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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 10^9-10^{10} 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 σ^E) is a transcriptional activator of the *pgaABCD* operon (Bossé *et al*., 2010).

Deletion of the gene encoding the negative regulator of the σ^E 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 autoagregation 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*

(Ramjeet *et al*., 2008). 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 *tolC*1 causes a reduction in surface adherence, auto-aggregation and biofilm production but the second *tolC* homologue, *tolC*2, does not have any effect on biofilm formation (Li *et al*., 2016a; Li *et al*., 2016b). The cell hydrophobicity is also changed in the *tolC*1 deletion mutant and *pgaA* and *cpxR* expression is down-regulated in the mutant (Li *et al*., 2016a). As a side note, the *tolC*2 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 dihydrouridine 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 competitve 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*

545 *al.*, 2014). These vaccines are widely distributed. However, these vaccines do not provide
546 complete protection against all serotypes of *A. pleuropneumoniae*.
547
548 Bacterins are typically prepared from bacteria grown as planktonic cells. Since biofilm cells
549 are known to exhibit phenotypes that are different than their planktonic counterparts
550 (Stewart and Franklin, 2008; O'May *et al*., 2009) and *A. pleuropneumoniae* form biofilm
551 aggregates during an infection (Tremblay *et al*., 2017), the vaccines described above may
552 not provide a full protection against *A. pleuropneumoniae* infections. Bacterins may help
553 the vaccinated pig developed a significant memory response against the planktonic form of
554 *A. pleuropneumoniae* but the antigenic nature of some targets are modified during growth
555 as biofilms. For example, the *A. pleuropneumoniae* lipid A molecular structure is modified
556 according to the mode of growth (Hathroubi *et al*., 2016b). Indeed, cells grown as a biofilm
557 have unique lipid A structures that are absent in planktonic cells including an increase in
558 higher molecular weight lipid A entities (Hathroubi *et al*., 2016b). Accordingly, it would
559 likely be best to create bacterins using both planktonic and biofilm cultures to provide a
560 better protection against *A. pleuropneumoniae* infections by presenting a larger set of
561 antigens that could be biologically relevant.
562
563 As with bacterins, commercially available recombinant vaccines based on Apx toxins
564 and/or other proteins have failed to provide a complete protection against every *A.*
565 *pleuropneumoniae* isolates (Del Pozo-Sacristán *et al*. 2014; Sjölund *et al.* 2010). The
566 development of new vaccines based on antigens specifically associated with *A.*
567 *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 loses 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.

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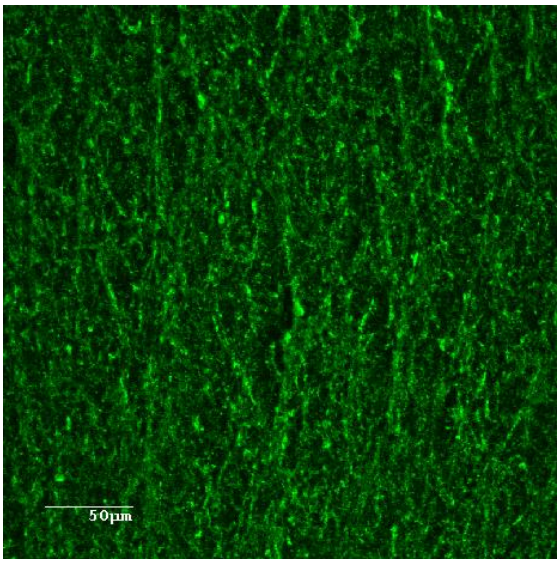

Figure 1 Confocal laser scanning microscopy image of *A. pleuropneumoniae* 4074 biofilm stained with WGA-Oregon Green 488.

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.

Bacterial species	Disease	Antigens	Reference
Acinetobacter baumannii	Nosocomial pathogen and causes severe infections such as bacteremia, pneumonia, meningitis, urinary tract and wound infections.	Biofilm associated protein (Bap), a 371 amino acid subunit.	Fattahian *et al*., 2011.
Acinetobacter baumannii	Nosocomial pathogen and causes severe infections such as bacteremia, pneumonia, meningitis, urinary tract and wound infections.	Bap with Outer Membrane Vesicles (without lipid A or Outer Membrane Protein A).	Badmasti *et al*., 2015.
Bordetella pertussis	Whooping cough or pertussis.	Bordetella intermediate protein A (BipA).	de Gouw *et al*., 2014.
Burkholderia pseudomallei	The causative agent of melioidosis (category B select agent).	mAbs namely BURK24 and BURK37.*	Peddayelachagiri *et al*., 2014.
Campylobacter jejuni	Food-borne bacterial gastroenteritis.	Oral vaccination with a recombinant attenuated *Salmonella enterica* strain synthesizing the *C. jejuni* Dps protein.	Theoret *et al*., 2012.
Enterococcus faecalis	Cause catheter-associated urinary tract infections.	Heteropolymeric surface long hair-like fiber known as the endocarditis-and biofilm-	Flores-Mireles *et al*., 2014.

		associated pilus (Ebp).	
Staphylococcus aureus and *Staphylococcus epidermidis*	Associated with biofilm-mediated infections (endocarditis, osteomyelitis, medical devices, etc.).	Phosphonate ABC transporter substrate binding protein (PhnD).	Lam *et al*., 2014
Staphylococcus aureus and *Staphylococcus epidermidis*	Associated with biofilm-mediated infections (endocarditis, osteomyelitis, medical devices, etc.).	The Major amidase (Atl-AM, amulti-functional non-covalently cell wall associated protein involved in biofilm formation).	Nair *et al*., 2015.
Staphylococcus aureus	Associated with biofilm-mediated infections (endocarditis, osteomyelitis, medical devices, etc.).	Exoproteome extract of an exopolysaccharide-dependent biofilm.	Gil *et al*., 2014.
Staphylococcus aureus	Persistent and chronic forms of mastitis in cows.	Formalin killed whole cell vaccine of *S. aureus* in a biofilm state.	Gogoi-Tiwari *et al*., 2015.
Staphylococcus aureus	Persistent and chronic forms of mastitis in cows.	Protein A (in biofilm formation contributing to the severity of *S. aureus* associated infections).	Gogoi-Tiwari *et al*., 2016.
Staphylococcus epidermidis	Medical implants associated infections.	Accumulation associated protein (Aap) C-terminal single B-repeat construct followed	Hu *et al*., 2011.

		by the 79-aa half repeat (AapBrpt1.5).	
Staphylococcus epidermidis	Medical implants associated infections.	Vaccination with a recombinant truncated SesC (hypothetical LPXTG motif-containing proteins).	Shahrooei *et al*., 2012.
Staphylococcus epidermidis	Medical implants associated infections.	Accumulation associated protein (Aap).	Yan *et al*., 2014
Streptococcus mutans	Predominant microorganism in the etiology and pathogenesis of dental caries.	DNA vaccine-induced salivary secretory immunoglobulin A (S-IgA) antibodies (DNA vaccine pGJA-P/VAX).	Huang *et al*., 2013.
Streptococcus equi ssp. zooepidemicus	Opportunistic pathogen infecting a wide variety of animals and human.	Recombinant chaperonin GroEL protein.	Yi *et al*., 2016.

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