ*apxIA* 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% H_2O_2), 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 H_2O_2, 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 cm^3, 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 Δ*apxIA* In contrast to the pigs inoculated with the parent strain, none of the pigs inoculated with mutant strain S4074 Δ*apxIA* 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

A. pleuropneumoniae strain	Clinical scores post infection[1]	Observations >40°C /total observations post infection	Mean increase WBC of all time points post infection (%)	Serum IL 6 at 12 hpi[3]		
				U/ml	Marked increase in number of pigs	Slight increase in number of pigs
S4074	6.75 (1.50)[2]	9/18	204	21,963 (42,895)[2]	3/4	1/4
S4074 Δ*apxIA*	2.67 (2.08)	5/15	261	132 (128)	2/3	0/3
S4074 Δ*apxIIA*	0.25 (0.50)	1/20	151	37 (40)	0/3	1/3
S4074 Δ*apxIA* Δ*apxIIA*	0.75 (0.96)	0/20	134	259 (478)	1/4	1/4
S4074 Δ*apxIBD*	0.50 (1.00)	0/20	127	235 (371)	1/4	2/4

[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] Expressed as mean, standard deviation in brackets
[3] 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

A. pleuropneumoniae strain	Intact *apxA* genes	Number of pigs with pneumonic lesions	Volumes of lung lesions in cm^3	Number of pigs from which the inoculation strain was reisolated
S4074	*apxI*, *apxII*, *apxIV*	4/4	7, 73, 255, 277	4/4
S4074 Δ*apxIA*	*apxII*, *apxIV*	2/3	25, 91	2/3
S4074 Δ*apxIIA*	*apxI*, *apxIV*	1[1]/4	14	1/4
S4074 Δ*apxIA* Δ*apxIIA*	*apxIV*	0/4	0	0/4
S4074 Δ*apxIBD*	*apxI*, *apxII*, *apxIV*	0/4	0	0/4

[1] Not accompanied with pleuritis

pneumonia with fibrinous pleuritis with an affected lung volume of 25 or 91 cm^3 (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 Δ*apxIIA* All pigs inoculated with mutant strain S4074 Δ*apxIIA* 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 cm^3 (Table 2). In contrast to the lesions induced by the parent strain or the mutant strain S4074 Δ*apxIA* 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 Δ*apxIA* Δ*apxIIA* All pigs inoculated with mutant strain S4074 Δ*apxIA* Δ*apxIIA* 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 *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 Δ*apxIBD* To confirm the results obtained with mutant strain S4074 Δ*apxIA* Δ*apxIIA*, the pathogenicity of strain S4074 Δ*apxIBD* 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 Δ*apxIBD*, 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 *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 Δ*apxIIA* (Fig. 1A) or S4074 Δ*apxIA*. The presence of lesions correlated with the isolation of *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 *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

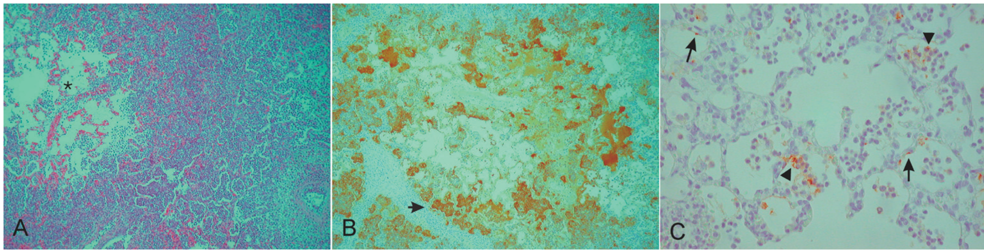

FIG. 1. Paraffin embedded tissue sections of fibrinonecrotic pneumonia in the lung of a pig after infection with S4074 Δ*apxIA*. A) The necrotic area (indicated by an asterisk) is surrounded by a demarcation zone with typical "streaming cells" and polymorphonuclear leucocytes (5 × objective). This demarcation zone is missing after inoculation with Apx toxins only (Kamp et al., 1997). B) Dark brown staining of *A. pleuropneumonia* antigen was located predominantly in the periphery of the necrotic lung areas and the demarcation zone (arrow, 5 × objective). C) *A. pleuropneumonia* antigen was sporadically detected outside of necrotic lung areas adhering to alveolar epithelium (arrows) and alveolar macrophages (arrowheads) (20 × objective). Note the tissue hyperaemia and the influx of leucocytes into the alveolar lumen. Staining in Fig. 1A was done with hematoxylin and eosin, *A. pleuropneumonia* antigen in Figs 1B and 1C was made visible with a specific antibody in conjunction with labelled streptavidin biotin immunoperoxidase staining and hematoxylin counterstain.

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 controle was not included, a reaction due to the inoculation procedure can not 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 Δ*apxIBD* 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 3

Adherence of *Actinobacillus pleuropneumoniae* to primary cultures of porcine lung epithelial cells

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ABSTRACT

To study adherence of *Actinobacillus pleuropneumoniae* to porcine lower respiratory epithelium, a cell culture model was developed using primary cultures of porcine lung epithelial cells (LEC). Adherence assays were performed and results were compared with data obtained with swine kidney cells (SK6). *A. pleuropneumoniae* efficiently adhered to LEC with up to 62 bacteria per cell after 2 h of incubation. Reference strain of serotype 3 (R3) adhered better to LEC than reference strains of serotypes 1 (R1), 7 (R7) and 8 (R8). Overall the adherence to LEC was more rapid and up to 30-fold more efficient than adherence to SK6 cells. In search for the mechanism involved in the adherence event, we tested the effect of LPS which has previously been demonstrated to cause adherence of the pathogen to upper respiratory epithelium. Adherence assays with LPS transposon mutants demonstrated unaltered (mutants with modification in core/lipid A moiety) or even three-fold more adherence (mutant lacking O-antigen) compared to the parent micro-organisms. Purified LPS of strains R1, R3, R7 and R8 did not inhibit adherence of R8 to LEC either, suggesting that LPS and particularly the O-antigen are not essential for adherence of *A. pleuropneumoniae* to LEC. The efficient, LPS-independent adherence of *A. pleuropneumoniae* to LEC cells indicates that *A. pleuropneumoniae* may carry different, cell type-specific adhesins and that infection of primary cultures of lower respiratory epithelium are valuable infection models in studying *A. pleuropneumoniae* pathogenesis.

INTRODUCTION

Actinobacillus pleuropneumoniae causes porcine pleuropneumonia, a disease that is distributed world-wide and causes severe economic losses to the pig rearing industry (Barnum, 1990; Nicolet, 1992). Based on differences in capsule composition, 12 serotypes of *A. pleuropneumoniae* have been identified (Nielsen, 1986). All serotypes of *A. pleuropneumoniae* can cause severe disease and death in pigs. *A. pleuropneumoniae* infections are host specific and mainly affect the lung. Several potential virulence factors have been identified, including polysaccharide capsule (Inzana, 1991; Tascon *et al.*, 1996; Haesebrouck *et al.*, 1997) and toxins (Frey, 1995; Kamp *et al.*, 1997). The molecular pathogenesis of the infection is not completely understood. In general, adherence of micro-organisms to epithelial cells is the initial step in the colonisation of the host and often involves specific interaction between bacterial adhesins and complementary receptors on host cells (Woods, 1988; Hoepelman and Tuomanen, 1992; Hultgren *et al.*, 1993). Host or tissue specificity in adherence can be explained by different receptors that interact with distinct adhesins present at the bacterial cell surface. *A. pleuropneumoniae* has been shown to adhere *in vitro* to porcine tracheal rings (Belanger *et al.*, 1990), frozen lung- and trachea-sections (Jacques *et al.*, 1991; Paradis *et al.*, 1994; Paradis *et al.*, 1999), and mucus of the respiratory tract (Belanger *et al.*, 1992; Belanger *et al.*, 1994). The adherence to porcine tracheal frozen sections was inhibited by the polysaccharide moiety of lipopolysaccharide (LPS) (Paradis *et al.*, 1994) and by monoclonal antibodies directed against the O-antigen of LPS (Paradis *et al.*, 1999) suggesting that LPS is an adherence factor. *In vivo* after intranasal inoculation, *A. pleuropneumoniae* has been shown to be associated with the cilia of terminal bronchioli and the epithelium of alveoli but only sporadically with the cilia or the epithelium of the bronchi and trachea (Dom *et al.*, 1994). *A. pleuropneumoniae* adherence to methanol-fixed alveolar epithelial cells has also been demonstrated (Haesebrouck *et al.*, 1996; Van Overbeke *et al.*, 2000), suggesting that lung cells may be an important infection target.

The purpose of this study was to search for an appropriate *A. pleuropneumoniae* infection system to study the adherence of this pathogen to lower respiratory tract epithelium. Here we report that primary cultures of lung epithelial cells (LEC) are an excellent model to study the pathogen–host interaction. Our data indicate rapid and efficient adherence of *A. pleuropneumoniae* to this cell system via an LPS-independent process.

MATERIALS AND METHODS

Bacterial strains and growth conditions *A. pleuropneumoniae* reference strains of serotypes 1 (S4047: R1), 3 (1421: R3), 7 (WF83: R7) and 8 (405: R8) were used. The LPS profiles of the strains were semirough (R1) or smooth (R3, R7 and R8). *Bordetella bronchiseptica* strain 92932, a highly adherent stain which served as a positive control, was isolated from a pig with atrophic rhinitis (Kamp and Kimman, 1988). *Pasteurella multocida* subspecies *multocida* Carter serotype D, strain NCTC 10325, a poor adherent strain, served as a negative control. *A. pleuropneumoniae* LPS transposon mutants #5.1, #15.1 and #24.1 with a defective core (#5.1) or O- antigen (#15.1, 24#1) and their nalidixic acid-resistant parent strain (4074 Nal^r) of serotype 1 were kindly provided by Dr. M. Jacques (University of Montreal, Canada). The construction of these LPS mutants has been described (Rioux *et al.*, 1999).

B. bronchiseptica and *P. multocida* were cultured on sheep blood agar (SB) plates. *A. pleuropneumoniae* was cultured on SB plates containing 0.01% β-nicotinamide adenine dinucleotide (NAD, Calbiochem, La Jolla, CA, USA). The parent 4047 Nal^r was cultured on brain heart infusion (BHI, Gibco BRL, Paisley, UK) agar plates supplemented with 0.05% NAD and 30 µg/ml nalidixic acid (Nal, Sigma Chemical Co., St. Louis, MO, USA), the transposon mutants were cultured on BHI-NAD-Nal agar plates containing 75 µg/ml kanamycin (Km, Sigma) as described (Rioux *et al.*, 1999). All plates were incubated at 37°C in a 5% CO_2 atmosphere. For adherence tests, bacteria were grown for 18 h on agar plates, harvested in phosphate buffered saline (PBS: 136.89 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 2.79 mM K_2HPO_4, pH 7.2), centrifuged for 10 min at 3700 × *g*, and suspended in 10 ml of Dulbecco's modified Eagles medium (DMEM, Gibco).

Isolation and analysis of lipopolysaccharides Lipopolysaccharides were isolated by the method of Westphal and Jann (1965). Briefly, bacteria were extracted with hot phenol and aqueous layers were dialysed against water. LPS was pelleted by ultracentrifugation, lyophilised and dissolved in water. Purified LPS was analysed by SDS-PAGE (15% gel) and silverstaining (Tsai and Frasch, 1982). LPS preparations were assayed for 2-keto-3-deoxyoctulosonic (KDO) acid released after hydrolisis of LPS using a colorimetric assay (Hanson and Philips, 1981) and contained 112 (R1), 152 (R3), 87 (R7), 100 (R8) and 134 (*Escherichia coli* O111:B4 (Sigma)) nmol KDO per µg LPS.

Isolation and culture of porcine lung epithelial cells (LEC) Lung epithelial cells were obtained from lungs of 6 weeks old pigs from our own specific pathogen free (SPF) herd. We used a modification of the technique of Dobbs (1990). Pigs were sedated by an intravenous injection of pentobarbital (Nembutal, 20 mg/kg of body weight). Additionally, heparin (350 IU/kg) was administered intravenously. Lungs were perfused with 2 l of PBS of 37°C containing 5 IU/ml heparin via the jugular vein until they were free of blood. The right cranial lobes were excised and washed 12 times via the main bronchus with 25 ml of solution I (140 mM NaCl, 5 mM KCl, 2.5 mM Na_2HPO_4, 0.2 mM EGTA (ethylene glycol bis (β-aminoethyl ether)-*N*,*N*,*N′*,*N′′*-tetraacetate, Sigma), 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid, Gibco) and 6 mM glucose, pH 7.4) and four times with 25 ml of solution II (140 mM NaCl, 5 mM KCl, 2.5 mM Na_2HPO_4, 2 mM $CaCl_2$ and 1.3 mM $MgSO_4$, 10 mM HEPES and 6 mM glucose, pH 7.4) to remove alveolar macrophages. Lobes were inflated with 40 ml of elastase (from porcine pancreas, Boehringer, Mannheim, Germany, 0.76 mg/ml dissolved in solution II) and incubated for 30 min at 37°C. To gain epithelial cells, the lobes were minced with a pair of sharp scissors in approximately 1 mm^3 cubes in the presence of 0.2 mg/ml DNAse I (Sigma) and suspended in solution II containing 10% foetal calf serum (FCS, Biochrom KG, Berlin, Germany) to block elastase activity. After gentle shaking, the suspension was filtered through 200 and 50 µm polyamidmono filters (Nybolt). Cells were centrifuged for 20 min at 150 × *g* and the cell pellet was resuspended in DMEM. To remove residual alveolar macrophages, the cell suspension was plated onto pig IgG-coated dishes as described by Dobbs *et al.* (1986). A porcine immunoglobulin G (Sigma) solution of 50 µg/ml was used to coat bacteriologic plastic dishes. After the plates were washed, 5 ml of the cell suspension was added to each plate. Plates were incubated for 2 h at 37°C in a 5% CO_2 atmosphere, and unattached cells were removed and seeded at 6.7×10^4 cells/cm^2. Cells were cultured in DMEM, 10% FCS, 2 mM glutamine (ICN, Aurora, OH, USA), 1 mM HEPES, 100 IU/ml penicillin and 100 µg/ml streptomycin (pen/strep) at 37°C in a 5% CO_2 atmosphere. When confluence was reached, cells were trypsinised, frozen in culture medium supplemented with 10% DMSO (Merck Diagnostica, Darmstadt, Germany), and stored in liquid nitrogen.

Cell characterisation Cultured lung cells were characterised by immuno-staining. Cells were fixed with methanol containing 0.3% H_2O_2 and washed with PBS. After blocking with normal rabbit serum (DAKO, Hamburg, Germany), cells were incubated (1 h, RT) with anti-pan cytokeratine (Clone C-11, Sigma, 1/400 dilution), anti-vimentin (Clone LN-6, Sigma,

1/1000) or anti-macrophage (mAb 517.2, 1/1000 Dominguez *et al.*, 1998). After washing, cells were incubated (1 h, RT) with rabbit anti-mouse horseradish peroxidase conjugate (DAKO, 1/1000). Bound antibodies were visualised by addition of hydrogen peroxide and 3,3´-diaminobenzidine tetrahydrochloride (Sigma) as chromogenic substrate. Cultured cells were immuno-stained after each isolation. For electron microscopy, porcine lung epithelial cells were cultured on carbon coated cover slips. Confluent cells were fixed using a rapid cold fixation method (Wagenaar *et al.*, 1993).

Culture of SK6 cells Epithelial swine kidney (SK6) cells (Kasza *et al.*, 1972) were grown in Eagle's minimal essential medium (EMEM, Gibco) supplemented with 5% FCS, 100 IU/ml penicillin and 100 µg/ml streptomycin (pen/strep) at 37°C in a 5% CO_2 atmosphere. Viability of cells was assessed by dye exclusion.

Adherence assay Epithelial cells of SK6 and LEC were thawed and seeded in 6-well plates in medium with pen/strep at 1.2×10^4 cells/cm^2 for LEC (first passage) and at 1.6×10^4 cells/cm^2 for SK6. LEC were used as confluent layers and SK6 cells were used at near confluence. The culture medium was replaced by culture medium without antibiotics 24 h prior to the adherence assay. Before incubation with bacteria, cells were washed with three times 3 ml of PBS to remove loose adherent cells and residual antibiotics, and fresh culture medium without antibiotics was added. One hundred µl of culture medium was tested for the absence of antibiotics by a disc diffusion assay on plate count agar with *Bacillus staerothermophilus*. Cell monolayers were incubated (37°C, 5% CO_2) with variable numbers of bacteria from an overnight culture on agar plates. At various times, non-adherent bacteria were removed by washing with four times 3 ml of PBS, and the cells were fixed with methanol (10 min, RT) and stained with water-diluted Giemsa stain (Merck). Bacterial adherence was scored by counting at least 20 (LEC) or 7 (SK6) randomly selected visual fields in an Olympus microscope (100 × objective). This yielded sufficient numbers of cells for statistical purposes in both cases. The average number of counted cells per field was 1.25 for large LEC and 7 for small LEC, and 31.7 for SK6. Adherence was expressed as the percentage of cells with adherent bacteria and as the average number of bacteria per cell (only cells with adherent bacteria were taken into account). Tests were rejected when *B. bronchiseptica* (positive control) adhered to less than 50% of the cells (8% of the tests) or when *P. multocida* (negative control) adhered to more than 40% of the cells (4% of the tests) after 2 h incubation with 10^8 or 10^7 colony forming units per ml (CFU/ml).

Effect of LPS on adherence To measure the effect of LPS on adherence, LEC were pre-incubated with dilutions of LPS isolated from *A. pleuropneumoniae* strains R1, R3, R7 and R8, and from *E. coli* O111:B4 (Sigma). After 30 min of incubation, *A. pleuropneumoniae* strain R8 was added to the wells and incubated for 2 h as described above.

Statistical analysis Preliminary analyses showed that variances of adherence clearly depended on the levels of means. Therefore generalised linear models were used to analyse the data. Means of the percentage of cells with adherent bacteria were analysed with a logistic link and a variance function proportional to pure binomial variance. Means of the number of bacteria per cell were analysed with a logarithmic link and a variance function proportional to the square of the mean. In accordance with these models pairwise comparisons between means were done at $P = 0.05$ significance level. All calculations were performed with the statistical programming language Genstat 5 (Genstat, 1993).

RESULTS

Characterisation of cultured lung cells Ninety percent of cultured lung cells stained positive with anti-cytokeratine specific for epithelial cells. The remaining 10% of the lung cells did not stain with anti-vimentin for fibroblasts or anti-macrophage for alveolar macrophages. Electron microscopy showed desmosomes between cells, large nuclei with little structure and no ciliary structures. Ciliary structures were not seen on freshly isolated lung cells either. Based on the reactivity with anti-cytokeratine and the morphology, we considered the cultured cells to be lung epithelial cells (LEC). Cultures of LEC contained two different cell phenotypes: small and large cells (Fig. 1). Both cell types stained similar in the immuno-staining, the small cells were more abundant. Small LEC grew in close contact with each other and were approximately 30 µm in diameter. Large LEC were large polymorphic, polynucleated flat cells and were approximately 100 µm in diameter. Cultures were confluent 5 days after seeding at 1.2×10^4 cells/cm^2. In all experiments with LEC, data have been collected for both small and large cells separately.

Adherence of *A. pleuropneumoniae* strain R7 to LEC *A. pleuropneumoniae* strain R7 produces only one toxin (ApxII) and has low cytotoxic activity. This strain was chosen to optimize the conditions for adherence. *B. bronchiseptica* (positive control) and *P. multocida*

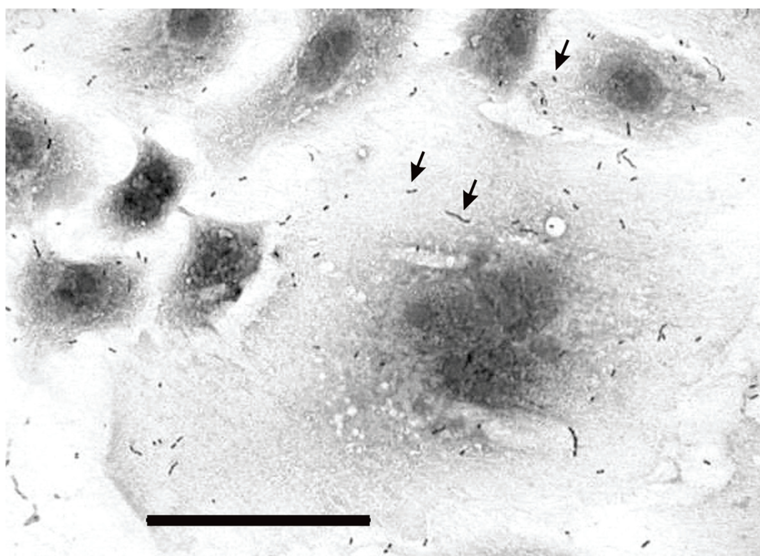

FIG. 1. Adherence of *A. pleuropneumoniae* strain R7 to large and small LEC in culture after an incubation time of 2 h and a bacterial inoculation of 10^8 CFU/ml (40 × objective). Shown is one large LEC surrounded by small LEC. Arrows indicate bound bacteria, bar is 100 µm.

(negative control) served to assess the quality of the assay. Variation of incubation time and bacterial concentration demonstrated that adherence of *A. pleuropneumoniae* R7 to LEC was time and dose related (Fig. 2 and 3). Adherence reached a plateau after 2 h incubation with 80–100% cells (Fig. 2) carrying on average seven bacteria (small LEC) and 62 bacteria (large LEC). The number of bacteria per small LEC was lower than the number of bacteria per large LEC, likely because of the smaller surface of small LEC. Lower bacterial concentrations led to decreased percentages of LEC with adherent bacteria (Fig. 3) and to decreased numbers of bacteria per small and large LEC (data not shown) after an incubation time of 2 h. At a concentration of 10^8 CFU/ml, strain R7 adhered to 77% of small LEC with nine bacteria per cell and to 100% of large LEC with 21 bacteria per cell. The kinetics of adherence for small and large LEC were similar. An incubation time of 2 h was chosen as the standard incubation time because saturation appeared to be reached at this time point. A bacterial concentration of 10^7 CFU/ml was used in subsequent assays to detect differences between highly adherent strains or between treatments.

Adherence of strain R7 to SK6 was also found to be time related (Fig. 2) and was markedly lower than adherence to LEC. After 3 h incubation, adherence of *A. pleuropneumoniae*

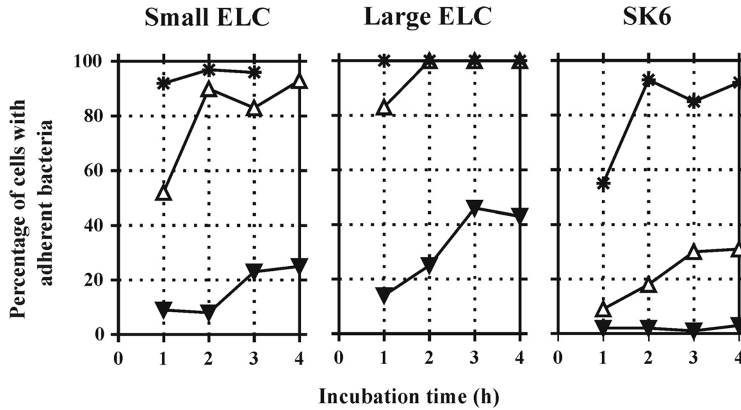

FIG. 2. Effect of incubation time on adherence of *A. pleuropneumoniae* strain R7 (△), *B. bronchiseptica* (✳) and *P. multocida* (▼) to small LEC, large LEC and SK6 with a bacterial concentration of 10^8 CFU/ml. Adherence is expressed as percentage of cells with adherent bacteria and the mean results of a three-fold (LEC) and a two-fold (SK6) experiment are shown.

reference strain of serotype 7 (R7) reached a plateau of 30% cells with adherent bacteria (Fig. 2). After 2 h incubation strain R7 adhered to 19% SK6 cells with 1.8 bacteria per cell. At a concentration of 10^8 CFU/ml, adherence of strain R7 to SK6 was markedly lower than

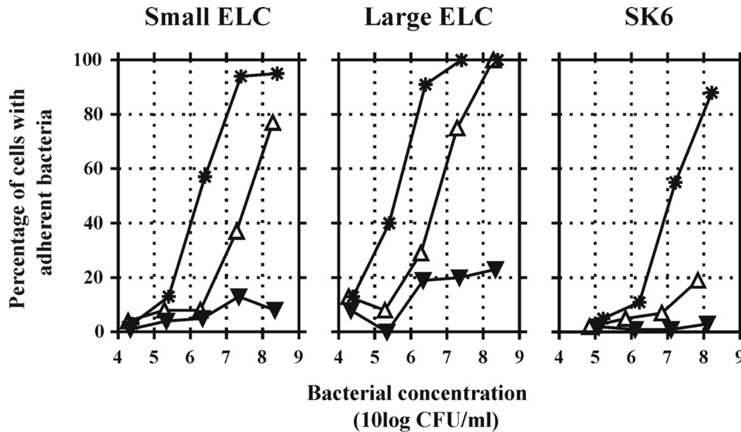

FIG. 3. Effect of bacterial concentration on adherence of *A. pleuropneumoniae* strain R7 (△), *B. bronchiseptica* (✳) and *P. multocida* (▼) to small LEC, large LEC and SK6 with an incubation time of 2 h. Adherence is expressed as percentage of cells with adherent bacteria and the results mean of a three-fold (LEC) and a two-fold (SK6) experiment are shown.

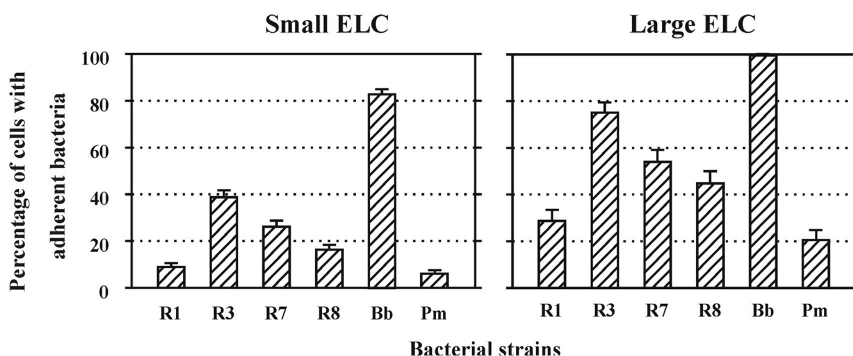

FIG. 4. Adherence of *A. pleuropneumoniae* strains R1, R3, R7 and R8, *B. bronchiseptica* (Bb) and *P. multocida* (Pm) to small and large LEC with an incubation time of 2 h. The bacterial concentrations ranged from 1.0 to 1.7 × 10^7 CFU/ml. The mean results and standard error of the mean (S.E.M.) of a six-fold experiment are shown.

adherence to LEC (Fig. 3). Lower concentrations led to decreased percentages of SK6 cells with adherent bacteria. Together, these data indicate that adherence of *A. pleuropneumoniae* to LEC cells was much more rapid and more effcient than to the swine kidney cell line.

Incubation of LEC with *A. pleuropneumoniae* reference strains R1, R3, R7 and R8 (10^8 CFU/ml, 2 h of incubation) did not result in significant changes in cell number or viability. Similar experiments with SK6 cells yielded similar results except that with strain R1 a reduction in cell number (11% of control, $P < 0.05$) was observed.

Adherence of *A. pleuropneumoniae* strains R1, R3, R7 and R8 Possible variability between *A. pleuropneumoniae* strains with respect to adherence was assessed by comparing the adherence of the reference strains R1, R3, R7 and R8 (Fig. 4). Strain R3 adhered to more LEC of both phenotypes and with more bacteria per cell than strains R1, R7 and R8 ($P < 0.05$). Strains R7 and R8 adhered to more LEC of both phenotypes than strain R1 ($P < 0.05$) and strain R7 adhered with more bacteria per cell to large LEC than strain R1 ($P < 0.05$).

Role of LPS in adherence of *A. pleuropneumoniae* LPS has previously been implicated to mediate adherence of *A. pleuropneumoniae* to tracheal epithelial cells (Belanger *et al.*, 1990; Paradis *et al.*, 1994). To test whether LPS is involved in adherence to lower respiratory tract epithelium, three different *A. pleuropneumoniae* LPS transposon mutants and their parent strain were tested for their adherence to LEC (Fig. 5). Mutant #5.1 which produces a modified core lipid A, adhered similarly as the parent. Mutants #15.1 and #24.1, which lacked O-antigen, adhered to more small LEC and with more bacteria per cell to large LEC

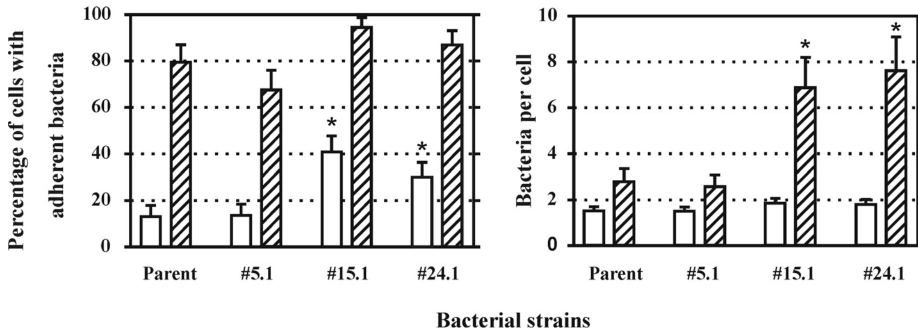

FIG. 5. Adherence of LPS transposon mutants of *A. pleuropneumoniae* serotype 1 and their parent strain to small and large LEC. An incubation time of 2 h and a bacterial concentration of 5 $\times 10^7$ CFU/ml were used. White bars represent small LEC and shaded bars represent large LEC. An asterisk indicates that a result differs significantly from that of the parent. The mean results and S.E.M. of a four-fold experiment are shown.

($P < 0.05$) compared to the parent strain (Fig. 5), suggesting that the O-antigen was not required for adherence in our model. To further corroborate this finding we evaluated the ability of purified LPS to inhibit adherence. Adherence of *A. pleuropneumoniae* strain R8 to LEC was unaltered ($P < 0.05$) when LEC were pre-incubated with LPS isolated from *A. pleuropneumoniae* strains R3 and R8 (Fig. 6). LPS from strains R1 and R7 had no significant effect on adherence of R8 either (data not shown).

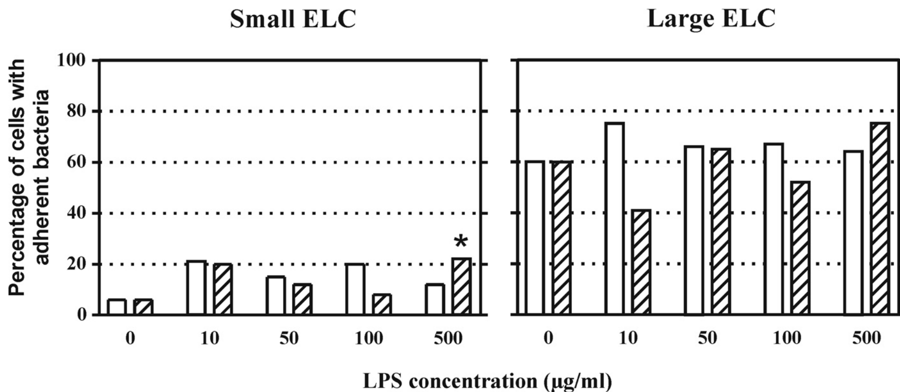

FIG. 6. Adherence of *A. pleuropneumoniae* strain R8 to small and large LEC in the presence of LPS from strains R3 and R8. An incubation time of 2 h and a bacterial concentration of 4.4 $\times 10^7$ CFU/ml were used. White bars represent LPS from strain R3 and shaded bars represent LPS from strain R8. An asterisk indicates that a result differs significantly from incubations without LPS. The mean results of a three-fold experiment are shown.

DISCUSSION

We have developed an adherence model for *A. pleuropneumoniae* using primary cultures of LEC. We tested the effect of incubation time and bacterial concentration on adherence of *A. pleuropneumoniae*. *A. pleuropneumoniae* reference strain R7 adhered at higher levels to LEC than to SK6 after shorter incubation times and at lower bacterial concentrations. This indicates that *A. pleuropneumoniae* has a higher affinity for LEC than for SK6 and stresses the importance of using organ specific cells in studying adherence of *A. pleuropneumoniae*. The highest adherence to LEC was observed for strain R3 and the lowest for strain R1. The cause of the differences between these *A. pleuropneumoniae* strains is unkown.

It has been demonstrated that bacteria adhere better to viable cells than to fixed cells and that adherence may involve a modulation of the host cell that requires *de novo* protein synthesis (Su *et al.*, 1998). Because an active role of host cells in adherence of *A. pleuropneumoniae* cannot be excluded, we decided to use viable cells for our adherence model. Previously, adherence of *A. pleuropneumoniae* serotypes 1, 2, 5 and 7 has been shown to frozen lung- and trachea sections (Jacques *et al.*, 1991; Paradis *et al.*, 1994; Paradis *et al.*, 1999) or methanol-fixed alveolar epithelial cells (Haesebrouck *et al.*, 1996; Van Overbeke *et al.*, 2000). Because living cells are sensitive to Apx toxins (Van Leengoed *et al.*, 1989; Serebrin *et al.*, 1991; Idris *et al.*, 1992; Jansen *et al.*, 1995) we tested the effect of *A. pleuropneumoniae* on cell-number and viability of cultured LEC and SK6 cells. The number and viability of cultured LEC were not affected by incubations with *A. pleuropneumoniae* strains R1, R3, R7 and R8. The number of SK6 cells was reduced by *A. pleuropneumoniae* strain R1 only, no effect was observed with strains R3, R7 and R8. From these results we conclude that this model is suitable to test adherence of *A. pleuropneumoniae* and that it can be used to identify factors that are involved in adherence.

A wide variety of adhesins is displayed by Gram-negative bacteria. Adhesins are responsible for recognizing and binding to specific receptors on the host cell. Various proteins and LPS have been identified that act as adhesins. In many cases, proteins are assembled into hair-like appendages called fimbriae. Fimbriae are produced by related species like *A. actinomycetemcomitans* (Kachlany *et al.*, 2001), *Haemophilus influenzae* (Van Ham *et al.*, 1995) and *P. multocida* (Doughty *et al.*, 2000). Up till now, little is known about fimbriae of *A. pleuropneumoniae*. The presence of fimbriae has been observed (Utrera and Pijoan, 1991; Dom *et al.*, 1994) and Zhang *et al.* (2000) isolated and identified type IV fimbriae of *A. pleuropneumoniae*. However, the role of fimbriae in adherence of *A. pleuropneumoniae*

to LEC is unknown.

Adherence of *A. pleuropneumoniae* to the respiratory tract has been reported to be related to LPS (Belanger *et al.*, 1990; Paradis *et al.*, 1994). Homologous LPS inhibited adherence to tracheal rings (89%–99%) (Belanger *et al.*, 1990) and frozen lung sections (70–80%) (Paradis *et al.*, 1994). The inhibition of adherence to frozen lung sections was found to be caused by the polysaccharide moiety of LPS comprising the O-chain and core oligosaccharide. To examine the role of LPS and particularly the O-antigen and the core region of LPS in adherence, three *A. pleuropneumoniae* LPS transposon mutants and their parent strain were tested for their ability to adhere to LEC. In accordance with data of Rioux *et al.* (1999), the O-antigen mutants showed increased binding efficiencies indicating that the O-antigen masks adhesins. In contrast to data of Rioux *et al.* (1999) no effect of core region alteration was observed on adherence to LEC. This indicates that LPS and particularly the O-antigen is not essential in adherence to LEC. This conclusion was confirmed by the finding that adherence of *A. pleuropneumoniae* could not significantly be inhibited by LPS. Apparently different adhesins are involved in adherence of *A. pleuropneumoniae* to living LEC or frozen trachea.

Here we have shown that differences between mutants and between treatments can be tested in this adherence model using living LEC. In future studies this model can be used to identify possible adhesins.

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Chapter 4

Expression of four type IV fimbrial genes of *Actinobacillus pleuropneumoniae* results in intact fimbriae

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ABSTRACT

The type IV fimbrial gene cluster of *Actinobacillus pleuropneumoniae* serotype 1 was cloned and characterised. The cluster was identified via screening of a genomic library with a part of the fimbrial subunit gene *apfA* obtained by touch down PCR with degenerate primers. Sequence analysis revealed that the cluster consisted of four genes that were designated as *apfA*, *apfB*, *apfC* and *apfD* based on their similarity with type IV fimbrial genes in other *Pasteurellaceae*. Sequencing of the *apfA* gene of forty-two strains of *A. pleuropneumoniae* showed a characteristic alanine residue at the position -1 from the cleavage site in ApfA which is different from the consensus glycine at this position in type IV subunits of other species. Southern blot hybridisation revealed that only a single copy of *apfA* is present in the *A. pleuropneumoniae* genome. Western blot analysis with an ApfA specific antiserum failed to demonstrate expression of the *apfA* gene for a number of *A. pleuropneumoniae* strains grown in different media. ApfA was detected when the *apfA* gene or the entire fimbriae cluster were placed under an active promoter in an expression vector. Electron microscopy demonstrated that transfer of the expression vector carrying the fimbrial operon into *A. pleuropneumoniae* resulted in fimbriae protruding from the bacterial surface despite the presence of the Ala residue at (-1) relative to ApfA cleavage site.

INTRODUCTION

Actinobacillus pleuropneumoniae causes porcine pleuropneumonia, a disease that is distributed world-wide and causes severe economic losses to the pig rearing industry (Barnum, 1990; Nicolet, 1992). Based on differences in capsule composition, 15 serotypes of *A. pleuropneumoniae* have been identified (Blackall *et al.*, 2002; Schaller *et al.*, 2001). All serotypes cause severe disease and death in pigs. The molecular pathogenesis of the infection is not completely understood. Several virulence factors have been described that enable the bacterium to survive *in vivo* (Haesebrouck *et al.*, 1997). Major virulence factors include the polysaccharide capsule (Inzana *et al.*, 1993; Rosendal and MacInnes, 1990; Ward and Inzana, 1994), several toxins (Frey, 1995; Kamp *et al.*, 1997), and lipopolysaccharide (LPS) (Udeze *et al.*, 1987). Capsule has been shown to protect against killing by antibody and complement, and against phagocytosis by polymorphonuclear leucocytes (Inzana *et al.*, 1988; Ward and Inzana, 1994). The toxins have been shown to be involved in the development of lesions (Kamp *et al.*, 1997). The bacterial lipopolysaccharide (LPS) has been shown to play a role in the adherence to mucus, tracheal rings and frozen lung sections (Belanger *et al.*, 1990; Belanger *et al.*, 1994; Paradis *et al.*, 1994). Several reports suggest that besides LPS additional adhesins may facilitate bacterial attachment to the mucosal surface. For adherence to alveolar epithelial cells both LPS and proteins have been shown to be required (Van Overbeke *et al.*, 2002), while adherence to primary cultures of lung epithelial cells appears to be an LPS-independent event (Boekema *et al.*, 2003). These data indicate the presence of other thus far unidentified adhesins at the bacterial cell surface.

Many bacterial pathogens carry fimbriae that mediate adherence of bacteria to host cells (Strom and Lory, 1993). Fimbriae or pili are filamentous polymeric structures that protrude from the cell surface (Wu and Fives-Taylor, 2001). Recently, fimbriae have been isolated from *A. pleuropneumoniae*. N-terminal amino acid sequencing of the major subunit indicated that the fimbriae belong to the type IV family of fimbriae (Zhang *et al.*, 2000). Type IV fimbriae form an unique class of multifunctional fimbriae defined by shared structural features and a conserved biogenesis pathway. They are found in extremely diverse groups of Gram-negative species (Strom and Lory, 1993) and can be involved in natural competence (Kennan *et al.*, 2001; Meier *et al.*, 2002; Wolfgang *et al.*, 1998), adherence (Hahn, 1997; Nassif *et al.*, 1997; Paranjpye *et al.*, 1998; Stone and Abu Kwaik, 1998; Strom and Lory, 1993), protein export (Fullner and Mekalanos, 1999; Hobbs and Mattick, 1993; Kennan *et al.*, 2001; Pepe *et al.*, 1996; Rossier and Cianciotto, 2001) and twitching motility (Mattick,

2002). The fimbriae are composed of subunits ±15−20 kDa in mass which are exported from the cell and polymerised to form the fimbrial strand. Assembly of fimbriae requires the processing of a hydrophilic leader peptide at a consensus cleavage site by a type IV prepilin peptidase during pilin secretion.

In the present study, we took advantage of the conservation of type IV subunits and cloned and characterised the type IV fimbriae operon of *A. pleuropneumoniae* serotype 1. The operon was found to contain four type IV fimbriae-like genes. Expression of fimbriae subunits and of intact fimbriae in *A. pleuropneumoniae* was established when the operon was cloned on a plasmid behind a constitutive promoter.

MATERIALS AND METHODS

Bacterial strains, growth conditions and plasmids Strains and plasmids used in this study are listed in Table 1. *A. pleuropneumoniae* strains were grown on sheep blood agar (SB) plates containing 0.1% NAD (Calbiochem, La Jolla, California, USA) or on brain heart infusion (BHI, Gibco BRL, Paisley, UK) medium containing 0.008% NAD (BHI+NAD) with or without 1.5% Bacto Agar (Becton Dickinson., Alphen a/d Rijn, The Netherlands). To study fimbriae expression, *A. pleuropneumoniae* was grown on Luria-Bertani (LB) agar plates containing 0.008% NAD (LB+NAD) or in 5 ml of chemically defined medium (CDM, Herriott *et al.*, 1970) in air, in CDM under micro-aerophilic conditions (composition 6% O_2, 7% CO_2, 7% H_2 and 80% N_2 was obtained with an Anoxomat WS8000 (Mart ® Microbiology B.V., Lichtenvoorde, The Netherlands)), in tryptic soy broth (TSB, Biotrading Benelux B.V., Mijdrecht, The Netherlands) + 0.008% NAD or in LB medium + 0.008% NAD. *A. actinomycetemcomitans* was grown on SB plates or in Todd Hewitt broth (code CM 189; Oxoid Ltd., Inc., Columbia, Maryland, USA). *P. multocida* strains were grown on SB plates and *H. influenzae* strains were grown on columbia base agar plates supplemented with 5% heated sheep blood. All *E. coli* strains were routinely grown in LB with or without 1.5% Bacto Agar (Becton Dickinson). When appropriate, ampicillin was added to the growth medium at a concentration of 100 µg/ml (*E. coli*) or 5 µg/ml (*A. pleuropneumoniae*). *E. coli* M15 (pREP4) was grown in the presence of 25 µg/ml kanamycin. Bacteria were grown at 37°C, unless indicated otherwise.

DNA transformation For use in electro-transformation, *A. pleuropneumoniae* reference strain S4074 (serotype 1) was grown in 5 ml of TSB with 0.008% NAD (TSB+NAD) at

TABLE 1. Bacterial strains and plasmids used

1A. *A. pleuropneumoniae* strains

Serotype[a]	Strain	Source	Serotype	Strain	Source
1	S4074	Reference	3	126023-3	ID-Lelystad
2	1536	Reference	5	J45	Inzana[c]
3	1421	Reference	6	125739	ID-Lelystad
4	M62	Reference	7	2827	ID-Lelystad
5a	K17	Reference	7	25535-2578	ID-Lelystad
5b	L20	Reference	7	HS30	Blackall
6	Femø	Reference	7	212:89-32159	Hilbink[d]
7	WF83	Reference	7	22:91-895	Hilbink
8	405	Reference	7	126398-165	ID-Lelystad
9	CVI13261	Reference	8	20044	ID-Lelystad
10	D13039	Reference	8	896	ID-Lelystad
11	56153	Reference	9	HS17	Blackall
12	8329	Reference	9	125943-191	ID-Lelystad
1[a]	N273	ID-Lelystad	10	3177/89	Nielsen[e]
2[a]	N282	ID-Lelystad	11	117559-1	ID-Lelystad
1	HS25	Blackall[b]	11	111290	ID-Lelystad
1	HS57	Blackall	11	20492	ID-Lelystad
2	126023-1	ID-Lelystad	11	126219-2	ID-Lelystad
3	117559-5	ID-Lelystad	12	6807/90	Nielsen
3	16169	ID-Lelystad	2[a]	118126G	ID-Lelystad
3	HS77	Blackall	2[a]	118126K	ID-Lelystad

[a]All listed strains are of biotype 1, except strains N273, N282, 118126G and 118126K (biotype 2)
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37°C with shaking at 120 rpm. After overnight growth, the culture was diluted ten-fold in TSB+NAD and incubated for 90 min at 37°C with shaking. Then, the bacteria were collected by centrifugation (5,500 × *g*, 10 min, 4°C), washed with 25 ml of chilled 274 mM sucrose, 15% glycerol, and resuspended in 274 mM sucrose, 15% glycerol to an OD_{600} of 6.0. Fifty microliters of this cell suspension (which was kept on ice) was mixed with plasmid DNA (1 μg) and transferred to a prechilled electroporation cuvette (Bio-Rad, Richmond, California,

TABLE 1 Continued

1B. Other strains		
Strain	Relevant characteristics	Reference or source
Actinobacillus actinomycetemcomitans	Positive for type IV fimbriae	ATCC 33384
Pasteurella multocida subspecies *multocida* Carter serotype D	Negative for type IV fimbriae	NCTC 10325
P. multocida subspecies *multocida* Carter serotype A	Positive for type IV fimbriae	NCTC 10322
Haemophilus influenzae Rd	Positive for type IV fimbriae	Van Ham, RIVM, Bilthoven, The Netherlands
H. influenzae AM30	Negative for type IV fimbriae	(Van Ham *et al.*, 1995)
Escherichia coli DH5αF'	Library of partially *Sau*3AI digested DNA fragments of *A. pleuropneumoniae* strain AP76 of serotype 7 in plasmids pGH432 and pGH433	Gerald F. Gerlach, Tierärtzliche Hochschule, Hannover, Germany (Baltes *et al*., 2003)
E. coli XL2-blue	Used for plasmid construction	Stratagene, La Jolla, California, USA
E. coli M15 (pREP4)	Used for analysis of expression vectors	Westburg, Leusden, The Netherlands

USA) with an electrode distance of 2 mm. Electrical charges (2,500 V, capacitance 25 µF, parallel resistor 200 Ohm) were delivered to ice-cold samples using a Gene-pulser (Bio-Rad). Immediately after the electrical charge 900 µl of SOC medium (Sambrook *et al.*, 1989) supplemented with 0.008% NAD was added and the cells were allowed to recover at 37°C for 3 hours with shaking. The cell suspension was plated onto BHI+NAD agar plates containing 5 µg/ml ampicillin (BHI+NAD+Amp). Transformants were grown overnight in 5 ml BHI+NAD+Amp and stored at -70°C in 50% glycerol in BHI. Transformation to *E. coli* was done according to the instructions supplied by the manufacturer.

PCR reactions Oligonucleotides used for PCR and DNA sequencing were obtained from Isogen Biosciences BV (Maarsen, The Netherlands) or Gibco. Relevant oligonucleotides are listed in Table 2. Touch down PCR was carried out by using the AmpliTaq® DNA polymerase kit reagents (Roche Molecular Systems, Inc., Branchburg, New Jersey, USA) according to the supplied protocol using primers 1024 and 1025. Each 50 µl PCR reaction

TABLE 1 Continued

1C. Plasmids	Relevant characteristics	Reference or source
pQE30	Expression vector	Westburg
pQE-ApfA	0.5 kb *Bam*HI-*Sph*I fragment containing the *A. pleuropneumoniae apfA* lacking part of the signal peptide in frame with N-terminal HIS-tag in pQE30	This study
pGEM7	Used for cloning	Promega
pGH432 & pGH433	Vectors used for construction of a library of partially *Sau*3AI digested DNA fragments of *A. pleuropneumoniae* strain AP76 of serotype 7	Gerald F. Gerlach (Baltes *et al.*, 2003)
pUC18	Used for cloning	Gibco
pSD2	Active SD2 promoter of *A. pleuropneumoniae* serotype 1 in a promoter-trap vector	Martha Mulks, Michigan State University, East Lansing, Michigan, USA (Doree and Mulks, 2001)
pUC-ApfABCD	3.9 kb *Xba*I-*Bam*HI fragment containing the *A. pleuropneumoniae apfABCD* operon including the RBS but lacking the promoter in pUC18	This study
pUC-SD2-ApfABCD	SD2 promoter upstream of the fimbriae operon in pUC-ApfABCD	This study
pGZRS19	*E. coli*-*A. pleuropneumoniae* shuttle vector	Susan West, University of Wisconsin-Madison, Madison, Wisconsin, USA (West *et al.*, 1995)
pGZRS-F1	Fimbriae operon downstream of SD2 promoter in pGZRS19	This study

contained 50 ng of template DNA, 15 pmol of (each) primer, 200 µM of deoxynucleoside triphosphate mix, 1× PCR buffer and 1.25 units of enzyme. Each sample was amplified using the following conditions: 10 min at 94°C, 10 cycles of 15 s at 94°C, 15 s at 55°C - 0.5°C per cycle and 10 s at 72°C, followed by 30 cycles of 15 s at 94°C, 15 s at 50°C and 1 min at 72°C, followed by 7 min at 72°C.

Amplification of the complete fimbriae operon was done by using the Expand™ High Fidelity kit (Roche) according to the supplied protocol using primers 25 and 26. Each 50 µl PCR reaction contained 50 of ng template DNA, 15 pmol of (each) primer, 200 µM of deoxynucleoside triphosphate mix, 1× buffer and 2.6 units of enzyme mix. Each sample was amplified using the following conditions: 2 min at 95°C, 10 cycles of 20 s at 94°C, 30 s at 55°C and 270 s at 68°C, followed by 20 cycles of 20 s at 94°C, 30 s at 55°C and 270 s + 5 s

TABLE 2. Oligonucleotides used in this study

Oligo-nucleotide	Location (nt)[a]	Use	Sequence (5' - 3')[b]
1024	1167-1198	Touch down PCR on fimbrial subunit	AAAAAAGGGTTTACATTAATCG
1025	1379-1354	Touch down PCR on fimbrial subunit	GCTIIAATICCITTTTGTCCICCII-TAC
FwG	1742-1768[c]	Insert PCR on pGH432 & pGH433	CGGCCAAGCTTACTCCCCATC-CCC
RevG	1947-1921[c]	Insert PCR on pGH432 & pGH433	CCACTCCCTGCCTCTGTCATCA-CG
8	135-156	Sequence analysis of cleavage site	TGTTCGGTCATGGCAAATACGC
9 *Bam*HI	1155-1175	Cloning of *apfA*	<u>CGGGATCCCG</u>TATTCGACCGCT-TACTAACGCG
10 *Sph*I	1642-1664	Cloning of *apfA*	<u>ACATGCATGCATG</u>TGCCACTGT-TCCTCGGAAATCCGG
25 *Xba*I	1037-1059	Cloning of*apfABCD*	<u>GCTCTAGA</u>GCGATACGGATCG-CAGAAGTCGG
26 *Bam*HI	4882-4902	Cloning of *apfABCD*	<u>CGGGATCCCG</u>CCGATTCCACC-GGTTAAACCG

[a]According to the operon sequence of *A. pleuropneumoniae* strain AP76 of serotype 7 determined for this paper

[b]I : inosine. Inosine was incorporated to reduce specificity. Underlined nucleotides are not exact matches to the sequence and were altered to add restriction enzyme sites

[c]Location in plasmids pGH432 and pGH433, used for sequence analysis of inserts of *A. pleuropneumoniae* genomic DNA

per cycle at 68°C, followed by 10 min at 72°C.

Standard PCR was carried out by using the Takara ExTaq™ kit reagents (Takara Shuzo Co., Ltd., Otsu, Shiga, Japan) according to the supplied protocol. Each 50 µl PCR reaction contained 50 of ng template DNA, 15 of pmol primer, 200 µM of deoxynucleoside triphosphate mix, 1× PCR buffer and 1.25 units of enzyme. Each sample was amplified using the following conditions: 10 min at 94°C, 30 cycles of 15 s at 94°C, 30 s at 60°C and 30 s at 72°C, followed by 7 min at 72°C. All PCR reactions were performed on a Primus 96 (MWG Biotech AG, Ebersberg, Germany).

DNA manipulations, Southern blotting and hybridisation Plasmid DNA was isolated by using the Miniprep or Midiprep Wizard kit (Promega Corporation, Madison, Wisconsin, USA). Genomic DNA was isolated as described by Sambrook *et al*. (1989). DNA ligations were done by using the rapid ligation kit (Roche Diagnostics GmbH, Roche Molecular Biochemicals, Mannheim, Germany). For use in Southern or spotblot hybridisation, PCR products were labeled with [α-^{32}P]CTP via random-primed labeling (Boehringer Mannheim). For spotblotting, 3 µl plasmid DNA or 3 µl culture was spotted on Genescreen Plus (NEN™ Life Science Products, Boston, Massachusetts, USA), denatured with 0.4 M NaOH, 1 M NaCl (two times 5 min), and neutralised in 2× SSC (composition of 1× SSC: 150 mM NaCl, 25 mM sodium citrate). For Southern blotting, approximately 20 µg of bacterial genomic DNA was digested with *Eco*RI, subjected to electrophoresis in a 0.8% agarose gel, and transferred to Genescreen Plus by standard procedures (Sambrook *et al.*, 1989). Radioactive labeled amplicons were boiled for 10 min, chilled in ice, and used as probes. Blots were incubated with the labeled probes for 16 h at 65°C in hybridisation solution (342 mM Na_2HPO_4, 158 mM NaH_2PO_4, 1 mM EDTA, 7% (wt/vol) sodium dodecyl sulfate (SDS)). The membranes were washed twice (30 min, 65°C) with washing solution (27 mM Na_2HPO_4, 13 mM NaH_2PO_4, 1 mM EDTA) containing 5% SDS, and twice (30 min, 65°C) with the same solution containing 1% SDS.

Cloning To construct an ApfA expression plasmid, primers 9 and 10 (Table 2, Fig. 1) were used to amplify the *apfA* gene. The resulting PCR product was cloned in pQE30 with *Bam*HI and *Sph*I generating pQE-ApfA. pQE-ApfA was transformed to *E. coli* M15 (pREP4). Expression was induced by the addition of 1 mM IPTG.

The entire fimbriae operon of *A. pleuropneumoniae* serotype 1 containing the putative ribosomal binding sequence (RBS) but lacking its own putative promoter sequence was amplified with primers 25 and 26 (Table 2, Fig. 1) and the High Fidelity kit. The resulting PCR product was cloned in pUC18 with *Xba*I and *Bam*HI generating pUC-ApfABCD. An *Eco*RI fragment from pSD2 containing the transcription terminator T4 and constitutive *A. pleuropneumoniae* promoter SD2 was subcloned in pGEM7 and a 600 bp fragment containing T4/SD2 was cloned with *Hin*dIII and *Xba*I upstream of the fimbriae operon in pUC-ApfABCD generating pUC-SD2-ApfABCD. The fragment containing T4/SD2 and the fimbriae operon was subsequently cloned in pGZRS19 with *Hin*dIII and *Bam*HI generating pGZRS-F1. pGZRS19 and pGZRS-F1 were transformed to *E. coli* XL2 as well as to *A. pleuropneumoniae* S4074. Sequence analysis was performed on inserts of pQE-ApfA and pUC-ApfABCD.

DNA sequencing and analysis DNA sequences were determined by using the Dye terminator cycle sequencing ready reaction kit (PE Biosystems, Warrington, UK) in an ABI 373A DNA sequencer (Applied Biosystems, Forster City, California, USA). Reactions contained 500 ng of template plasmid DNA or 20 ng PCR product, 8 µl of reaction mix, and 3.2 pmol of primer in a 20 µl volume. Alternatively, DNA sequences were determined by Plant Research International (Wageningen, The Netherlands) by using the BigDyeTerminator mix (version 2.0, Applied Biosystems). Reactions contained 500 ng of template plasmid DNA, 4 µl of reaction mix, and 10 pmol of primer in a 10 µl volume. Cycle sequencing reactions were performed on a Primus 96 (MWG Biotech). In all cases, both strands were sequenced. Primers FwG and RevG (Table 2) were used for sequence analysis of inserts in plasmids pGH432 and pGH433. Sequence analysis was performed using the DNAstar software package (DNAstar Inc., Madison, Wisconsin, USA). To search for homologies, the nucleotide and amino acid sequences were compared with sequences in the GenBank databases by using BLAST (Altschul *et al.*, 1990).

SDS-PAGE and Western blot analysis Production of fimbriae subunits was analysed by SDS-PAGE (17.5% polyacrylamide) and Western blotting. Blots were immunostained with 6×HIS monoclonal antibody (anti-HIS, Clontech Laboratories, Palo Alto, California, USA) or polyclonal anti-fimbriae peptide serum (Eurogentec, Seraing, Belgium). The anti-fimbriae peptide serum was raised in mice against a short synthetic peptide with amino acid sequence CSGGQNGVRKMTELR from ApfA (Eurogentec).

Electron microscopy Cultures were examined for the presence of fimbriae by negative staining. Bacteria were absorbed on carbon-coated collodion nickel grids from agar plates or suspensions. The grids were then floated three times for 5 s on a solution of 1% methylamine tungstate (Bio-Rad). After staining, the specimen were viewed with a Philips CM10.

Adherence assay The isolation and culture of porcine lung epithelial cells (LEC) were perfomed as described (Boekema *et al.*, 2003). Cell monolayers were incubated with approximately 10^8 CFU/ml bacteria from an overnight culture (37°C, 5% CO_2) supplemented with 5 µg/ml ampicillin. After 2 hours, non-adherent bacteria were removed by washing with four times with 3 ml of PBS, and the cells were fixed with methanol (10 min, RT) and stained with water-diluted Giemsa stain (Merck Diagnostica, Darmstadt, Germany). Bacterial adherence was scored by counting at least 20 randomly selected visual fields in an

Olympus microscope (100 × objective). This yielded sufficient numbers of cells for statistical purposes. Separate scores were obtained for the large and small cell phenotypes present in the LEC cultures. The average number of counted cells per field was 1.1 for large LEC and 11.4 for small LEC. Adherence was expressed as the percentage of cells with adherent bacteria and as the average number of bacteria per cell (only cells with adherent bacteria were taken into account).

Nucleotide sequence accession numbers The nucleotide sequences containing the type IV fimbriae operon of *A. pleuropneumoniae* serotype 1 (S4074) and serotype 7 are available at GenBank under accession no. AY235718 and AY235719, respectively.

RESULTS

Cloning of the *A. pleuropneumoniae* fimbrial gene cluster As a strategy to identify genes involved in fimbriae production in *A. pleuropneumoniae*, we amplified part of the major subunit gene with primers (1024 and 1025, Table 2). The primers were designed on the basis of the conserved fimbriae subunit sequence in *H. influenzae* and *A. actinomycetemcomitans* and the previously determined N-terminal amino acid sequence of an *A. pleuropneumoniae* subunit (Zhang *et al.*, 2000). Inosines were incorporated at seven positions in primer 1025 to reduce its specificity. Touch down PCR with these primers on genomic DNA from *A. actinomycetemcomitans*, *H. influenzae* Rd, *H. influenzae* AM30, *P. multocida* type D, *P. multocida* type A and *A. pleuropneumoniae* reference strains S4074, 1536 and WF83 (Table 1) yielded bands of the expected size (220 bp) at annealing temperatures ranging from 55−50°C for *H. influenzae* Rd and *A. actinomycetemcomitans*, 45−40°C for *P. multocida* type A, and 40−35°C for the three *A. pleuropneumoniae* strains. DNA sequencing and subsequent analysis of the PCR fragments obtained with *A. pleuropneumoniae* DNA revealed 55% similarity at the amino acid level with the type IV fimbriae subunits of *A. actinomycetemcomitans, H. influenzae* Rd and *P. multocida*.

In order to obtain the entire subunit gene (designated as *apfA*) and possible flanking fimbrial genes, a DNA library of *A. pleuropneumoniae* strain AP76 of serotype 7 was hybridised with the obtained *apfA* PCR fragments. Hybridising clones were collected and the entire DNA sequence of the inserts was determined. This procedure yielded a 5,303 bp DNA region that contained four complete and two partial open reading frames (ORFs) (Fig. 1).

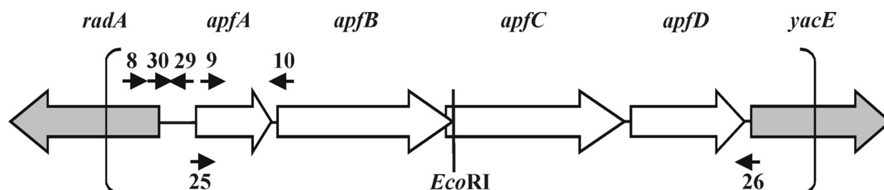

FIG. 1. Arrangement of the type IV fimbriae operon in *A. pleuropneumoniae*. The region between accolades was completely sequenced. Open arrows represent type IV fimbriae genes, filled arrows represent genes that are not involved in fimbriae biogenesis. Small black arrows with numbers indicate the positions of primers (Table 2).

Properties of the major fimbrial subunit gene, *apfA* Sequence analysis indicated that the first complete ORF of the identified region was the *apfA* gene. The gene was 444 bp in length and was predicted to encode a 15.9 kDa protein (Fig. 2). The putative protein was 81% similar to the fimbriae subunits of *H. influenzae*, A. *actinomycetemcomitans*, *P. multocida* (Fig. 2) and *Haemophilus somnus* and identical to the putative ApfA protein of *A. pleuropneumoniae* serotype 2 listed in the database (GenBank accession number AF302997). The amino terminus of ApfA appeared to contain a short positively charged signal peptide of 13 amino acids in length (Fig. 2) that was similar to the predicted signal peptides of the subunit proteins of *H. influenzae* and *P. multocida* (Doughty *et al.*, 2000; Fleischmann *et al.*, 1995) but 6 residues longer than the signal peptides of several other subunit proteins (Strom and Lory, 1993). The deduced protein sequence following this region corresponded to the amino acid sequence determined previously for the mature subunit (Zhang *et al.*, 2000). This region was hydrophobic and shared similarities with similar subunit sequences in other Gram-negative bacteria except for a remarkable Ala residue at position -1 relative to the cleavage site (Strom and Lory, 1993) (Fig. 2). Most known type IV prepilin-like leader sequences contain a glycine at this position (Strom and Lory, 1993) (Fig. 2). PCR with primers 8 and 10 (Fig. 1) and sequence analysis on fourty-two strains (Table 1A) including HS25, a strain which has been reported to produce fimbriae (Zhang *et al.*, 2000) confirmed the Ala residue at position -1 as an intrinsic trait of the *A. pleuropneumonia* subunit gene (data not shown). A stop codon instead of a glycine was found at residue 68 of the fimbriae subunit in *A. pleuropneumoniae* reference strain of serotype 7 (WF83). Further analysis of *apfA* indicated that the putative ApfA protein contained two pairs of cysteine residues as was reported for *H. influenzae*, *A. actinomycetemcomitans* and *P. multocida* (Doughty *et al.*, 2000; Fleischmann *et al.*, 1995; unfinished genome of *A. actinomycetemcomitans* available through GenBank). The first pair of cysteines was located at the amino acid positions 63 and

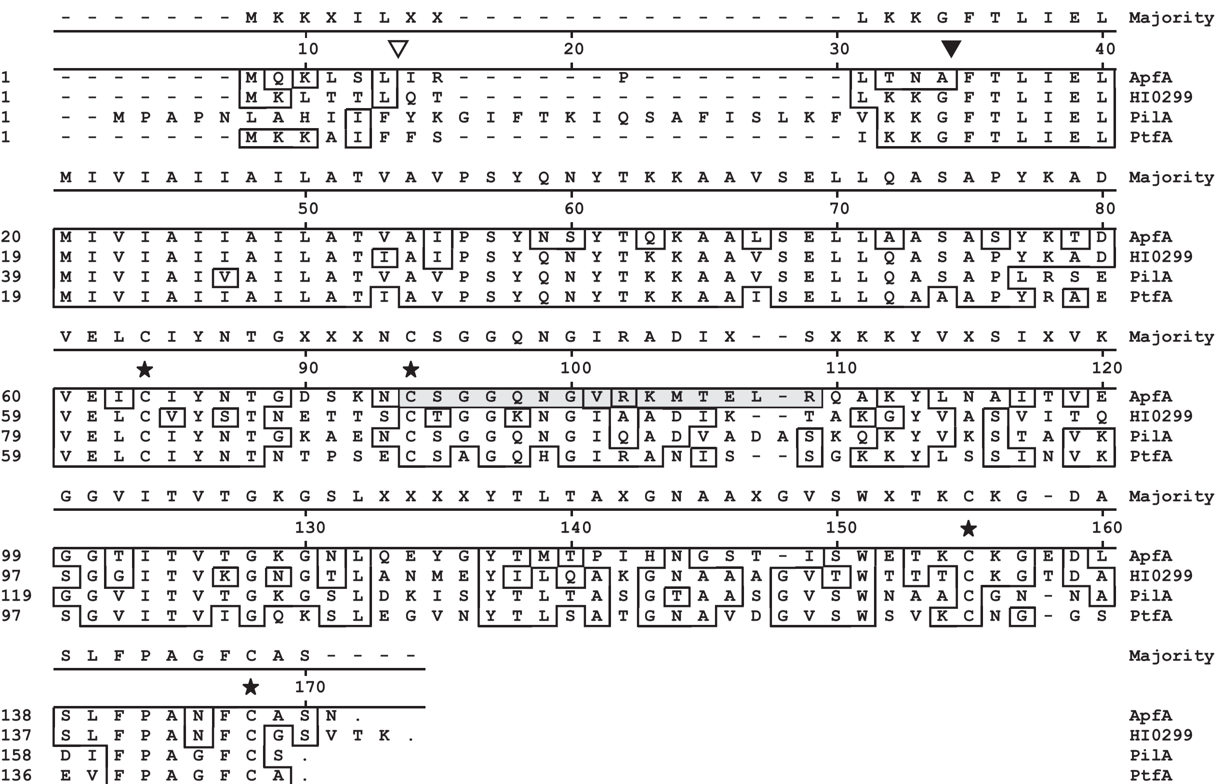

FIG. 2. Alignment of amino acid sequences of type 4 fimbriae subunits of *A. pleuropneumoniae* and three other *Pastereurellaceae*. Residues that are identical to the consensus are boxed. The putative cleavage site is indicated by a closed triangle. The position of the fusion of ApfA to the HIS-tag in pQE-ApfA is indicated by an open triangle. Conserved cysteine residues are indicated by asterisks. A synthetic peptide with the amino acid sequence of residues 73 to 87 (CSGGQNGVRKMTELR, shaded) of ApfA was used to immunize mice. GenBank accession numbers are: *A. pleuropneumoniae* ApfA: AY235719, *H. influenzae* Rd HI0299: AAC21963.1, *A. actinomycetemcomitans* PilA: AAF89188.1, *P. multocida* PtfA: AAF61196.1.

73 and the second pair at the residues 132 and 145.

Organisation and characterisation of the remaining of the fimbrial gene cluster
Seventeen bp downstream of the *A. pleuropneumoniae apfA* gene, a second ORF (designated as *apfB*) was identified. This gene was predicted to encode a 53.0 kDa protein that showed 78% similarity to the PilB analogues of *A. actinomycetemcomitans, P. multocida*, *H. somnus* and *H. influenzae* involved in fimbriae assembly (Turner *et al.*, 1993). The protein possessed the highly conserved Walker sequence, an ATP binding motif found in PilB like proteins (Turner *et al.*, 1993). Downstream of the *A. pleuropneumoniae apfB* gene, a third ORF (designated as *apfC*) was present that overlapped *apfB* by 17 bp. This ORF was predicted to encode a 45.6 kDa protein with 60% similarity to PilC analogues of *H. influenzae*, *A. actinomycetemcomitans*, *H. somnus* and *P. multocida*. Based on mutational analysis, PilC is thought to be required for fimbriae biogenesis (Koga *et al.*, 1993; Pepe *et al.*, 1996; Tonjum *et al.*, 1995). At 26 bp downstream of the *A. pleuropneumoniae apfC*, a fourth ORF (designated as *apfD*) was identified that was predicted to encode a 24.6 kDa protein with 45% similarity to PilD of *A. actinomycetemcomitans* but with very low similarity to *P. multocida* and *H. influenzae* sequences. *apfD* putatively encodes a prepilin peptidase that cleaves the positively charged N-terminal signal peptide of ApfA and methylates the exposed phenylalanine residue (Lory and Strom, 1997; Strom *et al.*, 1993b).

Analysis of the ORFs flanking *apfA-apfD* revealed a partial ORF at 181 bp upstream of *apfA* on the opposite strand that showed similarity to *radA*. This gene is involved in DNA repair and has no known relation with fimbriae biogenesis (Song and Sargentini, 1996). Downstream of *apfD*, a partial ORF was found that showed similarity to *yacE*. This gene encodes the enzyme dephosphocoenzyme A kinase that catalyses the final step in coenzyme A biosynthesis, the phosphorylation of the 3'-hydroxy group of the ribose sugar moiety (Mishra *et al.*, 2001). This gene also has no known relation with fimbriae biogenesis.

Analysis of the intergenic sequences indicated that the *apfA* gene was preceded at six bp upstream of the putative start codon by the sequence AGGAGA that resembled the AGGAGG consensus RBS for *A. pleuropneumoniae* (Doree and Mulks, 2001). A putative promoter with the sequence TTGAC (-35) and TATAAT (-10) with a spacing of 19 bp was identified at 180 bp from the ATG start codon. This promoter structure is similar to the consensus promoter structure TT(G/A)AA (-35) and TATAAT (-10) in *A. pleuropneumoniae* (Doree and Mulks, 2001). None of the different fimbriae genes was followed by a transcriptional terminator. This in conjunction with the spacing of the *apfA-apfD* genes suggests that the genes are

arranged in an operon and may be co-transcribed. A schematic representation of the fimbriae operon in *A. pleuropneumoniae* is given in Fig. 1.

***A. pleuropneumoniae* carries a single type IV fimbriae operon** To ascertain the presence of a single copy of *apfA* in the *A. pleuropneumoniae* genome, a Southern blot hybridization was performed. Genomic DNA, isolated from *A. pleuropneumoniae* reference strains of serotypes 1 and 7 (S4074 and WF83) and field isolates HS25 and HS77, was digested with *Eco*RI, separated on agarose gel and blotted. The blot was hybridised with a PCR product containing the first half of *apfA* as a probe. In all four strains, only one band hybridised with the probe (Fig. 3) indicating that only a single copy of *apfA* is present in the *A. pleuropneumoniae* genome of serotypes 1, 3 and 7. This was confirmed by homology searches using the complete ApfA or the signal peptide sequence of ApfA and the unfinished genome sequences of *A. pleuropneumoniae* serotypes 1, 5b and 7 (available through BLAST).

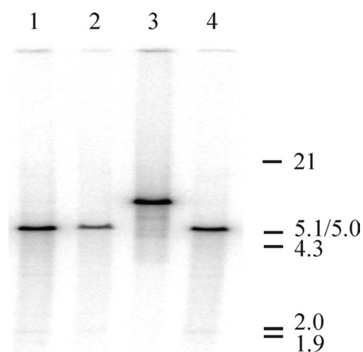

FIG. 3. Southern blot of *Eco*RI digested genomic DNA of *A. pleuropneumoniae*, probed with a PCR product containing *apfA*. Genomic DNA of *A. pleuropneumoniae* reference strains of serotype 1 (S4074, lane 1) and serotype 7 (WF83, lane 2) and field isolates HS25 (lane 3) and HS77 (lane 4) was used. Sizes in kilobases are indicated on the right.

Cloning and expression of the *apfA* gene Electron microscopy on *A. pleuropneumoniae* strains S4074, WF83, HS77, HS25 (Table 1A) grown on LB+NAD agar plates yielded no fimbriae-like structures protruding from the cell surface. Similar negative results were obtained when the production of ApfA in *A. pleuropneumoniae* strains grown in a diverse set of media including CDM was assessed on Western blots with an antiserum raised against a short synthetic peptide with the amino acid sequence CSGGQNGVRKMTELR (Fig. 2) in ApfA. These data suggest that fimbriae expression is either tightly regulated or that the identified operon is not functional.

In order to ensure that the cloned *apfA* gene can be translated into protein, the gene was cloned in frame with a HIS-tag (Fig. 2) in the expression plasmid pQE30, generating pQE-ApfA. Western blot analysis with an anti-HIS antibody indicated that upon induction with

IPTG, *E. coli* M15 (pREP4; pQE-ApfA) produced an additional protein of approximately 15 kDa (data not shown) which corresponded to the predicted size of the fusion product, ApfA-HIS. Similarly, the anti-fimbriae peptide serum reacted with the 15 kDa sized protein (Fig. 4, lanes 1 and 2).

Expression of recombinant fimbriae in *A. pleuropneumoniae* To investigate the ability of the operon placed under an active promoter to encode for the production of fimbrial subunits, a PCR product with primers 25 and 26 (Table 2, Fig. 1) containing the entire fimbriae operon of *A. pleuropneumoniae* S4074 but lacking its own promoter sequence was cloned in pGZRS19 downstream of the constitutive SD2 promoter. The resulting plasmid pGZRS-F1 was transformed to *E. coli* XL2. Western blot analysis of whole cell lysates of XL2 (pGZRS-F1) demonstrated the presence of an approximately 15 kDa protein (Fig. 4, lane 5) that was absent from *E. coli* carrying the empty plasmid pGZRS19 (Fig. 4, lane 4). These data indicate that fimbriae subunits were produced.

To investigate the ability of the operon placed under an active promoter to encode for the production and assembly of intact fimbriae in *A. pleuropneumoniae*, plasmid pGZRS-F1 was transferred to *A. pleuropneumoniae* strain S4074. Western blot analysis of whole cell lysates with the anti-fimbriae peptide serum indicated the synthesis of the 15 kDa subunit protein (Fig. 4, lane 8). Electron microscopy demonstrated straight fimbriae protruding from the bacterial cell surface from the recombinant strain carrying the fimbrial operon but not from the parent *A. pleuropneumoniae* S4074 (Fig. 5) or from *A. pleuropneumoniae* S4074 (pGZRS19). Together, the data indicate that *A. pleuropneumoniae* carries an intact fimbrial operon but that under routine laboratory growth conditions the promoter activity may be insufficient to stimulate the formation of intact fimbriae.

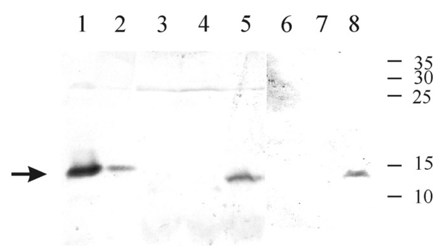

FIG. 4. Western blot analysis of fimbriae subunit ApfA expressed in *E. coli* and *A. pleuropneumoniae* clones, stained with anti-fimbriae peptide serum. Lanes 1 and 2: *E. coli* M15 (pREP4; pQE-ApfA) (2 µl and 0.2 µl), lane 3: *E. coli* XL2, lane 4: XL2 (pGZRS19), lane 5: XL2 (pGZRS-F1), lane 6: *A. pleuropneumoniae* strain S4074, lane 7: S4074 (pGZRS19) and lane 8: S4074 (pGZRS-F1). Arrow indicates the position of the fimbriae subunit ApfA (± 15 kDa). Molecular size markers are indicated on the right (in kDa).

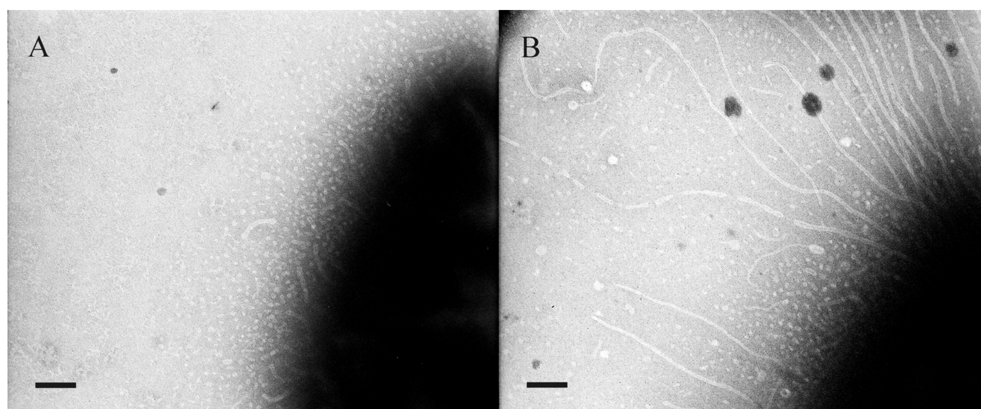

FIG. 5. Electron micrographs of *A. pleuropneumoniae* reference strain S4074 (A) and S4074 (pGZRS-F1) (B). Bacteria were stained with methylamine tungstate and examined by electron microscopy as described in materials and methods. Bars represent 200 nm.

Role fimbriae in adherence The role of type IV fimbriae of *A. pleuropneumoniae* in adherence was tested in an *in vitro* assay. LEC were cultured and incubated with *A. pleuropneumoniae* reference strain of serotype 1 S4074, S4074 (pGZRS19) and S4074 (pGZRS-F1). S4074 adhered to 88% of large LEC with 4.29 bacteria per cell and to 33% of small LEC with 1.46 bacteria per cell (Table 3). Adherence of S4074 was not significantly influenced by the expression of fimbriae on plasmid (pGZRS-F1) (Table 3).

TABLE 3. Adherence of *A. pleuropneumoniae* strains S4074, S4074 (pGZRS19) and S4074 (pGZRS-F1) to small and large LEC after 2 h of incubation. The bacterial inoculum varied from 2.8−5.0 × 10^8 CFU. Data represent the mean ± standard error of the mean of three-fold experiments.

Strain	Large LEC		Small LEC	
	Percentage of cells with adherent bacteria	Number of bacteria per cell[1]	Percentage of cells with adherent bacteria	Number of bacteria per cell
S4074	88% ± 9%	4.29 ± 1.31	33% ± 9%	1.46 ± 0.10
S4074 (pGZRS19)	76% ± 18%	6.38 ± 2.59	36% ± 10%	1.55 ± 0.15
S4074 (pGZRS-F1)	99% ± 1%	4.93 ± 2.02	37% ± 5%	1.65 ± 0.17

[1]Only cells with adherent bacteria are taking into account.

DISCUSSION

In the present study we identified and characterised the *A. pleuropneumoniae* gene cluster that facilitates the biogenesis of type IV fimbriae. The cluster consisted of four genes (*apfA-apfD*) separated by no or only small intergenic sequences and lacked apparent transcriptional terminator sequences. Although the overall organisation of the gene cluster resembled that of the related bacterial pathogens *H. influenzae*, *A. actinomycetemcomitans* and *P. multocida* (Doughty *et al.*, 2000; Fleischmann *et al.*, 1995; May *et al.*, 2001, unfinished genome of *A. actinomycetemcomitans* available through GenBank), the *A. pleuropneumoniae* cluster has several unique features both with regard to the sequence of the major subunit ApfA, its position in the genome, and its expression.

Sequence analysis of the major subunit ApfA indicated that it shared many of the typical features of type IV subunits including a short signal peptide followed by a hydrophobic domain and at least one pair of cysteines. The signal peptide is twice as long as in other subunits (Strom and Lory, 1993) and appears to be typical for *Pasteurellaceae*. Similarly, the predicted loop region between the pair of cysteine residues at the C-terminus, which comprises the epithelial cell-binding domain in *Pseudomonas aeruginosa* (Hahn, 1997), contains a motif with the sequence FPA(N/G)F that appears to be conserved among *Pasteurellaceae.* Interestingly, subunits of *H. influenzae* and *A. pleuropneumoniae* differ only in 2 out of 12 amino acids in this region.

The most striking finding with regard to ApfA however, was the presence of an Ala residue at position -1 relative to the ApfA cleavage site. This Ala (-1) was conserved among all 42 *A. pleuropneumoniae* isolates that were investigated. The consensus cleavage site of type IV fimbriae subunits consists of the the residues Gly (-1), Phe (+1) and Glu (+5) (Strom and Lory, 1993). In *P. aeruginosa*, all but one mutation at residue -1 resulted in lack of processing of the major subunit PilA (Strom and Lory, 1991). Partial processing of PilA was observed with a mutation to Ala (-1) but this did not result in production of intact pili (Strom and Lory, 1991). FimT of *P. aeruginosa* contains a prepilin-like leader sequence and also carries an Ala (-1). The *fimT* gene does not encode a fimbrial subunit but plays a role in fimbrial biogenesis (Alm and Mattick, 1996). Other proteins with type IV leader sequences with an Ala (-1) include the fimbrial subunit $PilE_L$ of *Legionella pneumophila* that can be assembled in intact fimbriae (Stone and Abu Kwaik, 1998) and five archeal proteins with type IV signal peptides (Albers and Driessen, 2002). The consequences of the presence of an Ala (-1) in the ApfA protein are not clear. In our hands, cloning of the fimbrial cluster onto an expression vector in

A. pleuropneumoniae did result in the expression of ApfA and the formation of fimbriae at the bacterial cell surface. This indicates that the Ala (-1) does not preclude fimbriae biogenesis. At this time we do not know whether the ApfD protein of *A. pleuropneumoniae* that cleaves the ApfA protein has unique characteristics with respect to cleavage activity in comparison with other prepilin peptidases or whether ApfA is cleaved at a reduced efficiency. The latter may affect the number or quality of the fimbriae. Putative prepilin peptidases of *Pasteurellaceae* appear to lack a cluster of Cys residues in the N-terminal half of the protein. Mutational analysis showed that the Cys residues are required for both cleavage and methylation activity of PilD (Strom *et al.*, 1993a). However, naturally occuring leader peptidases lacking the Cys residues can be fully functional (Hu *et al.*, 1995).

With regard to the position of the fimbrial gene cluster in the genome of *A. pleuropneumoniae*, it was remarkable that the *apfA* gene was preceded by *radA*, whereas in *A. actinomycetemcomitans*, *P. multocida* and *H. influenzae* the fimbriae subunit gene is preceded by *ampD* (Doughty *et al.*, 2000; Fleischmann *et al.*, 1995; unfinished genome of *A. actinomycetemcomitans* available through GenBank). The frequent clustering of *ampD* with fimbrial operons in other species and the fact that *radA* in other species is located in another section of the genome suggests that rearrangements have taken place in the *A. pleuropneumoniae* genome. Downstream, the fimbriae operon in *A. pleuropneumoniae* was flanked by *yacE* as was found in *A. actinomycetemcomitans, P. multocida, P. aeruginosa*, *Vibrio cholera*, *Vibrio vulnificus*, *Aeromonas hydrophila*, *E. coli* and *Neisseria gonorrhoeae* (Fullner and Mekalanos, 1999; May *et al.*, 2001; unfinished genome of *A. actinomycetemcomitans* available through GenBank). In *H. influenzae*, *yacE* is located in another section of the genome. Overall, the G+C percentage of the *A. pleuropneumoniae* type IV fimbriae genes was 38.44%. This value is slightly lower than the 42–43% G+C content of the flanking regions of the operon (including *yacE*) and of the *A. pleuropneumoniae* genome (Mannheim, 1984) and may indicate an exogenous origin of the fimbriae locus.

Another important conclusion from our work was that the *A. pleuropneumoniae* fimbriae cluster lacks an active promoter at least under the growth conditions employed. This has been noticed before (Stevenson *et al.*, 2003; Zhang *et al.*, 2000). Expression of the cluster from an expression vector did yield intact fimbriae indicating that the fimbrial genes were intact and that the *A. pleuropneumoniae* strain that was used as a host was capable to assemble fimbriae. In other species, type IV fimbriae expression is either constitutive or regulated by environmental factors (Winther-Larsen and Koomey, 2002). At this time, the exact conditions for fimbriae expression and the regulatory elements involved remain to be defined.

Expression of fimbriae in *A. pleuropneumoniae* S4074 (pGZSR-F1) from a constitutive promoter did not influence adherence to LEC. The most simple explanation for this is that the fimbriae-mediated adherence was masked by activity of other bacterial surface adhesins. Alternatively, it is possible that the type IV fimbriae of *A. pleuropneumoniae* exhibit cell type specificity and do not interact with LEC or that they function in other events e.g. modulation of the inflammatory response. Furthermore, it can be imagined that adherence requires the presence of additional fimbriae-associated proteins. In *Neisseria meningitidis* and *N. gonorrhoeae* adherence is mediated by PilC present at the tip of the fimbriae (Nassif *et al.*, 1994). A homologue of PilC is also found in *P. aeruginosa* and is required for pilus biogenesis (Alm *et al.*, 1996). A PilC homologue was not identified in the fimbriae operon of *A. pleuropneumoniae*. Future studies will be needed to discriminate between these possibilities.

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Chapter 5

The type IV fimbriae promoter of *Actinobacillus pleuropneumoniae* is active in vivo

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ABSTRACT

We recently demonstrated that the type IV fimbriae operon of *Actinobacillus pleuropneumoniae* is not expressed under routine laboratory conditions due to a lack of promoter activity. To study the conditions necessary for fimbriae expression, the fimbriae promoter region of *A. pleuropneumoniae* S4074 was cloned upstream of promoterless luciferase genes in the promoter trap vector pTF86. Fimbriae promoter activity depended on the bacterial growth phase and was seen only when bacteria were grown in a chemically defined medium. Variation in growth temperature and the availability of iron had only minor effects on promoter activity, whereas NAD had no effect. Promoter activity was observed when primary cultures of lung epithelial cells (LEC) were infected with bacteria carrying the promoter-reporter constructs. In these assays, promoter activity was found only in bacteria adhering to LEC ($P < 0.05$). Non-adherent bacteria in the culture supernatant exhibited no luciferase activity. To ascertain that fimbriae are also expressed *in vivo*, we assessed the activity of the fimbriae promoter after endobronchial inoculation of pigs. In pigs, the fimbriae promoter activity exhibited a 15-fold higher activity compared to *in vitro* conditions. These data indicate that the type IV fimbriae promoter of *A. pleuropneumoniae* is subject to regulation and is active upon cell contact as well as *in vivo*.

INTRODUCTION

Fimbriae or pili are filamentous polymeric structures that protrude from the cell surface (Wu and Fives-Taylor, 2001). Type IV fimbriae form an unique class of multifunctional fimbriae defined by shared structural features and a conserved biogenesis pathway. They can be involved in natural competence (Kennan *et al.*, 2001; Meier *et al.*, 2002; Wolfgang *et al.*, 1998), adherence (Hahn, 1997; Nassif *et al.*, 1997; Paranjpye *et al.*, 1998; Stone and Abu Kwaik, 1998; Strom and Lory, 1993), protein export (Fullner and Mekalanos, 1999; Hobbs and Mattick, 1993; Kennan *et al.*, 2001; Pepe *et al.*, 1996; Rossier and Cianciotto, 2001) and twitching motility (Mattick, 2002). Several genes have been identified to be involved in fimbriae biogenesis or regulation (Alm and Mattick, 1997). Regulation of type IV fimbriae expression differs between species (Strom and Lory, 1993; Winther-Larsen and Koomey, 2002). The best understood regulatory systems involve transcriptional modulation of the fimbriae subunit gene. In *Pseudomonas aeruginosa*, the PilS/R sensor-response regulator pair (Hobbs *et al.*, 1993) and the alternative sigma factor σ^{54} (Ishimoto and Lory, 1989) are essential for *pilA* transcription. In *Neisseria meningitidis*, *pilE* utilises a σ^{70} promoter (Carrick *et al.*, 1997) and expression of pili is down regulated upon cell contact by CrgA (Deghmane *et al.*, 2002).

Fimbriae have been found in extremely diverse groups of Gram-negative species (Strom and Lory, 1993) and their number is increasing. Recently, the type IV fimbriae operon of *Actinobacillus pleuropneumoniae* serotype 1 was identified (Chapter 4), although expression of the fimbriae subunit ApfA was not observed under the various growth conditions employed. Expression of the entire operon placed downstream a constitutive promoter resulted in the production of ApfA and the formation of intact fimbriae at the surface of *A. pleuropneumoniae*, indicating that the intrinsic *apfA* promoter is either defective or subject to regulation (Chapter 4).

The aim of this study was to investigate the conditions necessary for activity of the type IV fimbriae promoter of *A. pleuropneumoniae*. Activity of the fimbriae promoter was found only in chemically defined medium in bacteria grown to mid- to late-log phase. In addition, fimbriae promoter activity was induced in bacteria adhering to lung epithelial cells and to the lungs of pigs.

MATERIALS AND METHODS

Bacterial strains, growth conditions and plasmids *A. pleuropneumoniae* reference strain S4074 (serotype 1), was grown in brain heart infusion (BHI, Gibco BRL, Paisley, UK) medium containing 0.008% ß-nicotinamide adenine dinucleotide (NAD, Calbiochem, La Jolla, USA) with or without 1.5% Bacto Agar (Becton Dickinson B.V., Alphen a/d Rijn, The Netherlands). *Escherichia coli* strain XL2-blue (Stratagene, La Jolla, California, USA) was used for plasmid construction and analysis. When appropriate, ampicillin (Amp) was added to the growth medium at a concentration of 100 µg/ml (*E. coli*) or 5 µg/ml (*A. pleuropneumoniae*). Bacteria were grown at 37°C unless indicated otherwise.

Plasmids pTF86 and pSD2 (Doree and Mulks, 2001) were kindly provided by Dr. Martha H. Mulks (Michigan State University, East Lansing, Michigan, USA). pTF86 is a promoter-trap vector that contains, in sequence, the T4 terminator, a unique *Bam*HI site and a promoterless copy of the *V. harveyi luxAB* genes in the *E. coli-A. pleuropneumoniae* shuttle vector pGZRS19. pSD2 is based on pTF86 and contains the constitutive promoter SD2. pKUN (Konings *et al.*, 1987) was used for cloning.

Preparation of inocula For preparation of inocula, *A. pleuropneumoniae* strains were grown in 5 ml BHI + 0.008% NAD + Amp for 16 h. Bacteria were washed with phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 2.8 mM K_2HPO_4, pH 7.2) and diluted to approximately 2×10^6 colony forming units (CFU)/ml in PBS. The number of CFU before and after inoculation was determined by plating tenfold dilutions in threefold on BHI+NAD+Amp agar plates.

PCR reactions PCR was carried out by using the Takara ExTaq™ kit reagents (Takara Shuzo Co., Ltd., Otsu, Shiga, Japan) according to the supplied protocol. Each 50 µl PCR reaction contained 50 ng of template DNA, 15 pmol of primer, 200 µM of deoxynucleoside triphosphate mix, 1× PCR buffer and 1.25 units of enzyme. The amplification cycle used was 10 min at 94°C, 30 cycles of 15 s at 94°C, 30 s at 60°C, 30 s at 72°C, followed by 7 min at 72°C. All PCR reactions were performed on a Primus 96 (MWG Biotech AG, Ebersberg, Germany).

DNA manipulations Plasmid DNA was isolated by using the Miniprep or Midiprep Wizard kit (Promega Corporation, Madison, Wisconsin, USA). DNA ligations were done

by using the rapid ligation kit (Roche Diagnostics GmbH, Roche Molecular Biochemicals, Mannheim, Germany). Transformation of *A. pleuropneumoniae* was done as described (Chapter 4). Transformation of *E. coli* XL2 was done according to the instructions supplied by the manufacturer.

Cloning A PCR product with primers (5'-CGGGATCCCGAAACGCGTTAGTAAG CGG-TCG) and (5'-CGGGATCCCGCATATCCGCTGAAGCGGTCGC) containing the fimbriae promoter region of *A. pleuropneumoniae* S4074 was cloned in pKUN with *Bam*HI generating pKUN-F. Locations of the primers are 1177-1158 and 791-813 and correspond to the nucleotide sequence of the fimbriae operon (GenBank accession number AY235719). Underlined nucleotides are not exact matches to the sequence and were altered to add restriction enzyme sites. A 320 bp *Bam*HI fragment from pKUN-F was cloned in front of the promoterless *luxAB* genes in pTF86 generating pTF-F (orientation for the fimbriae promoter) and pTF-R (orientation for the *radA* promoter). The orientations of the inserts in pTF-F and pTF-R were confirmed by restriction analysis with *Eco*RI and *Bgl*II and sequence analysis.

DNA sequencing and analysis The DNA sequences were determined by Plant Research International (Wageningen, The Netherlands) by using the BigDyeTerminator mix (version 2.0, Applied Biosystems, Forster City, California, USA). Reactions contained 500 ng of template plasmid DNA, 4 µl of reaction mix, and 10 pmol of primer in a 10 µl volume. Cycle sequencing reactions were performed on a Primus 96 (MWG Biotech). In all cases, both strands were sequenced. Sequence analysis was performed using the DNAstar software package (DNAstar Inc., Madison, Wisconsin, USA).

Lux analysis Quantitative analysis of Lux expression was performed on a Victor™ 1420 multilabel counter (Wallac, Turku, Finland). N-decyl aldehyde (Sigma Chemical Co., St. Louis, MO, USA) substrate was made by dissolving 20 mg/ml Essentially Fatty Acid Free BSA (Sigma) in 1 ml of H_2O with 1 µl/ml N-decyl aldehyde. This mixture was incubated in a glass screw cap test tube for 30 min in a sonicating water bath at room temperature to disperse the N-decyl aldehyde into micelles. For Lux analysis, 20 µl of bacterial lysate was mixed with 20 µl of substrate in white polysorb luminescence plates (Nunc Gmbh & Co. KG, Wiesbaden, Germany). This mixture was then read with normal emission aperture, a delay of 5 s, and counting time of 10 s. Luminometer readings (counts per second (CPS)) were normalised to the number of bacteria in the sample as determined by plate counts on

selective media (µCPS/CFU) or to the OD_{600} for pure cultures of bacteria. An OD_{600} of 1.0 equals approximately 1×10^9 CFU/ml.

Promoter activity *in vitro* To investigate promoter activity *in vitro*, overnight cultures of *A. pleuropneumoniae* strains grown in BHI + 0.008% NAD + Amp were diluted ten times in 5 ml BHI + 0.008% NAD + Amp and incubated for 3 h at 37°C without shaking. Bacteria were washed once with test medium and resuspended in test medium. Test media included BHI + 0.008% NAD, chemically defined medium (CDM, Herriott *et al.*, 1970), CDM + 20 µM $FeSO_4$ and CDM + 0.03% NAD and all media were supplemented with 5 µg/ml Amp. Bacteria were incubated in test medium for 2 h at 37°C without shaking. OD_{600} was determined and 2.5 ml of culture was centrifuged for 10 min at 5,500 × *g* at 4°C and pellets were resuspended in 40 µl lysis buffer (50 mM KCl, 2.5 mM $MgCl_2$, 1.8 µM SDS, 15 mM Tris·HCl) and directly used for Lux quantitation.

Promoter activity in the presence of LEC The isolation and culture of porcine lung epithelial cells (LEC) were done as described (Boekema *et al.*, 2003). Overnight bacterial cultures were centrifuged and the pellets were resuspended in Dulbecco's modified Eagles medium (DMEM, Gibco). Approximately 10^8 CFU/ml were incubated with cell monolayers of LEC in the presence of 5 µg/ml Amp at 37°C in a 5% CO_2 atmosphere. After 2 hours, supernatant medium with non-adherent bacteria was removed and kept at 4°C. LEC were washed four times with 3 ml of PBS. Adherent bacteria were released by treating the cell monolayers with 1% Triton X-100 in PBS (for 1 min). Controls consisted of bacteria incubated with medium alone. For additional controls, bacteria that were incubated with medium alone, were centrifuged for 10 min at 5,500 × *g* at 4°C and were resuspended in 1% Triton X-100 in PBS (for 1 min). The numbers of CFU in supernatant medium and medium alone, and of Triton X-100 treated bacteria and adherent bacteria were determined by plating tenfold dilutions in threefold on BHI+NAD+Amp agar plates. One ml of each suspension was centrifuged for 10 min at 5,500 × *g* at 4°C. The pellets were resuspended in 20 µl lysis buffer and directly used for Lux quantitation.

Promoter activity *in vivo* Animal experiments were performed in three similar, consecutive trials in high health pigs free of *A. pleuropneumoniae*. The pigs were about five weeks of age and were housed in sterile stainless steel isolators. For endobronchial infection, pigs were anesthetised with a combination of azaperone (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. Three pigs per group were inoculated with approximately 10^7 CFU of *A. pleuropneumoniae* S4074 containing plasmids pTF86, pTF-F or pSD2. The average inocolum contained 8.5 10^6 CFU. Two hours post infection, pigs were anesthetised by intravenous injection of pentobarbital and exsanguinated. The lungs were excised and three tissue specimens of approximately 1 cm^3 were taken from both distal caudal lung lobes for Lux analysis. Tissues were minced with scalpels and 1.5 ml of PBS was added. Tissue suspensions were transferred to 5 ml tubes, mixed for 5 s and centrifuged for 5 min at 200 × *g* to remove large clumps of tissue. Bacterial concentrations of the supernatant were determined by plating tenfold dilutions on BHI+NAD+Amp agar plates. One ml of supernatant was centrifuged for 5 min at 10,000 × *g*. The pellets were resuspended in 100 µl lysis buffer and directly used for Lux quantitation. For Lux analysis, the bacterial lysate was mixed with 100 µl of N-decyl aldehyde.

All animal experiments were approved by the ethical committee of ID-Lelystad.

RESULTS

Cloning of the fimbriae promoter In order to find the molecular basis for the absence of type IV fimbriae in *A. pleuropneumoniae* under routine growth conditions (Chapter 4), we focussed on the promoter region of the fimbriae operon. The putative promoter region is likely located between the *apfA* gene and the *radA* gene that is located more upstream and directed in the opposite orientation as the fimbriae operon genes (Chapter 4). To determine possible promoter activity in this region, a PCR fragment containing this entire region of *A. pleuropneumoniae* S4074 was cloned with opposite orientations into the reporter vector pTF86 in front of the promoterless *luxAB* genes, generating pTF-F (orientation for the fimbriae promoter) and pTF-R (orientation for the *radA* promoter), respectively. As a control, we used plasmid pSD2 containing the constitutive *A. pleuropneumoniae* promoter SD2 in front of the *lux* genes (Doree and Mulks, 2001). All plasmids (pTF86, pSD2, pTF-F and pTF-R) were transformed to *A. pleuropneumoniae* S4074 and the level of expression of the *luxAB* genes was determined by measurement of Lux activity.

TABLE 1 *In vitro* promoter activity in *A. pleuropneumoniae* S4074 in BHI+NAD+ Amp and in CDM+Amp. The mean results of three experiments in threefold are shown.

Plasmid	BHI+NAD+Amp		CDM+Amp	
	Lux activity (µCPS/CFU)[a]	Relative activity[b]	Lux activity (µCPS/CFU)	Relative activity
pTF86	90	1	178	1
pSD2	7,307[c]	81.12	16,691[c]	93.41
pTF-R	5,426[c]	60.24	11,588[c]	64.85
pTF-F	77	0.85	4,678[c]	26.18

[a]Lux activity is expressed as CPS (counts per second) per OD_{600}. An OD_{600} of 1.0 equals approximately 1×10^9 CFU/ml.
[b]Relative Lux activity compared to pTF86.
[c]Significantly different from pTF86 in the same medium ($P < 0.05$).

Functionality of the fimbriae promoter The *in vitro* activity of the fimbriae promoter was initially assessed of bacteria carrying the various plasmids grown for 16 h in BHI+NAD+Amp or in CDM+Amp and of ten-fold dilutions of these cultures in both media grown for 1-4 h. Under these conditions no reproducible Lux activity was measured for strain S4074 carrying the pTF-F plasmid, while S4074 carrying pSD2 (positive control) and S4074 carrying pTF-R showed strong activity (data not shown). However, when bacteria at 3 h of exponential growth in BHI were collected by centrifugation, washed, and grown in CDM+Amp for an additional 2 h, S4074 carrying pTF-F did exhibit a luciferase activity of 4,678 µCPS/CFU. This was 26 times higher than the luciferase activity of S4074 (pTF86) that carried the promoterless *lux* genes ($P < 0.05$, Table 1) but lower than the activities of bacteria carrying plasmids pSD2 and pTF-R (Table 1). Similar experiments with bacteria grown in the final 2 h of incubation in BHI+NAD+Amp instead of in CDM indicated virtually no activity of the putative fimbriae promoter, although good activity was observed for the controls pSD2 and pTF-R (Table 1). Together, these data strongly suggest that the fimbriae promoter is intact and that its activity (in contrast to that of the *radA* promoter) requires distinct environmental conditions.

Environmental regulation of the fimbriae promoter The discovery of an intact and apparently regulated fimbriae promoter led us to assess several possible environmental conditions that may regulate the promoter activity. During our experiments, we noticed that when overnight cultures were diluted more than ten times in BHI prior to incubation in CDM, S4074 (pTF-F) expressed low or no Lux activity in CDM (data not shown) in contrast to the

controls S4074 (pSD2) and S4074 (pTF-R) (data not shown). This observation could indicate that the growth phase or a particular density of the bacteria was important for the induction of fimbriae promoter activity. One mechanism by which bacterial density may influence gene transcription is via the secretion of factors that influence gene transcription (quorum factors). The latter hypothesis was further tested by measurement of Lux activity after dilution (1:1) of cultures of S4074 (pTF86) and S4074 (pTF-F) grown in BHI with sterile culture supernatant of S4074 (pTF-F) grown in CDM. This procedure did not enhance Lux activity (data not shown), suggesting that the assumed factors (if present) were not sufficient to stimulate the fimbriae promoter.

TABLE 2 Influence of culture conditions on *in vitro* promoter activity in *A. pleuropneumoniae* S4074 in CDM.

Plasmid	0.03% NAD	20 µM $FeSO_4$	33°C
pSD2	1.21[a]	0.99	0.89
pTF-R	1.01	0.99	0.85
pTF-F	1.24	0.55[b]	1.60[b]

[a]Relative Lux activity compared to relative Lux activity in CDM.
[b]Significantly different from CDM ($P < 0.05$).

For *A. pleuropneumoniae* serotypes 5a, 9 and 10, fimbriae expression has been reported to be related with a low NAD (0.001%) concentration in the medium (Van Overbeke *et al.*, 2002). In our hands, growth in CDM containing NAD concentrations between 0.0004% and 0.03% NAD did not result in alteration of Lux activities for strains S4074 (pTF86), S4074 (pSD2), S4074 (pTF-R) or S4074 (pTF-F) ($P > 0.05$, 1 experiment in threefold, Table 2) indicating that NAD is not a critical determinant of fimbriae promoter activity.

Many bacterial pathogens respond to the low iron concentration encountered *in vivo* by expressing of a wide variety of virulence determinants. Therefore, the possible role of Fe^{2+} on fimbriae promoter activity in CDM was investigated. When iron limitation is involved in the regulation of fimbriae expression, fimbriae promoter activity should be abolished or greatly reduced in the presence of an excess of iron. Growth of the strains carrying the various plasmids in CDM in the presence of 20 µM $FeSO_4$ yielded unaltered Lux activities for strains S4074 (pTF86), S4074 (pSD2) and S4074 (pTF-R), while the relative Lux activity of S4074 (pTF-F) was only slightly decreased (by a factor 1.82) ($P < 0.05$, 3 experiments in threefold, Table 2). This suggests that iron availability may play a (minor) role in fimbriae expression.

To get further insight into the exact requirements for fimbriae expression, the effect of temperature on fimbriae promoter activity was tested. The Lux activity of S4074 (pTF86), S4074 (pSD2) and S4074 (pTF-R) was not affected by an incubation temperature of 33°C

TABLE 3 Influence of LEC on *in vitro* promoter activity in *A. pleuropneumoniae* S4074. The mean results of six experiments are shown.

Plasmid	Bacteria treated with medium alone		Bacteria in supernatant of LEC		Bacteria binding to LEC		Bacteria treated with Triton	
	Lux activity[a]	Relative activity[b]	Lux activity	Relative activity	Lux activity	Relative activity	Lux activity	Relative activity
pTF86	318	1	295	1	93	1	139	1
pSD2	15,893[c]	50.06	15,936[c]	53.95	17,432[c]	188.33	5,646[c]	40.52
pTF-R	9,229[c]	29.07	4,851[c]	16.42	5,970[c]	64.49	3,231[c]	23.19
pTF-F	214	0.67	221	0.75	1,523[c]	16.45	117	0.84

[a]Lux activity is expressed as µCPS/CFU.
[b]Relative Lux activity compared to pTF86.
[c]Significantly different from pTF86 in the same suspension ($P < 0.05$).

compared to 37°C (Table 2). The relative Lux activity of S4074 (pTF-F) at 33°C was slightly increased by a factor 1.60 compared to Lux activity at 37°C ($P < 0.05$, 2 experiments in threefold, Table 2), suggesting that also temperature may have a (minor) effect on fimbriae expression. The relative Lux acitivities of S4074 (pTF86), S4074 (pSD2), S4074 (pTF-R) were unaltered in the conditions tested (Table 2).

***In vitro* activity of the fimbriae promoter in the presence of LEC** The apparent strict growth conditions required for fimbriae promoter activity led us to test the activity of the promoter under more native conditions in the presence of lung epithelial cells (LEC). *A. pleuropneumoniae* S4074 containing plasmids pTF86, pSD2, pTF-R or pTF-F were incubated in the presence of LEC for 2 h and then Lux activities of the bacteria in the supernatant as well as of the adherent bacteria were determined (Table 3). As controls, Lux activities of bacteria in medium (without LEC) and of bacteria treated with Triton X-100 were determined. Lux activity of the negative control strain S4074 (pTF86) was low under all conditions and ranged from 93 to 318 µCPS/CFU (Table 3). Lux activities of the controls S4074 (pSD2) and S4074 (pTF-R) were high under all conditions and ranged from 3,231 to 17,432 µCPS/CFU. These activities were 16 to 188 times higher than luciferase activities of S4074 (pTF86) (Table 3). Lux activity of S4074 (pTF-F) was observed only in bacteria adhering to LEC ($P < 0.05$) and was 1,523 µCPS/CFU. This activity was substantially higher than that of the non-adherent bacteria in the supernatant and of the negative control strain S4074 (pTF86) (Table 3) irrespective of the presence of Triton X-100. Together, these data clearly indicate that the fimbriae promoter becomes active when bacteria adhere to the cell surface.

***In vivo* activity of the fimbriae promoter** To test the activity of the fimbriae promoter *in vivo*, *A. pleuropneumoniae* S4074 containing plasmids pTF86, pSD2 or pTF-F were used for endobronchial inoculation of pigs. Two hours after inoculation, pigs were sacrificed and the Lux activity of minced lung tissue was determined and related to the number of CFU (Table 4). As expected, the *in vivo* Lux activity of the strain with the promoterless *luxAB* genes, S4074 (pTF86), was low (326 µCPS/CFU, Table 4) and the *in vivo* Lux activity of the strain with the consitutive expressed *lux* genes in S4074 (pSD2) was very high (22,601 µCPS/CFU, Table 4). The *in vivo* Lux activity of S4074 (pTF-F), carrying the fimbriae promoter in the correct orientation was 1,176 µCPS/CFU (Table 4) and was substantially higher than the negative control ($P < 0.05$) (Table 4). The ratio of *in vivo* Lux activity to *in vitro* Lux activity in BHI was 15 for the fimbriae promoter (Table 4). The *in vivo* Lux activity of S4074 (pTF-F) was comparable to the Lux activity of S4074 (pTF-F) binding to LEC (Table 3). These results strongly suggest that the fimbriae promoter is active *in vivo* during infection of lung tissue.

TABLE 4 *In vivo* promoter activity in *A. pleuropneumoniae* S4074. The mean results of three tissue specimens of three pigs are shown.

Plasmid	Lux activity[a]	Relative activity[b]	Ratio *in vivo* to *in vitro* (BHI)
pTF86	326	1	3.62
pTF-F	1,176[c]	3.61	15.27
pSD2	22,601[c]	69.29	3.09

[a]Lux activity is expressed as µCPS/CFU.
[b]Relative Lux activity compared to pTF86.
[c]Significantly different from pTF86 ($P < 0.05$).

DISCUSSION

In the present work, we discovered that the promoter activity driving the production of type IV fimbriae of *A. pleuropneumoniae* is subject to environmental regulation. Fimbriae production is induced by contact with epithelial cells *in vitro* and during experimental infection of the lungs of pigs.

The strategy that was used to investigate whether the fimbriae promoter was intact and subject to regulation, involved the construction of a transcriptional fusion with cloning of the type IV fimbriae promoter region of *A. pleuropneumoniae* S4074 into a promoter trap vector carrying the *luxAB* reporter genes. This approach demonstrated that the DNA region preceding the fimbriae operon carried two promoters: the fimbriae promoter with variable activity dependent on the environmental conditions and, on the opposite strand, the *radA* promoter that appeared to be constitutively active and thus served as a control. The variable fimbriae promoter activity clearly showed that the fimbriae promoter was intact and that

the lack of type IV fimbriae observed after growth in routine growth media was caused by regulation of promoter activity rather than by an intrinsic defect in the promoter region.

The first evidence that the fimbriae promoter was subject to regulation was that the luciferase activity varied with the bacterial growth phase and the growth medium. Fimbriae promoter activity was found in cultures grown to mid- to late-log phase in chemically defined media but not when grown in BHI. These data may provide the genetic basis for the observations made by Zhang *et al.* (2000) who was only able to detect fimbriae when *A. pleuropneumoniae* were grown in CDM under microaerophilic conditions. It has been reported that in certain *A. pleuropneumoniae* serotypes (5a, 9 and 10) but not in others (serotype 2) NAD restriction is a critical factor for fimbriae production (Van Overbeke *et al.*, 2002). In our hands, variation in the concentration of NAD did not influence the activity of the fimbriae promoter which was derived from a serotype 1 strain. Together, the data suggest the existence of serotype specific differences in the regulation of fimbriae promoter activity.

The exact signals that drive fimbriae promoter activity in serotype 1 are unknown. We noticed that changes in temperature, which influence fimbriae expression in *Legionella pneumophila* (Liles *et al.*, 1998) and *Porphyromonas gingivalis* (Xie and Lamont, 1999), or the availability of iron had minor effects. These effects are probably not very specific and may well be related to concomitant changes in growth phase that appear to influence fimbriae promoter activity. We cannot conclude whether *apfA* induction was directly growth phase dependent or whether it was due to deprivation of a specific essential nutrient. A strong induction of fimbriae promoter activity was observed for *A. pleuropneumoniae* that were adherent to primary cultures of lung epithelial cells. This interesting finding indicates that contact with epithelial cells is a trigger for fimbriae production. The importance of a more natural setting for the induction of fimbriae promoter activity was further underlined by our finding that the fimbriae promoter was active *in vivo* after endobronchial inoculation of pigs. The *in vivo* Lux activity appeared less than that observed for the bacteria adherent to the cultured lung cells but this may be explained by the fact that we measured the total Lux activity in all bacteria (both adherent and non-adherent) present in the tissue samples. The 15-fold induction of fimbriae promoter activity *in vivo* compared to BHI in *A. pleuropneumoniae* is similar to inductions found in a recent study of *in vivo* induced promoters in *A. pleuropneumoniae* which were identified with an IVET system (Fuller *et al.*, 1999).

The fimbriae promoter activity *in vivo* suggests that fimbriae play a role in the pathogenesis. On the basis of the functions of type IV fimbriae in other bacterial pathogens, it is tempting to

speculate that the fimbriae of *A. pleuropneumonia*e play a role in the adherence and, possibly, at other stages of the infection e.g. in the regulation of the inflammatory response, and that these roles may require transient expression.

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Chapter 6

General Discussion

Actinobacillus pleuropneumoniae causes pleuropneumonia, a disease that has severe impact in the pig rearing industry world-wide. Although progress has been made in controlling the disease, current available whole cell or subunit vaccines do not provide full protection. These vaccines protect against clinical symptoms but they do not prevent colonisation and subsequent spread of the disease. Development of new, efficient tools for successful control and prevention requires better understanding of the pathogenesis. In the present work, we addressed this topic by studying the interaction of *A. pleuropneumoniae* with host cells in a novel sophisticated infection model of primary cultures of porcine lung epithelial cells and investigated the possible role of three potential virulence factors in the infection: fimbriae, lipopolysaccharide (LPS) and the RTX toxins. Fimbriae mediate adherence in a number of species and have been isolated recently from *A. pleuropneumoniae*. Bacterial adherence is believed to be essential for bacterial colonisation. LPS was investigated because of reports about its involvement in adherence of *A. pleuropneumoniae* to epithelial cells. The RTX toxins ApxI, ApxII and ApxIII are essential in the development of clinical symptoms and lesions typical for *A. pleuropneumoniae* infections. To assess the surplus value of the production of more than one toxin and the relative contribution of ApxI and ApxII to the infection, the ability of different Apx mutants of *A. pleuropneumoniae* to induce lesions was investigated.

Interaction of A. pleuropneumoniae with epithelial cells

To study the interaction of *A. pleuropneumoniae* with host cells in more detail, an *in vitro* model was developed using primary cultures of porcine lung epithelial cells (LEC) (Chapter 3). Primary cultures of LEC were used because shortly after intranasal infection, *A. pleuropneumoniae* was found to be predominantly associated with the alveolar epithelium and with cilia of the terminal bronchioli (Dom *et al.*, 1994), suggesting that cells of the lower respiratory tract may be an important infection target. Aerosol particles produced by sneezing or artificially are small enough to penetrate into the lower respiratory tract, bypassing the need for colonisation of the upper respiratory tract (Hensel *et al.*, 1995; Kaltrieder, 1976). In the LEC model, *A. pleuropneumoniae* did adhere to LEC indicating that adherence to epithelial cells in the lower respiratory tract may constitute an important step in the pathogenesis. Adherence results with LEC were compared with data obtained with cells of the swine kidney cell line (SK6). *A. pleuropneumoniae* demonstrated higher binding efficiencies to LEC than to SK6 stressing the importance of using organ specific

cells. Whether adherence of *A. pleuropneumoniae* to lower respiratory tract epithelium is also host-specific is unknown. Cell lines are commonly used to study adherence *in vitro* and represent a variety of hosts and tissues. They are easily cultured and highly homogenous. Only recently a cell line representing the porcine lower respiratory tract epithelium has become available but its use in adherence studies has to be determined yet (Seo *et al.*, 2001). Frozen or paraffin embedded tissue sections have been used to study adherence of *A. pleuropneumoniae* (Jacques *et al.*, 1991; Paradis *et al.*, 1994; Paradis *et al.*, 1999) but results with these models must be viewed critically because normally non-exposed domains present in these sections may distort results.

Isolated alveolar epithelial cells (AEC) used in adherence studies were characterised by the presence of lamellar bodies (Van Overbeke *et al.*, 2002). They represent differentiated alveolar type II (ATII) cells which are involved in surfactant production *in vivo* (Gil and Reiss, 1973; Goerke, 1974; Kikkawa *et al.*, 1975). Epithelial cells used for this thesis were isolated with a similar procedure as used to isolate AEC but were cultured until confluence was reached (Chapter 3). These cultured cells did not contain lamellar bodies but reacted with an epithelial cell marker and hence were termed lung epithelial cells (LEC). Incubations of viable LEC with *A. pleuropneumoniae* did not result in lysis of cells. Primary AEC had to be methanol-fixed for adherence studies to circumvent the lysis of cells observed after incubation of viable AEC with *A. pleuropneumoniae* or Apx toxins (Van de Kerkhof *et al.*, 1996; Van Overbeke *et al.*, 2002). Possibly, a difference in toxin susceptibility may be related to differences in cell culture conditions, cell differentiation state or exposed cell surfaces. Host cell-surface proteins have been demonstrated to participate in toxin activity of Lkt of *Mannheimia haemolytica* and Ltx of *Actinobacillus actinomycetemcomitans* (Deshpande *et al.*, 2002; Lally *et al.*, 1997; Narayanan *et al.*, 2002; Wang *et al.*, 1998). Similar proteins have not been described for Apx toxin activities but it can be speculated that these are lost upon culture of LEC making them more resistant to Apx toxins compared to AEC. Alternatively, bacterial culture conditions may influence toxin production. The resistance of viable LEC to Apx toxins makes the LEC culture system an attractive model to study the interaction of *A. pleuropneumoniae* with epithelial cells.

Role of LPS in A. pleuropneumoniae adherence

The role of LPS in adherence of *A. pleuropneumoniae* to LEC was assessed (Chapter 3). LPS and particularly the core region of LPS have been shown to be involved in adherence

to tracheal epithelial cells (Belanger *et al.*, 1990; Paradis *et al.*, 1994; Paradis *et al.*, 1999; Rioux *et al.*, 1999). In contrast, adherence of *A. pleuropneumoniae* to LEC was not affected by changes in the core region of LPS or by addition of purified LPS (Chapter 3) suggesting that different adhesins are involved in adherence to LEC and tracheal cells. Complementation of the mutant with an altered core region with the wild type sequence of the affected gene, *galU*, did restore adherence to tracheal epithelium (Rioux *et al.*, 1999). Interestingly, this complementation did not restore the core region of LPS leaving the role of LPS in adherence to tracheal epithelium unclear (Bosse *et al.*, 2002). Although *galU* mutants of *A. pleuropneumoniae*, *Haemophilus influenzae* and *Klebsiella pneumoniae* exhibit reduced virulence, this may be related to an increased sensitivity to non-immune serum (Chang *et al.*, 1996; Hood *et al.*, 1996; Rioux *et al.*, 1999).

Information on the role of LPS in adherence of other bacterial species is limited and knowledge about the adherence conferring moieties is rudimentary. LPS plays a role in adherence of *Pasteurella multocida*, *Haemophilus ducreyi* and *Pseudomonas aeruginosa* but not in adherence of *A. actinomycetemcomitans* or *H. influenzae* type b (Alfa and DeGagne, 1997; Gupta *et al.*, 1994; Jackson *et al.*, 1996; Jacques *et al.*, 1993; Mintz and Fives-Taylor, 1994). LPS of non-typeable *H. influenzae* and of commensal *Neisseria* spp. contains a phosphorylcholine (ChoP) moiety which mediates adherence and invasion (Serino and Virji, 2002; Swords *et al.*, 2000). ChoP binds the platelet activating factor receptor present on epithelial and endothelial cells. ChoP present on other structures than LPS also mediates adherence of *Streptococcus pneumoniae* and pathogenic *Neisseria* spp. and invasion of endothelial cells by *A. actinomycetemcomitans* (Berube *et al.*, 1999; Schenkein *et al.*, 2000; Serino and Virji, 2000; Sundberg-Kövamees *et al.*, 1996; Weiser *et al.*, 1998). Whether *A. pleuropneumoniae* adhesins contain ChoP is unknown. The major role of LPS in the pathogenesis of *A. pleuropneumoniae* most probably lies in blocking complement mediated killing, in scavenging free radicals and in the induction of inflammatory events (Bilinski, 1991; Lin *et al.*, 1994; Ulich *et al.*, 1991; Ward and Inzana, 1994). This is supported by reports on various pathogens where mutations in LPS biosynthesis genes resulted in increased serum-sensitivity and reduced survival *in vivo* (Chang *et al.*, 1996; Hood *et al.*, 1996; Rioux *et al.*, 1999; Spears *et al.*, 2000; West *et al.*, 2000).

As discussed, primary cultures of LEC are excellent infection models to study the interactions of *A. pleuropneumoniae* with host cells. For example the role of LPS in adherence to LEC was assessed *in vitro* by using purified LPS and LPS transposon mutants (Chapter 3). Advantages of the *in vitro* LEC culture system include ease and simplicity of

experimentation and the use of viable and host-specific cells. Disadvantages of the LEC culture system include a probable different profile of surface proteins compared to the *in vivo* situation and the laborious nature of microscopic counting of adherent bacteria. Microscopic counting, and not for example plating of adherent bacteria, was chosen because it yields data of specific (cell-associated) binding. While cell-contact-dependent regulation of bacterial genes and the role of individual adhesins can easily be tested in the LEC assay (Chapters 3 and 5), screening strategies for identifying putative adhesins require more elaborate methods. Cell cultures can be used to screen for factors contributing to adherence by applying a signature tagged mutagenesis system for *in vitro* screening (Badger *et al.*, 2000; Fuller *et al.*, 2000). Additionally, differential gene expression of pathogen and host can be investigated by using microarray analyses of whole bacterial or host genomes (Dietrich *et al.*, 2003; Grifantini *et al.*, 2002; Ichikawa *et al.*, 2000).

Role of fimbriae in A. pleuropneumoniae infection

An important conclusion from this thesis is that the *A. pleuropneumoniae* genome harbours a functional type IV fimbrial operon that is expressed *in vivo* (Chapters 4 and 5). The *in vivo* induced activity of the fimbriae promoter shortly after infection suggests that fimbriae play a role in the colonisation and the pathogenesis. This opens opportunities for testing the protective efficacy of purified fimbriae. Infection experiments with deletion mutants in pigs and challenge experiments with fimbriae immunised animals may provide insight into the vaccine potential of *A. pleuropneumoniae* fimbriae. The apparent tight regulation of fimbriae expression in *A. pleuropneumoniae*, led us to investigate the requirements for fimbriae (*apf*) promoter activity. The promoter trap vector that was used to investigate *apf* promoter activity has been proven to be a valuable tool in identifying putative virulence determinants (Chapter 5; Fuller *et al.*, 1999). It allows quantification of reporter protein expression and therefore the relative strength of the promoter driving the expression both *in vitro* and *in vivo* in *A. pleuropneumoniae*. To investigate the *in vivo* expression of fimbriae in *A. pleuropneumoniae*, pigs were endobronchially inoculated with *A. pleuropneumoniae* carrying promoter-reporter gene fusion constructs. We measured luciferase activity of the reporter protein of all bacteria present in homogenised tissue samples, including bacteria adhering to lower respiratory epithelium. These adhering bacteria may be especially important since contact to epithelial cells *in vitro* appears to be a trigger for fimbriae expression in *A. pleuropneumoniae*. Infection experiments of LEC with *A. pleuropneumoniae* carrying reporter constructs showed that the

apf promoter was active only in bacteria adhering to epithelial cells and not in bacteria in the culture supernatant. Whether the induced fimbriae expression is transient and/or requires viable cells remains to be elucidated.

Bacterial contact to eukaryotic cells can be a regulation signal for several virulence factors (Deghmane *et al.*, 2002; Dietrich *et al.*, 2003; Taha *et al.*, 1998; Tobe and Sasakawa, 2001). In for example *Neisseria meningitidis*, bacterial cell contact induces expression of the pilustip-located adhesin PilC1 and of the transcriptional regulator gene *crgA*, followed by down-regulation of fimbriae and capsule expression (Deghmane *et al.*, 2000; Deghmane *et al.*, 2002; Grifantini *et al.*, 2002; Taha *et al.*, 1998). Downregulation of fimbriae and capsule following cell contact enables the pathogen to switch from initial to intimate adherence. It can be speculated that fimbriae expression of *A. pleuropneumoniae* upon cell contact is transient and that *A. pleuropneumoniae* has a similar ability to switch from initial to intimate adherence. In *A. pleuropneumoniae*, expression of fimbriae is tightly regulated and is induced upon cell contact. Adherence to tracheal sections of non-encapsulated transposon mutants of *A. pleuropneumoniae* was increased compared to their parent strains (Rioux *et al.*, 2000), suggesting that capsule masks adhesins. In addition, homology searches using the unfinished genome of *A. pleuropneumoniae* (available through BLAST) revealed the presence of 13 genes in identical order and highly homologous to genes in the *tad*-cluster which are involved in tight adherence of *A. actinomycetemcomitans* and *H. ducreyi* (Kachlany *et al.*, 2000; Kachlany *et al.*, 2001; Nika *et al.*, 2002). Their putative function in *A. pleuropneumoniae* awaits further investigation.

While little is known about *in vivo* expression, the *in vitro* regulation of fimbriae expression has been studied in detail in several species (Larribe *et al.*, 1997; Winther-Larsen and Koomey, 2002; Wu and Kaiser, 1997). Many bacterial pathogens sense important environmental cues and respond by regulating the expression of various genes at the transcriptional level, enabling the bacterium to flexibly adapt to the different conditions in the host. Examples of signals regulating expression of virulence factors in several Gram-negative bacteria are temperature, pH, oxidative stress, host-specific inducers, nutrient deprivation or transition into the stationary growth phase (Fullner and Mekalanos, 1999; Iriarte *et al.*, 1995; Larribe *et al.*, 1997; Mellies *et al.*, 1997; Rosenshine *et al.*, 1996). The conditions necessary for expression of type IV fimbriae differ between species and probably reflect the different niches (Dougherty and Smith, 1999; Fullner and Mekalanos, 1999; Larribe *et al.*, 1997).

Although promoters similar to the putative fimbriae (*apf*) promoter sequence were highly active under routine growth conditions *in vitro* (Doree and Mulks, 2001), the *apf* promoter

required specific conditions for activity and was subject to environmental regulation (Chapter 5). The factors that control fimbriae expression in *A. pleuropneumoniae* are unknown. As discussed above, host factors can induce expression of fimbriae in *A. pleuropneumoniae* both *in vitro* and *in vivo*. In addition, fimbriae expression was found to be related to growth phase and medium. We cannot conclude whether fimbriae induction in *A. pleuropneumoniae* was directly growth phase dependent or whether it was due to deprivation of a specific essential nutrient. Quorum factors, temperature and iron or NAD concentration had no or minor effects on *apf* promoter activity. Quorum factors are specific extracellular substances that may influence gene transcription in a bacterial density dependent manner and are produced by many bacteria including *A. pleuropneumoniae* (Malott and Lo, 2002). The role of NAD concentration in fimbriae expression appears to be serotype specific. In *A. pleuropneumoniae* serotypes 5a, 9 and 10 but not in serotypes 1 and 2 NAD restriction is a critical factor for fimbriae expression (Chapter 5; Van Overbeke *et al.*, 2002).

Molecular mechanisms of regulation of gene transcription include two-component systems and alternative sigma factors. Sigma factors are specific RNA polymerase initiation factors which are essential for transcription of various genes. In the *apf* promoter region, a putative σ^{70} and not a putative σ^{54} promoter sequence was identified. Although most known type IV subunits are transcribed from σ^{54} promoters, several type IV subunits are transcribed from a σ^{70} promoter. The major subunit gene *pilE* in *Neisseria* species utilises a σ^{70} promoter and expression of PilE is regulated by PilA, PilB and RegF (De Reuse and Taha, 1997; Fyfe *et al.*, 1995; Larribe *et al.*, 1997). The environmentally regulated bundle-forming pili of enteropathogenic *Escherichia coli* are also transcribed from a conventional σ^{70} promoter. Here, expression of pili is regulated by BfpT (Martinez-Laguna *et al.*, 1999; Puente *et al.*, 1996). The *A. pleuropneumoniae* genome (unfinished genome available through BLAST) appears to contain homologues of PilR and PilS that are involved in the regulation of the major fimbriae subunit gene *pilA* of *P. aeruginosa*. PilS and PilR belong to the family of two-component transcriptional regulatory systems that have been described in many bacterial species. When stimulated by the appropriate environmental signals, the sensor protein PilS activates PilR through kinase activity. PilR then activates transcription of the major subunit gene *pilA*, probably by interacting with RNA polymerase containing σ^{54} (Alm and Mattick, 1997). As the *apf* genes do not appear to be under control of σ^{54}, the possible role of the PilR and PilS homologues in regulation of *A. pleuropneumoniae* fimbriae expression awaits further investigation.

Although expression of fimbriae in *A. pleuropneumoniae* is induced *in vivo* and upon

host-cell-contact, the function of these fimbriae is unknown. Fimbriae or pili can mediate adherence in a number of pathogens and are expressed by several disease causing species of the *Pasteurellaceae* (Kachlany *et al.*, 2001; Nika *et al.*, 2002; Ruffolo *et al.*, 1997; van Alphen, 1995). Adherence is regarded as an important first step in the microbial colonisation of the host. Type IV fimbriae form an unique class of fimbriae which can display many functions, but their contribution in virulence lies primarily in their ability to promote attachment to various types of receptors during tissue colonisation (Strom and Lory, 1993). Although expression of fimbriae was found in *A. pleuropneumoniae* adhering to LEC (Chapter 5), infection experiments with fimbriated recombinant *A. pleuropneumoniae* S4074 (pGZRS-F1) yielded similar adherence levels as obtained for the strain lacking fimbriae under the conditions employed (Chapter 4). The most simple explanation for this is that fimbriae are not involved in adherence. However, the induction of fimbriae expression upon cell contact suggests a role for fimbriae in adherence. The fimbriae-mediated adherence may be masked by activity of other bacterial surface adhesins. Another possibility is that fimbriae-mediated adherence of *A. pleuropneumoniae* requires a tip-located adhesin (Alm *et al.*, 1996; Nassif *et al.*, 1994; Serino and Virji, 2000) that is not encoded for in the operon expression construct described in Chapter 4. Another explanation may lay in the use of the LEC culture system for adherence studies. In contrast to our results, Van Overbeke *et al.* (2002) found a relationship between adherence of *A. pleuropneumoniae* to alveolar epithelial cells (AEC) and fimbriae expression. Although the type of these fimbriae is unknown, LEC and AEC may differ in their expression of cell surface structures that are exploited as receptors by *A. pleuropneumoniae* fimbriae. Alternatively, type IV fimbriae of *A. pleuropneumoniae* mediate adherence to other cell types (e.g. tracheal epithelial cells). Adherence is only one of many features displayed by type IV fimbriae. One other feature is the uptake of DNA in e.g. *H. influenzae* (Dougherty and Smith, 1999). As in *A. actinomycetemcomitans* (Wang *et al.*, 2002), natural transformation was not observed in *A. pleuropneumoniae*, irrespective of fimbriae expression (unpublished data). *In vitro* and *in vivo* experiments with *apfA* deletion mutants of *A. pleuropneumoniae* are necessary to learn more about the function of fimbriae of *A. pleuropneumoniae*.

The major subunit ApfA contained many of the characteristics shared by type IV subunits. The most striking difference of ApfA was the presence of an alanine residue instead of the consensus glycine at position -1 relative to the ApfA cleavage site. Although both amino acids are neutral, nonpolar and similar in structure (alanine contains an extra methyl group), substitution of Gly (-1) with Ala (-1) in mutational analysis of the major subunit PilE of *P. aeruginosa*, strongly reduced cleavage of prepilin monomers and blocked subsequent pilus

assembly (Strom and Lory, 1991). Substitutions of Gly (-1) with other residues resulted in a complete lack of subunit processing (Strom and Lory, 1991). The implications of the Ala (-1) in ApfA are not clear. Possibly, ApfA is cleaved at a reduced efficiency or the cleavage characteristics of the prepilin peptidase ApfD are different in comparison with prepilin peptidases of other bacteria. The latter is not unlikely since the homology between prepilin peptidases of different bacterial species is low. We found that expression of the *apfABCD* genes resulted in intact fimbriae and therefore we assume that ApfA subunits harbouring Ala (-1) can be processed and assembled into intact fimbriae. The major subunit $PilE_L$ from *Legionella pneumophila* also contains an Ala (-1) and can be assembled into fimbriae (Stone and Abu Kwaik, 1998). This suggests that the typical characteristics of the type IV pili biogenesis are perhaps less strict than previously assumed.

The amino acid sequences of type IV subunits identified in various species share similarities, which are mainly located in a small hydrophobic area within the amino terminus. Type IV pilus genes can be found throughout the genome or clustered in an operon although the orientation and location of the different genes can differ. In *A. pleuropneumoniae*, fimbriae biogenesis requires the products of the *apfABCD* genes and the genetic organisation was identical to fimbriae operons in *A. actinomycetemcomitans*, *H. influenzae* and *P. multocida*. Several type IV clusters contain numerous additional genes involved in regulation and biogenesis of fimbriae (Alm and Mattick, 1997; Marsh and Taylor, 1999; Srimanote *et al.*, 2002; Stone *et al.*, 1996; Tonjum and Koomey, 1997). Together with the wide spread distribution of type IV pilus genes in many unrelated species, these facts strongly suggest that an ancient gene common to many species has diverged, leaving little similarity among its variants except at the amino terminus.

Contribution of RTX toxins to A. pleuropneumoniae infection

In addition to factors contributing to colonisation *in vivo*, *A. pleuropneumoniae* produces several factors that counteract phagocytosis by host immune cells and killing by antibody and complement. Impairment of the host immune system by Apx toxins, is an important virulence trait of *A. pleuropneumoniae*. Killing of neutrophils and macrophages by Apx toxins probably disables the host to clear *A. pleuropneumoniae* from the lungs (Dom *et al.*, 1992a; Dom *et al.*, 1992b; Udeze and Kadis, 1992). In addition, Apx toxins cause tissue injury and subsequent lesions through lysis of host cells and induction of oxidative burst in neutrophils and

macrophages (Dom *et al.*, 1992a; Dom *et al.*, 1992b; Kamp *et al.*, 1997; Udeze and Kadis, 1992). The ability to produce more than one Apx toxin varies among the different serotypes of *A. pleuropneumoniae* and raises questions about their contribution to the pathogenesis. Particularly ApxII, which is produced by nearly all strains, is of interest. ApxII exhibits only weak toxicity *in vitro* and purified ApxII is much less potent to induce lesions *in vivo* than ApxI and ApxIII when instilled endobronchially (Kamp *et al.*, 1991; Kamp *et al.*, 1997). However, *A. pleuropneumoniae* strains of serotypes 7 and 12 and chemically or transposon induced mutant strains of serotypes 1 and 5 which produce *in vitro* ApxII only, are still able to cause severe disease with typical lung lesions (Prideaux *et al.*, 1999; Reimer *et al.*, 1995; Tascon *et al.*, 1994). Together, these data led us to asses the relative contribution of ApxI and ApxII in the pathogenesis by testing the ability of different toxin mutants to induce lesions and clinical disease (Chapter 2). Apx mutants, that produce *in vitro* either ApxI or ApxII only, are still able to cause lesions and clinical disease but not as consistently as is seen with the parent strain. A complete lack of virulence was observed in mutants lacking both ApxI and ApxII stressing the essential role of these toxins in the development of clinical symptoms and lesions.

The potency of the ApxI deletion mutant of serotype 1 (S4074 Δ*apxIA*) to induce clinical symptoms and lesions (Chapter 2) was unexpected in view of *in vivo* instillation experiments with purified toxins and *in vitro* toxicity (Kamp *et al.*, 1991; Kamp *et al.*, 1997). Moreover, strains of serotype 10 produce *in vitro* ApxI only and belong to the most virulent strains. In addition to a critical role of toxins in the pathogenesis, adhesins may contribute to the virulence of the tested strains. Close contact with target cells may lead to a local high concentration of toxins and thereby enhance survival of the bacteria *in vivo*. Interestingly, S4074 Δ*apxIA* induced *in vitro* a delayed but two times higher oxidative burst in neutrophils compared to the parent strain or the ApxII deletion mutant (S4074 Δ*apxIIA*) (Jansen *et al.*, 1995). The higher induced activity in neutrophils by S4074 Δ*apxIA* may be explained by the dependency of ApxII on export proteins encoded by Δ*apxIBD*. A lack of competition for export in S4074 Δ*apxIA* might result in a higher secretion of ApxII compared to the parent strain. Production of ApxI was unaltered in S4074 Δ*apxIIA* (Jansen *et al.*, 1995). However, S4074 Δ*apxIA* produced *in vitro* lower levels of ApxII than the parent strain (Jansen *et al.*, 1995). This suggests that the level of oxidative burst in neutrophils is inversely related to ApxII concentration. The higher reactivity in neutrophils induced by S4074 Δ*apxIA* might result in an overall higher production of oxygen radicals *in vivo* and thus more damage to host tissue. This idea is supported by observations that high concentrations of *A.*

pleuropneumoniae or cell-free culture supernatant were lytic, whereas low doses induced increased oxidative metabolism in neutrophils and alveolar macrophages (Dom *et al.*, 1992a; Dom *et al.*, 1992b; Udeze and Kadis, 1992). The inverse relation between Apx concentration and neutrophil activity is further supported by several reports on RTX toxins of *E. coli*, *M. haemolytica* and *A. actinomycetemcomitans*, where low concentrations of toxins exhibited profound effects on normal cell functions, including release of leukotrienes, interleukins and lysosomal content, and enhanced oxidative burst (Bhakdi and Martin, 1991; Cavalieri and Snyder, 1982; Konig *et al.*, 1994; Narayanan *et al.*, 2002; Welch, 1991; Welch *et al.*, 1995). Together, this can explain our observation that infection of pigs with S4074 Δ*apxIA* induced lesions in two out of three animals. However, since no information is available on the *in vivo* production level of ApxI and ApxII, we cannot exclude a difference in expression level of the various Apx toxins *in vivo* compared to *in vitro*. The data suggest that ApxI and ApxII act differently but are equally important in the pathogenesis and that the *in vivo* production level of each toxin may be a critical factor.

Concluding remarks

In this thesis we have shown that *A. pleuropneumoniae* efficiently adheres to lung epithelial cells suggesting that adherence to the lower respiratory tract epithelium is an important first step in the colonisation of the host. Although bacterial contact to eukaryotic cells induces expression of fimbriae, factors contributing to adherence remain to be elucidated. Infection experiments with Apx deletion mutants indicated that ApxII is as important as ApxI in the pathogenesis. Their different action and/or production level *in vivo* may be critical. In current available vaccines ApxI, ApxII and ApxIII are essential for protection against the clinical symptoms caused by *A. pleuropneumoniae* infections. However, they do not prevent colonisation. To obtain full protection additional proteins should be included into these vaccines. The type IV fimbriae of *A. pleuropneumoniae* could be a candidate to include in subunit vaccines: *i*) Fimbriae are expressed on the surface of the bacteria and are likely to be immunogenic. *ii*) Fimbriae are potential virulence factors and are induced *in vivo* and upon cell contact. This can be important to exclude selection of escape mutants under vaccine pressure. *iii*) The fimbrial subunit appears to be highly conserved among all serotypes suggesting that it is suitable for one vaccine against all serotypes. *iv*) Purified fimbriae of other pathogens have been shown to confer protection (Desjardins *et al.*, 1996; Evans *et al.*, 1992; Langermann *et al.*, 1997; Lepper *et al.*, 1992; O'Meara *et al.*, 1993; Palaszynski

et al., 1998; Sirakova *et al.*, 1994). Together, these data make the type IV fimbriae of *A. pleuropneumoniae* an attractive vaccine candidate.

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Chapter 7

Summary

Actinobacillus pleuropneumoniae causes porcine pleuropneumonia, a disease that occurs world-wide and affects growing pigs of all ages. Infection of pigs with *A. pleuropneumoniae* can result in high morbidity and mortality. Based on antigenic diversity of capsule and lipopolysaccharide [LPS] composition, 15 serotypes of *A. pleuropneumoniae* have been identified. All serotypes can cause severe disease and death in pigs. Currently available vaccines are based on whole cell bacterin or are subunit vaccines containing the RTX toxins ApxI, ApxII and ApxIII. As the available vaccines do not fully prevent the economic losses associated with the disease, work continues to improve vaccines that provide complete protection. Several factors have been identified that enable the bacterium to survive in the host but more knowledge concerning the role in the pathogenesis of virulence factors is required to improve available vaccines against *A. pleuropneumoniae*. In this thesis we addressed this topic by studying the role of RTX toxins, LPS and fimbriae in the pathogenesis.

The RTX toxins ApxI, ApxII and ApxIII are essential for the development of clinical symptoms and lesions typical for *A. pleuropneumoniae* infections. These Apx toxins are involved in impairment of host-defence mechanisms by their toxic effect on alveolar macrophages and neutrophils. Most serotypes of *A. pleuropneumoniae* produce *in vivo* more than one toxin. To assess the surplus value of the production of more than one toxin and the relative contribution of ApxI and ApxII to the infection, the ability of different Apx mutants of *A. pleuropneumoniae* serotype 1 to induce lesions was investigated (**Chapter 2**). Infection of pigs with isogenic strains unable to produce or secrete both ApxI and ApxII did not result in pathological lesions, indicating that both toxins are essential for the pathogenesis of pleuropneumonia. Infection with single toxin deletion mutants did not consistently result in lesions and clinical symptoms as was seen for the parent strain. Strikingly, ApxII seemed at least as potent as ApxI in 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. The results indicate that both ApxI and ApxII of *A. pleuropneumoniae* serotype 1 are necessary for full virulence and that ApxI and ApxII are equally important in the pathogenesis of *A. pleuropneumoniae* serotype 1.

Adherence to host tissue is regarded as an important first step in the colonisation of the host. *In vivo* after intranasal inoculation, *A. pleuropneumoniae* has been shown to be associated with lower respiratory epithelium. In this thesis, we describe an *in vitro* infection model to study the interaction of *A. pleuropneumoniae* with primary cultures of porcine lung epithelial cells [LEC] (**Chapter 3**). Results with LEC were compared with data obtained

with swine kidney cells [SK6]. Adherence of *A. pleuropneumoniae* to LEC was more rapid and 30-fold more efficient than adherence to SK6 cells stressing the importance of using organ specific cells. The efficient binding of *A. pleuropneumoniae* to LEC indicates that adherence to epithelial cells in the lower respiratory tract may constitute an important step in the pathogenesis. In search for the mechanism of the adherence event, the role of LPS was investigated because of reports about its involvement in adherence of *A. pleuropneumoniae* to frozen tracheal sections. Mutants lacking the O-antigen of LPS demonstrated increased adherence to LEC suggesting that O-antigens mask adhesins. Adherence of *A. pleuropneumoniae* to LEC was not affected by changes in the core region of LPS. These data indicate that LPS and particularly the O-antigen is not essential in adherence to LEC. This conclusion was confirmed by the finding that adherence of *A. pleuropneumoniae* could not significantly be inhibited by LPS. Apparently different adhesins are involved in adherence of *A. pleuropneumoniae* to living LEC or frozen trachea. The *in vitro* infection of primary cultures of lower respiratory epithelium is a valuable model in studying the pathogenesis of *A. pleuropneumoniae*.

In an attempt to identify factors involved in adherence of *A. pleuropneumoniae*, we focussed on fimbriae. Fimbriae mediate adherence in a number of species and have been isolated recently from *A. pleuropneumoniae*. The genome of *A. pleuropneumoniae* was found to contain at least four genes involved in type IV fimbriae biogenesis (**Chapter 4**). The first gene encodes the fimbrial subunit, the other genes encode proteins involved in processing and assemblage of subunits into intact fimbriae. A putative promoter sequence was identified upstream of the gene cluster. However, expression of fimbriae was not found in *A. pleuropneumoniae* under various growth conditions. To assess the functionality of the fimbriae genes, the complete gene cluster was placed under an active promoter in an expression vector and transferred to *A. pleuropneumoniae*. Expression of the fimbriae genes from this vector resulted in fimbrial subunits and in fimbriae protruding from the bacterial cell surface. These data indicate that *A. pleuropneumoniae* carries an intact fimbrial operon but that under routine laboratory growth conditions the promoter activity may be insufficient to stimulate the formation of intact fimbriae. The involvement of fimbriae in adherence to LEC could not be demonstrated.

The fimbrial subunit protein ApfA contains many of the features shared by type IV subunits but is remarkably different with respect to the cleavage site. ApfA contains an alanine at position -1 from the cleavage site in contrast to the consensus glycine at this position. Substitution of the consensus glycine (-1) with alanine (-1) in PilE of *Pseudomonas*

aeruginosa has been shown to block the assembly of subunits into intact pili. The presence of alanine (-1) appears to be an intrinsic trait of ApfA and does not preclude fimbriae biogenesis since expression of the complete operon resulted in intact fimbriae.

The apparent lack of fimbriae expression under routine laboratory growth conditions led us to investigate the conditions necessary for fimbriae promoter activity (**Chapter 5**). The fimbriae promoter region was cloned upstream of promoterless luciferase genes in a promoter trap vector. This promoter trap vector allows quantification of reporter protein expression and therefore the relative strength of the promoter driving the expression both *in vitro* and *in vivo* in *A. pleuropneumoniae*. The fimbriae promoter appears to be intact but is subject to regulation. Expression of the fimbriae promoter depended on the growth phase and was seen only in chemically defined medium. Variation of growth conditions had only minor or no effect. To test more native conditions, activity of the fimbriae promoter was tested in the presence of LEC. A strong induction of fimbriae promoter activity was observed for *A. pleuropneumoniae* that were adhering to primary cultures of LEC. This interesting finding indicates that contact with epithelial cells may be a trigger for fimbriae production. The importance of a more natural setting for the induction of fimbriae promoter activity was further underlined by our finding that the fimbriae promoter was active *in vivo* after endobronchial inoculation of pigs.

The present work contributes to the understanding of the pathogenesis of *A. pleuropneumoniae* by providing important novel insights into several major virulence traits of the pathogen. The production of more than one toxin enhances virulence of *A. pleuropneumoniae*. On the basis of our results with LEC, LPS seems not to be involved in adherence of *A. pleuropneumoniae* to cells of the lower respiratory tract. Type IV fimbriae have been identified as novel, potential virulence factors of *A. pleuropneumoniae*. These fimbriae may be very attractive candidates to include in subunit vaccines perhaps in combination with the different inactivated toxins. Further *in vivo* infection experiments with various mutants are needed to determine the role of the identified virulence factors in the pathogenesis of *A. pleuropneumoniae*. Vaccination challenge experiments are required to determine the potential of the identified virulence factors as vaccine components.

Chapter 8

Samenvatting

Actinobacillus pleuropneumoniae is een Gram-negatieve bacterie die longontsteking veroorzaakt bij varkens. Deze besmettelijke ziekte komt over de gehele wereld voor en veroorzaakt veel schade in de varkenshouderij. Infectie van biggen met *A. pleuropneumoniae* wordt beschouwd als een van de meest voorkomende oorzaken van acute luchtwegaandoeningen. Acute (klinische) uitbraken van de ziekte worden gekarakteriseerd door een hoog percentage zieke dieren en veel sterfte. De biggen kunnen beschermd worden tegen de ziekteverschijnselen door ze te vaccineren met bijvoorbeeld de toxinen van *A. pleuropneumoniae*. Echter, kolonisatie van biggen met *A. pleuropneumoniae* wordt door deze vaccins niet voorkomen. De bacterie kan zich daardoor blijven handhaven in de populatie en kan zo niet-gevaccineerde biggen ziek maken. Om de beschikbare vaccins te kunnen verbeteren, is meer kennis nodig van factoren van *A. pleuropneumoniae* die bijdragen aan het ziekteproces. Van *A. pleuropneumoniae* komen 15 verschillende serotypen voor die onderscheiden kunnen worden aan de hand van verschillende structuren op de buitenkant van de bacterie (kapsel en lipopolysaccharide [LPS]). Hoewel er verschillen bestaan in het vermogen waarmee deze serotypen ziekte kunnen veroorzaken (virulentie), zijn ze allemaal in staat ernstige ziekteverschijnselen te veroorzaken die zelfs tot de dood kunnen leiden. De moleculaire mechanismen die nodig zijn voor een succesvolle infectie met *A. pleuropneumoniae* zijn nog niet volledig bekend. Er zijn meerdere factoren van de bacterie beschreven die bijdragen aan de overleving en vermenigvuldiging van de bacterie in zijn gastheer en aan het ontstaan van ziekteverschijnselen. In dit proefschrift hebben we de rol in het ziekteproces van de toxinen, het LPS en de fimbriae nader onderzocht.

A. pleuropneumoniae produceert vier verschillende toxinen genaamd ApxI, ApxII, ApxIII en ApxIV. Drie van deze Apx toxinen zijn essentieel voor de ontwikkeling van klinische symptomen en beschadigingen in de long (laesies) die karakteristiek zijn voor infecties met *A. pleuropneumoniae*. De Apx toxinen kunnen longmacrofagen en neutrofielen doden en spelen op deze manier een belangrijke rol in het ontwijken en onschadelijk maken van het eerste verdedigingsmechanisme van de gastheer: fagocytose en afdoding. De bijdrage van de individuele toxinen aan het infectieproces is echter onduidelijk omdat verschillende serotypen verschillende combinaties van toxinen produceren. In **Hoofdstuk 2** hebben we de bijdrage bepaald van de toxinen ApxI en ApxII in de ontwikkeling van klinische symptomen en longlaesies. Hiervoor zijn mutanten van *A. pleuropneumoniae* serotype 1 gebruikt waarin één of twee toxine genen (*apxICA* en *apxIICA*) of de toxine export genen (*apxIBD*) zijn uitgeschakeld. Infectie van biggen met stammen die zowel ApxI als ApxII niet kunnen produceren of uitscheiden, resulteerde niet in de vorming van laesies en de bacteriën konden

niet geïsoleerd worden uit geïnfecteerde biggen. Dit geeft aan dat de toxinen essentieel zijn voor het ziekteproces. Stammen waarin slechts één van de twee toxine genen (*apxICA* of *apxIICA*) is uitgeschakeld, konden nog wel laesies en klinische symptomen opwekken maar niet zo consistent als de ouderstam die beide toxinen produceert. Uit deze resultaten blijkt dat beide toxinen van *A. pleuropneumoniae* serotype 1 nodig zijn voor een maximale capaciteit om ziekte te veroorzaken. Opmerkelijk is dat ApxII net zo goed laesies en klinische symptomen kan opwekken als ApxI. Uit eerdere studies met andere serotypen bleek dat ApxII minder bijdraagt dan ApxI aan de ontwikkeling van laesies in dierexperimenten met gezuiverd toxine en aan de toxiciteit *in vitro*. Een mogelijke verklaring is dat het productieniveau of de werking van deze toxinen in de long onderling verschilt.

Algemeen wordt aangenomen dat hechting van *A. pleuropneumoniae* aan luchtweg epitheel een belangrijke eerste stap is in de kolonisatie van de gastheer. Hechting verhoogt de mogelijkheid voor de bacteriën om zich te handhaven. In diverse *in vitro* studies is de binding van *A. pleuropneumoniae* onderzocht aan epitheel van de bovenste luchtwegen (trachea-epitheel) waarbij LPS een rol bleek te spelen. Echter, kort na infectie van biggen worden hechtende bacteriën voornamelijk gevonden aan epitheel van de onderste luchtwegen (longepitheel). Daarom hebben we een *in vitro* infectiemodel opgezet dat gebruik maakt van primaire kweken van longepitheel cellen [LEC] (**Hoofdstuk 3**). Resultaten verkregen met deze cellen hebben we vergeleken met data verkregen met varkensniercellen [SK6]. *A. pleuropneumoniae* bleek sneller en tot 30-maal efficiënter te binden aan LEC dan aan SK6. Deze resultaten benadrukken dat orgaanspecifieke cellen gebruikt moeten worden voor onderzoek naar bindingsfactoren (adhesines) van *A. pleuropneumoniae*. Daarnaast lijkt binding van *A. pleuropneumoniae* aan longepitheel een belangrijke factor te zijn in het infectieproces. De mogelijke rol van LPS in de binding aan LEC werd onderzocht met behulp van *A. pleuropneumoniae* mutanten waarin genen, die betrokken zijn bij de aanmaak van LPS, zijn uitgeschakeld. LPS bevindt zich aan de buitenkant van de bacterie en is opgebouwd uit O-antigeen (keten van suikers), lipid A (voor verankering in de membraan) en kerngedeelte (vormt de schakel tussen O-antigeen en lipid A). Mutanten die geen O-antigeen van het LPS produceren vertoonden een licht verhoogde binding aan LEC. Dit suggereert dat de O-antigenen adhesines maskeren. Verandering van het kerngedeelte van het LPS had geen effect op de bindingsefficiëntie. LPS en vooral het O-antigeen lijkt niet betrokken te zijn bij binding van *A. pleuropneumoniae* aan LEC. Verder bewijs voor de LPS-onafhankelijke binding aan LEC werd geleverd door de onveranderde binding van *A. pleuropneumoniae* aan LEC in aanwezigheid van gezuiverd LPS. Mogelijk produceert *A. pleuropneumoniae*

verschillende factoren die aan een specifiek cel type kunnen binden. Het gebruikte *in vitro* infectiemodel met LEC is waardevol in het onderzoek naar factoren die een rol spelen in het infectieproces van *A. pleuropneumoniae*.

Bacteriën kunnen zeer verschillende adhesines produceren. Haarvormige structuren op de oppervlakte van de bacterie genaamd fimbriae of pili zorgen voor binding aan gastheerweefsel in een aantal bacteriesoorten. Onlangs zijn fimbriae ook gevonden in *A. pleuropneumoniae*, zij bleken tot de type IV familie van fimbriae te behoren. Naast een rol in binding aan gastheerweefsel, hebben type IV fimbriae in andere bacteriesoorten verschillende andere functies, bijvoorbeeld de opname van DNA. Omdat de fimbriae mogelijk een rol spelen in *A. pleuropneumoniae* infecties, hebben we genen, die betrokken zijn bij de productie van deze fimbriae, opgespoord en onderzocht (**Hoofdstuk 4**). Het genoom van *A. pleuropneumoniae* bleek vier genen te bevatten die betrokken zijn bij de productie van fimbriae. Eén gen codeert voor de subunit waaruit de fimbriae zijn opgebouwd (ApfA), de andere genen coderen voor eiwitten die betrokken zijn bij de processing en assemblage van de fimbriae. Deze genen worden voorafgegaan door een mogelijke promotor. Een promotor bepaalt, afhankelijk van de omstandigheden, of genen aan of uit staan. Ondanks de aanwezigheid van fimbriae genen in het genoom, konden fimbriae niet aangetoond worden onder verschillende groeicondities van *A. pleuropneumoniae*. Om te bewijzen dat de gevonden genen wel degelijk betrokken zijn bij de productie van fimbriae, hebben we de vier genen geplaatst achter een sterke promotor op een plasmide. Deze promotor zorgde voor het aanschakelen van de fimbriae genen wat resulteerde in haarvormige structuren op de oppervlakte. Dit betekent dat de genen functioneel zijn en dat de eigen fimbriae promotor waarschijnlijk onderhevig is aan regulatie. De aanwezigheid van fimbriae had geen invloed op binding van *A. pleuropneumoniae* aan LEC. De functie van de fimbriae in *A. pleuropneumoniae* is nog niet bekend.

Het subunit ApfA heeft vele eigenschappen die karakteristiek zijn voor type IV subunits, maar verschilt van deze subunits rond de plaats waar het signaal peptide eraf geknipt wordt. Dit signaal peptide moet verwijderd worden voordat het subunit ingebouwd kan worden in fimbriae. Op positie -1 van de knipplek in ApfA zit het aminozuur alanine in plaats van glycine. In andere micro-organismen is gebleken dat dit een blokkade vormt voor het knipproces en dus voor de productie van fimbriae. Echter, in *A. pleuropneumoniae* werden fimbriae waargenomen nadat de vier fimbriae genen door een sterke promotor waren aangeschakeld. Blijkbaar vormt alanine op positie -1 van de knipplek in ApfA geen belemmering voor de productie van fimbriae in *A. pleuropneumoniae*.

Bacteriën zijn in staat zich snel aan te passen aan de veranderende omstandigheden in hun

omgeving. Dit doen ze door genen aan of uit te schakelen met behulp van een bijbehorende promotor. De fimbriae promotor lijkt onder normale groeiomstandigheden uit te staan. Om te bepalen of fimbriae van *A. pleuropneumoniae* belangrijk kunnen zijn voor het infectieproces, hebben we onderzocht onder welke condities de fimbriae promotor aanstaat ofwel fimbriae geproduceerd worden. Daarvoor hebben we gebruik gemaakt van luciferase genen. De eiwitten waarvoor deze genen coderen produceren in de aanwezigheid van een substraat licht. Door de fimbriae promotor voor de luciferase genen te plaatsen, kunnen we heel gemakkelijk de activiteit van die promotor bepalen onder verschillende omstandigheden (**Hoofdstuk 5**). Met dit systeem hebben we ontdekt dat de promotor van de fimbriae genen intact is maar dat deze alleen onder specifieke omstandigheden aanstaat, namelijk wanneer de bacteriën groeien in chemisch gedefinieerd medium. Om een meer natuurlijke situatie na te bootsen, is de activiteit van de promotor ook getest in de aanwezigheid van LEC. Hieruit bleek dat de promotor alleen aan stond in bacteriën die gehecht waren aan epitheel cellen. Blijkbaar is het contact van *A. pleuropneumoniae* met LEC een signaal om de fimbriae promotor aan te schakelen. Om te testen of de fimbriae promotor ook aan staat tijdens infectie, zijn enkele biggen geïnfecteerd met *A. pleuropneumoniae*. Kort na infectie bleek de fimbriae promotor ook aan te staan in bacteriën die aanwezig waren in longweefsel. Fimbriae worden blijkbaar tijdens infectie geproduceerd en zijn daarom mogelijk van belang voor het infectieproces.

Dit onderzoek laat zien dat de interactie van *A. pleuropneumoniae* met zijn gastheer zeer dynamisch is waarbij meerdere factoren betrokken zijn. De productie van meerdere toxinen verhoogt de virulentie van *A. pleuropneumoniae*. De efficiënte hechting van *A. pleuropneumoniae* aan epitheel cellen van de long draagt mogelijk ook bij aan het infectieproces. Deze interactie is onafhankelijk van LPS. Daarnaast wordt de productie van fimbriae aangeschakeld wanneer bacteriën hechten aan longepitheel cellen en tijdens infectie. De fimbriae vormen mogelijk een nieuwe virulentie factor van *A. pleuropneumoniae* en zijn een goede kandidaat om aan bestaande toxine vaccines toe te voegen. Uit de literatuur blijkt dat soortgelijke fimbriae van andere organismen bescherming kunnen geven tegen infecties veroorzaakt door die organismen. Of fimbriae van *A. pleuropneumoniae* ook bescherming bieden tegen *A. pleuropneumoniae* infecties moet nog onderzocht worden.

DANKWOORD

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Bouke

CURRICULUM VITAE

Bouke Boekema is geboren op 26 november 1971 te Eindhoven. In 1990 behaalde hij het VWO-diploma aan het van der Puttlyceum te Eindhoven. In datzelfde jaar begon hij de studie bioprocestechnologie aan de Landbouw Universiteit Wageningen. Na een onderbreking van een jaar waarin hij bestuurslid was bij Jongerenvereniging Unitas, deed hij een afstudeervak Industriële Microbiologie bij Dr. Frank Verhagen. Tijdens dit afstudeervak werd de productie van organohalogenen door schimmels bestudeerd. Dit afstudeervak werd gevolgd door een afstudeervak Moleculaire Biologie onder leiding van Ir. René Geurts, waar getracht werd plantgenen, die betrokken zijn bij de vorming van wortelknollen, op te sporen. Vervolgens deed hij een stage bij Introgene in Rijswijk onder leiding van Dr. Victor van Beusechem. Daar deed hij onderzoek naar de mogelijkheden om de gastheerspecificiteit van een virus te veranderen. In 1997 behaalde hij het diploma bioprocestechnologie in de cellulaire/ moleculaire oriëntatie.

In 1998 begon hij als Assistent in Opleiding aan de faculteit Diergeneeskunde van de Universiteit Utrecht aan het in dit proefschrift beschreven onderzoek. Dit onderzoek werd uitgevoerd bij de afdeling bacteriologie van het Instituut voor Dierhouderij en Diergezondheid (ID-Lelystad) in Lelystad. Per juni 2003 is hij aangesteld als post-doc bij de faculteit Biologie van de Universiteit Utrecht onder leiding van Prof. Dr. Jan Tommassen.