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To cite this version:


HAL Id: pasteur-02549248
https://pasteur.hal.science/pasteur-02549248

Submitted on 24 Apr 2020
Actinobacillus pleuropneumoniae biofilms: Role in pathogenicity and potential impact for vaccination development.

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Running title: A. pleuropneumoniae biofilm, reviewing a decade current research
ABSTRACT.

Actinobacillus pleuropneumoniae is a Gram-negative bacterium that belongs to the family Pasteurellaceae. It is the causative agent of porcine pleuropneumonia, a highly contagious respiratory disease that is responsible for major economic losses in the global pork industry. The disease may present itself as a chronic or an acute infection characterized by severe pathology including hemorrhage, fibrinous and necrotic lung lesions, and, in the worst cases, rapid death. A. pleuropneumoniae is transmitted via aerosol route, direct contact with infected pigs, and by the farm environment. Many virulence factors associated with this bacterium are well characterized. However, much less is known about the role of biofilm, a sessile mode of growth, that may have a critical impact on A. pleuropneumoniae pathogenicity. Here we review the current knowledge on A. pleuropneumoniae biofilm, factors associated with biofilm formation and dispersion, and the impact of biofilm on the pathogenesis A. pleuropneumoniae. We also provide an overview of current vaccination strategies against A. pleuropneumoniae and consider the possible role of biofilms vaccines for controlling the disease.

Keywords: Actinobacillus pleuropneumoniae, pleuropneumonia, biofilm, antimicrobial therapy and vaccine.
INTRODUCTION.

Respiratory diseases in pigs are common global problems for modern pork producers and are frequently associated with the porcine respiratory disease complex (PRDC) (Opriessnig et al., 2011). PRDC is a multifactorial syndrome caused by the interaction of bacteria, viruses and stresses associated with management practices, environmental conditions and genetic predispositions (Opriessnig et al., 2011; Schmidt et al., 2016). Within PRDC, *Actinobacillus pleuropneumoniae* is one of the most commonly identified bacterial pathogen that causes respiratory infections in pigs (Opriessnig et al., 2011; Dayao et al., 2016). *A. pleuropneumoniae* is a Gram-negative rod-shaped bacterium belonging to the *Pasteurellaceae* family (Chiers et al., 2010; Gómez-Laguna et al., 2014) and is the etiologic agent of porcine pleuropneumonia (Frey, 1995; Buettner et al., 2011). This respiratory infection is the major cause of morbidity and mortality, and is responsible for substantial economic losses worldwide (Chiers et al., 2010; Bossé et al., 2014). The disease is characterized by an exudative, fibrinous, hemorrhagic, and necrotizing pneumonia and associated pleuritis (Chen et al., 2011). Porcine pleuropneumonia is transmitted via aerosols or direct contact with infected animals including asymptomatic carriers (i.e. animals with a sub-clinical infection). Clinical infections may result into a chronic and persistent form, an acute form with the pathology described above or a peracute form associated with severe pathology and rapid death (Gottschalk, 2015).

In 1964, Shope was the first to described a disease affecting pigs in Argentina as porcine contagious pleuropneumonia (PCP) and he named the causative agent *Haemophilus pleuropneumoniae* (Shope, 1964; Shope et al., 1964). In 1983, Pohl and coworkers transferred the causative agents of PCP or similar infections to the genus *Actinobacillus*.
based on the higher DNA-sequence homology to the genus *Actinobacillus* (*Actinobacillus lignieresii*, 72-75%) (Pohl *et al*., 1983; Nicolet, 1988). In 1986, O'Reilly and Niven identified the pyridine nucleotides, the precursors that were needed to satisfied the V-factor requirement, and the nicotinamide adenine dinucleotide (NAD) was identified as a supplement that supported *in vitro* growth (O'Reilly and Niven, 1986). *A. pleuropneumoniae* is now divided into two biovars based of their NAD requirement for growth: biotype 1 is NAD-dependent, and biotype 2 is NAD-independent (Turni *et al*., 2014; Gottschalk, 2015; Ito, 2015).

*A. pleuropneumoniae* is further divided into 16 serotypes (or serovars) based on the antigenic properties of the capsular polysaccharides and the O-chain of the lipopolysaccharides (LPS) (Sárközi *et al*., 2015; Kim *et al*., 2016; Bossé *et al*., 2017). Serotypes 1 to 12 and 15 typically belong to biotype 1 whereas serotypes 13 and 14 are typically biotype 2 (Serrano *et al*., 2008; Gottschalk, 2015). The serotype 16 is not yet officially grouped in any biotype. However, this is not an absolute rule since variants of serotype 2, 4, 7, 9 and 11 have been identified as NAD-independent (biotype 2) (Perry *et al*., 2012). Furthermore, there has been an increase in the prevalence of isolates that are untypable (UT) (Morioka *et al*., 2016). Despite the global distribution of *A. pleuropneumoniae*, the prevalence of different serotypes varies between countries (Morioka *et al*., 2015). Specifically, serotypes 1, 5 and 7 are predominantly found in North America, serotype 2 is the most common type in Europe and serotypes 1, 3, 4, 5 and 7 are typically isolated in China (Buettner *et al*., 2011; Gottschalk and Lacouture; 2015; Morioka *et al*., 2016). For South America, serotypes 4, 6 and 7 are reported as the dominant serotypes in the region (Gómez-Laguna *et al*., 2014).
Infection and persistence of *A. pleuropneumoniae* is mediated by multiple virulence factors. Well characterized virulence factors of *A. pleuropneumoniae* include: the Apx toxins (ApxI, ApxII, ApxIII and ApxIV), lipopolysaccharide (LPS), capsule polysaccharide (CPS), proteases (e.g. LonA), urease, iron acquisition systems (e.g. transferrin-binding protein [Tbp], haemoglobin-binding protein [HbpA]), enzymes involved in anaerobic respiration (e.g. two-component signal transduction system [TCSTS] arcB and arcA), type IV pilus, Flp pilus, autotransporters (e.g. Trimeric Autotransporter Adhesin [TAA]), and more recently biofilms (Chiers *et al*., 2010; Tremblay *et al*., 2017). The role of biofilm in persistence, survival and pathogenesis of *A. pleuropneumoniae* is relatively new and the importance of biofilm is not fully understood. It has now been demonstrated that biofilms can develop during an infection and a recent report describes the growth of *A. pleuropneumoniae* as aggregates in lungs obtained from natural pig infections (Tremblay *et al*., 2017). In this review, our aim is to highlight and summarize the current knowledge on *A. pleuropneumoniae* biofilm formation and suggest its possible role in pathogenesis. Furthermore, we will also talk about vaccination and new strategies based on recent biofilm findings.

**BIOFILMS AND ANIMAL HEALTH**

It is well accepted by the scientific community that most bacteria can produce biofilms in their natural ecosystem as well as in artificial *in vitro* ecosystems (Briandet *et al*. 2012). Biofilms are defined as structured communities enclosed in a self-produced matrix that is attached to a surface (biotic or abiotic); however, recent evidences have demonstrated that *in vivo* biofilms and bacterial aggregates are not necessarily attached to the surface and are...
often embedded in host material (Bjarnsholt et al., 2013; Kragh et al., 2016). Our group has extensively reviewed biofilm formation by animal and zoonotic pathogens, and we will not cover general information about biofilm in this review (see Jacques et al., 2010). Several members of the Pasteurellaceae family, which include many important animal pathogens, are able to form biofilms and several studies in the past decade have demonstrated the ability of its members such as Haemophilus influenzae, Pasteurella multocida, Aggregatibacter actinomycetemcomitans, Mannheimia haemolytica Histophilus somni, and Haemophilus parasuis to produce a biofilm (Olson et al., 2002; Kaplan and Velliyagounder, 2004; Jin et al., 2006; Sandal et al., 2007; Wu et al., 2013; Bello-Ortí et al. 2014; Boukahil and Czuprynski, 2015). For several members of the Pasteurellaceae family, it has been suggested that biofilm formation is crucial for the persistence of these obligate inhabitants (Jin et al., 2006; Sandal et al., 2007; Bello-Ortí et al. 2014; Boukahil and Czuprynski, 2015). For example, non-virulent isolates of H. parasuis formed stronger and more robust biofilms than virulent isolates, suggesting that the biofilm phase favors colonization and the planktonic phase allows for the dissemination within the host (Jin et al., 2006; Bello-Ortí et al. 2014).

**Actinobacillus pleuropneumoniae Biofilms.**

The ability of A. pleuropneumoniae to form biofilms in vitro was first studied using a 96-well microtiter plate model (Coffey and Anderson, 2014) (Fig. 1). Kaplan et al. (2004) were the first to report that serotype 5b and 11 are producers of biofilms in vitro (Kaplan et al., 2004). A. pleuropneumoniae biofilms have also been assessed in glass tubes and under agitation. Biofilms form a ring at the air-liquid interface in this closed system model that incorporates shear force (Kaplan and Mulks, 2005). The ability to form biofilms appears to
be common among *A. pleuropneumoniae* isolates since studies demonstrate that isolates from every serotype are able to produce biofilms in microtiter plates and/or glass tubes (Kaplan and Mulks, 2005; Labrie et al., 2010). In the case of the newly reported serotype 16, the ability to form biofilms has yet to be studied.

**Biofilm formation in microtiter plates**

In general, the production of biofilm by *A. pleuropneumoniae* in microtiter plates is described as a rapid process with the detection of biomass as early as 3 hours for serotype 1 type strain S4074 and 6 hours for serotype 5b type strain L20 and clinical isolates (Labrie et al., 2010; Tremblay et al., 2013a). Interestingly, the biofilm cycle of serotype 1 type strain S4074 is completed within 8 hours. Specifically, biomass becomes detectable after 3 hours and reaches its peak at 5 hours, which correspond to the mature form of the biofilm (Tremblay et al., 2013a). Dispersion of the biofilm begins between 5-6 hours and the biomass is no longer detectable after 8 hours (Tremblay et al., 2013) (Fig. 2). The biofilm persistence can be extended if the spent medium is removed and fresh culture medium is added to a 4-hour old biofilm (i.e., a maturing biofilm). The change of growth medium can cause an increase in biomass and delay biofilm dispersion by 1 hour. This suggests that depletion of the culture medium or the accumulation of one or several signals molecules can activate biofilm dispersal (Tremblay et al., 2013a). These observations provide a good example for the limitations of closed biofilm systems.

**Biofilm formation in models with biologically relevant parameters**

To overcome the limitations of the microtiter plates, dynamic models are often used and these systems are thought to be more representative of the conditions encountered by
bacteria in their natural environment (Coenye and Nelis, 2010). For example, the “drip flow” reactor is a continuous flow system that continuously irrigates biofilms with fresh medium and allows biofilms to form on a coupon of choice (e.g., glass, stainless steel, PVC) that is deposited inside a sealed chamber (Goeres et al., 2009). In this model, biofilms are formed at the air-liquid interface in the presence of low shear forces that mimic the environment found in the lung and oral cavities (Goeres et al., 2009; Schwartz et al., 2010). Unlike the results obtained with the microtiter plates, *A. pleuropneumoniae* S4074 is able to establish and maintain a biofilm for up to 48 hours (Tremblay et al., 2013a). To grow biofilms under these conditions, the growth medium (Brain Heart Infusion [BHI] with NAD) is diluted to 50% and the flow can be set from 50 mL to 200 mL per hour per chamber (Tremblay et al., 2013a; Hathroubi et al., 2016a). After 24 hours, *A. pleuropneumoniae* forms an important biomass on a glass slide that is visible with the naked eye (Fig. 2). This biofilm contains 10⁹-10¹⁰ colonies forming units (CFU) per chamber with an average dry weight of 10 mg (Tremblay et al., 2013a; Hathroubi et al., 2016a). Although the “drip-flow” reactor provides a dynamic environment that resembles the lung cavity, the surface used was a microscopic slide, a substrate that *A. pleuropneumoniae* would never encounter *in vivo*.

In order to see if a biotic surface could be used by *A. pleuropneumoniae*, Tremblay and colleagues (2013b) investigated biofilm formation on a SJPL cell line by a non-hemolytic, non-cytotoxic mutant of strain S4074, called MBHPP147. This mutant has deletions in both the *apxIC* and *apxIIC* genes which prevents the acylation (and hence activation) of the protoxins ApxIA and ApxIIA. As observed with strain S4074, MBHPP147 is able to form a biofilm on polystyrene in microtiter plates. Furthermore, a robust biofilm is observed after
24 and 48 hours of contact with the SJPL cells (Tremblay et al., 2013b). These studies are consistent with the notion that *A. pleuropneumoniae* can form biofilms on biotic surfaces during host colonization.

Recently, *A. pleuropneumoniae* biofilm formation was studied using an embedded model created with 0.5% agarose. This porous substrate is thought to simulate the conditions found in the lungs during a natural infection (Tremblay et al., 2017). Biofilm formation in this model was tested with two clinical isolates of *A. pleuropneumoniae* (one serotype 5, and one serotype 7) that were previously shown to form biofilms in a 96-wells plates and aggregates in the lungs of naturally infected pigs. In the embedded models, both isolates developed aggregates ranging from 20-30 microns within the porous matrix formed by the agarose. The size of the aggregates (30-45 microns) and their structure were similar to those observed in the lungs of pigs naturally infected by either isolates (Tremblay et al., 2017). The use of this new model that mimic the pulmonary alveolus environment during an infection has a promising future and could provide a new platform to test the sensitivity of *A. pleuropneumoniae* biofilm to several antibiotics.

**Factors involved in the formation and dispersion of *A. pleuropneumoniae* biofilms.**

Several strategies have been used to identify genetic factors associated with biofilm formation. For example, a library of mini-Tn10 transposon mutants in *A. pleuropneumoniae* S4074 was screened in a 96-wells microplate assay and 16 genes affecting biofilm formation were identified (Grasteau et al., 2011). Otherwise, microarrays have also been used to gain insight into the transcriptome of maturing or dispersing biofilms formed under static or dynamic conditions (Tremblay et al., 2013a). These approaches provide different
insight into the biofilm formation process. The results are summarized in the sections below.

1) Composition of the biofilm matrix

Poly-N-acetyl-glucosamine (PGA) is the major component and an essential element of the A. pleuropneumoniae biofilm matrix regardless of the growth conditions and surfaces used (Fig. 1) (Izano et al., 2007; Bossé et al., 2010; Labrie et al., 2010; Tremblay et al., 2013a; Tremblay et al., 2013b; Hathroubi et al., 2015; Hathroubi et al., 2016a). The proteins responsible for PGA synthesis are encoded by the pgaABCD operon (Kaplan et al., 2004; Izano et al., 2007). This operon is highly prevalent among A. pleuropneumoniae serotypes and appears to have been preserved in every studied serotype (Izano et al., 2007). In studies by Izano et al. (2007), PCR analysis of the gene coding for the biosynthesis of PGA, pgaC, demonstrated that it was present in every reference strains investigated (serotypes 1 to 12) and in 76 of the 77 field isolates tested. The synthesis of PGA is essential for the biofilm formation process and deleting one gene in the operon, pgaC, completely abolishes the production of PGA and, thus, prevents biofilm formation (Izano et al., 2007; Bossé et al., 2010; Hathroubi et al., 2016a).

A. pleuropneumoniae can also control the degradation of the self-produced PGA polymers using a glycoside hydrolase, dispersin B (Izano et al., 2007). This enzyme can detach biofilms formed on difference surfaces, under different conditions and in different model systems (Izano et al., 2007; Labrie et al., 2010; Tremblay et al., 2013a; Tremblay et al., 2013b; Hathroubi et al., 2015; Hathroubi et al., 2016a).
Other components, such as extracellular DNA (eDNA) and proteins, may also provide building blocks for the matrix. Proteins and eDNA have been stained and observed by confocal microscopy in the biofilm formed by *A. pleuropneumoniae* (Wu *et al.*, 2013; Hathroubi *et al.*, 2016a). Under most conditions tested, these components do not appear to be required for the integrity of the biofilm matrix since proteinase K or DNase does not disperse pre-established biofilms (Grasteau *et al.*, 2011; Hathroubi *et al.*, 2016a). However, eDNA might contribute to the integrity of the biofilm under certain conditions such as in the presence of sub-minimal inhibitory concentration of penicillin B or in multi-species biofilms (Hathroubi *et al.*, 2016b; Loera-Muro *et al.*, 2016).

2) Growth medium and other conditions inducing biofilm formation

The composition of the culture medium affects *A. pleuropneumoniae* biofilm formation. For example, Li and collaborators in 2008 demonstrated that the reference strain S4074 only produced a biofilm in TSB (Tryptic Soy Broth) medium in the absence of serum although the mechanism of this inhibition remains to be determined (Li *et al.*, 2008). Later, Labrie *et al.* (2010) demonstrated that BHI medium favored biofilm formation of *A. pleuropneumoniae* S4074 when compared to TSB. Further, 54% serotypes 1, 5, 7 and 15 strains produced biofilms in BHI reinforcing the idea that BHI would better for the study of biofilms *in vitro*. However, the source of the BHI medium also has an impact on biofilm formation. For example, BHI from Oxoid enhanced the production of a robust biofilms whereas BHI from Difco does not promote biofilm formation (Labrie *et al.*, 2010).

When the composition of both media was analyzed the concentration of zinc was identified as a key difference with higher levels in BHI-Difco than BHI-Oxoid (Labrie *et al.*, 2010).
In support of these observations, researchers have shown that the addition of zinc to BHI-Oxoid inhibits biofilm formation in a dose-dependent manner without affecting bacterial growth (Labrie et al., 2010; Wu et al., 2013). Thus, zinc appears to specifically inhibit the production of biofilm by *A. pleuropneumoniae*. A similar inhibitory effect has also been observed for other porcine pathogens such as *Escherichia coli*, *Salmonella Typhimurium*, *Staphylococcus aureus* and *Streptococcus suis* (Wu et al., 2013). In *A. pleuropneumoniae*, the presence of zinc might interfere with the expression or biosynthesis of the major polymer found in the biofilm matrix, PGA, since the expression of the *pgaABCD* operon is up-regulated in BHI-Oxoid (Labrie et al., 2010) and zinc inhibits the activity of PgaB in *E. coli* (Little et al., 2012).

In addition to growth medium, anaerobic conditions also appear to induce biofilm formation (Li et al., 2014). Indeed, exposure to anaerobic conditions result in an increase in biofilm formation that is associated with the upregulation of the fine tangled pili major subunit gene (*ftpA*) and *pgaA* (Li et al., 2014).

Other growth conditions appear to induce the expression of biofilm-associated genes. For example, direct contact of *A. pleuropneumoniae* with epithelial cells results in an increased expression of the *pgaABCD* operons (Auger et al., 2009). Further, epinephrine and norepinephrine affect expression of *pgaB* and Apa1, an auto-transporter adhesin (Li et al., 2012). However, only norepinephrine induces enhance attachment to SJPL cells and neither catecholamine has an impact on biofilm formation (Li et al., 2012). It is conceivable that different factors play a role during the attachment of *A. pleuropneumoniae* to a biotic surface (e.g. SJPL cells) and an abiotic surface (e.g. polystyrene or glass). In support of this
statement, *A. pleuropneumoniae* does not form a biofilm on polystyrene when grown in a cell culture medium (Dulbecco’s modified Eagle’s medium [DMEM]) and was only able to form biofilm in the presence of SJPL in DMEM (Tremblay *et al.*, 2013b).

3) *The biofilm transcriptome*

The transcriptomes of maturing (static 4h), mature (drip-flow) and dispersing (static 6h) biofilms have been analysed and compared to each other and to their planktonic counterparts. In a study by Tremblay *et al.* (2013a), only 47 and 117 genes were differentially up or down-regulated in static biofilms when compared to planktonic cells. For example, biofilm bacteria down-regulated the expression of their energy metabolism gene when compared to planktonic bacteria (Tremblay *et al.*, 2013a). Indeed, the majority of energy metabolism genes such as the genes encoding the key enzymes of the anaerobic metabolism appeared to be repressed in the biofilm (Tremblay *et al.*, 2013a).

Major differences have also been observed when the maturing biofilm is compared to a dispersing biofilm. Specifically, 456 genes were differently regulated when a maturing biofilm and a dispersing biofilm were compared (Tremblay *et al.*, 2013a). Furthermore, the maturing biofilm appears to be under an iron-rich condition because several major genes in iron acquisition, including *tbpB*, are repressed in the maturing biofilm (Tremblay *et al.*, 2013a).

Interestingly, a comparative analysis reveals that the transcriptome of drip-flow biofilms share few differentially expressed genes with static biofilms. On the other hand, the drip-
flow transcriptome has several genes that has also been identified in natural or experimental infections of pigs (Tremblay et al., 2013a). Transcriptome and cross-referencing analyses indicate that biofilms formed in a drip-flow models require a different sub-set of genes than biofilms grown in microtiter plates (Tremblay et al., 2013a). Based on these results, it has been suggested that the drip-flow apparatus might provide a more relevant model to study biofilm formation by A. pleuropneumoniae (Tremblay et al., 2013a).

4) Regulators of biofilm formation

While environmental conditions and growth medium composition that are optimal for biofilm formation and induce production of PGA have been identified, other studies have identified potential regulators and molecular mechanism associated with biofilm formation. In addition to growth conditions, the expression of the pgaABCD genes and, consequently, PGA production are regulated by the histone type H-NS (histone-like protein), which acts as a repressor of expression and hence a suppressor of biofilm production (Dalai et al., 2009; Bossé et al., 2010; Grasteau et al., 2011). Tn insertions in the hns gene of A. pleuropneumoniae serotype 1 results in a sharp increase in biofilm formation and a loss of virulence (Dalai et al., 2009). Indeed, H-NS specifically represses the expression of the operon by binding sequences upstream the pgaA gene (Bossé et al., 2010). The importance of hns in repressing biofilm formation has also been independently confirmed in a screen that identified three Tn-mutants with an increase biofilm production (Grasteau et al., 2011). Unlike H-NS, the alternative sigma factor RpoE (or σE) is a transcriptional activator of the pgaABCD operon (Bossé et al., 2010).

Deletion of the gene encoding the negative regulator of the σE factor, RseA (regulator of
sigma-E), results in increased expression of the pgaABDC operon and higher biofilm production (Bossé, et al., 2010). Additionally, expression of the pgaABCD operon is also under the control of the RNA chaperone Hfq (Subashchandrabosea et al., 2013). Disruption of hfq decreases PGA production, biofilm formation, virulence and fitness (Subashchandrabosea et al., 2013).

Deletion of the quorum-sensing (QS) gene also results in an increase in pgaABC expression, a strong increase in biofilm production and a decrease in virulence (Li et al., 2008; Li et al., 2011). S-ribosylhomocisteine lyase (LuxS), is a protein involved in the production of the auto-inducer type 2 (AI-2) and in the QS mechanism. QS is involved in the biofilm formation in many bacteria (Prouty et al., 2002; Merritt et al., 2003; Ethapa et al., 2013). The increase biofilm production in A. pleuropneumoniae appears, however, to be independent of the production of AI-2 since the addition of AI-2 to the culture medium results in an increase biofilm production in the absence of LuxS (Li et al., 2011). Enhanced biofilm formation has also been observed in a mutant lacking the relA, a gene encoding the stringent response regulatory protein responsible for synthesis of (p)ppGpp (Li et al., 2015). This deletion results in the up-regulation of a fimbrial biogenesis protein and tight adherence protein, proteins thought be important for adhesion to surfaces (Li et al., 2015).

In addition to quorum sensing and the stringent response, two-component regulatory system also controls biofilm formation in A. pleuropneumoniae. For example, deletion of the ArcA, which belongs to the ArcAB two-component system, causes a defect in autoaggregation and biofilm formation (Buettner et al., 2008). Furthermore, the expression of the cpxA, a gene encoding the histidine kinase of the CpxRA stress response system, is
induced in bacteria grown in biofilm when compared to their planktonic counterparts (Tremblay, et al., 2013a). In *E. coli*, this system is induced during the biofilm maturation phase (Otto and Silhavy, 2002) and the CpxRA system can be activated by mechanical pressure (Vogt and Raivio, 2012). It has been suggested that such pressure could be encountered by bacteria during the initial attachment and biofilm formation and could activate the CpxRA stress response. Interestingly, an O-antigen mutant, which lost its ability to produce a biofilm, exhibits reduced expression of *cpxRA* (Hathroubi et al., 2016a). Furthermore, enhanced biofilm production induced by sub-MIC of penicillin G is associated with increased *cpxRA* expression (Hathroubi et al., 2015). In both cases described above, the expression of *pgaA* is also affected in the same direction suggesting a link between the CpxRA response and *pgaABCD* expression. Overall, activation of the *A. pleuropneumoniae* CpxRA system appears to occur during biofilm formation; however, the link between the CpxRA system, *pgaABCD* expression and biofilm formation requires further investigation before this could be said definitively.

5) *Surface-associated proteins and polysaccharides*

Proteins and polysaccharides located at the bacterium/surface interface are crucial for facilitating attachment, microcolonies formation, and/or subsequent maturation of the biofilm. Several proteins and polysaccharides have been identified and characterized as important for biofilm formation. In addition to the biofilm matrix polysaccharides, other surface polysaccharides have an impact on biofilm formation. For example, inactivation of *galU* results in an increase biofilm production (Grasteau et al., 2011). The *galU* gene encodes an UTP-α-D-glucose-1-phosphate uridylyltransferase, an enzyme involved in the biosynthesis of the lipopolysaccharide core oligosaccharide in *A. pleuropneumoniae*
Further, the *wecABD* operon and the genes encoding proteins involved in the biosynthesis of lipopolysaccharide O antigen are induced in a mature biofilm (Tremblay *et al*., 2013a).

Recently, it was demonstrated that the absence of the O antigen markedly reduces the ability of *A. pleuropneumoniae* to form a mature biofilm. This decrease is associated with a reduction in *pgaA* expression and, consequently, PGA production (Hathroubi *et al*., 2016a). Interestingly, LPS and O-antigen truncated LPS specifically bind PGA suggesting that interactions between LPS and PGA may help bacterial cells attached to the biofilm matrix. Taken together, these observations reinforce the idea that LPS may play a role in biofilm formation of *A. pleuropneumoniae*. Several studies have shown the importance of O chains in biofilm formation by other Gram negative such as *Stenotrophomonas maltophilia* (Huang *et al*., 2006), *Xanthomonas citri* ssp. *citri* (Li and Wang, 2011), *Xanthomonas oryzae* pv. *oryzicola* (Wang *et al*., 2013), and *Xylella fastidiosa* (Clifford *et al*., 2013).

Although LPS may have a key role in biofilm formation, the capsule polysaccharides do not appear to affect biofilm formation despite an increase in adherence to epithelial cells and polystyrene by a capsule mutant (Rioux *et al*., 2000; Hathroubi *et al*., 2016a). The capsule may mask critical adhesion factors such as adhesins. Several surface proteins have been associated with biofilm formation in *A. pleuropneumoniae*. For example, deletion of the autotransporter serine protease, AasP, results in increased adherence and biofilm formation (Tegetmeyer *et al*., 2009). The outer membrane protein VacJ is also involved in biofilm formation and outer membrane integrity (Xie *et al*., 2016a); deletion of this gene reduces the ability of *A. pleuropneumoniae* to form biofilms. Interestingly outer membrane
efflux proteins, such as TolC or a TolC-like homologue, have also been associated with biofilm formation. Moreover, it has been observed that the deletion of tolC1 causes a reduction in surface adherence, auto-aggregation and biofilm production but the second tolC homologue, tolC2, does not have any effect on biofilm formation (Li et al., 2016a; Li et al., 2016b). The cell hydrophobicity is also changed in the tolC1 deletion mutant and pgaA and cpxR expression is down-regulated in the mutant (Li et al., 2016a). As a side note, the tolC gene is up-regulated in dispersing biofilms and it has been suggested that this protein with MacAB-like proteins could mediate secretion of a dispersal signal (Tremblay et al., 2013a). Interestingly, the efflux pump inhibitor, phenylalanine-arginine beta-naphthylamide (PAβN), is able to repress biofilm formation of A. pleuropneumoniae and enhance the inhibitory effect of several antibiotics on pre-established biofilms (Li et al., 2016b).

Two trimeric autotransporter adhesins, Apa1 and Apa2, are also involved in autoaggregation and biofilm formation of A. pleuropneumoniae (Xiao et al., 2012; Wang et al., 2016). In the case of Apa1, the adhesion functional domain located at the head of the protein is required for autoaggregation, biofilm formation and adherence to SJPL (Wang et al., 2015). Apa1 is a Hsf-like trimeric autotransporter adhesin that has been identified to be differentially regulated under several conditions. For example, Apa1, also identified as APL_0443, is up-regulated when A. pleuropneumoniae is cultured in a growth medium favoring biofilm formation (Labrie et al., 2010), in the presence of norepinephrine (Li et al., 2012) and in the presence of porcine bronchoalveolar lavage fluid (Lone et al., 2009) while it is down-regulated in A. pleuropneumoniae attached to SJPL cells (Auger et al.,
2009), in a maturing biofilm (Tremblay et al., 2013a) and in the presence of epinephrine (Li et al., 2012). Based on these observations, it was suggested that APL_0443 is involved in the early reversible attachment step during biofilm formation of *A. pleuropneumoniae* (Tremblay et al., 2013a).

6) Other factors identified

Factors involved in biofilm formation are not limited to regulators and structures at the bacteria-surface interface; the periplasm and cytoplasm have also been identified as the location of key processes for biofilm formation. For example, ClpP, a protease of the CLP (caseinolytic protease) family, plays an important role in biofilm formation of *A. pleuropneumoniae*. Indeed, a *clpP* deletion mutant has been shown to have a defect in biofilm production (Xie et al., 2013). Other proteases also influence biofilm formation by *A. pleuropneumoniae*. Specifically, two homologues of the Lon proteases, LonA and LonC, have been identified but only the deletion of LonA results in decrease biofilm production (Xie et al., 2016b). The Lon proteases belong to a family of ATP-dependent proteases involved in the degradation of abnormal proteins created when bacteria are exposed to environmental stresses.

Furthermore, mutations in genes such *potD2*, a dihydouridine tRNA that binds polyamine/spermidine, and *rpmF*, a ribosomal L32 protein, caused a decrease in the production of *A. pleuropneumoniae* biofilm (Grasteau et al., 2011). Homologues of these genes have been associated with *Pseudomonas aeruginosa* biofilm and their mutations decrease biofilm production (Musken et al., 2010). Other genes such *pyrF* (decarboxylase orotidine-5-phosphate), *ptsI* (phosphotransferase) and *ribA* (synthesis of riboflavin), are
also associated with a decrease in biofilm formation in *A. pleuropneumoniae* (Grasteau et al., 2011). Also, riboflavin synthesis appears to be an important element in biofilm formation since the expression of certain genes in this pathway are modulated during biofilm formation (Tremblay et al., 2013a).

**BIOFILMS: ADVANTAGES AND BENEFITS FOR A. PLEUROPNEUMONIAE**

It is recognized that biofilms provide various advantages to bacteria including survival in harsh environments and resistance to stresses such as the presence of antibiotics or disinfectants (Jefferson, 2004; Nadell et al., 2015; Olsen, 2015; Hathroubi et al., 2017). For example, *A. pleuropneumoniae* gown as a biofilm is less sensitive to antibiotics and concentrations 100 to 30 000 times higher than the minimal inhibitory concentrations (MIC) required to kill planktonic cells (Archambault et al., 2012) are needed to kill biofilm cells. This decrease in sensitivity has been observed with antibiotics frequently used on pig farms including ampicillin, florfenicol, tiamulin and tilmicosin (Archambault et al., 2012).

It has been suggested that a decrease in sensitivity to antibiotics is due to the sequestration of antibiotics by extracellular matrix components such as PGA which is found in the biofilm matrix of *A. pleuropneumoniae* (Nadell et al., 2015; Olsen, 2015; Hathroubi et al., 2017). Indeed, pretreatment of biofilms with dispersin B increases the sensitivity of *A. pleuropneumoniae* cells to ampicillin suggesting that PGA can limit the diffusion of this antibiotic (Izano et al., 2007). In addition to decreasing antibiotic sensitivity, biofilms can also protect against the immune response or decrease the inflammatory response. With *A. pleuropneumoniae*, pro-inflammatory genes are down-regulated in porcine pulmonary alveolar macrophages exposed to biofilm cells when compared to planktonic cells (Hathroubi et al., 2016b). Furthermore, biofilm bacteria reduce the proliferation of porcine
peripheral blood mononuclear cells. Interestingly, biofilm cells modify their lipid A structures, and these modifications are absent in planktonic cells. Overall, the immune response towards cells isolated from *A. pleuropneumoniae* biofilms is weaker and this change could be partially driven by lipid A modification (Hathroubi *et al.*, 2016b).

The advantages conferred by biofilm formation might not be limited to stress resistance. During an infection or colonization, biofilms are generally formed as mixed population of several microorganisms resulting in competitive and/or mutualistic relationships (Peters, *et al.*, 2012; Willems *et al.*, 2016). In some cases, polymicrobial interactions in mixed biofilms can provide fertile ground for the exchange of resistance genes and/or increase survival and persistence (Harriott and Noverr, 2009; De Brucker *et al.*, 2015; Hathroubi *et al.*, 2017). Recently, it was demonstrated that *A. pleuropneumoniae* is able to form mixed biofilms with other swine pathogens such as *S. suis*, *Bordetella bronchiseptica* and *P. multocida* (Loera-Muro *et al.*, 2016). In this situation, *A. pleuropneumoniae* does not require the addition of the essential co-factor NAD to the medium for growth and biofilm formation. Furthermore, *S. suis*, *Bordetella bronchiseptica* and *P. multocida* form a weak biofilm that is at near the detection limit of the assay in BHI and in the absence of *A. pleuropneumoniae*. The association of *A. pleuropneumoniae* with other swine pathogens appears to benefit both partners. The swine pathogens provide an essential co-factor to *A. pleuropneumoniae* and, in exchange, *A. pleuropneumoniae* could provide components for the biofilm structure (e.g., PGA, eDNA, proteins, lipids) (Loera-Muro *et al.*, 2016).

The benefits of biofilm formation may not be limited to the host environment. Indeed, as an obligate parasite of the porcine respiratory tract, *A. pleuropneumoniae* can only survive for
a very short period of time outside its host and is unable to survive in the farm environment. However, a recent study detected *A. pleuropneumoniae* in biofilms from the drinking water found on swine farms in Mexico (Loera-Muro et al., 2013).

*A. pleuropneumoniae* biofilms may also be advantageous for other microorganisms such as important viral pathogens of pigs. Recently, it was demonstrated that the porcine reproductive and respiratory syndrome virus and type 2 porcine circovirus can persist inside an *A. pleuropneumoniae* biofilm for several days (Jacques et al., 2015).

On a final thought, biofilm may be a contributing factor, to some extent, to the high prevalence of *A. pleuropneumoniae* in both Canadian domestic pigs (70%) (MacInnes et al., 2008) and feral pigs in the United States (69.7%) by favoring persistent infections (Baroch et al., 2015).

**MANAGEMENT OF *A. PLEUROPNEUMONIAE* OUTBREAKS**

A wide variety of antimicrobial agents are used to treat *A. pleuropneumoniae*: β-lactams (amoxicillin, penicillin, ampicillin, and ceftiofur), tetracyclines (tetracycline and doxycycline), florfenicol, trimethoprim/sulfamethoxazole, tiamulin, lincomycin/spectinomycin, fluoroquinolones (danofloxacin and enrofloxacin) and gentamicin (Dayao et al., 2014; Dayao et al., 2016). In recent years, isolates with different levels of antibiotics resistance have started to arise worldwide (Archambault et al., 2011; Bossé et al., 2015; Dayao et al., 2014).

The direct link between biofilm formation and levels of antibiotic resistance in *A. pleuropneumoniae* is still unclear. However, it is worth mentioning that sub-MIC of
penicillin G may enhance biofilm production via the induction of PGA expression (Hathroubi et al., 2015). Since antibiotics are often used in North America at sub-therapeutic doses for growth promotion and prevention, and *A. pleuropneumoniae* biofilms are more tolerant to antibiotics (Archambault et al., 2012), the judicious use of antibiotic in pig production is highly advised.

Currently, antibiotics represent the most effective measure for controlling *A. pleuropneumoniae* outbreaks (Gottschalk, 2015). *A. pleuropneumoniae* biofilm should be taken into consideration for the development of new effective treatment strategies. These strategies should combine antimicrobials with anti-biofilm molecules such as zinc (Wu et al., 2013) or PAβN (Li et al., 2016b) to overcome persistent infections and reduce the cost of treatment.

**PREVENTION AND VACCINE STRATEGIES AGAINST *A. pleuropneumoniae***

In the last decade, several vaccines have been developed to protect against *A. pleuropneumoniae* infections. Most of vaccines are based on recombinant Apx toxins and membrane proteins (such as OMP and type 4 fimbrial proteins) and provide protection against some but not all serotypes (Shao et al. 2010; Lu et al., 2011; Shin et al. 2011; Sadilkova et al. 2012; Li et al., 2013; Hur and Lee, 2014; Yang et al., 2014; Hur et al., 2016; Kim et al., 2016; Li et al., 2016c; To et al., 2016). Inactivated/whole *A. pleuropneumoniae* cell based vaccines are also used in many countries to prevent porcine pleuropneumonia (Shao et al., 2010; Lu et al., 2011; Lee et al., 2014; Lopez-Bermudez et
These vaccines are widely distributed. However, these vaccines do not provide complete protection against all serotypes of *A. pleuropneumoniae*.

Bacterins are typically prepared from bacteria grown as planktonic cells. Since biofilm cells are known to exhibit phenotypes that are different than their planktonic counterparts (Stewart and Franklin, 2008; O’May *et al.*, 2009) and *A. pleuropneumoniae* form biofilm aggregates during an infection (Tremblay *et al.*, 2017), the vaccines described above may not provide a full protection against *A. pleuropneumoniae* infections. Bacterins may help the vaccinated pig developed a significant memory response against the planktonic form of *A. pleuropneumoniae* but the antigenic nature of some targets are modified during growth as biofilms. For example, the *A. pleuropneumoniae* lipid A molecular structure is modified according to the mode of growth (Hathroubi *et al.*, 2016b). Indeed, cells grown as a biofilm have unique lipid A structures that are absent in planktonic cells including an increase in higher molecular weight lipid A entities (Hathroubi *et al.*, 2016b). Accordingly, it would likely be best to create bacterins using both planktonic and biofilm cultures to provide a better protection against *A. pleuropneumoniae* infections by presenting a larger set of antigens that could be biologically relevant.

As with bacterins, commercially available recombinant vaccines based on Apx toxins and/or other proteins have failed to provide a complete protection against every *A. pleuropneumoniae* isolates (Del Pozo-Sacristán *et al*. 2014; Sjölund *et al*. 2010). The development of new vaccines based on antigens specifically associated with *A. pleuropneumoniae* biofilms in combination with the Apx toxins and other antigens could
help improve the protection but further investigations are required to identify relevant molecules expressed in biofilms and during infection.

Such strategies have been successful for the development of new vaccines against other pathogens. For example, a proteomic analysis of *Bordetella pertussis* biofilm and planktonic cells identified a biofilm-derived membrane protein called BipA as a potential vaccine antigen (de Gouw *et al*., 2014). Vaccination of mice with this antigen showed promising results that included induction of a specific antibodies response and a significant reduction in the colonization of lungs by *B. pertusis* (de Gouw *et al*., 2014). Moreover, anti-BipA antibodies have been detected in the serum of convalescent whooping cough patients (de Gouw *et al*., 2014). In another example, Gil *et al.* (2014) performed an intradermal administration of an exoproteome extract derived from an exopolysaccharide-dependent biofilm to develop an efficient antibiofilm vaccine against *Staphylococcus aureus*. The biofilm exoproteome induced a humoral immune response and elicited the production of interleukin (IL) 10 and IL-17 in mice. Furthermore, vaccination with the exoproteome extract significantly reduced the number of bacteria within biofilms and surrounding tissue in *in vivo* mesh-associated biofilm infection model (Gil *et al*., 2014).

The strategy of using biofilm-specific antigen is not limited to *B. pertussis* and *S. aureus*; others have begun to use similar strategies against bacterial pathogens of importance in veterinary and human health. These pathogens include: *S. aureus* (Speziale *et al*., 2014; Gogoi-Tiwari *et al*., 2015), *Campylobacter jejuni* (Theoret *et al*., 2012), *Mycobacterium tuberculosis*-complex (Flores-Valdez, 2016), *Streptococcus mutans* (Huang *et al*., 2013), *Staphylococcus epidermidis* (Shahrooei *et al*., 2012; Speziale *et al*., 2014), *Bacillus subtilis*
(Vogt et al., 2016), Acinetobacter baumannii (Fattahian et al., 2011), and Streptococcus equi ssp. zooepidemicus (Yi et al., 2016) (Table 1).

In the context of biofilm infections, two different types of antigens exist: bacterial cells within the biofilm and the biofilm matrix. The biofilm matrix may be composed of polysaccharides, proteins, and extracellular DNA and the composition of the matrix is dependent on the bacterial genera, species, and strains (Harro et al., 2010). Different studies have focused on identifying antigens from the bacteria, the matrix or both as the best strategy for the development of effective vaccines (Table 1).

Another factor that must be considered is that biofilm consortia typically exist as communities of bacteria, viruses, protozoans, and fungi and the overall biofilm architecture is affected by specific intermicrobial and host interactions (Harro et al., 2010). These consortia can allow colonization and subsequent infection by opportunistic pathogens that exploit unique niches found in these polymicrobial environments resulting in the development of polymicrobial infections.

Finally, vaccine research and design should take advantage of new techniques such as RNA sequencing, bioinformatics, proteomics, and lipidomics to identify molecules specifically expressed and/or secreted during biofilm formation. In our opinion, this should greatly improve the efficacy of future vaccines and ensure better protection of pigs against A. pleuropneumoniae.

CONCLUSION AND FUTURE CHALLENGES
Despite different strategies and years of prevention and control, *A. pleuropneumoniae* remains one of the main respiratory pathogens of pigs and is responsible for great economic losses to the worldwide pork industry. Although some countries such as the USA and Canada can manage *A. pleuropneumoniae*, this pathogen remains present in farms and, thus, resurgence in new outbreaks are always possible. These new outbreaks could emerge from isolates with increase resistance to antibiotics. Great efforts have been made to prevent infections with this pathogen through optimal farm management and through major investments in research and development of new and better vaccines. However, neither management nor vaccines have been 100% effective at controlling *A. pleuropneumoniae* infections. Fortunately, new research is shedding light on the pathogenesis of *A. pleuropneumoniae* and it is improving our understanding of this old acquaintance. Importantly, recent studies have revealed that *A. pleuropneumoniae* forms biofilm aggregates in the lung (Tremblay et al., 2017) and can form multi-species biofilms with other respiratory pathogens (Loera-Muro et al., 2016). Using these new findings, it will be possible to identify novel vaccine candidates to improve the next generation of vaccines and to develop better strategies to control *A. pleuropneumoniae*. These new developments could hopefully help prevent the persistent problems cause by this pathogen in the worldwide production of pigs for the last 50 years.

**Acknowledgements.**

This review was supported by a Discovery Grant (RGPIN-2016-04203 to MJ) from the Natural Sciences and Engineering Research Council of Canada (NSERC) and by CONACYT, Mexico (Cátedras CONACYT Program to ALM).
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Flores-Valdez MA (2016). Vaccines Directed Against Microorganisms or Their Products Present During Biofilm Lifestyle: Can We Make a Translation as a Broad Biological Model to Tuberculosis? *Frontiers in Microbiology* **7**: 14.


pleuropneumonia using the cubic phase of monoolein and purified toxins of *Actinobacillus pleuropneumoniae*. Vaccine **32**: 6805-6811.


pleuropneumoniae responsible for autoagglutination and host cell adherence. *Journal of Basic Microbiology* **52**: 598-607.


Figure 1 Confocal laser scanning microscopy image of *A. pleuropneumoniae* 4074 biofilm stained with WGA-Oregon Green 488.

Figure 2 Coupon with *A. pleuropneumoniae* 4074 biofilm from Drip flow system.
**Table 1.** Examples of vaccines based on biofilm-specific antigens produced by pathogenic bacteria of importance in veterinary and human health.

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>Disease</th>
<th>Antigens</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Acinetobacter baumannii</em></td>
<td>Nosocomial pathogen and causes severe infections such as bacteremia, pneumonia, meningitis, urinary tract and wound infections.</td>
<td>Biofilm associated protein (Bap), a 371 amino acid subunit.</td>
<td>Fattahian et al., 2011.</td>
</tr>
<tr>
<td><em>Acinetobacter baumannii</em></td>
<td>Nosocomial pathogen and causes severe infections such as bacteremia, pneumonia, meningitis, urinary tract and wound infections.</td>
<td>Bap with Outer Membrane Vesicles (without lipid A or Outer Membrane Protein A).</td>
<td>Badmasti et al., 2015.</td>
</tr>
<tr>
<td><em>Burkholderia pseudomallei</em></td>
<td>The causative agent of melioidosis (category B select agent).</td>
<td>mAbs namely BURK24 and BURK37.*</td>
<td>Peddayelachagiri et al., 2014.</td>
</tr>
<tr>
<td><em>Campylobacter jejuni</em></td>
<td>Food-borne gastroenteritis.</td>
<td>Oral vaccination with a recombinant attenuated <em>Salmonella enterica</em> strain synthesizing the <em>C. jejuni</em> Dps protein.</td>
<td>Theoret et al., 2012.</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em></td>
<td>Cause catheter-associated urinary tract infections.</td>
<td>Heteropolymeric surface long hair-like fiber known as the endocarditis-and biofilm-</td>
<td>Flores-Mireles et al., 2014.</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>Associated with biofilm-mediated infections (endocarditis, osteomyelitis, medical devices, etc.).</td>
<td>Phosphonate ABC transporter substrate binding protein (PhnD).</td>
<td>Lam et al., 2014</td>
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<tr>
<td>Staphylococcus epidermidis</td>
<td>Associated with biofilm-mediated infections (endocarditis, osteomyelitis, medical devices, etc.).</td>
<td>The Major amidase (Atl-AM, amulti-functional non-covalently cell wall associated protein involved in biofilm formation).</td>
<td>Nair et al., 2015</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>Associated with biofilm-mediated infections (endocarditis, osteomyelitis, medical devices, etc.).</td>
<td>Exoproteome extract of an exopolysaccharide-dependent biofilm.</td>
<td>Gil et al., 2014</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>Persistent and chronic forms of mastitis in cows.</td>
<td>Formalin killed whole cell vaccine of S. aureus in a biofilm state.</td>
<td>Gogoi-Tiwari et al., 2015</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>Persistent and chronic forms of mastitis in cows.</td>
<td>Protein A (in biofilm formation contributing to the severity of S. aureus associated infections).</td>
<td>Gogoi-Tiwari et al., 2016</td>
</tr>
<tr>
<td>Staphylococcus epidermidis</td>
<td>Medical implants associated infections.</td>
<td>Accumulation associated protein (Aap) C-terminal single B-repeat construct followed</td>
<td>Hu et al., 2011</td>
</tr>
</tbody>
</table>
by the 79-aa half repeat (AapBrpt1.5).

<table>
<thead>
<tr>
<th>Organism</th>
<th>Medical implants</th>
<th>Associated infections</th>
<th>Associated with</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td><em>Staphylococcus epidermidis</em></td>
<td>Medical implants</td>
<td>Associated infections</td>
<td>Vaccination with a recombinant truncated SesC (hypothetical LPXTG motif-containing proteins)</td>
<td>Shahrooei <em>et al.</em>, 2012.</td>
</tr>
<tr>
<td><em>Staphylococcus epidermidis</em></td>
<td>Medical implants</td>
<td>Associated infections</td>
<td>Accumulation associated protein (Aap)</td>
<td>Yan <em>et al.</em>, 2014</td>
</tr>
<tr>
<td><em>Streptococcus mutans</em></td>
<td>Predominant microorganism in the etiology and pathogenesis of dental caries.</td>
<td>DNA vaccine-induced salivary secretory immunoglobulin A (S-IgA) antibodies (DNA vaccine pGJA-P/VAX).</td>
<td>Huang <em>et al.</em>, 2013.</td>
<td></td>
</tr>
</tbody>
</table>

* Murine Monoclonal Antibodies (mAbs) against *Burkholderia pseudomallei* biofilms.