***Actinobacillus pleuropneumoniae* biofilms: Role in pathogenicity and potential impact for vaccination development.**

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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 be 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 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-ribosylhomocysteine 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 *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 *tolC2* 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 (PA β N), 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* (Grasteanu *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* grown 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 PA β N (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 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.

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References.

- Archambault M, Harel J, Gouré J, Tremblay YD and Jacques M (2012). Antimicrobial Susceptibilities and Resistance Genes of Canadian Isolates of *Actinobacillus pleuropneumoniae*. *Microbial Drug Resistance* **18**: 198-206.
- Auger E, Deslandes V, Ramjeet M, Contreras I, Nash J, Harel J, Gottschalk M, Olivier M and Jacques M (2009). Host-pathogen interactions of *Actinobacillus pleuropneumoniae* with porcine lung and tracheal epithelial cells. *Infection and Immunity* **77**: 1426-1441.
- Badmasti F, Ajdary S, Bouzari S, Fooladi AA, Shahcheraghi F and Siadat SD (2015). Immunological evaluation of OMV(PagL)+Bap(1-487aa) and AbOmpA(8-346aa)+Bap(1-487aa) as vaccine candidates against *Acinetobacter baumannii* sepsis infection. *Molecular Immunology* **67**: 552-558. doi: 10.1016/j.molimm.2015.07.031.
- Baroch JA, Gagnon CA, Lacouture S and Gottschalk M (2015). Exposure of feral swine (*Sus scrofa*) in the United States to selected pathogens. *Canadian Journal of Veterinary Research* **79**: 74-78.
- Bello-Ortí B, Deslandes V, Tremblay YD, Labrie J, Howell KJ, Tucker AW, Maskell DJ, Aragon V and Jacques M (2014) Biofilm formation by virulent and non-virulent strains of *Haemophilus parasuis*. *Veterinary Research* **45**: 104.
- Bjarnsholt, T., Alhede, M., Eickhardt-Sorensen, S.R., Moser, C., Kühl, M., Jensen, P.O., and Hoiby, N. (2013) The *in vivo* biofilm. *Trends in Microbiology* **21**: 466–474.
- Bossé JT, Sinha S, Li MS, O'Dwyer CA, Nash JH, Rycroft AN, Kroll JS and Langford PR (2010). Regulation of *pga* operon expression and biofilm formation in *Actinobacillus pleuropneumoniae* by σ^E and H-NS. *Journal of Bacteriology* **192**: 2414-2423. doi:10.1128/JB.01513-09

664 Bossé JT, Li Y, Angen Ø, Weinert LA, Chaudhuri RR, Holden MT, Williamson SM,
 665 Maskell DJ, Tucker AW, Wren BW, Rycroft AN and Langford PR (2014). Multiplex
 666 PCR assay for unequivocal differentiation of *Actinobacillus pleuropneumoniae*
 667 serovars 1 to 3, 5 to 8, 10, and 12. *Journal of Clinical Microbiology* **52**: 2380-2385.
 668 doi: 10.1128/JCM.00685-14.

669 Bossé JT, Li Y, Atherton TG, Walker S, Williamson SM, Rogers J, Chaudhuri RR, Weinert
 670 LA, Holden MT, Maskell DJ, Tucker AW, Wren BW, Rycroft AN, Langford PR and
 671 BRaDP1T consortium (2015). Characterisation of a mobilisable plasmid conferring
 672 florfenicol and chloramphenicol resistance in *Actinobacillus pleuropneumoniae*.
 673 *Veterinary Microbiology* **178**: 279-282. doi: 10.1016/j.vetmic.2015.05.020.

674 Bossé JT, Li Y, Sárközi R, Gottschalk M, Angen Ø, Nedbalcova K, Rycroft AN, Fodor L
 675 and Langford PR (2017). A unique capsule locus in the newly designated
 676 *Actinobacillus pleuropneumoniae* serovar 16 and development of a diagnostic PCR.
 677 *Journal of Clinical Microbiology* **55**: 902-907. doi: 10.1128/JCM.02166-16

678 Boukahil I and Czuprynski CJ (2015). Characterization of *Mannheimia haemolytica*
 679 biofilm formation *in vitro*. *Veterinary Microbiology* **175**: 114-122

680 Briandet R, Fechner L, Naïtali M and Dreanno C (2012). Biofilms, quand les microbes
 681 s'organisent. Editions Quae, France.

682 Buettner F, Konze S, Maas A and Gerlach G (2011). Proteomic and immunoproteomic
 683 characterization of a DIVA subunit vaccine against *Actinobacillus*
 684 *pleuropneumoniae*. *Proteome Science* **9**: 1-23.

685 Chen Z, Chien MS, Chang NY, Chen TH, Wu CM, Huang C, Lee WC and Hsuan SL
 686 (2011). Mechanisms underlying *Actinobacillus pleuropneumoniae* exotoxin ApxI

687 induced expression of IL-1b, IL-8 and TNF-a in porcine alveolar macrophages.
 688 *Veterinary Research* **42**: 2-10.

689 Chiers K, De Waele T, Pasmans F, Ducatelle R and Haesebrouck F (2010). Virulence
 690 factors of *Actinobacillus pleuropneumoniae* involved in colonization, persistence and
 691 induction of lesions in its porcine host. *Veterinary Research* **41**: 65.

692 Clifford JC, Rapicavoli JN and Roper MC (2013). A rhamnose-rich O-antigen mediates
 693 adhesion, virulence and host colonization for the xylem-limited phytopathogen,
 694 *Xylella fastidiosa*. *Molecular Plant-Microbe Interactions* **26**: 676-685.
 695 doi:10.1094/MPMI-12-12-0283-R

696 Coenye T and Nelis HJ (2010). *In vitro* and *in vivo* model systems to study microbial
 697 biofilm formation. *Journal of Microbiology Methods* **83**: 89-105.

698 Coffey BM and Anderson GG (2014). Biofilm formation in the 96-well microtiter plate.
 699 *Methods Molecular Biology* **1149**: 631-641.

700 Dalai B, Zhou R, Wan Y, Kang M, Li L, Li T, Zhang S and Chen H (2009). Histone-like
 701 protein H-NS regulates biofilm formation and virulence of *Actinobacillus*
 702 *pleuropneumoniae*. *Microbial Pathogenesis* **46**: 128-134.

703 Dayao DA, Gibson JS, Blackall PJ and Turni C (2014). Antimicrobial resistance in bacteria
 704 associated with porcine respiratory disease in Australia. *Veterinary Microbiology*
 705 **171**: 232-235. doi: 10.1016/j.vetmic.2014.03.014.

706 Dayao D, Gibson JS, Blackall PJ and Turni C (2016). Antimicrobial resistance genes in
 707 *Actinobacillus pleuropneumoniae*, *Haemophilus parasuis* and *Pasteurella multocida*
 708 isolated from Australian pigs. *Australian Veterinary Journal* **94**: 227-31. doi:
 709 10.1111/avj.12458.

710 De Brucker K, Tan Y, Vints K, De Cremer K, Braem A, Verstraeten N, Michiels J,
 711 Vleugels J, Cammue BP and Thevissen K (2015). Fungal beta-1,3-glucan increases
 712 ofloxacin tolerance of *Escherichia coli* in a polymicrobial *E. coli/Candida albicans*
 713 biofilm. *Antimicrobial Agents and Chemotherapy* **59**: 3052-3058.

714 De Gouw D, Serra DO, de Jonge MI, et al. (2014). The vaccine potential of *Bordetella*
 715 *pertussis* biofilm-derived membrane proteins. *Emerging Microbes & Infections* **3**:
 716 e58-. doi:10.1038/emi.2014.58.

717 Del Pozo-Sacristán R, Michiels A, Martens M, Haesebrouck F and Maes D (2014).
 718 Efficacy of vaccination against *Actinobacillus pleuropneumoniae* in two Belgian
 719 farrow-to-finish pig herds with a history of chronic pleurisy. *Veterinary Record* **174**:
 720 302.

721 Ethapa T, Leuzzi R, NgYK, Baban ST, Adamo R, Kuehne SA, Scarselli M, Minton NP,
 722 Serruto D and Unnikrishnan M (2013). Multiple factors modulate biofilm formation
 723 by the anaerobic pathogen *Clostridium difficile*. *Journal of Bacteriology* **195**: 545-
 724 555.

725 Fattahian Y, Rasooli I, Gargari SLM, Rahbar MR, Astaneh SDA and Amani J (2011).
 726 Protection against *Acinetobacter baumannii* infection via its functional deprivation of
 727 biofilm associated protein (Bap). *Microbial Pathogenesis* **51**: 402-406.

728 Flores-Mireles AL, Pinkner JS, Caparon MG and Hultgren SJ (2014). EbpA vaccine
 729 antibodies block binding of *Enterococcus faecalis* to fibrinogen to prevent catheter-
 730 associated bladder infection in mice. *Science Translational Medicine* **6**: 254ra127.
 731 doi: 10.1126/scitranslmed.3009384.

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.

Frey J (1995). Virulence in *Actinobacillus pleuropneumoniae* and RTX toxins. *Trends in Microbiology* **3**: 257-261.

Gil C, Solano C, Burgui S, Latasa C, García B, Toledo-Arana A, Lasa I and Valle J (2014). Biofilm Matrix Exoproteins Induce a Protective Immune Response against *Staphylococcus aureus* Biofilm Infection. *Infection and Immunity* **82**: 1017-1029.

Goeres D, Hamilton M, Beck N, Buckingham-Meyer K, Hilyard J, Loetterle L, Lorenz L, Walker D and Stewart P (2009). A method for growing a biofilm under low shear at the air-liquid interface using the drip flow biofilm reactor. *Nature Protocols* **4**: 783-788.

Gogoi-Tiwari J, Williams V, Waryah CB, Eto KY, Tau M, Costantino P, Tiwari HK and Mukkur T (2015). Comparative studies of the immunogenicity and protective potential of biofilm vs planktonic *Staphylococcus aureus* vaccine against bovine mastitis using non-invasive mouse mastitis as a model system. *Biofouling* **31**: 543-54. doi: 10.1080/08927014.2015.1074681.

Gogoi-Tiwari J, Williams V, Waryah CB, Mathavan S, Tiwari HK, Costantino P and Mukkur T (2016). Intramammary Immunization of Pregnant Mice with Staphylococcal Protein A Reduces the Post-Challenge Mammary Gland Bacterial Load but Not Pathology. *PLoS One* **11**: e0148383. doi: 10.1371/journal.pone.0148383.

Gómez-Laguna J, Islas A, Muñoz D, Ruiz A, Villamil A, Carrasco L and Quezada M (2014). Infection dynamics and acute phase response of an *Actinobacillus*

756 *pleuropneumoniae* field isolate of moderate virulence in pigs. *Veterinary*
757 *Microbiology* **173**: 332-339. doi: 10.1016/j.vetmic.2014.08.015.

758 Gottschalk M (2015). The challenge of detecting herds sub-clinically infected with
759 *Actinobacillus pleuropneumoniae*. *The Veterinary Journal* **206**: 30-8. doi:
760 10.1016/j.tvjl.2015.06.016.

761 Gottschalk M and Lacouture S (2015). Canada: Distribution of *Streptococcus suis* (from
762 2012 to 2014) and *Actinobacillus pleuropneumoniae* (from 2011 to 2014) serotypes
763 isolated from diseased pigs. *The Canadian Veterinary Journal* **56**: 1093-1094.

764 Grasteau A, Tremblay YD, Labrie J and Jacques M (2011). Novel genes associated with
765 biofilm formation of *Actinobacillus pleuropneumoniae*. *Veterinary*
766 *Microbiology* **153**: 134-143. doi:.10.1016/j.vetmic.2011.03.029

767 Harriott MM and Noverr MC (2009). *Candida albicans* and *Staphylococcus aureus* form
768 polymicrobial biofilms: effects on antimicrobial resistance. *Antimicrobial Agents and*
769 *Chemotherapy* **53**: 3914-3922.

770 Harro JM, Peters BM, O'May GA, Archer N, Kerns P, Prabhakara R and Shirtliff ME
771 (2010). Vaccine development in *Staphylococcus aureus*: taking the biofilm phenotype
772 into consideration. *FEMS Immunology and Medical Microbiology* **59**: 306-323. doi:
773 10.1111/j.1574-695X.2010.00708.x.

774 Hathroubi S, Fontaine-Gosselin SÈ, Tremblay YD, Labrie J and Jacques M (2015). Sub-
775 inhibitory concentrations of penicillin G induce biofilm formation by field isolates of
776 *Actinobacillus pleuropneumoniae*. *Veterinary Microbiology* **179**: 277-86.

777 Hathroubi S, Hancock MA, Bossé JT, Langford PR, Tremblay YD, Labrie J and Jacques M
778 (2016a). Surface Polysaccharide Mutants Reveal that Absence of O Antigen Reduces

779 Biofilm Formation of *Actinobacillus pleuropneumoniae*. *Infection and Immunity* **84**:
780 127-37.

781 Hathroubi S, Beaudry F, Provost C, Martelet L, Segura M, Gagnon CA and Jacques M
782 (2016b). Impact of *Actinobacillus pleuropneumoniae* biofilm mode of growth on the
783 lipid A structures and stimulation of immune cells. *Innate Immunity* **22**: 353-62.

784 Hathroubi S, Mekni MA, Domenico P, Nguyen D and Jacques M (2017). Biofilms:
785 Microbial shelters against antibiotics. *Microbial Drug Resistance* **23**: 147-156.
786 doi: 10.1089/mdr.2016.0087.

787 Hu J, Xu T, Zhu T, Lou Q, Wang X, Wu Y, Huang R, Liu J, Liu H, Yu F, Ding B, Huang
788 Y, Tong W and Qu D (2011). Monoclonal antibodies against accumulation-associated
789 protein affect EPS biosynthesis and enhance bacterial accumulation of
790 *Staphylococcus epidermidis*. *PLoS One* **6**: e20918. doi:
791 10.1371/journal.pone.0020918.

792 Huang TP, Somers EB and Wong AC (2006). Differential biofilm formation and motility
793 associated with lipopolysaccharide/exopolysaccharide-coupled biosynthetic genes in
794 *Stenotrophomonas maltophilia*. *Journal of Bacteriology* **188**: 3116-3120.
795 doi:10.1128/JB.188.8.3116-3120.2006

796 Huang L, Xu Q, Liu C, Fan M and Li Y (2013). Anti-caries DNA vaccine-induced
797 secretory immunoglobulin A antibodies inhibit formation of *Streptococcus mutans*
798 biofilms *in vitro*. *Acta Pharmacologica Sinica* **34**: 239-246.

799 Hur J and Lee JH (2014). Optimization of immune strategy for a construct of *Salmonella*-
800 delivered ApxIA, ApxIIA, ApxIIIA and OmpA antigens of *Actinobacillus*
801 *pleuropneumoniae* for prevention of porcine pleuropneumonia using a murine model.
802 *Veterinary Research Communications* **38**: 87-91.

803 Hur J, Eo SK, Park SY, Choi Y and Lee JH (2016). Immunological study of an attenuated
 804 *Salmonella Typhimurium* expressing ApxIA, ApxIIA, ApxIIIA and OmpA of
 805 *Actinobacillus pleuropneumoniae* in a mouse model. *The Journal of Veterinary*
 806 *Medical Science* **77**: 1693-1696.

807 Ito H (2015). The genetic organization of the capsular polysaccharide biosynthesis region
 808 of *Actinobacillus pleuropneumoniae* serotype 14. *The Journal of Veterinary Medical*
 809 *Science* **77**: 583-586. doi: 10.1292/jvms.14-0174.

810 Izano, E. A., I. Sadovskaya, E. Vinogradov, M. H. Mulks, K. Velliyagounder, C. Ragunath,
 811 W. B. Kher, N. Ramasubbu, S. Jabbouri, M. B. Perry and J. B. Kaplan (2007). Poly-
 812 N-acetylglucosamine mediates biofilm formation and antibiotic resistance in
 813 *Actinobacillus pleuropneumoniae*. *Microbial Pathogenesis* **43**: 1-9.

814 Jacques M, Grenier D, Labrie J, Provost C and Gagnon C (2015). Persistence of porcine
 815 reproductive and respiratory syndrome virus and porcine circovirus type 2 in bacterial
 816 biofilms. *Journal of Swine Health & Production* **23**: 132-136.

817 Jefferson KK (2004). What drives bacteria to produce a biofilm? *FEMS Microbiology*
 818 *Letters* **236**: 163-173.

819 Jin H, Zhou R, Kang M, Luo R, Cai X and Chen H (2006). Biofilm formation by field
 820 isolates and reference strains of *Haemophilus parasuis*. *Veterinary Microbiology*
 821 **118**(1-2): 117-123.

822 Kaplan JB, Velliyagounder K, Ragunath C, Rohde H, Mack D, Knobloch JK and
 823 Ramasubbu N (2004). Genes involved in the synthesis and degradation of matrix
 824 polysaccharide in *Actinobacillus actinomycetemcomitans* and *Actinobacillus*
 825 *pleuropneumoniae* biofilms. *Journal of Bacteriology* **186**: 8213-8220.
 826 doi:10.1128/JB.186.24.8213-8220.2004

827 Kaplan JB and Mulks MH (2005). Biofilm formation is prevalent among field isolates of
 828 *Actinobacillus pleuropneumoniae*. *Veterinary Microbiology* **108**(1-2): 89-94.
 829 Kim MY, Kim TG and Yang MS (2016). Production and immunogenicity of *Actinobacillus*
 830 *pleuropneumoniae* ApxIIA protein in transgenic rice callus. *Protein Expression and*
 831 *Purification* **S1046-5928**: 30088-2.
 832 Kragh, K.N., Hutchison, J.A., Melaugh, G., Rodesney, C., Roberts, A.E.L., Irie, Y., et al.
 833 (2016) Role of multicellular aggregates in biofilm formation. *mBio* 7: e00237–16.
 834 Labrie J, Pelletier-Jacques G, Deslandes V, Ramjeet M, Auger E, Nash JH and Jacques M
 835 (2010). Effects of growth conditions on biofilm formation by *Actinobacillus*
 836 *pleuropneumoniae*. *Veterinary Research* **41**: 03.
 837 Lam H, Kesselly A, Stegalkina S, Kleanthous H and Yethon JA (2014). Antibodies to
 838 PhnD inhibit staphylococcal biofilms. *Infection and Immunity* **82**: 3764-3774.
 839 doi:10.1128/IAI.02168-14
 840 Lee SH, Lee S, Chae C and Ryu DY (2014). A recombinant chimera comprising the R1 and
 841 R2 repeat regions of *M. hyopneumoniae* P97 and the N-terminal region of *A.*
 842 *pleuropneumoniae* ApxIII elicits immune responses. *BMC Veterinary Research* **10**:
 843 43.
 844 Li L, Zhou R, Li T, Kang M, Wan Y, Xu Z and Chen H (2008). Enhanced biofilm
 845 formation and reduced virulence of *Actinobacillus pleuropneumoniae luxS* mutant.
 846 *Microbial Pathogenesis* **45**(3): 192-200.
 847 Li J and Wang N (2011). The wxacO gene of *Xanthomonas citri* ssp. *citri* encodes a protein
 848 with a role in lipopolysaccharide biosynthesis, biofilm formation, stress tolerance and
 849 virulence. *Molecular Plant Pathology* **12**: 381-396.

850 Li L, Xu Z, Zhou Y, Li T, Sun L, Chen H and Zhou R (2011). Analysis on *Actinobacillus*
851 *pleuropneumoniae* LuxS regulated genes reveals pleiotropic roles of LuxS/AI-2 on
852 biofilm formation, adhesion ability and iron metabolism. *Microbial Pathogenesis* **50**:
853 293-302

854 Li L, Xu Z, Zhou Y, Sun L, Liu Z, Chen H and Zhou R (2012). Global effects of
855 catecholamines on *Actinobacillus pleuropneumoniae* gene expression. *PLoS One* **7**:
856 e31121.

857 Li L, Sun C, Yang F, Yang S, Feng X, Gu J, Han W, Langford PR and Lei L (2013).
858 Identification of proteins of *Propionibacterium acnes* for use as vaccine candidates to
859 prevent infection by the pig pathogen *Actinobacillus pleuropneumoniae*. *Vaccine* **31**:
860 5269-5275.

861 Li L, Zhu J, Yang K, Xu Z, Liu Z and Zhou R (2014). Changes in Gene Expression of
862 *Actinobacillus pleuropneumoniae* in Response to Anaerobic Stress Reveal Induction
863 of Central Metabolism and Biofilm Formation. *Journal of Microbiology* **52**: 473-481.

864 Li G, Xie F, Zhang Y, Bossé JT, Langford PR and Wang C (2015). Role of (p)ppGpp in
865 Viability and Biofilm Formation of *Actinobacillus pleuropneumoniae* S8. *PLoS ONE*
866 **10**: e0141501

867 Li Y, Cao S, Zhang L, Yuan, Lau GW, Wen Y, Wu R, Zhao Q, Huang X, Yan Q, Huang Y
868 and Wen X (2016a). Absence of TolC Impairs Biofilm Formation in *Actinobacillus*
869 *pleuropneumoniae* by Reducing Initial Attachment. *PLoS One* **11**: e0163364.

870 Li Y, Cao S, Zhang L, Lau GW, Wen Y, Wu R, Zhao Q, Huang X, Yan Q, Huang Y and
871 Wen X (2016b). A TolC-Like Protein of *Actinobacillus pleuropneumoniae* Is
872 Involved in Antibiotic Resistance and Biofilm Formation. *Frontiers in Microbiology*
873 **7**: 1618.

874 Li HS, Shin MK, Singh B, Maharjan S, Park TE, Kang SK, Yoo HS, Hong ZS, Cho CS and
 875 Choi YJ (2016c). Nasal immunization with mannan-decorated mucoadhesive
 876 HPMCP microspheres containing ApxIIA toxin induces protective immunity against
 877 challenge infection with *Actinobacillus pleuropneumoniae* in mice. *Journal of*
 878 *Controlled Release* **233**: 114-125.

879 Little DJ, Poloczek J, Whitney JC, Robinson H, Nitz M and Howell PL (2012). The
 880 structure- and metal-dependent activity of *Escherichia coli* PgaB provides insight into
 881 the partial de-N-acetylation of poly-beta-1,6-N-acetyl-D-glucosamine. *Journal of*
 882 *Biological Chemistry* **287**: 31126-31137

883 Loera-Muro V, Jacques M, Tremblay Y, Avelar-González F, Loera-Muro A, Ramírez E,
 884 Medina A, González H and Guerrero-Barrera AL (2013). Detection of *Actinobacillus*
 885 *pleuropneumoniae* in drinking water from pig farms. *Microbiology* **159**:536-544. doi:
 886 10.1099/mic.0.057992-0.

887 Loera-Muro A, Jacques M, Avelar-González FJ, Labrie J, Tremblay YD, Oropeza-Navarro
 888 R and Guerrero-Barrera A (2016). Auxotrophic *Actinobacillus pleuropneumoniae*
 889 grows in multispecies biofilms without the need for nicotinamide-adenine
 890 dinucleotide (NAD) supplementation. *BMC Microbiology*. **16**: 128.
 891 doi:10.1186/s12866-016-0742-3.

892 Lone AG, Deslandes V, Nash JH, Jacques M and Macinnes JI (2009). Modulation of gene
 893 expression in *Actinobacillus pleuropneumoniae* exposed to bronchoalveolar fluid.
 894 *PLoS One* **4**: e6139.

895 Lopez-Bermudez J, Quintanar-Guerrero D, Lara-Puente H, Tórtora-Perez J, Suárez F,
 896 Ciprián-Carrasco A and Mendoza S (2014). Oral immunization against porcine

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

899 Lu YC, Li MC, Chen YM, Chu CY, Lin SF and Yang WJ (2011). DNA
 900 vaccine encoding type IV pilin of *Actinobacillus pleuropneumoniae* induces strong
 901 immune response but confers limited protective efficacy against serotype 2 challenge.
 902 *Vaccine* **29**: 7740-7746.

903 MacInnes JI, Gottschalk M, Lone AG, et al. (2008). Prevalence of *Actinobacillus*
 904 *pleuropneumoniae*, *Actinobacillus suis*, *Haemophilus parasuis*, *Pasteurella*
 905 *multocida*, and *Streptococcus suis* in representative Ontario swine herds. *Canadian*
 906 *Journal of Veterinary Research* **72**: 242-248.

907 Merritt J, Qi F, Goodman SD, Anderson MH and Shi W (2003). Mutation of *luxS* affects
 908 biofilm formation in *Streptococcus mutans*. *Infection and Immunity* **71**: 1972-1979.

909 Morioka A, Shimazaki Y, Uchiyama M and Suzuki S (2016). Serotyping reanalysis of
 910 unserotypable *Actinobacillus pleuropneumoniae* isolates by agar gel diffusion test.
 911 *Journal of Veterinary Medical Science* **78**: 723-725. doi: 10.1292/jvms.15-0538.

912 Musken M, Di Fiore S, Dotsch A, Fischer R and Haussler S (2010). Genetic determinants
 913 of *Pseudomonas aeruginosa* biofilm establishment. *Microbiology* **156**:431-441.

914 Nadell CD, Drescher K, Wingreen NS and Bassler BL (2015). Extracellular matrix
 915 structure governs invasion resistance in bacterial biofilms. *ISME Journal* **9**: 1700-
 916 1709.

917 Nair N, Vinod V, Suresh MK, Vijayrajratnam S, Biswas L, Peethambaran R, Vasudevan
 918 AK and Biswas R (2015). Amidase, a cell wall hydrolase, elicits protective immunity
 919 against *Staphylococcus aureus* and *S. epidermidis*. *International Journal of*
 920 *Biological Macromolecules* **77**: 314-321. doi:10.1016/j.ijbiomac.2015.03.047

921 Nicolet J (1988). Taxonomy and Serological Identification of *Actinobacillus*
 922 *pleuropneumoniae*. *Canadian Veterinary Journal* **29**: 578-80.

923 O'May GA, Jacobsen SM, Longwell M, Stoodley P, Mobley HL and Shirtliff ME (2011).
 924 The high-affinity phosphate transporter Pst in *Proteus mirabilis* HI4320 and its
 925 importance in biofilm formation. *Microbiology* **155**: 1523-1535.

926 O'Reilly T and Niven DF (1986). Defining the metabolic and growth responses of porcine
 927 haemophili to exogenous pyridine nucleotides and precursors. *Journal of General*
 928 *Microbiology* **132**: 807-818.

929 Olsen I (2015). Biofilm-specific antibiotic tolerance and resistance. *European Journal of*
 930 *Clinical Microbiology & Infectious Diseases* **34**: 877-886

931 Olson ME, Ceri H, Morck DW, Buret AG and Read RR (2002). "Biofilm bacteria:
 932 formation and comparative susceptibility to antibiotics. *Canadian Journal of*
 933 *Veterinary Research* **66**: 86-92.

934 Opriessnig T, Giménez-Lirola LG and Halbur PG (2011). Polymicrobial respiratory disease
 935 in pigs. *Animal Health Research Review* **12**: 133-148.

936 Otto K and Silhavy TJ (2002). Surface sensing and adhesion of *Escherichia coli* controlled
 937 by the Cpx-signaling pathway. *Proc Natl Acad Sci USA* **99**: 2287-2292.

938 Peddayelachagiri BV, Paul S, Makam SS, Urs RM, Kingston JJ, Tuteja U, Sripathy MH
 939 and Batra HV (2014). Functional Characterization and Evaluation of In Vitro
 940 Protective Efficacy of Murine Monoclonal Antibodies BURK24 and BURK37
 941 against *Burkholderia pseudomallei*. *PLoS ONE* **9**: e90930.
 942 doi:10.1371/journal.pone.0090930

943 Perry MB, Angen O, Maclean LL, Lacouture S, Kokotovic B and Gottschalk M (2012). An
 944 atypical biotype I *Actinobacillus pleuropneumoniae* serovar 13 is present in North
 945 America. *Veterinary Microbiology* **156**: 403-410.

946 Peters BM, Jabra-Rizk MA, O'May GA, Costerton JW and Shirtliff ME (2012).
 947 Polymicrobial interactions: impact on pathogenesis and human disease. *Clinical*
 948 *Microbiology Reviews* **25**: 193-213.

949 Pohl S, Bertschinger HU, Frederiksen W and Mannheim W (1983). Transfer of
 950 *Haemophilus pleuropneumoniae* and the *Pasteurella haernolytica*-Like Organism
 951 Causing Porcine Necrotic Pleuropneumonia to the Genus *Actinobacillus*
 952 (*Actinobacillus pleuropneumoniae* comb. nov.) on the Basis of Phenotypic and
 953 Deoxyribonucleic Acid Relatedness. *International Journal of Systematic*
 954 *Bacteriology* **33**: 510-514.

955 Prouty AM, Schwesinger WH and Gunn JS (2002). Biofilm formation and interaction with
 956 the surfaces of gallstones by *Salmonella* spp. *Infection and Immunity* **70**: 2640-2649.

957 Ramjeet M, Cox AD, Hancock MA, Mourez M, Labrie J, Gottschalk M and Jacques M
 958 (2008). Mutation in the LPS outer core biosynthesis gene, galU, affects LPS
 959 interaction with the RTX toxins ApxI and ApxII and cytolytic activity of
 960 *Actinobacillus pleuropneumoniae* serotype 1. *Molecular Microbiology* **70**: 221-235.

961 Rioux S, Galarneau C, Harel J, Kobisch M, Frey J, Gottschalk M and Jacques M (2000).
 962 Isolation and characterization of a capsule-deficient mutant of *Actinobacillus*
 963 *pleuropneumoniae* serotype 1. *Microbial Pathogenesis* **28**: 279-289.

964 Sadilkova L, Nepereny J, Vrzal V, Sebo P and Osicka R (2012). Type IV fimbrial subunit
 965 protein ApxA contributes to protection against porcine pleuropneumonia. *Veterinary*
 966 *Research* **43**: 2.

967 Sandal I, Hong W, Swords WE and Inzana TJ (2007). Characterization and comparison of
 968 biofilm development by pathogenic and commensal isolates of *Histophilus somni*.
 969 *Journal of Bacteriology* **189**: 8179-8185.

970 Sárközi R, Makrai L and Fodor L (2015). Identification of a proposed new serovar of
 971 *Actinobacillus pleuropneumoniae*: Serovar 16. *Acta Veterinaria Hungarica* **63**: 444-
 972 450. doi: 10.1556/004.2015.041.

973 Schmidt C, Cibulski SP, Andrade CP, Teixeira TF, Valera APM, Scheffer CM, Franco AC,
 974 Almeida LL and Roehle PM (2016). Swine influenza virus and association with the
 975 porcine respiratory disease complex in pig farms in southern Brazil. *Zoonoses and*
 976 *Public Health* **63**: 234-240.

977 Schwartz K, Stephenson R, Hernandez M, Jambang N and Boles BR (2010). The use of
 978 drip flow and rotating disk reactors for *Staphylococcus aureus* biofilm analysis.
 979 *Journal of Visualized Experiments* **46**: e2470.

980 Serrano L, Tenorio-Gutiérrez V, Suárez F, Reyes-Cortés R, Rodríguez-Mendiola M, Arias-
 981 Castro C, Godínez-Vargas D and de la Garza M (2008). Identification of
 982 *Actinobacillus pleuropneumoniae* biovars 1 and 2 in pigs using a PCR assay.
 983 *Molecular and Cellular Probes* **22**: 305-312.

984 Shahrooei M, Hira V, Khodaparast L, Khodaparast L, Stijlemans B, Kucharíková S,
 985 Burghout P, Hermans PWM and Elderea JV (2012). Vaccination with SesC
 986 Decreases *Staphylococcus epidermidis* Biofilm Formation. *Infection and Immunity*
 987 **80**: 3660-3668.

988 Shao M, Wang Y, Wang C, Guo Y, Peng Y, Liu J, Li G, Liu H and Liu S (2010).
 989 Evaluation of multicomponent recombinant vaccines against *Actinobacillus pleuropn*
 990 *eumoniae* in mice. *Acta Veterinaria Scandinavica* **52**: 52.

991 Shin MK, Jung MH, Lee WJ, Choi PS, Jang YS and Yoo HS (2011). Generation of
 992 transgenic corn-derived *Actinobacillus pleuropneumoniae* ApxIIA fused with the
 993 cholera toxin B subunit as a vaccine candidate. *Journal of Veterinary Science* **12**:
 994 401-403.

995 Shope RE (1964). Porcine Contagious Pleuropneumonia. I. Experimental Transmission,
 996 Etiology, and Pathology. *Journal of Experimental Medicine* **119**: 357-68.

997 Shope RE, White DC and Leidy G (1964). Porcine Contagious Pleuropneumonia. II.
 998 Studies of the Pathogenicity of the Etiological Agent, Hemophilus
 999 pleuropneumoniae. *Journal of Experimental Medicine* **119**: 369-375.

1000 Sjölund M and Wallgren P (2010). Field experience with two different vaccination
 1001 strategies aiming to control infections with *Actinobacillus pleuropneumoniae* in a
 1002 fattening pig herd. *Acta Veterinaria Scandinavica* **2010**: 52:23.

1003 Speziale P, Pietrocola G, Foster T and Geoghegan J (2014). Protein-based biofilm matrices
 1004 in Staphylococci. *Frontiers in Cellular and Infection Microbiology* **4**: 171.

1005 Stewart PS and Franklin MJ. Physiological heterogeneity in biofilms (2008). *Nature*
 1006 *Review Microbiology* **6**: 199-210.

1007 Subashchandrabosea S, Leveque RM, Kirkwoodc RN, Kiupela M and Mulksa MH (2013).
 1008 *Infection and Immunity* **81**: 2952-2961.

1009 Tegetmeyer H, Fricke K, and Baltes N (2009). An isogenic *Actinobacillus*
 1010 *pleuropneumoniae* AasP mutant exhibits altered biofilm formation but retains
 1011 virulence. *Veterinary Microbiology* **137**: 392-396.

1012 Theoret JR, Cooper KK, Zekarias B, Roland KL, Law BF, Curtiss R and Joensa LA (2012).
 1013 The *Campylobacter jejuni* Dps Homologue Is Important for *In Vitro* Biofilm

1014 Formation and Cecal Colonization of Poultry and May Serve as a Protective Antigen
 1015 for Vaccination. *Clinical and Vaccine Immunology* **19**: 1426-1431.

1016 To H, Nagai S, Iwata A, Koyama T, Oshima A and Tsutsumi N (2016). Genetic and
 1017 antigenic characteristics of ApxIIA and ApxIIIA from *Actinobacillus*
 1018 *pleuropneumoniae* serovars 2, 3, 4, 6, 8 and 15. *Microbiology and Immunology* **60**:
 1019 447-458.

1020 Tremblay YDN, Deslandes V and Jacques M (2013a). *Actinobacillus pleuropneumoniae*
 1021 genes expression in biofilms cultured under static conditions and in a drip-flow
 1022 apparatus. *BMC Genomics* **14**: 364.

1023 Tremblay YDN, Lévesque C, Segers RP and Jacques M (2013b). Method to grow
 1024 *Actinobacillus pleuropneumoniae* biofilm on a biotic surface. *BMC Veterinary*
 1025 *Research* **9**: 213.

1026 Tremblay YDN, Labrie J, Chénier S and Jacques M (2017). *Actinobacillus*
 1027 *pleuropneumoniae* grows as aggregates in the lung of pigs: is it time to refine our in
 1028 vitro biofilm assays? *Microbial Biotechnology*. 10: 756-760. doi: 10.1111/1751-
 1029 7915.12432.

1030 Turni C, Singh R, Schembri MA and Blackall PJ (2014). Evaluation of a multiplex PCR to
 1031 identify and serotype *Actinobacillus pleuropneumoniae* serovars 1, 5, 7, 12 and 15.
 1032 *Letters in Applied Microbiology* **59**: 362-369. doi: 10.1111/lam.12287.

1033 Vogt SL and Raivio TL (2012). Just scratching the surface: an expanding view of the Cpx
 1034 envelope stress response. *FEMS Microbiology Letters* **326**: 2-11. doi: 10.1111/j.1574-
 1035 6968.2011.02406.x.

1036 Vogt MC, Schraner EM, Aguilar C and Eichwald C (2016). Heterologous expression of
1037 antigenic peptides in *Bacillus subtilis* biofilms. *Microbial Cell Factories* **15**: 137. doi:
1038 10.1186/s12934-016-0532-5

1039 Wang L, Vinogradov EV and Bogdanove AJ (2013). Requirement of the
1040 lipopolysaccharide O-chain biosynthesis gene *wxocB* for type III secretion and
1041 virulence of *Xanthomonas oryzae* pv. *Oryzicola*. *Journal of Bacteriology* **195**: 1959-
1042 1969. doi:10.1128/JB.02299-12

1043 Wang L, Qin W, Yang S, Zhai R, Zhou L, Sun C, Pan F, Ji Q, Wang Y, Gu J, Feng X, Du
1044 C, Han W, Langford PR and Lei L (2015). The Adh adhesin domain is required for
1045 trimeric autotransporter Apa1-mediated *Actinobacillus pleuropneumoniae* adhesion,
1046 autoaggregation, biofilm formation and pathogenicity. *Veterinary Microbiology* **177**:
1047 175-83.

1048 Wang L, Qin W, Zhang J, Bao C, Zhang H, Che Y, Sun C, Gu J, Feng X, Du C, Han W,
1049 Richard PL and Lei L (2016). Adh enhances *Actinobacillus pleuropneumoniae*
1050 pathogenicity by binding to OR5M11 and activating p38 which induces apoptosis of
1051 PAMs and IL-8 release. *Scientific Reports* **6**: 24058. doi: 10.1038/srep24058.

1052 Willems HM, Xu Z and Peters BM (2016). Polymicrobial Biofilm Studies: From Basic
1053 Science to Biofilm Control. *Current Oral Health Reports* **3**: 36-44.

1054 Wu C, Labrie J, Tremblay YD, Haine D, Mourez M and Jacques M (2013). Zinc as an
1055 agent for the prevention of biofilm formation by pathogenic bacteria. *Journal of*
1056 *Applied Microbiology* **115**: 30-40.

1057 Xiao L, Zhou L, Sun C, Feng X, Du C, Gao Y, Ji Q, Yang S, Wang Y, Han W, Langford
1058 PR and Lei L (2012). Apa is a trimeric autotransporter adhesin of *Actinobacillus*

1059 *pleuropneumoniae* responsible for autoagglutination and host cell adherence. *Journal*
1060 *of Basic Microbiology* **52**: 598-607.

1061 Xie F, Zhang Y, Li G, Liu S and Wang C (2013). The ClpP protease is required for the
1062 stress tolerance and biofilm formation in *Actinobacillus pleuropneumoniae*. *PLoS*
1063 *One* **8**: e53600.

1064 Xie F, Li G, Zhang W, Zhang Y, Zhou L, Liu S, Liu S and Wang C (2016a). Outer
1065 membrane lipoprotein VacJ is required for the membrane integrity, serum resistance
1066 and biofilm formation of *Actinobacillus pleuropneumoniae*. *Veterinary Microbiology*
1067 **183**: 1-8.

1068 Xie F, Li G, Zhang Y, Zhou L, Liu S, Liu S and Wang C (2016b). The Lon protease
1069 homologue LonA, not LonC, contributes to the stress tolerance and biofilm formation
1070 of *Actinobacillus pleuropneumoniae*. *Microbial Pathogenesis* **93**: 38-43. doi:
1071 10.1016/j.micpath.2016.01.009.

1072 Yan L, Zhang L, Ma H, Chiu D and Bryers JD (2014). A Single B-repeat of
1073 *Staphylococcus epidermidis* accumulation-associated protein induces protective
1074 immune responses in an experimental biomaterial-associated infection mouse model.
1075 *Clinical and Vaccine Immunology* **21**: 1206-1214. doi:10.1128/CVI.00306-14

1076 Yang F, Ma Q, Lei L, Huang J, Ji Q, Zhai R, Wang L, Wang Y, Li L, Sun C, Feng X and
1077 Han W (2014). Specific humoral immune response induced by *Propionibacterium*
1078 *acnes* can prevent *Actinobacillus pleuropneumoniae* infection in mice. *Clinical and*
1079 *Vaccine Immunology* **21**: 407-416.

1080 Yi L, Wang Y, Ma Z, Lin HX, Xu B, Grenier D, Fan HJ and Lu CP (2016). Identification
1081 and characterization of a *Streptococcus equi* ssp. *Zooepidemicus* immunogenic

1082 GroEL protein involved in biofilm formation. *Veterinary Research* **47**: 50. doi:
1083 10.1186/s13567-016-0334-0
1084

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
<i>Acinetobacter baumannii</i>	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 <i>et al.</i> , 2011.
<i>Acinetobacter baumannii</i>	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 <i>et al.</i> , 2015.
<i>Bordetella pertussis</i>	Whooping cough or pertussis.	Bordetella intermediate protein A (BipA).	de Gouw <i>et al.</i> , 2014.
<i>Burkholderia pseudomallei</i>	The causative agent of melioidosis (category B select agent).	mAbs namely BURK24 and BURK37.*	Peddayelachagiri <i>et al.</i> , 2014.
<i>Campylobacter jejuni</i>	Food-borne bacterial gastroenteritis.	Oral vaccination with a recombinant attenuated <i>Salmonella enterica</i> strain synthesizing the <i>C. jejuni</i> Dps protein.	Theoret <i>et al.</i> , 2012.
<i>Enterococcus faecalis</i>	Cause catheter-associated urinary tract infections.	Heteropolymeric surface long hair-like fiber known as the endocarditis-and biofilm-	Flores-Mireles <i>et al.</i> , 2014.

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

				by the 79-aa half repeat (AapBrpt1.5).	
<i>Staphylococcus epidermidis</i>	Medical implants associated infections.			Vaccination with a recombinant truncated SesC (hypothetical LPXTG motif-containing proteins).	Shahrooei <i>et al.</i> , 2012.
<i>Staphylococcus epidermidis</i>	Medical implants associated infections.			Accumulation associated protein (Aap).	Yan <i>et al.</i> , 2014
<i>Streptococcus mutans</i>	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 <i>et al.</i> , 2013.
<i>Streptococcus equi ssp. zooepidemicus</i>	Opportunistic pathogen infecting a wide variety of animals and human.			Recombinant chaperonin GroEL protein.	Yi <i>et al.</i> , 2016.

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