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REVIEW ARTICLE

Adhesion mechanisms of *Actinobacillus pleuropneumoniae* to the porcine respiratory system and biofilm formation

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ABSTRACT. *Actinobacillus pleuropneumoniae* is a Gram-negative bacterium and the causative agent of porcine pleuropneumonia, a highly contagious disease of pigs characterised by fibrinohaemorrhagic necrotising pneumonia. Although it has been well controlled in some developed countries, outbreaks can occur in pigs of all ages in contact with asymptomatic carriers, leading to significant economic losses to the swine industry due to the high morbidity and mortality rates. Adhesion is a critical step in the colonisation of the swine respiratory tract and the pathogenesis of the porcine pleuropneumonia; however, a literature review of this process is not available to date. Therefore, this review aims to provide information regarding the molecules that have been described in the adhesion of *A. pleuropneumoniae* to cells and tissues of the porcine respiratory tract. Since adhesion is the first step in biofilm formation, we included a section to describe the genes involved in this process; some of these genes could participate directly or indirectly in the adhesion of *A. pleuropneumoniae* to the porcine respiratory system. Although the role of biofilms in porcine pleuropneumonia is still not clear, these molecules could be considered in the future as candidates for vaccine development.

Keywords: *Actinobacillus pleuropneumoniae*, porcine pleuropneumonia, adhesion mechanisms, biofilm formation.

INTRODUCTION

Porcine pleuropneumonia is a highly contagious disease in pigs of worldwide distribution. *Actinobacillus pleuropneumoniae* (AP) is a small encapsulated, gram-negative rod and the etiological agent of this disease, which is characterised by fibrinohaemorrhagic and necrotising pneumonia that often follows a fatal course during acute presentations (Chiers *et al.*, 2010). AP can be found in the nostrils, tonsils and lungs of infected pigs, and it can also be found in asymptomatic carriers previously infected or with a subclinical infection (Sidibé *et al.*, 1993; Chiers *et al.*, 2001). It is also known that pigs with chronic infection have deficient feed conversion and weight gain (Sassu *et al.*, 2018). In addition, research on naturally and experimentally infected animals suggests that the natural course of infection starts with the presence of AP in the upper respiratory tract, progressing all the way from the nasal cavities to the lungs; here the bacterium induces lesions and the production of neutralising antibodies. Also, tonsils seem to act as a reservoir of AP (Chiers *et al.*, 2001). Interestingly, it has been shown that there is no detection of neutralising antibodies in the serum of pigs that were positive for the presence of AP in the nasal cavity and/or tonsils, but negative for the presence of infected lung lesions, indicating a subclinical infection of pigs carrying the bacterium (Chiers *et al.*, 2002). Based on the requirement of nicotinamide adenine dinucleotide (NAD), two biotypes of AP have been described. In addition, there are 19 serotypes of AP based on differences in the antigenic properties of the capsular polysaccharides (Stringer *et al.*, 2021). It is known that there is a predominant serotype in herds endemically infected, however, more than one serotype has been isolated in some herds (Sidibé *et al.*, 1993). In addition, some of the described virulence factors are AP involved in adhesion, nutrient acquisition, induction of lung lesions, evasion of the immune system and persistence in the host (Chiers *et al.*, 2010). Furthermore, the severity of the disease is not only influenced by the bacterium, but also due to intrinsic factors such as the nutritional status and the immune system of the host and extrinsic factors related to environmental stress (Chiers *et al.*, 2010). There are some variations in the virulence among serotypes, and this could be in part attributed to the production of different combinations of Apx exotoxins, which differ among them in their cytotoxic and hemolytic activities (Hernández-Cuellar *et al.*, 2021). In this regard, ApxI expressed in serotypes 1, 5a, 5b, 9, 10, 11, 14, and 16 is highly haemolytic and cytotoxic, ApxII expressed in all serotypes but 10 and 14 are slightly haemolytic and moderately cytotoxic, and ApxIII expressed in serotypes 2, 3, 4, 6, 8, and 15 is non-haemolytic but highly cytotoxic (Sassu *et al.*, 2018). Also, it has been recently described that AP can internalise not only to phagocytic cells but

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to non-phagocytic endothelial cells (Plasencia-Muñoz *et al.*, 2021). However, the first step for the bacteria to colonise the porcine respiratory system is the adhesion to epithelial cells or extracellular matrix components. There is limited information available in the literature related to the adhesion mechanisms of AP even though this event represents the initial step in the establishment of the infection (Jacques & Paradis 1991). Therefore, the next section aims to present the most relevant findings regarding the adhesion molecules in AP described to date.

ADHESION MECHANISMS OF AP TO THE PORCINE RESPIRATORY TRACT

BINDING TO EXTRACELLULAR MATRIX COMPONENTS

Adhesion to porcine respiratory tract mucus was initially evaluated for 17 AP isolates. It was observed that ~70% of the isolates showed affinity to the mucus and this feature was independent of the serotype. It was also found that the presence of a capsule or a high capsular thickness decreased the adherence to the mucus (Bélanger *et al.*, 1992). In a similar study, AP serotype 1 was able to bind *in vitro* to swine-lung collagen in a Ca^{2+}-dependent manner. By using an overlay assay, it was shown that an unknown 60 kDa outer-membrane protein was able to bind to collagen and fibrinogen, but not to fibronectin and laminin (Enriquez-Verdugo *et al.*, 2004). Hammer-Barrera *et al.* (2004) showed that adhesion by AP serotype 1 was higher to swine buccal epithelial cells (BEC) in comparison with their cell counterparts of human or rat origin. Treatment with proteolytic enzymes and periodate highly decreased the adherence to swine BEC, suggesting the participation of cell-surface glycoproteins in the adhesion of AP to these cells. Interaction with fibrinogen or fibronectin resulted in reduced adherence to swine BEC, suggesting also the adhesion of bacteria to these extracellular matrix components (Hamer-Barrera *et al.*, 2004). We suggest that these extracellular matrix surfaces may be helpful for AP, an extracellular bacterium, to attach and progress from the upper to the lower porcine respiratory tract.

ROLE OF LPS AND CAPSULE IN THE ADHESION

Using porcine tracheal rings with ciliated epithelial cells maintained in culture, it was found that isolated AP serotypes 1, 2, 5, and 7 had variation in the adhesion capacity among serotypes or even within isolates of the same serotype. To analyse the role of the capsule on the adhesion, two capsulated isolates and their unencapsulated variants were tested and no differences were found in the adhesion index, suggesting that capsule was not involved in this process. There were differences in the lipopolysaccharide pattern of the bacterial isolates when a whole-cell lysate was subjected to a treatment with proteinase K. Based on this, isolates with a smooth-type lipopolysaccharide (75% of isolates with serotype 2 and 7) adhered in large number to porcine tracheal rings while isolates with a semi rough-type lipopolysaccharide (serotypes 1 and 5) adhered poorly (Bélanger *et al.*, 1990). It is worth mentioning that lipopolysaccharide varies in structure among bacteria but possessed three different regions attached covalently, the lipid A, an oligosaccharide core, and the O-antigen. Lipopolysaccharide with O-antigen is referred to as smooth type, while rough-type lipopolysaccharide does not contain O-antigen (Steimle *et al.*, 2016). Interestingly, purified lipopolysaccharides from homologous AP reference strains inhibited the bacterial adhesion to the porcine tracheal rings. Therefore, lipopolysaccharides were proposed for the first time as molecules important for the adhesion of AP to ciliated epithelial cells of the trachea (Bélanger *et al.*, 1990).

Similar research related to the adhesion capacity of lipopolysaccharides in AP showed by flow cytometry and electron microscopy that these molecules were well exposed at the surface of the encapsulated AP analysed. In addition, immunostaining showed that the lipopolysaccharide extracted from AP serotype 1 and 2 adhered to lung vascular endothelium and tracheal epithelium when incubated with porcine lung or tracheal frozen sections, respectively. To know which part of the lipopolysaccharides had the adhesion capacity, an extract of lipopolysaccharides from AP was obtained and hydrolysed. Through an adhesion-inhibition assay, it was found that the polysaccharide moiety was responsible for the adhesion of AP, while the lipid A was dispensable in this process (Paradis *et al.*, 1994). In addition, it was shown in an experiment trying to simulate the adhesion to cell membranes that AP serotype 1, 5b, and 7 were able to bind to phosphatidylethanolamine (PE) but not to other phospholipids. It was suggested that the lipopolysaccharide O-antigen was responsible for the binding to PE (Jeannotte *et al.*, 2003).

Contrary to the findings showing that lipopolysaccharides play an important role in the adhesion of AP to tracheal and lung epithelial cells, a different workgroup analysed the adhesion of several strains of AP to primary cultures of porcine lung epithelial cells (LEC). It was found that adhesion of AP was faster and up to 30-fold more efficient for LEC than for swine kidney cells. However, adhesion to LEC did not change for a transposon mutant with a modification in the lipid A moiety of the lipopolysaccharide or even resulted in a three-fold more adhesion for a mutant lacking O antigen compared to the parent strains. Furthermore, lipopolysaccharides purified from AP serotype 1, 3, 7, and 8 did not alter the adhesion of AP serotype 8 to LEC (Boekema *et al.*, 2003). These results clearly show that the mechanisms of adhesion for AP could be different depending on the surface of the porcine respiratory system.

In another study, 23 AP isolates were evaluated on their ability to adhere *in vitro* to porcine tracheal epithelial cells or frozen lung sections. Different to the frozen lung sections, adherence to the tracheal epithelial cells was very

poor and, in both cases, there was no correlation of the adherence with the serotype of the AP isolates. However, contrary to the aforementioned study, two unencapsulated variants adhered in greater numbers to the lung sections compared to the capsulated parent strains (Jacques *et al.*, 1991). Similarly, using a transposon mutagenesis system to generate an AP serotype 1 capsule-deficient mutant, it was found that the mutant strain showed more adhesion to porcine tracheal frozen sections than the parent strain. However, it was less virulent in pigs and it did not induce mortality. It was described that the product of mutation was the protein CpxC involved in polysaccharide transport across the cytoplasmic membrane during the biosynthesis of capsular polysaccharides. It was also concluded that the capsule was not important for adherence and may even mask an outer membrane protein important for adhesion (Rioux *et al.*, 2000).

OUTER MEMBRANE PROTEINS INVOLVED IN ADHESION

Adhesion of AP serotypes 2, 5a, 9, and 10 to alveolar epithelial cells showed that optimal adherence was obtained in NAD-restricted medium for strains 5a, 9, and 10. Interestingly, under this condition, it was expressed an outer membrane protein of 55 kDa and the presence of fimbriae was observed by electron microscopy. However, the sequence of the N-terminal of this outer membrane protein did not correspond to any known protein. Bacterial adhesion was significantly reduced when treated with proteolytic enzymes. This finding suggested that besides lipopolysaccharides, proteins are also important for the adhesion of AP. Furthermore, treatment of AP with a combination of pronase and sodium metaperiodate produced a higher inhibition of the adherence to alveolar epithelial cells compared to reagents being used separately. Therefore, glycoproteins could also be involved in the adhesion of AP to these cells (Overbeke *et al.*, 2002).

It was found that 170 genes were differentially expressed in AP attached to St. Jude porcine lung cell line (SJPL) compared with detached bacteria in the medium (planktonic). Two genes called *TadB* and *rcpA*, potentially involved in adhesion and biofilm formation were upregulated. Also, a gene (APL_0443) with high homology to the Hsf autotransporter adhesin of *Haemophilus influenzae* was upregulated (Auger *et al.*, 2009). This Hsf-like autotransporter called Apa1 (AP antigenic protein) was previously found to be expressed by AP in necrotic porcine lung tissue (Baltes *et al.*, 2004). Sequence analysis of the C-terminal region of Apa1 showed a translocator domain and six conserved HsfBD1-like or HsfBD2-like binding domains among different strains of AP. Adhesion to SJPL cell monolayers was tested by confocal microscopy through a GST fusion protein methodology in which GST was bound to the six ApaBD (HsfBD-like) domains. GST-ApaBD3 showed strong fluorescence while the other five domains had only basal fluorescence. It was confirmed the adhesion ability of ApaBD3 to epithelial cells through an adherence inhibition assay with a recombinant *E. coli*-ApaBD3 that expresses the domain on the surface (Xiao *et al.*, 2012). It was shown later an extra N-terminal domain (residues 124-612) of the trimeric autotransporter Apa1 called Adh that was required for adhesion, autoaggregation, and biofilm formation (Wang *et al.*, 2015).

In another study, the outer membrane protein Lip40 was described to mediate adherence of AP to SJPL cells using a mutant strain, Δ*lip40*. Interestingly, the mutant strain had also a reduced ability to invade the lungs of infected mice. Also, in an infection assay with pigs, the mutant strain produced fewer clinical signs (dyspnea, lethargy, and fever) and lung invasion than the wild-type or complemented strain (Liu *et al.*, 2018). These findings suggest a critical role of the adhesion process in the virulence of this bacterium.

GENES INVOLVED IN FIMBRIAE FORMATION

The presence of fimbriae in AP has been previously described (Utrera *et al.*, 1991, Dom *et al.*, 1994, Overbeke *et al.*, 2002) with the identification of ApfA, a 17 kDa type 4 fimbrial subunit protein (Zhang *et al.*, 2000). An operon (*apfABCD*) consisting of four genes involved in type 4 fimbrial biogenesis was also proposed (Stevenson *et al.*, 2003, Boekema *et al.*, 2004). ApfA was found to be highly conserved among the different serotypes of AP. Also, it was suggested as an adhesin since its expression was greatly upregulated upon contact of AP with the SJPL cell line. Adhesion to SJPL cell line and porcine iliac artery endothelial cell line (PIEC cells) decreased significantly for AP $4074^{\Delta apfA}$, a mutant strain deficient in ApfA. Furthermore, recombinant ApfA blocked the adhesion of AP to those cell lines. Interestingly, it was shown that ApfA mediates colonisation of AP to the lungs of infected mice, as the mutant strain AP $4074^{\Delta apfA}$ had reduced bacterial loads in lungs compared with mice infected with the wt AP strain 4074. Also, using a purified recombinant ApfA protein, it was found an elevated humoral immune response and protection against AP in an infection model in mice, proposing this fimbrial subunit as a promising vaccine candidate (Zhou *et al.*, 2013).

Two component systems (TCS) play important roles in adaptation to changes in the environment. Through genomic analysis, it has been described that AP have five pairs of TCS: ArcA/ArcB, CpxR/CpxA, NarP/NarQ, PhoB/PhoR, and QseB/QseC. It was analysed through a microarray the changes in the gene expression profile between a QseB/QseC deficient AP strain and the corresponding parent strain AP 4074. The expression of 44 genes was shown to be different, with 27 of them being up-regulated and 17 down-regulated. The expression levels of some of these genes, such as *PilM* were validated using qRT-PCR. Also, with an electrophoretic mobility shift assay (EMSA), it was shown that a phosphorylated recombinant QseB (rQseB-P) was able to bind to the promoter sequence of

PilM. An AP deficient in the expression of *PilM* showed a significant decrease in the adherence to SJPL cell line and was less virulent in pigs (Liu *et al.*, 2015). It was later found that the *apfABCD* and *PilMNOPQ* gene clusters were operons conserved in all the AP serovars and their products (*apfA*, *apfB*, *apfC*, *apfD*, *pilM*, *pilN*, *pilO*, *pilP*, and *pilQ*) are required for Tfp (a type IV pili) biogenesis, biofilm formation, and adhesion to SJPL cells (Liu *et al.*, 2018).

The presence of the *flp* operon consisting of 14 genes (*flp1-flp2-tadV-rcpCAB-tad-ZABCDEFG*) was described in AP. In reference strains with serotypes 1, 4, 5, 7, 12, and 13, the complete operon was identified. However, the *flp* promoter was absent in serotypes 2, 3, 6, 9, and 11, and for serotypes 10 and 15, the *flp1* gene was truncated resulting in the absence of pilus as observed by transmission electron microscopy. Adherence to SJPL cells resulted to be higher for piliated strains (Li *et al.*, 2012). Later, it was shown that the genes *flp1* and *tadD* were essential for Flp pilus biosynthesis using AP mutants in which biofilm formation and adherence to SJPL and porcine iliac artery endothelial (PIEC) cell lines was reduced. Also, those mutants lacking *flp1* and *tadD* resulted in deficient colonisation with reduced bacterial loads in the lungs of infected mice and pigs (Li *et al.*, 2019).

GLYCOSYLATION SYSTEMS

It was reported in AP the crystal structure of HMW1C, a glycosyltransferase of the GT41 family that was previously described in *Haemophilus influenzae*. HMW1C creates N-glycosidic linkages on HMW1, an adhesin that mediates adherence to respiratory epithelial cells (Kawai *et al.*, 2011). A recent study described the role of the cytoplasmic *N*-linked glycosylation system of AP (NGT) in the adhesion to A549 cells, human adenocarcinoma lung epithelial cells. A putative NGT locus consisting of *rimO* (methylthiotransferase) was proposed and the glycosyltransferases *ngt* and *agt*. Using AP strain HS143 to generate mutants deficient in *agt* and *ngt*, it was shown that the adhesion to cells was almost abrogated for the mutant strains HS143Δagt and HS143Δngt (Cuccui *et al.*, 2017). Table 1 summarises all the molecules that have been described in the adhesion of AP to surfaces related to the porcine respiratory system.

BIOFILM FORMATION

Biofilms are defined as communities of microbes embedded in an extracellular matrix, conferring them protection against environmental stress, host defence, and antibiotics (Hathroubi *et al.*, 2018). Most of the experimental studies in biofilm formation have been on abiotic surfaces such as polystyrene microplates. Although the ability to form biofilms has been associated with the virulence of AP, it is still not clear how this process contributes *in vivo* to the pathogenesis of the porcine pleuropneumoniae (Hathroubi *et al.*, 2018). In this respect, the presence of AP aggregates in the lungs of pigs naturally infected has been reported (Tremblay *et al.*, 2017). In addition, it was described the ability of AP to form biofilms on a biotic surface, using a monolayer culture of SJPL cells in which the bacterium formed biofilms at later times (~24h) in comparison with the highest biofilm formation in microplates at 4h. This biofilm formation was associated with an increase in the adhesion number of bacteria to the cells, and PNAG (a polymer of N-acetyl-D-glucosamine residues in beta (1,6) linkage) was shown to be an important component necessary for biofilm formation (Tremblay *et al.*, 2013). In addition, it was shown that medium replenishment was important to increase the biofilm biomass and delay bacterial dispersion. Using a drip flow system with constant nutrient supplementation, it was found that AP forms larger and more stable biofilms. In case of biofilm formation in microplates under static conditions, genes involved in energy metabolism were downregulated while genes involved in transport were upregulated in biofilm cells compared with planktonic cells, suggesting the need for an active nutrient supplementation of AP in biofilms. Also, it seems to be that the dispersion of AP in biofilms after 4h is driven by stress-related genes while at a growing phase, the bacterium expressed genes involved in transport and energy metabolism. For bacteria in biofilms coming from the drip flow system, genes involved in protein synthesis were upregulated in comparison with effluent bacteria (Tremblay *et al.*, 2013). In a different approach looking for genes involved in biofilm formation by AP, it was found 16 genes from a transposon library with around 1200 mutants. The genes associated with an increase in the biofilm formation were of unknown function while those associated with a deficient biofilm formation encoded proteins involved in transport, protein and nucleic acid synthesis (Grasteau *et al.*, 2011). Interestingly, it was found that sub-minimum inhibitory concentrations of penicillin G, an antibiotic used to control AP outbreaks, induced biofilm formation on polystyrene microplates in 9 out of 13 AP field isolates. These biofilms contained more PNAG, extracellular DNA and proteins compared with the control biofilms. Also, the expression of *pgaA* and genes of the envelope-stress two-component system CpxRA were up-regulated in AP under the presence of sub-minimum inhibitory concentrations of penicillin G, suggesting that the stress induced by the antibiotic on the cell wall of AP is associated with increased production of PNAG and the biofilm formation (Hathroubi *et al.*, 2015).

On the other hand, we have described in this review the adherence of AP to cells and tissues of the porcine respiratory system; however, adhesion is also the first step in biofilm formation. In this regard, fimbriae assembly in AP through the operons apfABCD, pilMNOPQ, and flp were required for cell adhesion, biofilm formation, and to confer virulence *in vivo* (Liu *et al.*, 2018; Li *et al.*, 2019).

Table 1. Adhesion mechanisms of *Actinobacillus pleuropneumoniae* to the porcine respiratory system.

Surface of adhesion	Type of experiment	Mechanism of Adhesion	Reference
Porcine respiratory tract mucus	*In vitro*	Presence of capsule or a high capsular thickness decreased the adherence	Bélanger *et al*. 1992
Swine-lung collagen (Type I, III, IV, and V) and fibrinogen	*In vitro*	Ca^{2+}-dependent/ A 60 kDa Outer-membrane protein	Enriquez-Verdugo *et al*. 2004
Swine buccal epithelial cells (BEC), fibronectin, and fibrinogen	*In vitro*	Cell-surface glycoproteins as treatment with proteolytic enzymes and periodate highly decreased the adherence to swine BEC	Hamer-Barrera *et al*. 2004
Porcine tracheal rings	*In vitro*	LPS (smooth type, O Antigen)	Bélanger *et al*. 1990
Lung vascular endothelium and tracheal epithelium	*In vitro*	LPS (Polysaccharide moiety)	Paradis *et al*. 1994
Phosphatidylethanolamine	*In vitro*	LPS (O antigen)	Jeannotte *et al*. 2003
Porcine lung epithelial cells	*In vitro*	LPS-independent	Boekema *et al*. 2003
Frozen lung sections	*In vitro*	Noncapsulated strains showed higher adhesion	Jacques *et al*. 1991
Porcine tracheal frozen sections	*In vitro/In vivo*	A capsule-deficient mutant showed higher adhesion	Rioux *et al*. 2000
Alveolar epithelial cells	*In vitro*	Fimbriae and a 55 kDa outer-membrane protein expressed in NAD-restricted medium	Overbeke *et al*. 2002
Not tested	*Microarray/ Transcriptomic profile*	*TadB*, *rcpA and Apa1* genes upregulated	Auger *et al*. 2009
St. Jude porcine lung cell line	*In vitro*	Apa1	Xiao *et al*. 2012, Wang *et al*. 2015
St. Jude porcine lung cell line	*In vitro/In vivo*	Lip40	Liu *et al*. 2018
Epithelium of alveolar cells and cilia of the terminal bronchioli epithelia	*In vitro*	Not tested but presence of fimbriae in AP was described	Dom *et al*. 1994
Not tested	*In silico/sequence analysis*	Fimbriae, Identification of ApfA protein by purification and amino acid sequence analysis	Zhang *et al*. 2000
PK-15 cells and St. Jude porcine lung cell line	*In vitro*	Operon *apfABCD* and *PilMNOPQ,* important for adhesion and biofilm formation	Liu *et al*. 2018
Not tested	*Cloning and In silico/sequence analysis*	ApfA and Operon (*apfABCD*) consisting of four genes involved in type 4 fimbrial biogenesis	Boekema *et al*. 2004, Stevenson *et al*. 2003
St. Jude porcine lung cell line and porcine iliac artery endothelial cell line	*In vitro/In vivo*	ApfA	Zhou *et al.* 2013
St. Jude porcine lung cell line	*In vitro/In vivo*	PilM	Liu *et al*. 2015
St. Jude porcine lung cell line	*In vitro*	Fimbriae, flp Operon consisting of 14 genes (flp1-flp2-tadV-rcpCAB-tadZABCDEFG)	Li *et al*. 2012, Li *et al*. 2019
Not tested	*In silico/sequence analysis and crystal structure*	ApHMW1C, a glycosyltransferase	
A549 cells, human adenocarcinoma lung epithelial cells	*In vitro* and *In silico*/ sequence analysis	*Agt* and *Ngt*, glycosyltransferases	Cuccui *et al*. 2017

Also, O-antigen, a key component of lipopolysaccharides was not only important for cell adhesion but also to form biofilms (Hathroubi *et al.*, 2015). Finally, the trimeric autotransporter adhesin Apa1 participated in cell adhesion and biofilm formation (Wang *et al.*, 2015). Table 2 summarises more genes involved in biofilm formation for AP and their biological role; however, for most of those genes, it is not known whether they could be involved directly or indirectly in the process of adhesion of AP to cells and tissues of the porcine respiratory system. It is worth mentioning that many of the genes described in table 2 belong to stress-responding genes whose products may be important in the adaptation of AP to different environmental changes, for example, the two component systems (TCS) proteins CpxA, CpxR, and ArcA (Li *et al.*, 2018, Buettner et al 2008), the quorum sensing LuxS/

Table 2. Genes involved in biofilm formation by *Actinobacillus pleuropneumoniae*.

Gene	Function	Consequence of the mutation in AP	Reference
apf and *pil* operon genes	Formation of fimbriae (Type IV pilus, Tfp)	Deficient biofilm formation	Liu *et al*. 2018
flp1 and *tadD* (flp operon genes)	Formation of fimbriae (Type IVb pilus)	Deficient biofilm formation and attenuated virulence in mice and pigs.	Li *et al*. 2019
ArcA	Two-component systems (Metabolic adaptation to anaerobiocity)	Deficient biofilm formation. Attenuated virulence in the acute infection of pigs.	Buettner *et al*. 2008
CpxA/CpxR	Two-component systems	Deficient biofilm formation. Decreased expression of *RpoE* and *pgaC*. Attenuated virulence in mice.	Li *et al*. 2018
LuxS	Quorum sensing through AI-2 signaling molecule	Increased biofilm formation. Attenuated virulen in a mouse model/ Biofilm formation genes *pgaABC* were upregulated in early exponential phase. Some genes involved in adhesion were repressed at late exponential phase such as *apfABC* genes.	Li *et al*. 2008
hfq gene	RNA chaperone and posttranscriptional regulator, Hfq	Deficient biofilm formation, decreased level of *pgaC* transcript and PNAG content. Attenuated virulence in pigs.	Subashchandrabose *et al*. 2013
relA	relA-depedent (p)ppGpp-mediated stringent response, activated in nutritional starvation	Increased biofilm formation	Li *et al*. 2015
r*seA (mclA)* and *hns*	HN-S is a gene regulator and RseA is a negative regulator of the extracytoplasmic stress response sigma factor RpoE/σE	Increased biofilm formation. Attenuated virulence in mice/ Regulate the expression of the pgaABC operon.	Bossé *et al*. 2010, Dalai *et al*. 2009
TolC	An outer membrane channel, component of multidrug efflux pumps and type I secretion systems	Deficient initial adherence and biofilm formation. Decreased *pgaA* and *cpxR* expression. Decreased PNAG content in biofilm.	Li *et al*. 2016
TolC1, a TolC-like protein	An outer membrane channel, component of multidrug efflux pumps and type I secretion systems	Deficient biofilm formation and increased drug sensitivity	Li *et al*. 2016
VacJ	Outer membrane lipoprotein	Deficient biofilm formation	Xie *et al*. 2016b
Apa1 (Adh domain)	Trimeric Autotransporter serine protein	Deficient biofilm formation and adhesion. Attenuated virulence in piglets.	Wang *et al*. 2015
Apa1/Apa2	Trimeric Autotransporter serine protein	Deficient biofilm formation and adhesion to RAW246.7 macrophages. Attenuated virulence in mice.	Xiao *et al*. 2012
Aasp	An autotransporter serine protease	Deficient in biofilm formation	Tegetmeyer *et al.* 2009
Lon A	ATP-dependent protease (degradation of abnormal proteins in bacteria/stress tolerance)	Deficient biofilm formation. Attenuated virulence in mice.	Xie *et al*. 2016
ClpP	ClpP, the catalytic core of the Clp proteolytic complex. Stress tolerance.	Deficient in biofilm formation	Xie *et al*. 2013
pgaABC operon	PNAG or PGA (Polymer of N-acetyl-D-glucosamine residues in beta (1,6) linkage). Intercellular adhesion and attachment of cells to abiotic surfaces	Deficient biofilm formation	Izano *et al*. 2007

AI-2 system (Li *et al.*, 2008), the stress tolerance proteases LonA and ClpP (Xie *et al.*, 2013, Xie *et al.*, 2016a), the multidrug efflux channel protein TolC (Lie *et al.*, 2016), the RNA chaperone and posttranscriptional regulator Hfq (Subashchandrabose *et al* 2013), rseA, a regulator of sigma E (Bossé *et al.*, 2010), and RelA, an enzyme that participates in the (p)ppGpp-mediated stringent response (Li *et al.*, 2015). PNAG was shown to be an important component of the AP biofilm matrix, and AP strains able to form biofilms failed in this process when treated with

dispersin B, a PNAG-hydrolysing enzyme (Izano *et al.*, 2007). Dispersin B is an enzyme that induces the release of adherent cells from mature biofilms through catalysing the hydrolysis of linear polymers of N-acetyl-D-glucosamines (Kaplan *et al.*, 2004). Interestingly, the *pgaABC* operon genes encoding for PNAG were regulated by the TCS CpxA/CpxR (Li *et al* 2018), TolC (Li *et al.*, 2016), Hfq (Subashchandrabose *et al.*, 2013), LuxS (Li *et al.*, 2008), rseA, and the histone-like nucleoid structuring protein H-NS (Bossé *et al.*, 2010). Although the mutation of most of these genes in AP resulted in a deficient *in vitro* biofilm formation and an *in vivo* attenuated virulence, for *LuxS* and H-NS, however, it was an increased biofilm formation with attenuated virulence (Bossé *et al.*, 2010, Li *et al.*, 2008). These differences challenge the notion of the association of the *in vitro* biofilm formation on abiotic surfaces with the pathogenicity in an infection model. It is worth mentioning that some of these differences may be due to the animal model used, the experimental infection route, and the role of the genes in the pathogenicity independently of the contribution to form biofilms. Figure 1 describes the molecules involved in biofilm formation and/or adhesion to the porcine respiratory system, as well as the regulation of the *pgaABCD* operon.

CONCLUDING REMARKS

In this review, we have described the adhesion features of AP to the porcine extracellular matrix components such as mucus, collagen, fibronectin, and fibrinogen. These surfaces may be the initial contact of AP (an extracellular bacterium) to progress throughout the porcine respiratory system till reaching the lungs. Lipopolysaccharides were also initially proposed as molecules involved in adhesion to cells of the upper porcine respiratory tract. However, as we mentioned before, these molecules were dispensable for adhesion in a different cell model. We suggest that this difference could be explained not only because of a different model but the former protocols of lipopolysaccharides extraction that were contaminated with proteins and other cellular products. We consider that new studies with purified lipopolysaccharides or mutant strains deficient in the synthesis of these molecules will be helpful to analyse their exact role in the adherence of AP. Interestingly, unencapsulated AP strains and capsule-deficient mutants showed higher adhesion capacity to lung and tracheal epithelial tissue, thus, the capsule is not only dispensable in cell adhesion, but it seems that it masks other molecules important for adhesion such as

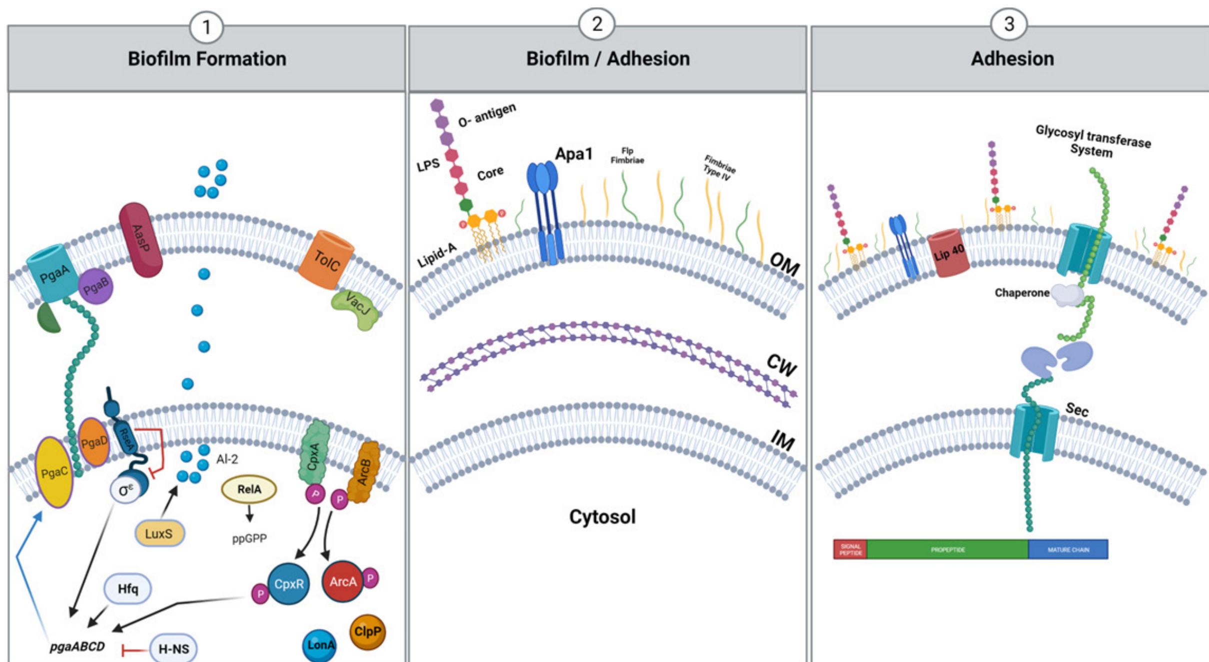

Figure 1. Molecules involved in biofilm formation and/or adhesion to the porcine respiratory system by Actinobacillus pleuropneumoniae. PNAG (Polymer of N-acetyl-D-glucosamine residues in beta (1,6) linkage) is a key component of biofilms. pgaABCD operon is necessary for PNAG synthesis and its expression is regulated by the chaperone Hfq, the global gene regulator H-NS, the two-component system (TCS) CpxA/CpxR, and RseA (the repressor of RpoE/σE). Other molecules involved in biofilm formation are the proteases ClpP, LonA, and Aasp, the outer membrane proteins VacJ and TolC, the relA hydrolase, the TCS ArcA, and the LuxS/Al-2 quorum-sensing system. The lipoprotein Lip40 and the glycosyltransferase system NGT participate in the adhesion to porcine respiratory cells. It is suggested that HMW1C, another glycosyltransferase may be involved in this process. Flp and type IV fimbriae, the trimeric autotransporter Apa1, and the lipopolysaccharide (o-antigen) were shown to participate in biofilm formation and adhesion to the porcine respiratory epithelial cells. OM, outer membrane; IM, inner membrane; CW, cell wall. This figure was created by BioRender software (https://www.biorender.com).

outer membrane proteins. However, how these molecules are exposed in AP with an intact capsule is not clear. In this case, the adhesion of AP must be through the type IV pilus and Flp pilus. It is worth mentioning that most of the studies were performed *in vitro*. Furthermore, most of the research related to the adhesion of AP employed the SJPL cell line which was mistakenly classified as being of simian instead of pig origin, as previously thought. Therefore, a more appropriate cell line model must be considered in the future. On the other hand, biofilm formation, a sessile mode of growth, has been associated with the virulence of bacteria. Although it is still not clear the role of biofilms during infection by AP, it was shown that AP can grow as aggregates on porcine respiratory tissue and biotic surfaces such as cell monolayers. We have presented information related to the genes involved in biofilm formation by AP at different stages, under different conditions, and comparing with the planktonic bacteria. For those genes, many of them with unknown functions, it is not known whether they are involved in adhesion to the porcine respiratory tract or in a different biological process affecting biofilm formation. Finally, most of the commercially available vaccines for porcine pleuropneumonia are based on the use of whole-cell bacterins (first-generation vaccines), an attenuated form of a specific AP serovar or a combination of serovars. One of the limitations of these vaccines is a partial protection against heterologous serovars and the lack of important virulence factors produced in live bacteria such as the Apx toxins. Although lipopolysaccharides of AP are known to participate in the adhesion to the porcine respiratory system, the potential as antigens to generate vaccines was not as expected due to the high heterogeneity of LPS among serotypes and the same was true for capsular polysaccharides. Because of this, it seems to be that the most promising antigens to generate vaccines in AP are outer membrane proteins and lipoproteins with conserved sequences among serotypes. In this regard, this review may be helpful to find conserved molecules with antigenic properties involved directly or indirectly in the adhesion of AP to the porcine respiratory tract or in biofilm formation to develop new vaccines that may confer protection against porcine pleuropneumonia.

CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest.

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