Role of *Actinobacillus suis* adhesins in host-pathogen interactions in the tonsils of the soft palate of swine

by

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Tonsils are the immune system’s first line of defence against antigens entering the upper respiratory tract. Paradoxically, they are also an important site of colonisation and can be a portal of entry for many pathogens. Despite their significant role, virtually nothing is known about how bacteria attach to or the factors that control invasion of tonsils. To address these questions, I have used the gram-negative facultative anaerobe, *Actinobacillus suis*, as a model. *A. suis* is a common resident of swine tonsils, but it sometimes invades the bloodstream, leading to systemic disease and death. Using a combination of bioinformatic and expression studies, I have shown that *A. suis* expresses different adhesins in growth conditions that mimic the environment of the tonsils and the biofilm and planktonic stages of the bacterium that might lead to host invasion. Three knockout mutants were created to better understand mechanisms of attachment of *A. suis* to tonsils, and experiments to identify host molecules involved in infection were completed. I found that disrupting adhesins involved in attachment and cell integrity (*ompA*), attachment to host connective tissues (*comEI*), and biofilm formation (*flp1*) resulted in reduced binding of *A.
*A. suis* to purified extracellular matrix components (fibronectin, vitronectin, laminin, collagen I, collagen IV), as well as reduced its ability to form biofilms. Together, these studies provide insight into the pathogenesis of *A. suis* and other important invasive pathogens known to colonise the tonsils of swine and other species.
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Dedication

To my parents, Paul and Cynthia Bujold:

Mom, you have been unwaiveringlly behind me, at times cheering me on, at times holding me up, and often both in equal measure. The high personal bar that you set, your passion, and your selflessness inspire me so much. Thank you for being my first and most devoted cheerleader.

Dad, you have modelled the attributes of a scholar and person that I strive every day to possess: a fair and measured assessment of the facts, an unquenchable curiosity for knowledge, and the integrity to stand up and speak truth under all circumstances. Your calm demeanour, dry wit, and humour have grounded me and buoyed me, whichever was required at the time. Also, I suspect you set me on this path at the age of four, when we lay on the floor reading a Wrinkle in Time and learned about folding time. Now that my time has converged on this particular point, I share this accomplishment with you.

To my partner and best friend, Anne Laarman: I don’t know where to begin. I admire and look up to you in so many ways I can’t even enumerate. You inspire me, you ground me, you support me, and you love me. Thank you for sharing the load and walking beside me, now and always.
Declaration of Work Done

I declare that this thesis, entitled “Role of Actinobacillus suis adhesins in host-pathogen interactions in the tonsils of the soft palate of swine”, is entirely my own work and that where any material could be construed as the work of others, it is fully cited and referenced, with appropriate acknowledgement given. All experiments were designed and executed solely by me, with the following exceptions: screening additional A. suis isolates for the presence of adhesin genes by real-time PCR, where technical assistance was provided by Glenn Soltes; generating A. suis isogenic mutants, where technical assistance was provided by Sarah Walker; conducting biofilm assays, where technical assistance was provided by Josée Labrie; and preparing tonsil samples for mass spectrometry, where technical assistance was provided by Kearin Devlin.

Signature:

Name of Student: Adina R. Bujold

Name of advisor: Janet I. MacInnes
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<th>Definition</th>
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<tr>
<td>AALS</td>
<td>Anionic Acid Labile Surfactant</td>
</tr>
<tr>
<td>AMP</td>
<td>Antimicrobial peptide</td>
</tr>
<tr>
<td>ANI</td>
<td>Average nucleotide identity</td>
</tr>
<tr>
<td>AT</td>
<td>Autotransporter</td>
</tr>
<tr>
<td>BALF</td>
<td>Bronchoalveolar fluid</td>
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<tr>
<td>BAP</td>
<td>Blood agar plate</td>
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<tr>
<td>BHI</td>
<td>Brain heart infusion</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>Capsule serotype</td>
<td>K serotype</td>
</tr>
<tr>
<td>CEACAM1</td>
<td>Carcinoembryonic antigen-related cell adhesion molecule 1</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>Cm</td>
<td>Chloramphenicol resistant</td>
</tr>
<tr>
<td>CPS</td>
<td>Capsular polysaccharide</td>
</tr>
<tr>
<td>DAP</td>
<td>Diaminopimelic acid</td>
</tr>
<tr>
<td>DPS</td>
<td>DNA protection during starvation</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EmaA</td>
<td>Extracellular matrix protein adhesin A</td>
</tr>
<tr>
<td>FHA</td>
<td>Filamentous haemagglutinin</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence in situ hybridisation</td>
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LC-MS .......................................................... Liquid chromatography-mass spectrometry
LPS ......................................................................Lipopolysaccharide
M cell ...................................................................... Membrane or microfold cell
MALT ...................................................................... Mucosal-associated lymphoid tissue
MHC .................................................................. Major histocompatibility complex
mpa .........................................................................Minutes post-attachment
mpi ......................................................................... Minutes post-inoculation
NAD ........................................................................Nicotinamide adenine dinucleotide
NTHi ........................................................................ Nontypeable Haemophilus influenzae
O serotype .............................................................. Lipopolysaccharide O-chain serotype
OMP ......................................................................... Outer membrane protein
ONPG ........................................................................ o-Nitrophenyl-β-D-galactopyranoside
PBMEC ...................................................................... Porcine brain microvascular endothelial cell
PBS ........................................................................ Phosphate-buffered saline
PCR ........................................................................ Polymerase chain reaction
PGS ........................................................................ Poly-β-1,6-N-acetyl-D-glucosamine
PMN ......................................................................... Polymorphonuclear leucocyte
qPCR ........................................................................ Semi-quantitative real-time PCR
RGD motif ................................................................ Arginyl-glycyl-aspartic acid motif
SCOTS ..................................................................... Selective capture of transcribed sequences
SEM ........................................................................ Standard error of the mean
SJPL cell ................................................................... St. Jude porcine lung cell
SLRP ................................................................. Small leucine-rich proteoglycan
Sodium dodecyl sulphate .............................................................. SDS
STM ............................................................... Signature-tagged mutagenesis
TAA ................................................................. Trimeric autotransporter adhesin
tad ........................................................................ Tight adherence
TBS ................................................................. Tris-buffered saline
WT ........................................................................ Wild type
wt/vol ................................................................... Weight per volume
Chapter 1: Literature Review

1.1 Preamble

The porcine tonsils of the soft palate are not only involved in immune surveillance of foreign antigens entering the host, but are also a site of colonisation and entry for many bacterial and viral pathogens. In order to gain a better understanding of the pathogenesis of *Actinobacillus suis*, a common resident of the upper respiratory tract of swine and an important opportunistic pathogen, the porcine tonsils of the soft palate will be reviewed, as well as *A. suis*.

1.2 Tonsils

The tonsils are secondary lymphoid organs in the oral cavity that are part of the mucosal-associated lymphoid tissue (MALT). They form a ring in the pharyngeal wall, coined the Waldeyer ring (Perry and Whyte, 1998), and are comprised of clusters of lymphoid cells in the mucosa of the nasopharynx, the laryngopharynx and the oropharynx (Casteleyn et al., 2011a). This positioning at the entry of the digestive and respiratory tracts allows for immune surveillance of materials entering the body through the mouth or nares (Horter et al., 2003), and provides a first line of defense against foreign antigens (Casteleyn et al., 2011a). Paradoxically, many pathogens are able to invade the host through the tonsils.

1.2.1 Types of tonsils

In mammals, six types of tonsils, named for their anatomical locations, have been described: lingual, paraepiglottic, pharyngeal, tubal, palatine tonsils, and the tonsils of the soft palate (Figure 1.1). All but the palatine tonsils are present in swine. Some reports in the literature use
the term palatine tonsil synonymously with the tonsils of the soft palate in pigs; however, this is a misnomer (Casteleyn et al., 2011b). The lingual tonsil is located at the root of the tongue and possesses a few isolated tonsillar follicles. The paraepiglottic tonsils can be found bilaterally at the base of the epiglottis and also possess only a few tonsillar follicles. The pharyngeal tonsil is an irregularly raised tissue at the caudal end of the pharyngeal septum. The tubal tonsils are large and flat and can be found where the auditory tubes open into the pharynx. The tonsils of the soft palate are bilateral oval plaques found on the ventral side of the soft palate in pigs. The size of each tonsil of the soft palate in adult pigs is approximately 5 cm long by 3 cm wide, while in young pigs it is approximately 3 cm long by 2 cm wide (Belz and Heath, 1996). Trautmann and Fiebiger (1952) categorised the porcine palatine tonsils (tonsils of the soft palate), the tubal tonsils, and the paraepiglottic tonsils as follicular, and the pharyngeal tonsil as non-follicular; Horter et al. (2003) suggested that the lingual tonsil should also be classified as non-follicular. A more recent examination by Casteleyn et al. (2011a), however, found all porcine tonsils to be follicular, though to varying degrees, with the tonsils of the soft palate possessing by far the most follicles.

The tonsils of the soft palate are the largest and most developed tonsils of the pig and also the best studied. The rest of this review will focus on the tonsils of the soft palate and, for comparison, the functionally equivalent palatine tonsil in other species will be discussed.

1.2.2 Anatomy

On the ventral side of the soft palate of the pig along the oral cavity, there are between 160 and 180 circular or oval tonsillar fossules 90-500 µm in diameter that are oriented cranio-
caudally (Belz and Heath, 1996). These fossules are the openings into the tonsillar crypts that infiltrate the tonsils of the soft palate. The crypts run deep into the tonsil, branch extensively, and terminate blindly in the lymphoid tissue beneath (Belz and Heath, 1996; Horter et al., 2003). Figure 1.2 illustrates the anatomy of a tonsillar crypt.

The lymphoid follicles are closely associated with the crypts and are surrounded by diffuse lymphoid tissue (Casteleyn et al., 2011a). The follicles surrounding the crypts are separated from one another by connective tissue septa (Belz, 1998). Belz and Heath (1996) described the lymphoid tissue in the tonsils of the soft palate as belonging to one of three categories: lymphoid follicles, which are typically found within domed epithelial areas, parafollicular tissue, which separates adjacent follicles, or subepithelial lymphoid tissue, which connects to the basal interface of the epithelium.

Connective tissue permeates the tonsils of the soft palate (Belz and Heath, 1996) beneath the mucosal layer in the oral cavity as well as around the lymphatic vessels at the bottom of the tonsil (Belz, 1998). From the tonsillar fossules on the surface of the tonsil, the crypts penetrate a layer of dense connective tissue that segregates the epithelium of the mucosa from the immediately subjacent lymphoid tissue. This lymphoid tissue is comprised of several secondary lymphoid follicles. The top layer of connective tissue contours the crypts and diffusely penetrates the subepithelial connective tissue. A capsule made of connective tissue also surrounds the lymphoid tissue and contains lymphatic and blood vessels. In addition, the capsule is associated with the underlying skeletal muscle and, along with the connective tissue that underlies the
“palatine” mucosa, branches out into septa that further penetrate the lymphoid tissue. The connective tissue contains nerves, blood vessels, and lymphatic vessels (Horter et al., 2003).

1.2.3 Histology

1.2.3.1 Epithelium

The surface of the follicular tonsils such as the tonsils of the soft palate is covered with avascular, non-keratinised stratified squamous epithelium, as are the openings of the crypts (Trautmann and Fiebiger, 1952). This non-reticular epithelium is characterised by tightly-packed layers of cells with pyknotic flattened nuclei on the surface and flattened and polygonal cells with fine microplications on the luminal side (Belz and Heath, 1996). The upper epithelial layers of the tonsils of the soft palate stain positively for glycogen and contain cytoplasmic glycogen granules (Belz and Heath, 1996), which is thought to be consistent with the notion that they may be involved in the energetically costly processes of absorption and secretion (Perry, 1994).

Deeper within the tonsil, the crypts are lined with lymphoepithelium. This reticular layer is composed of squamous epithelium that is thinner than on the surface of the tonsil (Belz and Heath, 1996). Belz and Heath (1996) identified three different categories of epithelium in the lymphoepithelium. The first type, the epithelium overlying the apex of lymphoid follicles, is mainly made up of squamous epithelial cells with intermittent “membrane” or “microfold” cells (M cells) and is frequently infiltrated by lymphoid cells. The second type, the epithelium overlying the periphery of follicles, has a cobblestone-like appearance due to the protrusion of individual polygonal epithelial cells. A greater number of M cells, goblet cells, and intraepithelial lymphoid cells are present in this epithelium type. The third type of epithelium,
adjacent to the thin layer of connective tissue that supports the underlying subepithelial lymphoid tissue, is of variable thickness, between two and five layers, and contains cuboidal or columnar cells. These layers, along with goblet cells and a few intraepithelial lymphocytes, overlie the basal cell layer and are supported by a fairly complete basement membrane.

Belz and Heath (1996) also examined the fine structure of the epithelium of the tonsillar crypts and found it to resemble that of the epithelium overlying the lymphoid follicles, but lacking the substantial leucocyte infiltration observed in the latter region. These authors also described three distinct layers observed in the crypt epithelium. The basal layer is a single row of cells with cuboidal morphology and large nuclei, and is thought to support and adjoin the cells of the next layer. The intermediate layer contains larger cells that are polyhedral in shape and support the top layer. Unlike the other two layers, the superficial layer is not homogeneous, containing cells with both squamous and polygonal morphology. The squamous cells are elongated and form a layer, one to two cells thick, that separates the intraepithelial lymphocytes from the lumen of the crypt. Some polygonal cells are laced with blunt microplications while others have short microvilli. These folds and microvilli are thought to play a role in increasing available surface area for transport.

The lymphoepithelium contains numerous microscopic folds and is occasionally interrupted by M cells with adjacent goblet cells (Horter et al., 2003). The surface of the M cells contains microplications as well as glycocalyx-coated microvilli of varying sizes and orientations (Belz and Heath, 1996). Belz and Heath (1996) suggest that the varying sizes of the microvilli may be due to an ability of these cells to extend and retract into the crypt lumen in response to certain
macromolecules or antigenic stimulation, but this has not been substantiated. M cells adjoin their
neighbouring epithelial cells by desmosomes (Belz and Heath, 1996). They can be hard to
visualise because their close association with the surrounding epithelial cells often conceals them
in tissue depressions, and their morphology is variable (Belz and Heath, 1996). That said, they
are usually identifiable because they are typically found closely associated with intraepithelial
lymphocytes; however, there is currently no known cellular marker to unambiguously
differentiate these cells from the surrounding epithelium (Perry and Whyte, 1998).

Goblet cells are often found close to M cells in the middle and deeper regions of the crypts in
the tonsils of the soft palate of swine (Belz and Heath, 1996). They contain several mucous
granules and have sparse microvilli on their surface. They are found on the periphery of the
lymphoepithelium above the lymphoid follicles and are typically located among the polygonal
cells. Goblet cells produce mucus that aids in clearing debris from the crypts (Ramos et al.,
1992). Belz and Heath (1996) suggest that goblet cells may be present in swine, but absent in
many other domestic mammals. This, they posit, is because there is a necessity for extra
measures to clear out the far more numerous crypts present in the tonsils of the soft palate in
swine. While this seems possible, there is no evidence to support this hypothesis. These authors
further indicate that the close proximity of goblet cells to M cells hints at other important roles
for these cells, since previous research has found mucus release to be stimulated by immune
complexes and by antigen (Lake et al., 1979; Walker et al., 1977). However, those studies were
conducted in the intestine rather than the tonsil. Moreover, these data are consistent with removal
of debris and foreign material from the crypts as being the main function of goblet cells. Other
work by Bland et al. (1995) suggests that goblet cells are able to protect swine ileal epithelial
cells damaged by cytotoxins in swine dysentery; Belz and Heath (1996) suggest that this may also occur in the tonsil. Clearly, further work remains to be done in order to establish the roles of goblet cells and M cells in the tonsil.

1.2.3.2 Leucocyte populations

Several subpopulations of leucocytes are present in the tonsils of the soft palate. For example, professional phagocytes such as macrophages and neutrophils are found in the lymphoepithelium and tonsillar crypts of the palatine tonsils of humans (Yamamoto et al., 1988) as well as in the tonsils of the soft palate in pigs (Belz and Heath, 1996); however, there have been few enumeration studies. Berndt et al. (2000) reported that monoclonal antibodies against porcine macrophages are able to detect macrophages in follicles, the interfollicular area, and the subcapsular sinuses, but the type of tonsil used in this study was not specified. Dendritic cells have also been observed in pathogen-free pigs by immunohistochemistry and flow cytometry, and are primarily localised to the interfollicular area of the tonsil in close proximity to T cells (Jamin et al., 2006). Again it is unclear if this study made use of tissue from the tonsils of the soft palate. In addition, no work has been done in pigs with stimulated immune systems, so the proportion of dendritic cells present in a conventionally reared animal is unknown. In the tonsillar crypts of human palatine tonsils, Howie (1982) also observed a small number of mast cells; however, this cell type has not been demonstrated in swine tonsil.

There have been several studies that attempt to characterise the lymphocyte subpopulations in the porcine tonsils of the soft palate, but the results of these studies vary (Boeker et al., 1999; Salles and Middleton, 2000; Terzic et al., 2002). This variability may be attributed to differences
in the age, disease status or breed of the animals used, to name a few, but this has not yet been proven. Perry (1994), however, hypothesised that in the human palatine tonsil each crypt has its own microenvironment, with different antigenic stimulation, and as a result possesses different leucocyte populations. While this may be true, the various lymphocytes present in the tonsil mature away from the crypts, so a more fitting explanation may be that the population of lymphocytes present in a tonsillar crypt at any given time is a result of what foreign materials or antigens are present, though this remains to be demonstrated.

According to Horter et al. (2003), the lymphocyte population of the tonsillar microenvironment is comprised of approximately 75% T lymphocytes and just under 25% B lymphocytes in pigs. T cells are found primarily in the parafollicular regions of the tonsil, but can infiltrate the tonsillar crypt as well (Horter et al., 2003). In human palatine tonsils, Nave et al. (2001) reported that T cells are mainly found in the extrafollicular region of the tonsil and are primarily helper cells. In early studies, Zuckermann and Gaskins (1996) reported that CD4⁺CD8⁺ T cells are the dominant subpopulation in the tonsils of 6 to 7 month old pigs, with a slightly lower number of CD4⁺ and CD8⁺ T cells; γδ T cells were not quantified. In another study by Zuckermann and Husmann (1996), it was found that CD4⁺ T cells are present in the tonsils of 3 day old pigs, but CD4⁺CD8⁺ T cells are absent; again, γδ T cells were not quantified. Taken together, Zuckermann and Husmann (1996) and Zuckermann and Gaskins (1996) suggested that their findings imply that CD4⁺CD8⁺ T cells develop with age and represent mature, antigen-experienced cells. In later work by Boeker et al. (1999), it was found that among the T lymphocytes in the tonsils of 9 to 12 month old pigs, a fairly large proportion are CD8⁺ or CD4⁺CD8⁺ T cells, and a much smaller number are CD4⁺. The remainder of the T cell
population is comprised of γδ T cells. Salles and Middleton (2000) reported that in 6 month old pigs from a minimal disease herd, CD4+ cells are found abundantly in the perifollicular area as well as in the follicles, while γδ T cells are less numerous and are usually situated subjacent to the crypt epithelium, but rarely found in the follicles. Furthermore, these authors found CD8+ cells to be low in number, sparsely distributed in the crypt epithelium, and only sporadically observed in the perifollicular area and the follicles; CD4+CD8+ T cells were not quantified individually.

B cells in the tonsil are mainly found in the lymphoid follicles (Horter et al., 2003), but there are also immunoglobulin-secreting plasma cells in the tonsillar crypts (Belz and Heath, 1996). Of the approximately 25% of the lymphocyte population that B cells comprise, Boeker et al. (1999) reported that 14% of these cells express IgM antibody on their surface, while the remaining 10% of B cells are about 5% IgA- and 5% IgG-expressing cells. B cells expressing IgM are likely antigen-naïve cells that have not yet undergone isotype switching.

1.2.4 Role in immunity

The tonsils of the soft palate in swine are considered to be a first line of defense against foreign materials entering through the nose or mouth. As such, this site is equipped with both innate and adaptive immune components to fend off infection by both humoral and cellular means.

1.2.4.1 Innate immunity

The first barrier a foreign antigen encounters in the tonsil would likely be mucus. Mucus contains the glycoprotein mucin that can prevent bacteria from adhering to epithelial cells (Wu et
al., 1994). In the lymphoepithelium, goblet cells secrete mucus and possess microvilli that entrap foreign material in the tonsillar crypts (Belz and Heath, 1996).

The next barrier is the epithelium. In their description of the tonsils of the soft palate of swine, Belz and Heath (1996) describe the epithelial cells that line the surface and crypts of the tonsil as providing a physical barrier against the entry of pathogens into the host. While this may be true for some organisms, it is possible that certain pathogens are able to invade the epithelial cells and penetrate this line of defense, perhaps by means of the M cells.

The presence of complement has not been demonstrated in the tonsil, but Horter et al. (2003) speculate that it may be present based on the identification of complement activation components in secondary lymphoid follicles in humans (Zwirner et al., 1989). Activated complement components play a role in triggering inflammation and directing phagocytes via chemotaxis, in addition to lysing and opsonising bacteria (Cooper, 1985).

As previously mentioned, professional phagocytes, namely macrophages and neutrophils, have been found in the lymphoepithelium and the tonsillar crypts (Belz and Heath, 1996; Yamamoto et al., 1992). These cells phagocytose microorganisms they encounter, but also take part in adaptive immunity by presenting antigen to lymphocytes.

Neutrophils, other phagocytes, and mucosal epithelial cells can also synthesise cationic molecules called antimicrobial peptides (AMPs or CAMPs) that play a significant role in innate immunity (Zhang et al., 2000a). AMPs promote and regulate immune responses and stimulate chemotaxis for neutrophils and T cells (Hancock and Scott, 2000). They are also natural antibiotics with broad microbicidal activity (Zhang et al., 2000a). AMPs have not been studied in
porcine tonsils, but Horter et al. (2003) suggest that they may be found in the tonsils of the soft palate based on a study that identified a porcine AMP expressed in the epithelial cells that line the dorsal tongue and buccal mucosa (Shi et al., 1999), and in another study that characterised human β-defensin 1 in saliva coating the oral cavity, the tonsillar epithelium, and lymphoepithelium (Sahasrabudhe et al., 2000). Clearly, further work remains to be done in order to determine if AMPs are present in porcine tonsils.

1.2.4.2 Adaptive immunity

While the innate immune response provides immediate protection in a non-specific and broad spectrum manner, the adaptive immune response is antigen-specific and takes a minimum of 4 to 7 days to develop (Horter et al., 2003). In the tonsil, antigen that is not effectively cleared by the innate immune response is subject to the adaptive immune response. In studies conducted using human palatine tonsils, it has been found that, upon entering the tonsillar crypts, foreign antigen makes contact with the M cells of the lymphoepithelium and is transported across the epithelial barrier to the parafollicular area where it is sampled by antigen-presenting cells such as macrophages or dendritic cells (Brandtzaeg et al., 1999; Jahnsen and Brandtzaeg, 1999). Further studies into M cells and antigen presentation in the porcine tonsils would be valuable in elucidating the early steps of the adaptive immune response in swine.

It is believed that naïve B lymphocytes in human tonsils are stimulated in the extrafollicular area to undergo terminal differentiation to plasma cells (Jahnsen and Brandtzaeg, 1999). This process is thought to require the help of activated CD4+ T cells, but B cells are also able to interact with the native antigen directly using their own receptors (Jahnsen and Brandtzaeg,
In addition, a small amount of native antigen is thought to be retained on follicular dendritic cells in the germinal centres in order to maintain B cell memory (Brandtzaeg et al., 1999; Jahnsen and Brandtzaeg, 1999). Brandtzaeg et al. (1999) speculate that, once activated, emigration of B cells from germinal centres is mediated by chemokines, and that the memory B cells appear to migrate quickly to extrafollicular regions such as the tonsillar crypts, where it is thought that they can continue to present antigen and to survey the environment. While it is plausible that this may also occur in the porcine tonsils of the soft palate, no studies have confirmed these findings to date. However, early work in swine by Bradley et al. (1976) shows that IgA-containing cells are closely associated with the crypt epithelium while IgM- and IgG-containing cells are more generally distributed between the lymphoid follicles and are often found near the germinal centres. Again, a greater understanding of the roles of B cells in the porcine tonsil is needed.

T cells are thought to play an important regulatory role in adaptive immunity in the tonsils of the soft palate, as well as at the systemic level through cytokine production resulting in the activation and direction of lymphocytes, antigen-presenting cells, and neutrophils (Zuckermann, 1999). Salles and Middleton (2000) reported that the helper T CD4 cells and γδ T cells are the most abundant subsets of the T cell population within the tonsils of the soft palate in swine. CD4^+CD8^+ T cells are concentrated primarily in the parafollicular area of the tonsil, with many cells in the follicles (Salles and Middleton, 2000), and they likely constitute a mature memory cell population (Pescovitz et al., 1994; Zuckermann and Husmann, 1996). Contrary to findings by Salles and Middleton (2000), CD4^+ cells, or helper T cells, are reported to constitute a very small proportion of the T cell population (Boeker et al., 1999). These differing results may be
partially due to the fact that the methods used by Salles and Middleton (2000) did not
differentiate CD4+ cells from CD4+CD8+ cells. Previous work suggests that antigenic stimulation
of CD4+ T cells causes them to become CD4+CD8+ T cells (Pescovitz et al., 1994; Saalmuller et
al., 1987). This is supported by evidence that although the tonsils of 3 day old pigs are
predominantly populated with CD4+ cells, this population shifts towards CD4+CD8+ cells with
age and antigen exposure (Pescovitz et al., 1994; Zuckermann and Husmann, 1996). This
assertion is further supported by the fact that CD4+CD8+ cells can recognise antigen that the host
has previously encountered (Zuckermann and Husmann, 1996).

Based on a study using 6 month old pigs, approximately half of the T cell population found
in the crypt epithelium is comprised of γδ T cells (Salles and Middleton, 2000). Unlike CD4 and
CD8 cells, γδ T cells do not require antigen-processing for recognition, nor do they require major
histocompatibility complex (MHC) presentation (Salles and Middleton, 2000). Furthermore, γδ T
cells may have a regulatory role in the early immune response to an infection, earlier than CD4
and CD8 cell activation, by releasing cytokines (Boismenu and Havran, 1997). In their review,
these authors further note that γδ T cells recognise antigens found on both stressed host epithelial
cells and on microorganisms. These findings would explain their abundance in the tonsillar
crypts where microorganisms and, presumably, epithelial cell damage abound.

Relatively little work has been done on CD8+ T cells in porcine tonsils beyond preliminary
enumeration studies. CD8+ cells, or cytotoxic T cells, are found in low numbers in the crypt
epithelium, the perifollicular area, and the follicles of 6 month old pigs (Salles and Middleton,
2000). Quantification of these cells can be easily overestimated since other cells such as natural
killer cells and even some γδ T cells may also possess the CD8 receptor at low densities (Salles and Middleton, 2000). In order to gain a greater understanding of the proportion of lymphocyte subsets in porcine tonsils, the impact of confounding variables such as age, breed, health status, and inter-animal variation must be carefully assessed.

### 1.2.5 Role in disease

Despite their role in immunity, the tonsils of the soft palate have also been identified as a portal of entry for microbes as well as a site of replication, persistence, and infection. Bacteria persist mainly in the tonsillar crypts, while viruses are more often localised to the lymphoepithelium and the lymphoid follicles (Horter et al., 2003). Within the crypts, bacteria are often found as communities, and it is postulated resources are pooled, allowing microorganisms to persist in the inhospitable host environment.

Both commensal and pathogenic organisms have been identified in the tonsils of the soft palate (Kernaghan et al., 2012). Indeed, organisms that are normally considered to be commensals may, under the right conditions, become pathogens. For example, in high health status herds, because the animals encounter fewer potentially pathogenic microbes compared to conventional herds, they are immunologically naïve and are therefore often more susceptible to diseases caused by bacteria that are typically considered to be commensals (Patrick and Larkin, 1995). It has been suggested that this may explain the recent emergence of *A. suis* disease among high health status herds.

The microbial community of the porcine tonsils has received some attention in recent years, although much remains to be done. The characterisation of bacterial communities can be done
using culture-dependent as well as culture-independent techniques. Baele et al. (2001) characterised the gram-positive microflora of healthy piglets using culture-dependent methods and found that pig tonsils contain streptococci and staphylococci before and after weaning, as well as increased numbers of lactobacilli after weaning. Other species such as *Trueperella pyogenes* and *Actinomyces hyovaginalis* were also identified. While this study provided some insight into the tonsil microbiome of several pigs, findings were limited both by the scope of the study (i.e., healthy animals only, gram-positives only) and by the sole use of culture-dependent methods.

In recent work by Lowe et al. (2011), both culture-dependent and -independent methods were used although the results from the different methods were somewhat dissimilar. Using culture-dependent methods, the authors identified a number of streptococci, enterococci, and staphylococci, similar to findings by Baele et al. (2001), as well as gram-negative isolates belonging to the families *Enterobacteriaceae* and *Pasteurellaceae*. In the same study, using culture-independent methods that relied on 16S rRNA sequence libraries, many of the same members of *Enterobacteriaceae* and *Pasteurellaceae* were observed, but very few gram-positive cocci were identified. Conversely, other genera were observed in the culture-independent method that were not found by culture at least in part because anaerobic culture was not done and the tonsillar crypts are likely to be low oxygen environments. These findings emphasise the variability of results depending on the method used and draw attention to the difficulty of definitively characterising a microbial population using just one technique. In addition, it is worth mentioning that this study was conducted using the tonsils of healthy pigs from two herds—one high health status herd and one conventional herd—and that the presence and
prevalence of microbes in each of these herds was quite different. For example, Actinobacillus species were the most prevalent in the high health status herd, while Pasteurella species were the most prevalent in the other herd. Finally, this study used a very small sample size, making it difficult to reliably extrapolate findings. The authors suggest that this study may point to the effects of management practices on the microbial community. While this hypothesis is intriguing and certainly plausible, a larger sample size is needed in order to support or refute such a statement. Further work in this area would provide a better idea of the normal flora of the tonsil, which would in turn allow for a better understanding of the organisms associated with healthy and diseased animals.

In other recent work by O’Sullivan et al. (2011), the tonsils of the soft palate from normal and abnormal swine carcasses were screened for pathogens at slaughter using a culture-dependent method. These authors also found streptococcal species to be frequently present, most notably the opportunistic pathogen Streptococcus suis. They also reported Trueperella pyogenes and Pasteurella multocida as common residents of the tonsils of the soft palate in pigs. While this study identified some streptococci and staphylococci as being more prevalent in the tonsils from carcasses of abnormal animals, they point out that carcasses could be deemed abnormal for any number of reasons, including ones that may be irrelevant to disease status. While this work identified some of the same organisms found in the study by Lowe et al. (2011), such as members of the family Pasteurellaceae, other organisms, namely members of the family Enterobacteriaceae, were not detected. It is unclear whether these organisms were not detected simply because they were not screened for, or because they were indeed absent from the tonsils.
Together, these studies demonstrate the need for further study of the tonsillar microbiome in order to gain a greater understanding of how pathogenic and commensal bacteria interact with the tonsil and how this tissue may play a role in health and in disease. Moreover, it is evident that thorough characterisation of microbial communities likely requires the use of a variety of techniques, both culture-dependent and -independent to obtain an accurate representation of the bacterial populations present. In the future, it would also be useful to employ techniques such as fluorescence in situ hybridisation (FISH) or immunohistochemical analyses that would allow for the localisation of the various bacteria to specific regions of the tonsil to better characterise host-pathogen interactions.

1.3 *Actinobacillus suis*

1.3.1 Characteristics

*Actinobacillus* species are small gram-negative pleomorphic coccobacillary rods that are nonsporulating and nonmotile. They are facultative anaerobes that grow best in the presence of 5% CO$_2$ and have an optimum growth temperature of 37°C. Members of this genus are urease and β-galactosidase positive, indole negative, and are able to reduce nitrates. These bacteria have complex nutritional requirements and apart from *A. pleuropneumoniae* and some strains of *A. suis* (MacInnes and Lally, 2006), most are able to grow on MacConkey agar. Members of the genus *Actinobacillus* are associated with mucous membranes of the respiratory, alimentary, and genital tracts of their respective hosts, and their host range is usually limited. While typically considered to be commensals, members of this genus are also able to cause disease, particularly in domestic animals.
In addition to the above mentioned characteristics, *A. suis* is typically able to grow on slightly simpler substrates than other *Actinobacillus* species. It is catalase, oxidase, esculin, alkaline phosphatase, and o-Nitrophenyl-β-D-galactopyranoside (ONPG) positive, as well as β-haemolytic on blood agar, although less so than *A. pleuropneumoniae* (Van Ostaaijen et al., 1997). In addition, it is nicotinamide adenine dinucleotide (NAD) independent and can ferment xylose, sucrose, glucose, maltose, D-mannose, lactose, and trehalose, but is not able to ferment mannitol. *A. suis* produces small, sticky colonies that become stickier with prolonged incubation (MacInnes and Lally, 2006).

### 1.3.2 Taxonomy

*A. suis* is a member of the family *Pasteurellaceae*. Recent (phylo)genetic investigations suggest that the main genera of the family (*Actinobacillus, Haemophilus, Pasteurella, and Mannheimia*) are scattered among different clades as a result of several species being named based on phenotypic characteristics such as serology and nutritional requirements (Christensen et al., 2007). As a result, the taxonomy of the family *Pasteurellaceae* has been undergoing revision, and new guidelines for this family have been proposed (Christensen et al., 2007). As of 2010, the family *Pasteurellaceae* was comprised of 64 other named species belonging to 13 genera (Bonaventura et al., 2010); however, genera and species continue to be described (Hansen et al., 2016).

The genus *Actinobacillus* has undergone revision in recent years. The ninth edition of Bergey’s Manual of Determinative Bacteriology listed the following species as belonging to the genus *Actinobacillus*: *A. actinomycetemcomitans, A. capsulatus, A. equuli, A. hominis, A.*
lignieresii, A. muris, A. rossi, A. seminis, A. ureae, A. pleuropneumoniae and A. suis (Bergey and Holt, 1994). In 1996, A. delphinicola, A. minor, A. porcinus, and A. indolicus were added to the genus (Foster et al., 1996; Moller et al., 1996). Over the years, other species came to be included as well, but the guidelines for inclusion were poorly defined as a result of overlapping genotypic and phenotypic characteristics among isolates. In 2004, Christensen and Bisgaard proposed a revised list of members for the genus *Actinobacillus* based on a combination of DNA-DNA hybridisation, phenotypic analysis and 16S rRNA sequence-based phylogenetic results.

According to this work, *Actinobacillus sensu stricto* should be restricted to a monophyletic group that includes *A. lignieresii*, *A. equuli* subsp. *equuli*, *A. equuli* subsp. *haemolyticus* (formerly taxon 11 of Bisgaard), *A. hominis*, *A. ureae*, *A. arthitidis* (formerly taxon 9 of Bisgaard), *A. pleuropneumoniae*, *A. suis*, *Actinobacillus* genomospecies 1 and 2, and the taxa 8 and 26 of Bisgaard. The authors go on to suggest that isolates formerly classified as *Actinobacillus* species but not belonging to the *sensu stricto* group should be assigned to other genera.

Among the *Actinobacillus* species, there has been some confusion as to the taxonomic positions of *A. equuli* and *A. suis*. Despite strong host specificity, *A. equuli* isolates have been reported in swine and *A. suis* isolates in horses. In the past, these organisms were found to be nearly indistinguishable due to similarities in outer membrane proteins, antigenic cross-reactivity, and 16S rRNA profiles. Confusion was further created by some isolates being haemolytic while others were not. Based on 16S rRNA and DNA-DNA hybridisation results, Christensen et al. (2002) proposed that *A. equuli* be subdivided into *A. equuli* subsp. *equuli* and *A. equuli* subsp. *haemolyticus*. Work by Berthoud et al. (2002) supported this division by finding two distinct 16S rRNA groups among *A. equuli* isolates examined and by determining that all
haemolytic strains possess the RTX toxin-encoding \textit{aqx} operon. Even though their 16S rRNA gene sequence is 98\% identical (Dewhirst et al., 1992), Kuhnert et al. (2003) determined that representative haemolytic isolates of \textit{A. suis} and \textit{A. equuli} are distinguishable based on their RTX toxins, where \textit{A. suis} possesses \textit{apx} genes, while haemolytic \textit{A. equuli} possesses the \textit{aqx} genes. In recent studies, Huang et al. (2015) reported that the \textit{A. equuli} subsp. \textit{equuli} 16S genes are 99\% identical to those of both \textit{A. suis} H91-0380 and the \textit{A. suis} type strain ATCC 33415, and that the genome structure of \textit{A. equuli} subsp. \textit{equuli} is very similar to that of \textit{A. suis}, again suggesting that \textit{A. equuli} subsp. \textit{equuli} and \textit{A. suis} are the same species. However, when genomes of the two \textit{A. suis} strains were compared with that of \textit{A. equuli} subsp. \textit{equuli}, the average nucleotide identity (ANI) value of both comparisons was found to be 93.82\%, which is lower than the recommended cutoff value of 95\% for delineating species. \textit{In silico} DNA-DNA hybridisation studies done using a Genome Blast Distance Phylogeny also demonstrated differences between \textit{A. equuli} and \textit{A. suis}. Based on these analyses, these authors concluded that \textit{A. suis} and \textit{A. equuli} subsp. \textit{equuli} are related but distinct species, and that care must be taken to identify them correctly.

In addition, there have been many other reports of non-porcine isolates of \textit{A. suis}-like organisms apart from the supposed equine isolates. Such early reports are typically of sporadic disease in a variety of birds and mammals, and without proper genotypic characterisation, these claims cannot be confirmed. However, there is evidence that some strains of \textit{A. suis} are able to infect mammals other than pigs (Christensen and Bisgaard, 2004; Jeannotte et al., 2002), including a recent report of a case of meningitis and sepsis in a human, caused by \textit{A. suis} or \textit{A. equuli} (Montagnani et al., 2015).
1.3.3 Epidemiology

*A. suis* is a commensal bacterium found in the nasal cavity and tonsils of healthy pigs of all ages, and also colonises the vaginas of apparently healthy sows (Ross et al., 1972). A recent study by MacInnes et al. (2008) revealed that *A. suis* is likely present in almost all Ontario herds. Since its original description in 1962, sporadic cases of infections caused by *A. suis* have been reported, primarily manifesting as septicaemia in suckling or recently weaned pigs (MacInnes, 2010; Taylor, 2006). However, over the past 30 years, there has been a shift in pig production from smaller to larger operations, and from conventional herds to minimal disease or high health status herds (Miniats et al., 1989; Sanford et al., 1990). It is suspected that these changes in management have resulted in the increasing number of severe outbreaks of *A. suis* disease (Bilkei, 1995). Since the beginning of the 1990s, a number of severe *A. suis* outbreaks in pigs of all ages have been reported in Canada, the United States, and Australia (MacInnes and Desrosiers, 1999; Taylor, 2006; Wilson and McOrist, 2000), as well as some reports of abortion in sows in different parts of Europe (Bilkei, 1995; Mauch and Bilkei, 2004). It has been speculated that the exclusion of many primary pathogens from high health status herds has allowed for commensal organisms such as *A. suis* to assume a more pathogenic role (MacInnes and Desrosiers, 1999; Patrick and Larkin, 1995). Furthermore, current rearing practices can result in largely immunologically naïve herds, which in turn leads to more severe disease when an organism is first encountered because the immune system is not primed to ward off infection (MacInnes and Lally, 2006). However, it has been found that once herd immunity against an organism has been established, there is a decrease in the number of subsequent outbreaks (Taylor, 2006).
Early work on A. suis collected from both healthy and diseased pigs suggested a population with little diversity based on genotypic and phenotypic results (Bada et al., 1996; Van Ostaaijen et al., 1997). Later studies conducted by Slavic et al. (2000a; 2000b) demonstrated the presence of two O (lipopolysaccharide O-chain) serotypes of A. suis and at least three K (capsule) types. The O2 serotype was associated with strains thought to be more virulent (Slavic et al., 2000a); however, further studies suggested that the capsular polysaccharide (CPS) may play an even greater role than the O-antigen in determining pathogenicity (Deutschmann et al., 2011; Slavic et al., 2000a; Slavic et al., 2000b).

In a study by Slavic et al. (2000b), the prevalence of the various A. suis serotypes was assessed among isolates collected from healthy and diseased pigs from several locations in North America, and it was revealed that the O2:K3 strains were more common in Quebec and Western Canada while the less virulent O1:K1 strains were more often found in Ontario. The O1:K1 and O2:K3 strains were both found in equal prevalence among isolates from Kansas. This was not a systematic study and more strains, especially from healthy pigs, are needed before definitive conclusions can be drawn. In addition, the studies by Slavic et al. (2000a; 2000b) revealed that cross-reactivity among surface antigens of A. suis precludes development of simple slide agglutination tests and makes serological characterisation of this organism largely uninformative except by immunoblotting.

1.3.4 Transmission, disease, and control

Transmission of A. suis infection occurs via aerosol or close contact between uninfected animals and asymptomatic carriers or diseased animals. The organism is also able to gain entry through bite wounds and open sores (e.g., following castration). In neonates and suckling pigs, A.
* suis can cause an acute septicaemia that may be fatal within 15 hours (MacInnes, 2010; MacInnes and Lally, 2006). The most common signs of disease in affected animals include sudden death, fever, respiratory distress, neurological disturbances, petechial haemorrhage, cyanosis, and lameness (MacInnes and Desrosiers, 1999). Mortality is less common in weaned pigs, and the disease is typically less severe. These animals are often poor doers and may present with fever, persistent cough, and anorexia (MacInnes, 2010). Mature animals with *A. suis* disease often exhibit signs that are easily confused with erysipelas such as erythematous skin lesions and inappetance, as well as metritis and abortion in sows (Miniats et al., 1989). Moreover, sequelae to septicaemia may develop in older animals including pneumonia, meningitis, and arthritis (MacInnes and Desrosiers, 1999; Yaeger, 1996).

Once *A. suis* enters the bloodstream, it can form microcolonies on vessel walls, which leads to localised haemorrhage and necrosis. In this instance, gross lesions are often seen in the skin, lungs, heart, kidneys, intestines, and spleen of affected animals (MacInnes, 2010; Taylor, 2006). The lungs can also fill with serous or serofibrinous exudate, causing them to resemble those of animals with pleuropneumonia (MacInnes, 2010; Yaeger, 1996). The mechanism by which *A. suis* reaches the bloodstream is poorly understood, but once in the host it is thought that capsule, LPS, toxin production, and serum resistance assist the organism in evading host responses.

Control of *A. suis* disease outbreaks relies heavily on good management practices (Maes et al., 2001). Presently there are no commercial vaccines; however, autogenous bacterins have been used with varying degrees of success (Lapointe et al., 2001; Taylor, 2006). It has been postulated that some *A. pleuropneumoniae* vaccines may provide cross-protection against *A. suis* infections due to cross-reactivity between the Apx toxins and outer membrane proteins of the two species.
(MacInnes and Rosendal, 1987). *A. suis* isolates are typically sensitive to antibiotics; however, due to the often sudden onset of disease in infected animals, antimicrobial therapy is largely impractical (MacInnes, 2010; Taylor, 2006). In older pigs, where disease does not usually lead to death, treatment with penicillin, ampicillin, tetracycline, or streptomycin has proven quite effective (Taylor, 2006). However, it is worth monitoring tetracycline resistance since plasmid-mediated resistance to this drug has been described (MacInnes, 2010).

### 1.3.5 Virulence Factors

Little is known about the pathogenesis or virulence factors of *A. suis*. However, the closely related swine pathogen *A. pleuropneumoniae* has been quite well studied. Therefore, the review of the virulence factors of *A. suis* will be supplemented with a discussion of the virulence factors of *A. pleuropneumoniae* to gain insight into how *A. suis* may cause disease, but it is important to note that *A. pleuropneumoniae* causes disease in the lower respiratory tract, whereas *A. suis* mainly causes systemic disease. Both *A. suis* and *A. pleuropneumoniae* colonise the porcine tonsils of the soft palate and it is thought that colonisation of this site may be a preliminary step to the development of their respective diseases. It is further hypothesised that *A. suis* has virulence factors not present in *A. pleuropneumoniae* that allow it to invade the bloodstream.

The process of pathogenesis of most bacterial pathogens is believed to occur in four basic steps: attachment and colonisation of the host, nutrient acquisition, evasion of host defences, and damage to the host. Ultimately, these steps often lead to the eventual transmission to other hosts. In this review of the literature, the main emphasis will be the process of initial attachment and colonisation.
1.3.5.1 Attachment and colonisation

Little is known about the critical first steps of attachment and colonisation of the host by A. suis. However, phenotypic similarities between A. suis and A. pleuropneumoniae suggest that A. suis may express homologues of many of the described A. pleuropneumoniae adhesins. In addition, adhesins described in other members of the family Pasteurellaceae may also be present.

Adhesins can be categorised in many ways, but for the purpose of this review, they will be subdivided into fimbrial adhesins, afimbrial adhesins, and biofilm-related adhesins.

1.3.5.1.1 Fimbrial adhesins

Putative fimbrial adhesins of A. pleuropneumoniae have been described in the literature (Table 1.1), but none have been described in A. suis to date.

Type IV fimbriae have been observed (Dom et al., 1994; Utrera and Pijoan, 1991; Van Overbeke et al., 2002) and isolated (Zhang et al., 2000b) in many serotypes of A. pleuropneumoniae. Proteins for the synthesis of type IV fimbriae are encoded by the apfABCD operon. Stevenson et al. (2003) described apfA as a structural gene that likely encodes a 16 kDa polypeptide, the first 30 residues of which are highly conserved among other members of the group A type IV fimbriae family. The remaining genes in the operon are predicted to share homology with type IV fimbrial biogenesis genes from other species such as Haemophilus ducreyi; the last gene, apfD is a putative leader specific peptidase. In A. pleuropneumoniae, the apf genes are induced in vitro by contact with epithelial cells, and their expression has been detected in vivo during lung infection (Boekema et al., 2003; Boekema et al., 2004) suggesting a
potential role in adhesion. These studies, along with the conservation of this operon among other closely related members of the family *Pasteurellaceae* (Doughty et al., 2000; Fleischmann et al., 1995), suggest that there is a good chance that type IV fimbriae would be present in *A. suis* and have a role in attachment.

Baltes and Gerlach (2004) used selective capture of transcribed sequences (SCOTS) analysis to identify *A. pleuropneumoniae* genes that were up-regulated in necrotic porcine lung tissue. In this study, a homologue of the FlpD fimbria-like protein of *Haemophilus ducreyi* was identified. In *H. ducreyi*, this protein has been shown to be critical in virulence and microcolony formation in humans (Nika et al., 2002; Spinola et al., 2003). The highly conserved nature of this protein suggests that it may be important in *A. pleuropneumoniae*, and perhaps *A. suis*, virulence as well.

Negrete-Abascal et al. (2004) described *fliC*, the gene that encodes a 65 kDa flagellar protein with sequence identity to *E. coli*, *Salmonella* spp., and *Shigella* spp. flagellins. However, there has been no evidence for motility in either *A. suis* or *A. pleuropneumoniae*, therefore it seems unlikely that this gene would have a role in motility or attachment as has been demonstrated in other organisms (Erdem et al., 2007). Moreover, there is no further discussion of FliC in the literature, nor has the *fliC* gene been found in any of the reported complete genomes of *A. pleuropneumoniae*.

### 1.3.5.1.2 Afimbrial adhesins

Putative afimbrial adhesins of *A. pleuropneumoniae* have been identified in a number of studies (Table 1.2). For example, Fuller et al. (2000b) identified the putative outer membrane
protein (OMP) adhesin gene pomA in *A. pleuropneumoniae* by signature-tagged mutagenesis (STM), and suggested that it was essential for virulence of this organism. Baltes and Gerlach (2004) also found pomA to be up-regulated in *A. pleuropneumoniae* recovered from necrotic lung tissue. In addition, homologues have been reported in many other members of the family *Pasteurellaceae*, e.g., PomA in *Pasteurella haemolytica* (Zeng et al., 1999), OMP P5 in nontypeable *Haemophilus influenzae* (Sirakova et al., 1994), and OmpA2 and MOMP in *Haemophilus ducreyi* (Klesney-Tait et al., 1997). *H. influenzae* also encodes a filamentous, fimbria-like structure, dubbed OMP P5, that adheres to mucin and epithelial cells (Miyamoto and Bakaletz, 1996; Reddy et al., 1996; Sirakova et al., 1994).

As well, a 55 kDa OMP has been isolated from an outer membrane preparation of *A. pleuropneumoniae* (Van Overbeke et al., 2002). Based on N-terminal amino acid sequence analysis, it does not possess homology with any known sequence, but the 55 kDa protein is expressed in bacteria exhibiting strong adhesion to alveolar epithelial cells. In the studies of Van Overbeke et al. (2002), adhesion could be attenuated by proteolytic cleavage, heat, and sodium metaperiodate. The authors further suggested that not only does the 55 kDa OMP play a role in adhesion, but that the attachment process likely involves other adhesins, possibly ones containing carbohydrates (Van Overbeke et al., 2002).

In a similar study by Enriquez-Verdugo et al. (2004), a 60 kDa OMP of *A. pleuropneumoniae* that binds swine collagens and fibrinogens *in vitro* was identified. These interactions were lessened in the presence of convalescent serum, suggesting the potential importance of this protein during the course of disease (Enriquez-Verdugo et al., 2004). However, neither this OMP
nor the 55 kDa OMP mentioned above has been characterised genetically, so identification of homologues in *A. suis* might be challenging.

In 2005, Ojha et al. (2010) identified 30 colonisation-essential genes of *A. suis*. Further characterisation of the OMP gene *ompA* revealed its involvement in attachment to porcine tonsil explants and the brain microvascular endothelial cell line PBMEC/C1-2. The authors speculated that OmpA may play a role in the invasion process for this organism, but due to toxicity of the Apx toxins to the PBMECs, this has yet to be confirmed. It is, however, noteworthy that *A. pleuropneumoniae* (which is non-invasive) does not adhere to PBMECs, while *H. parasuis* (which is invasive) does (Vanier et al., 2006). Apart from OmpA, no other adhesins of *A. suis* have been described.

In *Yersinia* spp., the prototypical trimeric autotransporter YadA plays an important role in attachment and invasion (Lyskowski et al., 2011). A YadA homologue was identified in the outer membrane fraction of *A. pleuropneumoniae* by proteomic analysis (Chung et al., 2007), but no further characterisation was conducted. Because *A. pleuropneumoniae* is not thought to be invasive, this YadA homologue may function as an adhesin, but this remains to be demonstrated.

*In vitro* studies by Auger et al. (2009) using immortalised porcine epithelial cell lines derived from trachea and lung revealed that a putative adhesin with homology to the Hsf autotransporter of *H. influenzae* was up-regulated in *A. pleuropneumoniae*. In *H. influenzae*, Hsf is associated with adherence to human epithelial cells (St. Geme and Cutter, 1995), and is thought to play a significant role in colonisation of the human respiratory tract (St. Geme et al., 1996). The Hsf homologue in *A. pleuropneumoniae* contains a region with a high degree of homology with two
proteins. The first of these is the extracellular matrix protein adhesin A (EmaA) of *Aggregatibacter actinomycetemcomitans*, an oligomeric autotransporter with a YadA domain that binds collagen (Tang et al., 2007). The second is the putative Hsf protein of *Mannheimia haemolytica* which appears to be an autotransporter similar to the high-molecular-weight Hia and the Hsf adhesins of nontypeable strains of *H. influenzae* (Lo, 2001). The function of the Hsf autotransporter homologue in *A. pleuropneumoniae* has yet to be elucidated; however, the authors suggest that, given the fact that the gene is up-regulated in planktonic cells over St. Jude porcine lung (SJPL) cells, Hsf may play a role in the initial adhesion of the organism to host tissues (Auger et al., 2009).

In a study by Mullen et al. (2008a), a ComE1 homologue was identified in *A. pleuropneumoniae*. This protein binds fibronectin and double-stranded DNA in a non-sequence-specific manner. It also affects transformation efficiency of the organism when it is knocked out. ComE1 was originally described in *P. multocida* (Mullen et al., 2007), and homologues have been found in all *Pasteurellaceae* species examined to date. Accordingly, it is reasonable to assume that it will be present in *A. suis* as well, and that it may be involved in attachment to host cells.

TufA, another putative fibronectin-binding protein in other species (Dalio et al., 2002), is encoded by *A. pleuropneumoniae* and expressed in necrotic lung tissue (Baltes and Gerlach, 2004); however, its potential role in attachment remains to be determined.

Based on several studies reviewed by Jacques (2004), LPS has been identified as an important adhesin in *A. pleuropneumoniae*. Jacques suggests that the inner core structure, which
is conserved across all serovars of *A. pleuropneumoniae*, is critical for intimate binding to the host cell surface proteins likely by low affinity interactions with host molecules such as phosphatidylethanolamine.

1.3.5.1.3 *Biofilm genes*

The tight adherence (*tad*) locus has been identified in *A. pleuropneumoniae*. It is found in many bacterial species including *Aggregatibacter (Actinobacillus) actinomycetemcomitans* and other members of the family *Pasteurellaceae* (Planet et al., 2003; Tomich et al., 2007). This locus, encoded on the Widespread Colonisation Island, is comprised of 14 genes in *A. pleuropneumoniae* organised in an operon (Kaplan and Mulks, 2005; Tomich et al., 2007). The genes of the *tad* locus encode fimbrial structures similar to type IV pili that are required for surface attachment, autoaggregation, and biofilm formation (Kaplan and Mulks, 2005). The importance of the *tad* locus in virulence and biofilm formation has been confirmed in several studies (Auger et al., 2009; Baltes and Gerlach, 2004; Izano et al., 2007; Lone et al., 2009).

There is also evidence of an operon (*pgaABCD*) encoding genes involved in poly-β-1,6-N-acetyl-D-glucosamine (PGA) biofilm biosynthesis in field isolates of *A. pleuropneumoniae* (Izano et al., 2007). The genes of the *pga* operon are up-regulated in *A. pleuropneumoniae* in the presence of SJPL cells (Auger et al., 2009), as well as in the presence of bronchoalveolar lavage fluid (BALF) (Lone et al., 2009). Together, these results support the notion that not only does PGA play an important role in biofilm formation, but that this function may be relevant to the colonisation and pathogenesis of *A. pleuropneumoniae*.
Tegetmeyer et al. (2009) found the autotransporter serine protease AasP to be expressed by *A. pleuropneumoniae* in necrotic porcine lung tissue. Using an isogenic mutant, the authors conducted a biofilm assay *in vitro* and an aerosol infection model *in vivo*, and determined that while *aasP* is involved in biofilm formation, it does not appear to be critical to virulence. Based on these findings, it was suggested that this gene may not be important to *A. pleuropneumoniae* pathogenicity. Further characterisation of the AasP autotransporter, including a search for homologues in *A. suis*, may provide further insight into the function of this adhesin in pathogenesis, if any.

1.3.5.2 **Nutrient acquisition**

Once a pathogen has successfully attached to the host cells, it needs to obtain nutrients. *A. suis* possesses several mechanisms to acquire iron in porcine tissues, which contain very little free iron. Like *A. pleuropneumoniae, A. suis* uses TbpA and TbpB to bind porcine transferrin in a species-specific manner (MacInnes, 2010). These two proteins are very similar to those of *A. pleuropneumoniae* (Bahrami et al., 2003). *A. suis* also expresses a haemoglobin-binding protein, HgbA, that is much like the one in *A. pleuropneumoniae* (Bahrami and Niven, 2005). Apart from these iron acquisition genes identified in *A. suis*, it is unclear if this organism makes use of other mechanisms for iron acquisition. Much work remains to be done to gain a clearer understanding of this and other nutrient acquisition systems in *A. suis*.

*A. suis* is also urease-positive. It is plausible that urease may be used by this organism for nitrogen acquisition, as has been suggested for *A. pleuropneumoniae* (Bosse et al., 2002), but no work has been done to directly address this in *A. suis*. 
1.3.5.3  *Evasion of host defences*

It is not understood how *A. suis* invades the host, but once it enters the bloodstream, it likely makes use of LPS and CPS to evade and survive the host defence mechanisms. Moreover, in a study by Slavic et al. (2000a), all field isolates tested, and the ATCC 15557 strain, were found to be serum resistant.

Recent studies by Deutschmann et al. (2011) have gone a long way to improve our understanding of the surface polysaccharides of *A. suis*. These authors were able to show that *A. suis* strains with an intermediate or high degree of virulence have a CPS (K2 type) decorated with fructose and lack \((1\rightarrow6)\)-\(\beta\)-D-glucan. It was postulated that, since \((1\rightarrow6)\)-\(\beta\)-D-glucan is ubiquitous in the environment and is also a component of the CPS of K1 strains, there may be low levels of antibodies in the animals against it. It was further hypothesised that a host immune response to \((1\rightarrow6)\)-\(\beta\)-D-glucan may reduce the probability of O1:K1 isolates causing disease. Moreover, since fructosylation is relatively uncommon in bacterial CPS, this may confer upon *A. suis* K2 strains an additional ability to evade host detection. Finally, Deutschmann et al. (2011) also demonstrated that the O-chain of the O2 type LPS is sialylated. In other species, sialylation of bacterial polysaccharides has been shown to contribute to host evasion by inhibiting complement activation, interfering with phagocytosis, and camouflaging the pathogen from host detection (Ram et al., 1998). Together, these modifications to the CPS and LPS of virulent *A. suis* strains may contribute to host evasion.

*A. suis* produces RTX toxins that are also likely to be involved in evasion of the host immune response. ApxI and ApxII in *A. suis* and *A. pleuropneumoniae* are nearly identical, but the
expression levels in A. suis are lower than those of A. pleuropneumoniae (Van Ostaaijen et al., 1997). In contrast to A. pleuropneumoniae, A. suis does not express ApxIV, and does not possess the genes for it (Schaller et al., 1999). The relatively low levels of RTX toxin expression in A. suis have been postulated to allow the organism to enter the bloodstream via tonsillar leucocytes (MacInnes, 2010); however, this has yet to be proven. In A. pleuropneumoniae, RTX toxins are thought to aid the pathogen in avoiding non-specific clearance by phagocytes in the lung (Bosse et al., 2002). Sublytic levels of A. pleuropneumoniae RTX toxins may also impair phagocytosis by macrophages and polymorphonuclear leucocytes (PMNs) (MacInnes, 2010). While it is not evident how this might translate to survival of A. suis in the tonsil and the bloodstream, it is possible that subtle differences between A. pleuropneumoniae and A. suis toxin expression, secretion, or potency may exist.

1.3.5.4 Damage to the host

Very little is known with regards to how A. suis damages the host. Based on findings in A. pleuropneumoniae, host damage can occur in a variety of ways, such as direct or indirect toxicity, induction of an inflammatory response, proteolytic damage, and apoptosis.

In addition to playing a role in host evasion when present at sublytic levels, RTX toxins are also thought to directly contribute to host damage. While the exact concentrations of toxin are unknown, it has been found that higher levels of A. pleuropneumoniae RTX toxins are cytotoxic to alveolar macrophages and PMNs (MacInnes, 2010). Due to the similarities between the RTX toxins of these two species, it is assumed that A. suis Apx toxins may also be cytotoxic.
*A. pleuropneumoniae* Apx toxins can also have an indirect effect on host tissues by promoting an inflammatory response through the activation of phagocytes (MacInnes, 2010). In addition, macrophage activation leads to the release of toxic oxygen species, cytokines and proteolytic enzymes, as well as induction of apoptosis (Pabst, 1996), resulting in further damage to host tissues.

Like *A. pleuropneumoniae*, *A. suis* strains are strongly urease-positive (Bergey and Holt, 1994). Urease may play a role in pathogenesis not only by providing a nitrogen source for the pathogen but also by potentially impairing the local immune response (Baltes et al., 2001; Bosse et al., 2002).

LPS, alone or in concert with Apx toxins, has been implicated in causing lesions during *A. pleuropneumoniae* infection (Bosse et al., 2002; Frey, 1995; Jacques, 2004). Most of the studies of *A. suis* LPS have focused on diagnostic methods rather than virulence, but the widespread implication of LPS in virulence of other bacterial species suggests that this might hold true for *A. suis*, as well.

*A. pleuropneumoniae* has been found to secrete a 24 kDa protease as well as an autocatalytic 101 kDa zinc metalloprotease in diseased lung tissue (Garcia Gonzalez et al., 2004). Their role in pathogenesis, however, remains to be elucidated. It has been postulated that these proteases may degrade immunoglobulins, contribute to complement inactivation, or play a role in inflammatory cascades (MacInnes, 2010), but this has yet to be confirmed.

An examination of the *A. suis* genome for these and additional virulence factors would be worthwhile in providing deeper insight into the pathogenesis of this organism.
Figure 1.1. Anatomic locations of the porcine tonsils

The lingual tonsil of pigs is situated at the root of the tongue (green); the paraepiglottic tonsils are on each side of the base of the epiglottis (pink); the pharyngeal tonsil is located at the caudal end of the pharyngeal septum (red); the tubal tonsils are located where the auditory tubes open into the pharynx (blue), and the tonsils of the soft palate are located on the ventral side of the soft palate (purple). Figure modified from Biologycorner.com (2011) under Creative Commons licence.
Figure 1.2. Composite diagram (a) and haematoxylin and eosin staining (b) of a cross section through a region of the tonsil of the soft palate of swine

The fossule (F) opens into the tonsillar crypt (C). The surface of the soft palate and the crypt neck are lined with a thick layer of non-keratinised stratified squamous epithelium (E) with underlying connective tissue (T). Lymphoid follicles (L) are interspersed in the parenchyma and surrounded by diffuse lymphoid tissues (D). Figure from Kernaghan et al. (2012), used with permission of Cambridge University Press.
Table 1.1. Fimbrial adhesins of *A. pleuropneumoniae* described in the literature

<table>
<thead>
<tr>
<th>Adhesin</th>
<th>Gene</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type IV fimbriae (operon <em>apfABCD</em>)</td>
<td><em>apfA</em></td>
<td>Group A fimbrial subunit</td>
<td>(Stevenson et al., 2003; Zhang et al., 2000b)</td>
</tr>
<tr>
<td></td>
<td><em>apfB</em></td>
<td>Fimbrial biogenesis</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>apfC</em></td>
<td>Fimbrial biogenesis</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>apfD</em></td>
<td>Leader specific peptidase</td>
<td></td>
</tr>
<tr>
<td>FlpD homologue</td>
<td><em>flpD</em></td>
<td>Homologue of <em>H. ducreyi</em> FlpD fimbria-like protein</td>
<td>(Baltes and Gerlach, 2004)</td>
</tr>
<tr>
<td>Flagellin</td>
<td><em>fliC</em></td>
<td>65 kDa protein with homology to <em>E. coli</em>, <em>Salmonella</em>, and <em>Shigella</em> flagellins</td>
<td>(Negrete-Abascal et al., 2003)</td>
</tr>
</tbody>
</table>
### Table 1.2. Afimbrial adhesins of *A. pleuropneumoniae* described in the literature

<table>
<thead>
<tr>
<th>Adhesin</th>
<th>Gene</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>OMP</td>
<td><em>pomA/momp/ompp5</em> homologue</td>
<td>Identified by STM; Homologues in several <em>Pasteurellaceae</em></td>
<td>(Baltes and Gerlach, 2004; Fuller et al., 2000b)</td>
</tr>
<tr>
<td>55 kDa OMP</td>
<td>?</td>
<td>Found in OMP fraction of serotypes 2, 5a, 9 and 10 strains grown in NAD-restricted conditions</td>
<td>(Van Overbeke et al., 2002)</td>
</tr>
<tr>
<td>60 kDa OMP</td>
<td>?</td>
<td>Found in OMP fraction of most serotypes; Serotype 1 66kDa OMP adheres to immobilised swine-lung collagen</td>
<td>(Enriquez-Verdugo et al., 2004)</td>
</tr>
<tr>
<td>Autotransporter (Hsf)</td>
<td><em>hia</em> homologue</td>
<td>Homologue of <em>H. influenzae</em> Hia adhesin</td>
<td>(Baltes and Gerlach, 2004)</td>
</tr>
<tr>
<td>Autotransporter</td>
<td>NZ_AACK01000066</td>
<td>Uncharacterised</td>
<td>(Baltes and Gerlach, 2004)</td>
</tr>
<tr>
<td>Autotransporter</td>
<td>NZ_AACK01000041</td>
<td>Uncharacterised</td>
<td>(Baltes and Gerlach, 2004)</td>
</tr>
<tr>
<td>ComE1 homologue</td>
<td>APL_1406</td>
<td>Binds fibronectin and DNA; Homologues found in other <em>Pasteurellaceae</em> species</td>
<td>(Mullen et al., 2008a)</td>
</tr>
<tr>
<td>LPS</td>
<td>Various</td>
<td>Considered important adhesin; Oligosaccharide core involved in adhesion</td>
<td>(Jacques, 2004)</td>
</tr>
</tbody>
</table>
Chapter 2: Thesis objectives and hypotheses

2.1 Purpose

The porcine tonsils of the soft palate are a secondary lymphoid tissue that is situated in the oral cavity at the junction between the respiratory and gastrointestinal tracts, providing an ideal location for the surveillance and sampling of antigens that enter through the mouth and nares. The tonsils offer a first line of immune surveillance for the animal. Several microbes are known to colonise this site, and it has been demonstrated that some pathogens use the tonsils as a point of entry into the host.

*Actinobacillus suis* is a common commensal of swine that colonises the tonsils of the soft palate. Particularly in high health status herds, it is also an important opportunistic pathogen that can cause septicaemia, pleuropneumonia, and meningitis in animals of all ages and abortion in sows. Little is known about the pathogenesis of this organism including the critical first steps of host colonisation. Therefore, the aim of this work was to identify and characterise *A. suis* adhesins, proteins involved in attachment, colonisation, and biofilm formation. The information garnered from this research will provide a framework for further study of porcine tonsillar pathogens.

2.2 Summary of objectives

The primary goal of this thesis was to identify and characterise the putative adhesins of *A. suis* H91-0380, a virulent O2:K2 clinical isolate.
To do this, I began by using a bioinformatic approach to identify adhesin genes in the complete genome of *A. suis* strains H91-0380 and ATCC 33415T and in the draft genomes of three other *A. suis* isolates (Chapter 3).

I next characterised the expression of adhesin genes under conditions that mimicked the nutrient and oxygen levels present in the tonsils of the soft palate of swine (Chapters 4 and 5). In these studies, *A. suis* H91-0380 was grown in brain heart infusion (BHI) to exponential and stationary phases under aerobic growth conditions in the presence and absence of a physiological level of epinephrine (50 µM), and under anoxic static growth conditions. The expression of 12 putative adhesin genes, chosen to represent the various classes of adhesins identified in Chapter 3, was measured by semi-quantitative real-time PCR (qPCR; Chapter 5).

In the final study (Chapter 6), I identified extracellular matrix (ECM) components present in the tonsils of the soft palate of swine and measured the attachment of *A. suis* H91-0380 and three isogenic adhesin gene mutants to five common ECM components: collagen types I and IV, fibronectin, laminin, and vitronectin. For this study, tonsils were homogenised, lysed, and/or fractionated and subjected to liquid chromatography-mass spectrometry and comparative bioinformatics to identify proteins. For the attachment assays, the ability of *A. suis* wild type and isogenic mutants to adhere to 96-well plates pre-coated with purified ECM components was measured. The isogenic mutants used had unmarked deletions in the genes for Flp1 (pilin and first gene of the tight adherence locus that encodes for type IVb; ∆flp1), OmpA (outer membrane protein that has previously been demonstrated to participate in attachment of *A. suis* to tonsil
explants and to brain microvascular endothelial cells; ∆ompA), and ComE1 (homologue of a fibronectin-binding protein that also participates in natural competence; ∆comE1).

2.3 Experimental design summary

Specific objectives:

1. Use a bioinformatic approach to identify adhesins
2. Determine optimal in vitro growth conditions for expression of each adhesin
3. Generate knockout mutants of selected adhesin genes
4. Identify receptors and ECM components of the tonsils of the soft palate
5. Characterise mutants for biofilm formation and attachment to ECM components

Objective 1: Use a bioinformatic approach to identify adhesins

Hypothesis: There will be adhesin genes of A. suis that will share homology with genes of other members of the family Pasteurellaceae, and the highest homology will be with the adhesins of the genetically similar primary pathogen, A. pleuropneumoniae.

Methods:

Two approaches were used to identify putative adhesins:

1. The recently annotated A. suis H91-0380 genome sequence was searched for putative fimbrial and afimbrial adhesins (pili, autotransporters, outer membrane proteins, miscellaneous)
   - Homologues in other species were identified using blastx in GenBank

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2. Adhesins of *Pasteurellaceae* described in the literature (genes and proteins) were found
   
   • Homologues in *A. suis* H91-0380 were identified using local blastx and blastp

**Objective 2: Determine the effects of selected in vitro growth conditions on expression of each adhesin gene**

**Hypothesis:** There will be differential expression of adhesin genes in response to different growth conditions, and the presence of the stress hormone epinephrine will up-regulate the expression of some adhesin genes.

**Methods:**

*A. suis* H91-0380 was cultured in brain heart infusion (BHI) under the following conditions:

- Aerobic cultures grown at 37°C with shaking at 200 rpm
- Epinephrine cultures grown at 37°C with shaking at 200 rpm, with 50 µM epinephrine in the growth medium
- Anoxic cultures grown at 37°C + 5% CO₂ without shaking
- Each of the above conditions was sampled at two time points: exponential and stationary phase (to account for early and late expression)

RNA samples were collected, reference genes were validated, and adhesin gene expression was assessed by semi-quantitative real-time PCR (qPCR).
Objective 3: Generate knockout mutants of putative adhesins that are expressed *in vitro*

Methods:

Isogenic mutants of 3 putative adhesin genes were generated using the *sacB* trans-conjugation system (Oswald et al., 1999) (Supplementary figure 5.1S). The isogenic mutants generated were:

- \(\Delta flp1\): pilin gene and first gene of tight adherence locus
- \(\Delta ompA\): porin gene
- \(\Delta comE1\): gene encoding fibronectin-binding protein

Generation of isogenic mutants was carried out as follows:

1. 5′ and 3′ ends of gene from *A. suis* H91-0380 were amplified by PCR and cloned into pEMOC2 vector
2. *E. coli* carrying modified pEMOC2 vector were mated with *A. suis* H91-0380 and plasmid co-integrates were identified by PCR
3. Counter-selection for mutants was conducted and confirmed by PCR and sequencing

Objective 4: Identify receptors and ECM components of the tonsils of the soft palate

Hypothesis: There will be several ECM components present in the tonsils that are able to interact with colonising and invading bacteria.
Methods:

- Tonsils from healthy animals were homogenised, lysed, and/or fractionated
- Peptides were identified by liquid chromatography-mass spectrometry (LC-MS)
- Peptides were used to identify proteins of the tonsils using comparative bioinformatics

Objective 5: Characterise mutants for biofilm formation and attachment to ECM components

Hypothesis: *A. suis* H91-0380 will attach to some of the extracellular matrix (ECM) components, while the isogenic mutants will have a reduced ability to attach to the ECM components during the phase of growth where each gene was found to be up-regulated in Chapter 5.

Methods:

Biofilm formation was measured for *A. suis* H91-0380 and three isogenic mutants using a static assay and staining with crystal violet, as described by Labrie et al. (2010). Two separate experiments were conducted:

1. Overnight cultures were diluted in fresh medium and added to wells of a 96-well plate, where they were incubated for 6 or 24 h
2. Cultures were grown to exponential and stationary phase, then added to wells of a 96-well plate, where they were incubated for an additional 6 or 24 h
Attachment to ECM components was also measured for *A. suis* H91-0380 and three isogenic mutants, as follows:

1. Cultures were grown to exponential and stationary phase, then added to wells of a 96-well plate pre-coated with purified human collagen I, collagen IV, fibronectin, laminin, and vitronectin

2. Wells were incubated for 0, 15, 30, 45, 60, and 120 minutes, then stained with crystal violet
Chapter 3: Identification of putative adhesins of

*Actinobacillus suis* and their homologues in other
members of the family *Pasteurellaceae*

A version of this chapter was previously published in BMC Research Notes:

**Bujold AR, MacInnes JI. Identification of putative adhesins of Actinobacillus suis**

and their homologues in other members of the family *Pasteurellaceae*. BMC Res Notes. 8: 675.
3.1 Abstract

*Actinobacillus suis* disease has been reported in a wide range of vertebrate species, but is most commonly found in swine. *A. suis* is a commensal of the tonsils of the soft palate of swine, but in the presence of unknown stimuli it can invade the bloodstream, causing septicaemia and sequelae such as meningitis, arthritis, and death. It is genotypically and phenotypically similar to *A. pleuropneumoniae*, the causative agent of pleuropneumonia, and to other members of the family *Pasteurellaceae* that colonise tonsils. At present, very little is known about the genes involved in attachment, colonisation, and invasion by *A. suis* (or related members of the tonsil microbiota).

Bioinformatic analyses of the *A. suis* H91-0380 genome were done using BASys and blastx in GenBank. Forty-seven putative adhesin-associated genes predicted to encode 24 putative adhesins were discovered. Among these are 6 autotransporters, 25 fimbriae-associated genes (encoding 3 adhesins), 12 outer membrane proteins, and 4 additional genes (encoding 3 adhesins). With the exception of 2 autotransporter-encoding genes (*aidA* and *ycgV*), both with described roles in virulence in other species, all of the putative adhesin-associated genes had homologues in *A. pleuropneumoniae*. However, the majority of the closest homologues of the *A. suis* adhesins are found in *A. ureae* and *A. capsulatus*—species not known to infect swine, but both of which can cause systemic infections.

*A. suis* and *A. pleuropneumoniae* share many of the same putative adhesins, suggesting that the different diseases, tissue tropism, and host range of these pathogens are due to subtle genetic differences, or perhaps differential expression of virulence factors during infection. However, many of the putative adhesins of *A. suis*
share even greater homology with those of other pathogens within the family Pasteurellaceae. Similar to A. suis, these pathogens (A. capsulatus and A. ureae) cause systemic infections and it is tempting to speculate that they employ similar strategies to invade the host, but more work is needed before that assertion can be made. This work begins to examine adhesin-associated factors that allow some members of the family Pasteurellaceae to invade the bloodstream while others cause a more localised infection.

3.2 Introduction

Actinobacillus suis, a member of the family Pasteurellaceae, is a gram-negative, facultative anaerobe, and a common commensal of the tonsils of the soft palate of swine (Kernaghan et al., 2012). However, under unknown conditions, it can invade the bloodstream of animals of all ages, resulting in septicaemia and sequelae such as meningitis, arthritis, and pneumonia (MacInnes and Desrosiers, 1999). A. pleuropneumoniae is a primary pathogen of swine that also colonises the upper respiratory tract and causes a contagious pleuropneumonia (Bosse et al., 2002). A. pleuropneumoniae and A. suis share many of the same virulence factors, including virtually identical ApxI and ApxII toxins (though there are differences in the apxIBD transport genes), iron acquisition proteins including transferrin-binding proteins, urease, lipopolysaccharide, and adhesins (MacInnes, 2010). Despite many similarities, A. pleuropneumoniae and A. suis cause different diseases in swine, and A. suis has a broader host range (Jeannotte et al., 2002).

Little is known about the virulence factors of A. suis, particularly the adhesins. Therefore, the objective of this study was to use bioinformatics tools to mine the newly annotated genome of a clinical isolate of A. suis (MacInnes et al., 2012) and identify adhesin-associated genes that may be involved in the early stages of
pathogenesis of this organism. Adhesins play an important role in the pathogenesis of most bacteria by allowing them to attach to, colonise, and invade their hosts. In addition to host-pathogen interactions, adhesins are also critical in adherence to abiotic surfaces, auto-aggregation to other bacteria, and in the early stages of biofilm formation (Klemm and Schembri, 2000; Ofek and Doyle, 1994; Soto and Hultgren, 1999). Adhesins are often classified as either fimbrial or afimbrial, where fimbrial adhesins are composed of multiple copies of one protein assembled into long appendages such as pili, and afimbrial adhesins are single proteins (e.g., autotransporters or outer membrane proteins) that have adhesive properties (Linke and Goldman, 2011).

In the current study, we have identified proteins belonging to four different classes of adhesin-associated genes present in the \textit{A. suis} genome (one fimbrial and three afimbrial) and provided a brief summary of their described roles in attachment in other members of the family \textit{Pasteurellaceae}, with special emphasis on species in the genus \textit{Actinobacillus}.

3.3 Methods

3.3.1 Bioinformatics

To identify putative adhesin-associated genes in \textit{Actinobacillus suis} H91-0380, a virulent O2:K2 isolate (MacInnes et al., 2012), a manual search of the annotations of the \textit{A. suis} H91-0380 genome assigned by the BASys pipeline (Van Domselaar et al., 2005) and GenBank (http://www.ncbi.nlm.nih.gov/) was done to identify putative adhesin-associated genes; blastx was used to find homologues in other species with a described or annotated role in attachment, colonisation, or invasion. Genes or proteins
described in the literature in other members of the family *Pasteurellaceae* were also analysed by blastx or blastp to find homologues in *A. suis*.

Further analysis of selected putative adhesin-associated genes was done using Pfam ([http://pfam.xfam.org/](http://pfam.xfam.org/)) to determine if conserved amino acid motifs characteristic of described protein families were present. When motifs were not identified, sequence identity and query coverage alone were used to classify genes.

### 3.3.2 Bacterial strains and growth media

Bacterial isolates (Table 3.6) were cultured from glycerol stocks onto Columbia agar plates containing 5% sheep’s blood (Oxoid Co., Nepean, ON), and in the case of the *A. pleuropneumoniae* isolate, supplemented with 0.01% (wt/vol) nicotinamide adenine dinucleotide (Sigma-Aldrich, St. Louis, MO). Plates were incubated overnight at 37°C in an atmosphere of 5% CO₂.

### 3.3.3 Real-time PCR

Crude genomic DNA was prepared by picking isolated colonies and dispersing them in Instagene matrix (Bio-Rad Laboratories Ltd., Hercules, CA), mixing by vortex, incubating at 56°C for 30 minutes, mixing again by vortex, incubating at 100°C for 8 minutes, centrifuging at 5000 X g for 2 minutes, and using the supernatant as template for PCR. At least two biological replicates were done for each strain and gene tested.

PCR primers were designed using Primer3 as previously described (Thornton and Basu, 2011), and are listed in Table 3.7. The total reaction volume was 20 μL, which contained 10 μL LightCycler 480 SYBR Green I Master mix (Roche Diagnostics Co., Indianapolis, IN), 0.4 μL each of the forward and reverse primers to a final concentration of 1 μM, 4.2 μL nuclease-free water, and 5 μL template.
Real-time PCR was done in a LightCycler 480 (Roche Diagnostics Co., Indianapolis, IN) using a program with an initial denaturation of 95°C for 5 minutes followed by 45 cycles of 95°C for 10 seconds, 54°C for 20 seconds, and 72°C for 12 seconds. Stepwise melt curves were done at the end of each run to confirm that only one template was amplified.

3.3.4 Sequencing additional isolates

A. suis strains ATCC 15557, H89-1173, and H91-0406 were sequenced at the Advanced Analytics Centre at the University of Guelph using MiSeq, and pseudogenomes were assembled with SeqMan Pro (DNASTAR Inc., Madison, WI) followed by progressiveMauve (Darling et al., 2010), and annotated using the BASys pipeline (Van Domselaar et al., 2005).

3.4 Results and discussion

Forty-seven putative adhesin-associated genes predicted to encode 24 adhesins were identified in the A. suis H91-0380 genome. These genes were categorised as encoding autotransporter (Table 3.1), fimbriae-associated adhesins (Table 3.2), outer membrane proteins (OMPs; Table 3.3), and miscellaneous (Table 3.4).

3.4.1 Autotransporters

Six autotransporter- genes were identified in the A. suis genome (Table 3.1). Among these, 4 encode proteins that belong to the subfamily known as trimeric autotransporter adhesins (TAAs). Autotransporters are large proteins with three domains—an N-terminal signal domain (present in the immature form of the protein, cleaved from the mature protein), a passenger domain, and a C-terminal translocator domain. In the case of TAAs, the translocator domain is short, and the adhesin structure is formed by a homotrimerisation of the encoded protein (Cotter et al.,
Examples of classic TAAs include Hia in *Haemophilus influenzae* (Surana et al., 2004) and YadA in *Yersinia enterocolitica* (Hoiczyk et al., 2000; Roggenkamp et al., 2003), and they are characterised by a conserved YadA domain and resistance to proteolytic degradation. All TAAs described to date have adhesive properties and bind to different host components including epithelial cells, extracellular matrix components, and circulating molecules (e.g., complement inhibitory proteins, immunoglobulins) (Cotter et al., 2005).

The four genes encoding TAAs identified in the *A. suis* genome, ASU2_04675, ASU2_06645, ASU2_07040, and ASU2_11275, are all well conserved in *A. capsulatus* (E value = 0.0). They also have homologues in *A. pleuropneumoniae* (E values = 0.0 - 5e-25), but the top homologues are found in different serovars. These TAAs also share homology with genes in distant species (E values ranging from 2e-14 to 2e-90). Given that many of the distant species (e.g., *Collimonas, Megasphaera, Advenella, Acinetobacter* spp.) with homologues of the *A. suis*-encoded TAAs are environmental isolates, this may hint that these TAAs are well conserved throughout evolution.

The other two autotransporter- genes identified in the *A. suis* genome encode putative conventional autotransporters. These proteins have the same domains as TAAs, but have a longer translocator domain. In addition to being adhesins that play important roles in attachment and biofilm formation, these autotransporters can have additional properties such as cytotoxic, proteolytic or lipolytic activity, and may play a role in serum resistance (Cotter et al., 2005). In the *A. suis* genome, the putative conventional autotransporter-encoding genes, ASU2_07665 and ASU2_11100, are annotated as *ycgV* and *aidA*, respectively. While the *ycgV* gene is well conserved in *A. capsulatus* (E-value = 0.0) and *aidA* is quite well conserved in *A. ureae* (E value = 5e-
132), there were no close homologues in *A. pleuropneumoniae*. It is also noteworthy that in a search for motifs in *aidA* done using Pfam, no conserved motifs, including the hallmark domains of conventional autotransporters, were detected. Therefore, the classification of this gene as an autotransporter-encoding gene relied solely on homology to other autotransporter-encoding genes in GenBank and annotation by BASys. The top homologue of *aidA* identified in species outside the family *Pasteurellaceae* was in the gram-positive bacterium *Streptococcus suis*. However, almost all *aidA* homologues in *Streptococcus* species are annotated as hypothetical proteins (with the exception of one homologue which is annotated as the LPXTG-motif cell wall anchor domain protein), and the E value (3e-11), coverage (53%), and identity (31%) of the top *Streptococcus suis* homologue suggest that the degree of conservation of this gene is low. The homology of the *A. suis aidA* gene with species such as streptococci that share a common environment in the upper respiratory tract of swine may hint at convergent evolution or horizontal gene transfer, but further studies would have to be done to rigorously test such assertions.

### 3.4.2 Fimbriae-associated adhesins

Twenty-five putative fimbriae-associated adhesin genes were also identified (Table 3.2). These included 14 genes predicted to be part of a tight adherence (*tad*) locus, a type IV pilus operon (4 genes), another type IV pilus biogenesis locus containing 6 genes, and another pilus-associated gene.

The *pilF* gene (ASU2_00450) annotated as a putative fimbrial biogenesis and twitching motility protein, is well conserved in *Pasteurellaceae*. It is less well conserved outside the family, but *pilF* homologues are present in *Pseudomonas aeruginosa* and in *Neisseria meningitidis* (*pilW*) and are thought to encode a protein that is critical for pilus stability and function, including attachment to human cells.
Pfam analysis revealed TPR repeats in the *A. suis* *pilF* gene. In other species, these repeats are thought to play a role in protein-protein interactions in both prokaryotic and eukaryotic cells, and contribute to virulence of bacterial pathogens by aiding in attachment to and invasion of host cells and circumventing host defences (Cerverny et al., 2013).

The *tad* locus is a conserved widespread colonisation island (Planet et al., 2003) that plays an important role in the pathogenesis, biofilm formation, and colonisation of several organisms, including members of the family *Pasteurellaceae* (Fuller et al., 2000a; Spinola et al., 2003; Tomich et al., 2006; Tomich et al., 2007). The *tad* locus encodes the machinery needed to assemble the fimbrial low-molecular-weight protein (Flp) pilin into long, bundled type IVb pili (Tomich et al., 2006; Tomich et al., 2007). The *A. suis* genome contains a *tad* locus comprised of homologues of *flp1-flp2-tadV-rcpC-rcpA-rcpB-tadZ-tadA-tadB-tadC-tadD-tadE-tadF-tadG*. The two putative pilin genes, *ASU2_04295* and *ASU2_04300*, are predicted to encode *flp1* and *flp2*, respectively; however, it may be noted that *flp2* is not expressed in *Aggregatibacter actinomycetemcomitans* (Tomich et al., 2007). Neither of these genes is very highly conserved within the family *Pasteurellaceae* and is even less so in more distant species. This may reflect the fact that the *flp1* and *flp2* putative pilin genes in *A. suis* have adapted for colonisation of different hosts or different host cell receptors. A genetic analysis of the *tad* locus by Li et al. (2012b) revealed that *flp1* is truncated or missing altogether in some strains of *A. pleuropneumoniae*. In the same study, these authors found that *tadC* is the best conserved among *A. pleuropneumoniae* strains tested and *tadG* the least, findings that were not observed in this work when the same genes in the *A. suis* genome were compared to other species. However, many of the biogenesis components of the *tad* locus of *A. suis* are well conserved in *A.*
pleuropneumoniae and other members of the family Pasteurellaceae such as A. capsulatus, and much less well conserved outside the family.

In addition to the tad locus, the A. suis genome also has two other loci for type IV pilus biogenesis: a type IV pilus locus (pilABCD/apfABCD) and a homologue of the comABCDEF locus. Type IV pili are important virulence factors in many gram-negative organisms, including other members of the family Pasteurellaceae such as nontypeable Haemophilus influenzae (NTHi) (Bakaletz et al., 2005; Carruthers et al., 2012; Jurcisek and Bakaletz, 2007; Jurcisek et al., 2007), Pasteurella multocida (Hunt et al., 2000), and A. pleuropneumoniae (Boekema et al., 2003; Boekema et al., 2004; Zhou et al., 2013). In these species, type IV pili have demonstrated roles in biofilm formation, attachment to epithelial cells, twitching motility, competence, and interactions with phage (Craig et al., 2004; Hansen and Forest, 2006; Wall and Kaiser, 1999). In A. pleuropneumoniae, the apfA pilin gene is present in all strains and is well conserved in all serovars (Zhou et al., 2013). The homologue of this gene in A. suis (ASU2_05045) is the least well conserved gene in the pilABCD locus, but is still homologous to genes in both A. ureae (1e-70) and A. pleuropneumoniae (5e-37). Of the biogenesis genes, pilBCD, pilB, which encodes the ATPase, is the best conserved (E values = 0.0), and has well conserved homologues outside the family Pasteurellaceae (e.g., in Plesiomonas shigelloides, E value = 5e-20). On the other hand, the pilD gene, predicted to encode the fimbrial leader peptidase, is not conserved in species outside the family Pasteurellaceae (e.g., Enterococcus faecium, E value = 0.014).

Like pilABCD/apfABCD, the comABCDEF competence locus is predicted to encode the biogenesis components for type IV pilus assembly; however, no pilin gene is associated with this operon in the A. suis genome, and the comF gene
(ASU2_11115) is not linked with the rest of the *com* locus, unlike other species such as NTHi (Carruthers et al., 2012). In a recent study of NTHi, Carruthers et al. found that all of the products of both the *pil* and *com* operons, including *comF*, are essential for proper type IV pilus construction and formation (Carruthers et al., 2012). Taken together, these results suggest that the proteins encoded by the *pil* and *com* loci may work together to produce type IV pili in *A. suis*, and that the *pilA* homologue (ASU2_05045) may encode the major pilin protein.

### 3.4.3 Outer membrane proteins

Genes predicted to encode twelve outer membrane proteins (OMPs) were identified, including homologues of *ompA*, *ompP2*, and *ompP5* porin genes (Table 3.3). OMPs are described as multifunctional proteins. Many OMPs have been demonstrated to form porins in the outer membrane of gram-negative bacteria, which can contribute to nutrient acquisition, antibiotic resistance, attachment, invasion, and complement resistance, to name a few (Galdiero et al., 2012).

Most of the OMPs of *A. suis* are highly conserved when compared to other members of the family *Pasteurellaceae*. Two members of the OmpA family were identified in the *A. suis* genome, ASU2_09940 and ASU2_09935. In our previous studies, the OmpA homologue ASU2_09940 was identified by signature-tagged mutagenesis as an important virulence factor of *A. suis*, with a demonstrated role in attachment to swine tonsil explants and to porcine brain microvascular endothelial cells (Ojha et al., 2010; Ojha et al., 2005). The other member of the OmpA family of OMPs, an *ompP5* homologue (ASU2_09935), is adjacent to the *ompA* homologue ASU2_09940 in the *A. suis* genome. It is also highly conserved (E value = 0.0) in members of the family *Pasteurellaceae* and has a high degree of homology with OMPs outside the family. In NTHi, OmpP5 has been shown to bind to human mucin
(Novotny et al., 2000) and to carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) (Hill et al., 2001); however, the precise role of OmpP5 and most other A. suis OMPs in pathogenesis remains to be demonstrated.

Two ompP2 genes (ASU2_00030 and ASU2_00525) and one ompP2-like gene (ASU2_03810) were identified in the A. suis genome. In addition to conferring antibiotic resistance (Regelink et al., 1999), providing a pore for general diffusion and transport of specific substrates (Andersen et al., 2003), the OmpP2 of NTHi has also been shown to play a role in attachment in the host environment through interactions with mucin (Reddy et al., 1996). The ompP2 gene (ASU2_00030) is predicted to encode a protein that is very similar to an OmpP2 homologue in A. capsulatus (E=0.0) while the ASU2_00525 gene encodes a protein that is well conserved in A. ureae (9E-159). The ompP2 homologues identified in A. suis are well conserved in A. pleuropneumoniae, but the serovar of the top homologues in A. pleuropneumoniae is different with each gene, as is the degree of conservation. Of the OMPs identified in A. suis, the ompP2 gene ASU2_00525 and the plp4 homologue ASU2_02415 have the least homology with proteins encoded by organisms outside of the family Pasteurellaceae. The GC content of ASU2_00525 differs markedly from that of the A. suis genome (36% vs. 40%), which may suggest that this ompP2 gene was recently acquired by A. suis.

Because of the multifunctional nature of the OMPs, it would be premature to predict that all OMPs identified in this study play a role in attachment or invasion, and further studies should be done to characterise each gene and its potential role in bacterial pathogenesis for A. suis.
3.4.4 Miscellaneous adhesins

Four additional genes from three different loci were identified that could play a role in bacterial attachment, colonisation, or invasion for *A. suis* (Table 3.4).

A filamentous haemagglutinin (FHA) locus consisting of two genes (ASU2_06635 and ASU2_06640) is also found in the *A. suis* genome. The *fhaB* gene encodes the adhesin structure while *fhaC* encodes the transporter. FHA has been demonstrated to play a role in bacterial attachment to integrins, carbohydrates present on macrophages, cilia, epithelial cells, and extracellular matrix components including heparin (Locht et al., 2001), and is thought to contribute to colonisation and biofilm formation by important pathogens such as *Histophilus somni*, *Bordetella bronchiseptica*, *Acinetobacter baumannii*, and *Pasteurella multocida* (Darvish Alipour Astaneh et al., 2014; Nicholson et al., 2009; Sandal et al., 2009; Verma et al., 2013). The *A. suis* *fhaB* gene has highly conserved (E values = 0.0) homologues in *A. pleuropneumoniae*, *Pasteurellaceae*, and in other species outside of the family *Pasteurellaceae*. The *fhaC* gene is also predicted to encode highly conserved homologues in members of the family *Pasteurellaceae* but to a slightly lower degree (E value = 1e-168). It is also interesting to note that the TAA-encoding ASU2_06645 gene is linked to the filamentous haemagglutinin locus, though the relevance of this finding, if any, remains to be elucidated.

A fine-tangled pili gene, *ftpA*, is also present in the *A. suis* genome. This gene lacks a cleavable signal sequence (Brentjens et al., 1996); however, no biogenesis genes for the translocation and assembly of this structure were identified. In other species, fine-tangled pili are assigned to the DNA protection during starvation (DPS) family of proteins. DPS proteins are thought to confer protection of DNA from environmental stressors such as low pH, Fe$^{2+}$, and hydrogen peroxide (Haikarainen
and Papageorgiou, 2010). Further, these proteins have been shown to be involved in bacterial adhesion to and invasion of host cells, and in auto-aggregation (Haikarainen and Papageorgiou, 2010; Huergo et al., 2013; Pang et al., 2012; Theoret et al., 2011; Theoret et al., 2012), though it is not clear whether the mechanisms of these actions are via a direct or indirect adhesive function of the Dps homologue. In *A. suis*, the *ftpA* gene (ASU2_09130) is well conserved in both *A. pleuropneumoniae* (E value = 2e-119) and other members of *Pasteurellaceae* (*A. ureae*, E value = 2e-130), and to a lesser extent in other species (*Jonesia denitrificans*, E value = 2e-61).

Finally, a homologue of *comE1*, originally described in *Pasteurella multocida* (Mullen et al., 2007), was also identified in *A. suis*. In addition to its roles in DNA-binding and uptake, this gene encodes a protein involved in bacterial attachment of five different members of the family *Pasteurellaceae* to the extracellular matrix component fibronectin (Mullen et al., 2008a; Mullen et al., 2008b). The closest homologue of the *comE1* gene in *A. suis* (ASU2_10345) is found in *A. capsulatus* (E value = 1e-75). Less well conserved homologues are also present in *A. pleuropneumoniae* (E value = 6e-34), other members of *Pasteurellaceae*, and even in other species outside the family. Given the role of this gene in fibronectin-binding in other members of *Pasteurellaceae*, it would be interesting to assess whether it plays a similar function in *A. suis*.

### 3.4.5 Adhesins in other *A. suis* strains

To determine whether putative adhesin genes are conserved in other *A. suis* isolates, real-time PCR was done on 9 additional isolates, including *A. pleuropneumoniae* L20, a serovar 5b isolate (Table 3.5). Ten genes were chosen for characterisation, with representatives from each of the classes of adhesins described. All *A. suis* isolates tested were positive for the selected adhesin genes, while the *A.*
*pleuropneumoniae* isolate was only positive for the putative *ompP2* gene. Upon closer inspection of the *A. pleuropneumoniae* L20 genome sequence, the only adhesin gene tested without a homologue was *ycgV* (ASU2_07665); however, despite there being homologues of the other genes, the sequence conservation in the primer binding sites in all but the *ompP2* gene was poor.

The pseudogenomes of three additional *A. suis* strains—ATCC 15557, H89-0406, and H91-1173 were annotated using BASys, and the genome sequence of ATCC 33416T was obtained from GenBank (Calcutt et al., 2014). These four genome sequences were used to determine whether putative adhesin genes were conserved in different *A. suis* isolates using blastn for direct nucleotide sequence comparisons (Additional file 1). Homologues of all adhesin genes identified in the *A. suis* H91-0380 genome were found in the four additional genomes, and were, for the most part, highly conserved (most >99% sequence identity). Some gene lengths varied among isolates, with the most notable differences seen in the ASU2_04675 autotransporter-encoding homologue found in ATCC 33415 and ATCC 15557, the ASU2_11275 autotransporter-encoding homologue in ATCC 15557, and the truncated but highly conserved *flp1* (ASU2_04295) homologue in H89-1173. The OMP homologue ASU2_01965 in the ATCC 33415 isolate shared only 67% nucleotide identity with H91-0380, despite 90% sequence coverage. Overall, however, putative adhesin genes were highly conserved in all *A. suis* isolates examined, which may suggest a clonal population, though other classes of genes, particularly virulence-associated genes, should also be compared.
3.5 Conclusions

Attachment and colonisation of the host environment are important steps in the early stages of bacterial colonisation and pathogenesis (Ofek and Doyle, 1994). As virtually nothing was known about these early steps in *A. suis*, the purpose of this study was to identify putative adhesins that may contribute to these processes in the genomes of several *A. suis* strains. Our analysis revealed that *A. suis* shares many of the same putative adhesins as *A. pleuropneumoniae*, an important primary pathogen of swine that is also known to colonise the upper respiratory tract. It may therefore be hypothesised that the different tissue tropisms and diseases caused by *A. suis* and *A. pleuropneumoniae* might be attributed, at least in part, to subtle differences in the adhesins of these organisms or to differential expression of adhesins at different stages of the infection process.

The adhesins identified in the *A. suis* genome are also well conserved in several other members of the family *Pasteurellaceae*. It is perhaps noteworthy that *Pasteurellaceae* that cause similar diseases but in different hosts, such as *A. ureae* and *A. capsulatus*, have nearly all the same adhesins that are present in *A. suis*. Of particular note are the autotransporter-encoding genes *ycgV* and *aidA* that are present in *A. suis*, *A. ureae*, and *A. capsulatus*, but which are missing in *A. pleuropneumoniae*. It may be hypothesised that these organisms employ similar strategies to invade the host, but more work is needed to characterise such host-pathogen interactions.

Together, these data begin to identify attachment and colonisation factors that allow some members of the family *Pasteurellaceae* to invade the bloodstream while others cause more localised infections. Future research on the expression of adhesins in *A. suis* and other organisms will help in elucidating the mechanisms of attachment
and colonisation, and should eventually lead to a better understanding of critical host-pathogen relationships.
Table 3.1. Putative autotransporter-encoding genes identified by bioinformatics

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<th>(Possible) gene name</th>
<th>GenBank Annotated protein function</th>
<th>Top App homologue/E value</th>
<th>Top <em>Pasteurellaceae</em> homologue/E value</th>
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<td>- a</td>
<td>autotransporter adhesin</td>
<td>Ser. 6 str. Femo</td>
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<td>0.0 Acinetobacter sp. ANC 4105</td>
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</table>

( ) indicates a suggested name that was not present in the annotation

aFunction assigned by conserved motifs

bIdentified by BASys

cClassified by description of homologues
Table 3.2. Putative fimbriae-associated genes identified by bioinformatics

<table>
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<tr>
<th>ASU2 locus tag</th>
<th>GenInfo (GI) number</th>
<th>(Possible) gene name</th>
<th>Annotated protein function</th>
<th>Top App homologue/E value</th>
<th>Top Pasteurellaceae homologue/E value</th>
<th>Top other homologue/E value</th>
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<td>407387739</td>
<td>pilF</td>
<td>putative fimbrial biogenesis and twitching motility protein PilF-like protein</td>
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<td>flp1</td>
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<tr>
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<td>(flp2)</td>
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<tr>
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<td>407388506</td>
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<td>flp operon protein B; Flp pilus assembly protein, protease CpaA</td>
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<td>407388507</td>
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<td>ASU2_04330</td>
<td>407388511</td>
<td>tadA</td>
<td>tight adherence protein A; Flp pilus assembly protein, ATPase CpaF</td>
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<td>Actinobacillus capsulatus</td>
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<td><strong>tadC</strong></td>
<td>tight adherence protein C; Flp pilus assembly protein TadC</td>
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<td>ASU2_04345</td>
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<td><strong>tadD</strong></td>
<td>tight adherence protein D; Flp pilus assembly protein TadD, contains TPR repeats</td>
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<td>Mannheimia haemolytica</td>
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<tr>
<td>ASU2_05030</td>
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<td>(<strong>pilD</strong>)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>fimbrial leader peptidase; Type II secretory pathway, prepilin signal peptidase PulO and related peptidases</td>
<td>Ser. 1 str. 4074</td>
<td>3e-56</td>
<td><strong>Actinobacillus ureae</strong></td>
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<tr>
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<td>pili/fimbriae biogenesis protein; Type II secretory pathway, component PulF</td>
<td>Ser. 7 str. AP76</td>
<td>5e-112</td>
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<tr>
<td>ASU2_05040</td>
<td>407388653</td>
<td>(<strong>hofB</strong>, <strong>pilB</strong>)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>fimbrial biogenesis protein; Type II secretory pathway, ATPase PulE/Tfp pilus assembly pathway, ATPase PilB</td>
<td>Ser. 5b str. L20</td>
<td>0.0</td>
<td><strong>Actinobacillus ureae</strong></td>
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<tr>
<td>ASU2_05045</td>
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<td><strong>apfA</strong> (<strong>ppdD</strong>, <strong>pilA</strong>)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>type 4 prepilin subunit; Tfp pilus assembly protein, major pilin PilA</td>
<td>Ser. 12 str. 1096</td>
<td>5e-37</td>
<td><strong>Actinobacillus ureae</strong></td>
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<td>407388868</td>
<td>hofQ (comE)</td>
<td>type II secretory pathway, component HofQ</td>
<td>Ser. 5b str. L20</td>
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<tr>
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<td>ASU2_06120</td>
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<td>hypothetical protein</td>
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<tr>
<td>ASU2_06130</td>
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<td>(comB)</td>
<td>hypothetical protein</td>
<td>Ser. 3 str. JL03</td>
<td>2e-65</td>
<td>Actinobacillus capsulatus</td>
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<tr>
<td>ASU2_06135</td>
<td>407388872</td>
<td>(comA)</td>
<td>hypothetical protein</td>
<td>Ser. 10 str. D13039</td>
<td>7e-106</td>
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<tr>
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<td>407389857</td>
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<td>competence</td>
<td>Ser. 10 str. D13039</td>
<td>9e-97</td>
<td>Actinobacillus capsulatus</td>
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</tbody>
</table>

( ) indicates a suggested name that was not present in the annotation

*a Function assigned by conserved motifs

*b Identified by BASys

*c Classified by description of homologues
<table>
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<tr>
<th>ASU2 locus tag</th>
<th>GenInfo (GI) number</th>
<th>(Possible) gene name</th>
<th>Annotated protein function</th>
<th>Top App homologue/E value</th>
<th>Top Pasteurellaceae homologue/E value</th>
<th>Top other homologue/E value</th>
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<tr>
<td>ASU2_00030</td>
<td>407387657</td>
<td>ompP2</td>
<td>outer membrane protein P2; porin</td>
<td>Ser. 5b str. L20 0.0</td>
<td>Actinobacillus capsulatus 0.0</td>
<td>Neisseria sp. oral taxon 014 str. F0314 6e-131</td>
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<tr>
<td>ASU2_00525</td>
<td>407387754</td>
<td>ompP2</td>
<td>outer membrane protein P2; porin</td>
<td>Ser. 4 str. M62 6e-42</td>
<td>Actinobacillus ureae 9E-159</td>
<td>Neisseria sp. oral taxon 014 str. F0314 4e-08</td>
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<tr>
<td>ASU2_01965</td>
<td>407388042</td>
<td>(ompP1)</td>
<td>long-chain fatty acid outer membrane transporter</td>
<td>Ser. 7 str. AP76 9e-142</td>
<td>Actinobacillus ureae 0.0</td>
<td>Serratia proteamaculans 568 4e-98</td>
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<td>ASU2_02415</td>
<td>407388132</td>
<td>b,c</td>
<td>hypothetical protein</td>
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<td>Actinobacillus ureae 2E-132</td>
<td>Pantoea sp. A4 6e-06</td>
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<td>ASU2_03005</td>
<td>407388248</td>
<td>plp4</td>
<td>lipoprotein; small protein A (tmRNA-binding)</td>
<td>Ser. 5b str. L20 0.0</td>
<td>Actinobacillus capsulatus 0.0</td>
<td>Pelistega sp. HM-7 8e-107</td>
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<td>ASU2_03810</td>
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<td>b</td>
<td>outer membrane protein P2-like protein; porin</td>
<td>Ser. 3 str. JL03 0.0</td>
<td>Actinobacillus ureae 0.0</td>
<td>Neisseria sp. oral taxon 014 7e-27</td>
</tr>
<tr>
<td>ASU2_05520</td>
<td>407388749</td>
<td>palA</td>
<td>outer membrane protein and related peptidoglycan-associated (lipo)proteins</td>
<td>Ser. 3 str. JL03 2E-89</td>
<td>Actinobacillus ureae 2E-89</td>
<td>Morganella morganii subsp. morganii KT 1e-46</td>
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<td>ASU2_05735</td>
<td>407388792</td>
<td>ompW</td>
<td>outer membrane protein</td>
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<tr>
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<td>hypothetical protein</td>
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<td>outer membrane protein P5</td>
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<td>ASU2_09940</td>
<td>407389623</td>
<td><em>(ompA)</em></td>
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<td>Ser. 6 str. Femo</td>
<td><strong>0.0</strong></td>
<td><em>Actinobacillus capsulatus</em></td>
</tr>
</tbody>
</table>

( ) indicates a suggested name that was not present in the annotation

aFunction assigned by conserved motifs

bIdentified by BASys

cClassified by description of homologues
Table 3.4. Miscellaneous other putative adhesin-associated genes identified by bioinformatics

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<tr>
<th>ASU2 locus tag</th>
<th>GenInfo (GI) number</th>
<th>(Possible) gene name</th>
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<th>Top App homologue/E value</th>
<th>Top Pasteurellaceae homologue/E value</th>
<th>Top other homologue/E value</th>
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</thead>
<tbody>
<tr>
<td>ASU2_06635</td>
<td>407388972</td>
<td>(fhaB)^f</td>
<td>filamentous haemagglutinin outer membrane protein</td>
<td>Ser. 6 str. Femo 0.0</td>
<td>Actinobacillus capsulatus 0.0</td>
<td>Acinetobacter bohemicus 0.0</td>
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<tr>
<td>ASU2_06640</td>
<td>407388973</td>
<td>(fhaC)^f</td>
<td>haemolysin activation/secretion protein</td>
<td>Ser. 4 str. M62 0.0</td>
<td>Actinobacillus capsulatus 0.0</td>
<td>Ralstonia solanacearum 1e-168</td>
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<tr>
<td>ASU2_09130</td>
<td>407389463</td>
<td>(fipA, dps)^c</td>
<td>fine tangled pili major subunit; DNA-binding ferritin-like protein (oxidative damage protectant); DNA protection during starvation</td>
<td>Ser. 3 str. JL03 2e-119</td>
<td>Actinobacillus ureae 2E-130</td>
<td>Jonestia denitrificans DSM 20603 2e-61</td>
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<tr>
<td>ASU2_10345</td>
<td>407389704</td>
<td>(comE1, comEA, ybaV)^f</td>
<td>DNA uptake protein; DNA uptake protein and related DNA-binding proteins; transporter</td>
<td>Ser. 7 str. AP76 6e-34</td>
<td>Actinobacillus capsulatus 1E-75</td>
<td>Vibrio nigripulchritudo 4e-21</td>
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</table>

( ) indicates a suggested name that was not present in the annotation

^aFunction assigned by conserved motifs

^bIdentified by BASys

^cClassified by description of homologues
Table 3.5. Real-time PCR detection of selected putative adhesin genes in the genomes of several *A. suis* and one *A. pleuropneumoniae* strains using primers specific to *A. suis* H91-0380

<table>
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<tr>
<th>Isolate</th>
<th>ycgV  (ASU2_07665)</th>
<th>flp1  (ASU2_04295)</th>
<th>tadG  (ASU2_04360)</th>
<th>pilA  (ASU2_05045)</th>
<th>ompP2 (ASU2_00030)</th>
<th>ompA  (ASU2_09940)</th>
<th>plp4  (ASU2_11270)</th>
<th>fhaB (ASU2_06635)</th>
<th>ftpA  (ASU2_09130)</th>
<th>comE1 (ASU2_10345)</th>
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</tr>
</tbody>
</table>

+ indicates detection of a gene in a specific isolate

– indicates no detection of a gene in a specific isolate
Table 3.6. Bacterial strains used to identify homologous putative adhesin genes by bioinformatics and real-time PCR

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<tr>
<th>Bacterial strain</th>
<th>Characteristic(s)</th>
<th>GenBank accession number and Reference</th>
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</thead>
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<tr>
<td><em>Actinobacillus suis</em> H91-0380</td>
<td>O2:K2 clinical isolate</td>
<td>CP003875; (MacInnes et al., 2012; Van Ostaaijen et al., 1997)</td>
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<tr>
<td><em>Actinobacillus suis</em> ATCC 33415</td>
<td>Untyped clinical isolate</td>
<td>CP009159; (Calcutt et al., 2014)</td>
</tr>
<tr>
<td><em>Actinobacillus suis</em> ATCC 15557</td>
<td>O1:K1 isolate</td>
<td>(Slavic et al., 2000a; Van Ostaaijen et al., 1997)</td>
</tr>
<tr>
<td><em>Actinobacillus suis</em> H89-1173</td>
<td>O2:K3 clinical isolate</td>
<td>(Slavic et al., 2000a; Van Ostaaijen et al., 1997)</td>
</tr>
<tr>
<td><em>Actinobacillus suis</em> H91-0406</td>
<td>O2:K3 clinical isolate</td>
<td>(Slavic et al., 2000a; Van Ostaaijen et al., 1997)</td>
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<tr>
<td><em>Actinobacillus suis</em> SO4 Na†</td>
<td>O1:K1 isolate</td>
<td>(Slavic et al., 2000a; Van Ostaaijen et al., 1997)</td>
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<td><em>Actinobacillus suis</em> VSB 3714</td>
<td>Rough:K? isolate</td>
<td>(Slavic et al., 2000a; Van Ostaaijen et al., 1997)</td>
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<tr>
<td><em>Actinobacillus suis</em> C84</td>
<td>O1:K2 isolate</td>
<td>(Slavic et al., 2000a; Van Ostaaijen et al., 1997)</td>
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<tr>
<td><em>Actinobacillus suis</em> Q95-6256</td>
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<td>(Slavic et al., 2000a; Van Ostaaijen et al., 1997)</td>
</tr>
<tr>
<td><em>Actinobacillus suis</em> H93-1250</td>
<td>Untyped clinical isolate</td>
<td>(Slavic et al., 2000a; Van Ostaaijen et al., 1997)</td>
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<tr>
<td><em>Actinobacillus pleuropneumoniae</em> L20</td>
<td>Serovar 5b</td>
<td>(Foote et al., 2008)</td>
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</tbody>
</table>
Table 3.7. Primers designed based on the *A. suis* H91-0380 genome and used to screen for adhesin genes in *A. suis* and *A. pleuropneumoniae* strains by real-time PCR

<table>
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<th>Primer name</th>
<th>Class</th>
<th>Locus tag</th>
<th>Sequence (5’–3’)</th>
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</thead>
<tbody>
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<td>ASU2-ycgV-F1</td>
<td>Autotransporter</td>
<td>ASU2_07665</td>
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<tr>
<td>ASU2-ycgV-R1</td>
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Supplementary table 3.1S. blastn comparison of *A. suis* H91-0380 adhesin-associated genes to adhesin-associated genes in four additional *A. suis* strains

Spreadsheet of blastn results showing gene sizes, locations, query coverage, E value, and sequence identity for adhesin-associated genes in *A. suis* H91-0380 compared to *A. suis* ATCC 33415, H91-0406, ATCC 15557, and H89-1173 available at

Chapter 4: Validation of reference genes for quantitative real-time PCR (qPCR) analysis of *Actinobacillus suis*

A version of this chapter was previously published in BMC Research Notes:

**Bujold AR, MacInnes JI. 2015. Validation of reference genes for quantitative real-time PCR (qPCR) analysis of *Actinobacillus suis*. BMC Res Notes. 8: 86.**
4.1 Abstract

Quantitative real-time PCR is a valuable tool for evaluating bacterial gene expression. However, in order to make best use of this method, endogenous reference genes for expression data normalisation must first be identified by carefully validating the stability of expression under experimental conditions. Therefore, the objective of this study was to validate eight reference genes of the opportunistic swine pathogen, *Actinobacillus suis*, grown in aerobic cultures with (Epinephrine) or without (Aerobic) epinephrine in the growth medium and in anoxic static cultures (Anoxic), and sampled during exponential and stationary phases.

Using the RefFinder tool, expression data were analysed to determine whether comprehensive stability rankings of selected reference genes varied with experimental design. When comparing Aerobic and Epinephrine cultures by growth phase, *pyk* and *rpoB* were both among the most stably expressed genes, but when analysing both growth phases together, only *pyk* remained in the top three rankings. When comparing Aerobic and Anoxic samples, *proS* ranked among the most stable genes in exponential and stationary phase data sets as well as in combined rankings. When analysing the Aerobic, Epinephrine, and Anoxic samples together, only *gyrA* ranked consistently among the top three most stably expressed genes during exponential and stationary growth as well as in combined rankings; the *rho* gene ranked as least stably expressed gene in this data set.

Reference gene stability should be carefully assessed with the design of the experiment in mind. In this study, even the commonly used reference genes *16S rRNA* demonstrated large variability in stability depending on the conditions studied and how the data were analysed. As
previously suggested, the best approach may be to use a geometric mean of multiple genes to normalise qPCR results. As researchers continue to validate reference genes for various organisms in multiple growth conditions and sampling time points, it may be possible to make informed predictions as to which genes may be most suitable to validate for a given experimental design, but in the meantime, the reference genes used to normalise qPCR data should be selected with caution.

4.2 Introduction

*Actinobacillus suis* is a gram-negative facultative anaerobe which is a frequent member of the normal microbiome of swine tonsils of the soft palate (Kernaghan et al., 2012). It is also an important pathogen in pigs of all ages, where it can cause septicaemia and sequelae such as meningitis, arthritis, and pleuropneumonia (MacInnes and Desrosiers, 1999). However, little is known about the pathogenesis of *A. suis*, including the expression of virulence-associated genes.

Quantitative real-time PCR (qPCR) is a sensitive method for the determination of bacterial gene expression. In most qPCR studies, endogenous reference genes are used to control for sample-to-sample variations that may arise due to differences in cell number and efficiency of RNA extraction and cDNA synthesis, among other factors (Huggett et al., 2005). Further, using a reference gene permits for normalisation of multiple genes to a common control, allowing for more robust data comparison. However, several recent studies suggest that rather than relying on commonly used genes, reference genes should be carefully selected and rigorously validated (Derveaux et al., 2010; Dheda et al., 2005; Huggett et al., 2005; Radonic et al., 2004). Also, it has been suggested that using the geometric mean of data collected from multiple reference
genes is more appropriate than relying on a single reference gene for normalisation (Vandesompele et al., 2002).

Therefore, the objective of this work was to validate reference genes of a clinical isolate of A. suis, H91-0380, grown in different conditions and sampled during different growth phases. Eight reference genes were selected for evaluation based on published expression studies of other members of the family Pasteurellaceae (Klitgaard Nielsen and Boye, 2005; Ren et al., 2013) and the analysis of the A. suis genome for the presence of commonly used reference genes.

4.3 Methods

4.3.1 Bacterial strains and growth media

Actinobacillus suis H91-0380, a virulent O2:K2 clinical isolate collected in Southwestern Ontario, Canada, from a pig with septicaemia (MacInnes et al., 2012; Van Ostaaijen et al., 1997) (Table 4.1), was grown in brain heart infusion (BHI) (BD, Sparks, MD); epinephrine (Sigma-Aldrich, St. Louis, MO) was added to the growth medium at the time of inoculation to a final concentration of 50 µM.

4.3.2 Growth conditions

Aerobic cultures of A. suis H91-0380 (+/- epinephrine) were grown in BHI at 37°C with shaking at 200 rpm. Anoxic static cultures were grown without shaking in BHI at 37°C + 5% CO₂ in 1 mL aliquots in sealed 1.5 mL microcentrifuge tubes.

Growth curves were done in triplicate by measuring the OD₆₀₀ of A. suis every 30 minutes from the time of inoculation until stationary phase was achieved, and then three or more additional times. Sampling time points for early exponential and early stationary phases of
growth were determined, and the number of CFU/mL was calculated by plating 10-fold serial dilutions of the cultures on Columbia agar with 5% sheep blood (Oxoid Co., Nepean, ON).

4.3.3 RNA extraction

Samples of ~1x10^8 CFU were collected from aerobic cultures at 60 and 180 minutes post-inoculation (mpi), and from anoxic cultures at 60 and 210 mpi (representing exponential and stationary phases, respectively). Cells were pelleted at 6000 X g for 5 minutes at 4°C and the supernatant was decanted. Cells were lysed as previously described (Deslandes et al., 2010). Briefly, the pellet was suspended in 100 µL pre-warmed SDS lysis solution (2% SDS, 16 mM EDTA) and heated to 100°C for 5 minutes. After addition of 1 mL TRIzol, the lysate was incubated for 5 minutes at room temperature, and then frozen at -70°C until RNA was extracted.

RNA extraction was done from four independent biological replicates of each culture at the two sampling time points using the Direct-zol RNA MiniPrep Kit (Zymo Research Co., Irvine, CA). RNA was then precipitated with 2.5 M lithium chloride (Amresco, Solon, OH), re-suspended in nuclease-free water, and treated with DNase I (Invitrogen, Carlsbad, CA) for 30 minutes at 37°C. Ethylenediaminetetraacetic acid (EDTA; 2.3 mM) was then added and the samples were heat-inactivated at 65°C for 10 minutes. RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

cDNA was synthesised from 500 ng of total cellular RNA by random priming using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) in the presence of RNase inhibitor as per the manufacturer’s instructions.
4.3.4 Semi-quantitative real-time PCR

Primers were designed using Primer3 as previously described (Thornton and Basu, 2011), and are listed in Table 4.2. The amplification efficiencies of all primer pairs were between 95 and 102%. Three technical replicates were done per sample in a total reaction volume of 10 µL, which contained 5 µL of PerfeCTa® SYBR® Green FastMix® (Quanta BioSciences, Inc., Gaithersburg, MD), 2.5 µL of a forward/reverse primer mix with 1.6 µM of each primer, and 2.5 µL of cDNA diluted 1:15.

Real-time PCR was done in a StepOnePlus Thermocycler (Applied Biosystems, Foster City, CA) using a program with an initial denaturation step at 95°C for 30 seconds followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. Stepwise melt curves were done at the end of each run to confirm that only one template was amplified.

4.3.5 Reference gene validation

The stability of eight reference genes (Table 4.1) was assessed using RefFinder (http://www.leonxie.com/referencegene.php), a web-based tool that integrates the algorithms for geNorm, Normfinder, BestKeeper, and the comparative ∆Ct methods to rank candidate reference genes from most to least stable. A composite score is then assigned to each gene by taking into account the rankings of the various algorithms employed.

4.4 Results

4.4.1 Growth curves to determine sampling time points

To determine the times of early exponential and early stationary growth phase, growth curves of aerobic cultures with (Epinephrine) or without (Aerobic) 50 µM epinephrine added to the
growth medium at the time of inoculation, and anoxic static cultures (Anoxic) (Figure 4.1) were done. Aerobic cultures, with or without epinephrine, grew to a higher optical density than the Anoxic cultures. However, the presence of epinephrine in the growth medium did not affect the rate of growth of these cultures relative to Aerobic cultures.

To control for sample variability (Bustin et al., 2005), once the exponential and early stationary growth phase time points were identified, the volume of culture sampled was adjusted to ensure that an approximately equal number of cells was collected for RNA extraction each time; cell numbers were also enumerated by plate counting.

4.4.2 Reference gene validation based on growth condition and growth phase

Comparisons of comprehensive stability rankings of reference genes were determined for the epinephrine study (Aerobic and Epinephrine samples) and the anoxic study (Aerobic and Anoxic samples). For overall comparison, samples from both the epinephrine and the anoxic studies at each time point (exponential and stationary) and combined samples (collected during both exponential and stationary growth) were also evaluated.

When comparing gene stability for samples collected during exponential growth phase in the epinephrine study (Table 4.3), the presence of epinephrine in the growth media affected the order of the gene stability rankings. In the Aerobic cultures, \textit{ackA} was the most stably expressed gene, while \textit{glyA} was the least stably expressed. Conversely, in the Epinephrine cultures, \textit{ackA} was the least stably expressed gene and \textit{glyA} was the second most stable gene. In both Aerobic and Epinephrine cultures, \textit{pyk} ranked in the top two most stably expressed genes, so it was not surprising that it was the most stably expressed gene when all samples collected during
exponential growth in the epinephrine study were combined. With the exception of $ackA$ and $glyA$, the overall order of the stability rankings for the Aerobic and Epinephrine samples was comparable.

In the anoxic study, the stability of reference genes in samples collected during exponential growth for both Aerobic and Anoxic cultures were compared (Table 4.4). The $16S$ rRNA gene was the most stably expressed in Anoxic cultures, but it ranked second to last in the Aerobic samples. The second and third most stably expressed genes in the Anoxic samples, $proS$ and $gyrA$, ranked in the top three most stably expressed genes in the Aerobic cultures, as well. Interestingly, $rpoB$, the least stably expressed gene in the Aerobic cultures, ranked third in the combined stability rankings of reference genes for exponential samples collected from both Aerobic and Anoxic cultures, while the second most stably expressed gene in the Aerobic samples, $glyA$, was ranked as the least stably expressed gene in the combined scores.

When comparing the stability of reference genes from samples collected in the epinephrine study during stationary phase (Table 4.5), there was less variation among the top three most stably expressed genes compared to exponential samples. In stationary growth, both $pyk$ and $rpoB$ ranked in the top three for Aerobic, Epinephrine, and combined samples. The expression of $16S$ rRNA was less stable in the Aerobic samples than in the Epinephrine samples, while the opposite was true for $gyrA$. The least stably expressed gene, $ackA$, was consistent in all rankings, and $glyA$ was also found in the bottom three rankings for all analyses of these samples.

In the samples collected for the anoxic study at stationary phase, the stability rankings differed in Aerobic samples and Anoxic samples (Table 4.6). The stability scores of $proS$, $16S$
rRNA, and rho did not differ substantially between these different growth conditions. The scores of rpoB, pyk, glyA, gyrA, and ackA, however, varied markedly, resulting in very different ranking orders for reference genes measured in the Aerobic and Anoxic cultures.

When the data for all samples collected during exponential and stationary growth in the epinephrine study were analysed together, the overall stability rankings of the top three genes was similar in both growth conditions, and this was reflected in the combined rankings (Table 4.7), with proS, pyk, and glyA being the most stably expressed. With the exception of rho, which consistently ranked as the least stably expressed gene, the difference in stability scores of the remaining 4 reference genes was not substantial.

Comprehensive rankings of the combined exponential and stationary samples of the anoxic study (Table 4.8) had similar trends to those observed in the epinephrine study, with proS and pyk ranking high in stability. The only exception to this was with glyA and gyrA, where stability in Aerobic and Anoxic cultures differed enough to affect both their stability scores and their overall rankings in each growth condition.

Finally, data from samples collected in both the epinephrine and anoxic studies were combined in order to determine overall comprehensive rankings and the effect of multiple growth conditions on gene stability rankings (Table 4.9). During exponential phase, 16S rRNA, gyrA, and ackA were the most stable reference genes, with comparable stability scores. However, in stationary phase, only gyrA remained in the top ranking reference genes for stability, with 16S rRNA and ackA ranking near the bottom of the list. The other two most stably expressed genes were rpoB, which ranked 5th for exponential samples, and pyk, which ranked 4th. In the combined
comprehensive ranking, the most stably expressed reference genes were *pyk, glyA*, and *gyrA*. When comparing these genes to the combined comprehensive rankings from the other analyses, it is interesting to note that at least one of these genes, and sometimes two, ranked among the top three most stably expressed genes in all other conditions. Using geNorm’s calculation of pairwise variation to determine the optimal number of reference genes, it was found that three reference genes were adequate for effective data normalisation (data not shown).

In summary, in the epinephrine study, where only aerobic cultures with or without epinephrine in the growth media were compared, two of the same reference genes (*pyk* and *rpoB*) ranked in the top three most stably expressed genes in samples collected during exponential and stationary phases (Tables 4.3 and 4.5). However, when the comprehensive stability rankings of all samples in the epinephrine study collected during both phases of growth were compared (Table 4.7), only *pyk* remained among the top three most stably expressed genes. Similarly, in the anoxic study, where Aerobic and Anoxic samples were grouped together in the experimental design, *proS* and *rpoB* both ranked among the most stably expressed reference genes in the combined rankings for each of the exponential (Table 4.4) and stationary (Table 4.6) phase samples, whereas only *proS* ranked among the top three most stably expressed genes of the combined scores for all samples of the anoxic study collected during both growth phases (Table 4.8).

4.5 Discussion

To date, no studies have been done to characterise reference genes for *A. suis* and our preliminary studies suggested that reference genes used to study closely related organisms were
not appropriate. Therefore, the expression stability of eight reference genes was assessed in
different growth conditions, growth phases, and with various methods of data analysis. No single
reference gene was suitable for normalisation of qPCR results in all growth conditions, sampling
time points, or experimental designs. Depending on how the data were analysed, the overall
stability rankings of all the reference genes evaluated varied markedly.

Some of the reference genes validated in this study were studied in previous work done by
Klitgaard Nielsen and Boye (Klitgaard Nielsen and Boye, 2005) in *Actinobacillus
pleuropneumoniae*. Similar to *A. pleuropneumoniae*, *glyA* and *pyk* were stably expressed in most
conditions and time points in *A. suis*. On the other hand, *rho* ranked low in stability in nearly all
cases, often at or near the bottom of the list of genes characterised, and demonstrated several C_{t}
differences between exponential and stationary phase samples, and between Aerobic and Anoxic
samples in the anoxic studies (data not shown).

When choosing appropriate reference genes, consideration should be given to the design of
the study, as the number of sampling time points and the different growth phases in which
samples are collected can impact the choice of reference genes for data normalisation
downstream. Likewise, if the experimental design includes samples from numerous growth
conditions with changes in variables such as degree of aeration (shaken vs. static), overall levels
of oxygen (aerobic vs. anoxic), different additives in the growth media (presence or absence of
epinephrine), and phase of growth when sampling (exponential vs. stationary), the impact on the
comprehensive stability rankings of potential reference genes can be drastic. A thorough
reference gene validation study of *Staphylococcus epidermidis* by Vandecasteele et al.
(Vandecasteele et al., 2001) found that gene expression of purported reference genes, particularly that of 16s rRNA, varied in response to different growth conditions. In this study, when combining all samples from both the epinephrine study and the anoxic study collected at both exponential and stationary phases (Table 4.9), gyrA was the only reference gene that ranked among the top three most stably expressed genes during exponential phase, stationary phase, and combined sampling time points.

The determination of whether to keep or discard a reference gene can be made based on the stability scores assigned by the different algorithms that go into determining the composite score. If a gene is found to rank consistently low in stability by most or all of the individual algorithms for a given experimental design, the composite score of this gene will reflect this due to the weighted calculation employed in its determination. Similarly, the cut-off between a suitable or unsuitable reference gene can be considered in the context of the individual algorithms depending on the design of the study. BestKeeper and geNorm employ similar techniques of pairwise comparisons of reference genes while considering the dataset as a whole rather than considering the possible effects of comparing different time points or replicates collected (Hildyard and Wells, 2014). On the other hand, NormFinder takes into account these latter types of variation, and compares each gene to the mean derived from the dataset and so it is better able to identify the gene(s) with the greatest stability in the conditions included in the dataset. To benefit from the strengths of each algorithm, and to limit the inherent biases from the assumptions employed by these different methods of reference gene validation, the composite score assigned by RefFinder reflects the geometric mean of the weighted ranking of a gene from the different algorithms. It is also valuable to employ GeNorm’s calculation of pairwise
variations in normalisation factors for different combinations of reference genes in order to determine the optimum number of reference genes recommended for accurate normalisation (Vandesompele et al., 2002). Employing this method in addition to the stability rankings from the algorithms and the composite scores from RefFinder allows for a reasonable validation of the most stably expressed reference genes as well as the ideal number of reference genes suited to a given experimental design based on the genes tested.

There have been few reference gene validation studies published for members of the family Pasteurellaceae. In two studies of Haemophilus ducreyi, qPCR was used to validate expression of a subset of genes from RNA-Seq or microarray findings. In the RNA-Seq study (Gangaiah et al., 2013), dnaE was used to normalise qPCR results, but no mention was made as to why this gene was chosen or if its stability was validated by qPCR. In the microarray study (Labandeira-Rey et al., 2009), gyrB was used to normalise the cDNA per sample because transcript levels of this gene did not change during DNA microarray experiments. In a study of the expression of Pasteurella multocida virulence genes during experimental infection of mice, 16S rRNA was used to normalise qPCR results (Ren et al., 2013), but it was not explicitly stated as to why this gene was chosen or if it was validated for this study. In a study of A. pleuropneumoniae biofilms cultured under static and planktonic conditions and sampled at different time points, qPCR was used to validate microarray results (Tremblay et al., 2013). In this study, the results were normalised using rluC based on its constant signal in the microarrays, though it is not clear whether this gene was also validated independently by qPCR. In a study of Aggregatibacter actinomycetemcomitans, qPCR was used to look at whether the expression of selected genes from in vivo-induced antigen technology in human infections was consistent with the expression
of the same genes during epithelial cell interaction. In this work, 16S rRNA and gapdh were used for normalisation (Richardson et al., 2005). While these reference genes were validated, the authors mentioned that their expression was variable and that they were differentially expressed under experimental conditions. Despite this, Longo et al. (Longo et al., 2013) used gapdh to normalise expression data in a later study. Finally, in a study looking at gene expression of Mannheimia haemolytica at two time points during experimental infection of calves and at early log phase of bacteria grown in vitro, dnaN was used to normalise qPCR results based on its apparent stable expression in a previous microarray study (Sathiamoorthy et al., 2012); however, no mention was made as to whether this gene was specifically validated for qPCR.

There have been many studies where 16S rRNA has been used as the sole reference gene for data normalisation with little or no data provided regarding its suitability. Others have observed variability in the stability of 16s rRNA for various organisms grown under different conditions, and even strain-to-strain variation among members of the same species (Metcalf et al., 2010; Vandecasteele et al., 2001). In the current study, 16S rRNA ranked as the most stably expressed gene in three instances (Tables 4.4, 4.5, and 4.9), and in the top three most stably expressed genes for combined data once (Table 4.5). This is not to say that 16S rRNA is not suitable for some studies, but caution should be taken in assuming that this gene is stably expressed in all growth conditions and growth phases, and its suitability should be assessed for each study and experimental design.
4.6 Conclusions

The current study demonstrated the relative stability rankings of eight reference genes in *A. suis* in different growth conditions and growth phases. This work lends further support to the notion that reference genes must be carefully assessed with all of the experimental conditions in mind, and that one should not rely on commonly used genes without first demonstrating the stability of their expression under the specific conditions under study. As more qPCR studies are reported, it may be possible to make informed predictions as to which reference genes might be useful to select for validation studies; however, in the meantime, caution is warranted. Finally, these data support the recommendations of Vandesompele et al. (2002) that the best approach to normalise qPCR results may be to use the geometric mean of multiple reference genes.
Table 4.1. Bacterial strain studied and reference genes characterised by qPCR

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<td><em>Actinobacillus suis</em> H91-0380</td>
<td>O2:K2 clinical isolate</td>
<td>(MacInnes et al., 2012; Van Ostaaijen et al., 1997)</td>
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<tr>
<td><strong>Genes</strong></td>
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<td></td>
</tr>
<tr>
<td><em>16S rRNA</em></td>
<td>16S ribosomal subunit</td>
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<td>Acetate kinase A</td>
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<tr>
<td><strong>glyA</strong></td>
<td>Glycine/serine hydroxymethyltransferase</td>
<td>ASU2_01625</td>
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<td><strong>gyrA</strong></td>
<td>DNA gyrase subunit A</td>
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<td>Prolyl-tRNA synthetase</td>
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<td><strong>rho</strong></td>
<td>Transcription termination factor Rho</td>
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<td><strong>rpoB</strong></td>
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Table 4.2. Primers used to conduct reference gene validation by qPCR

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<td>ASU2-16SrRNA-R1</td>
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</tr>
<tr>
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<tr>
<td>ASU2-pyk-R1</td>
<td>AACTAACGCAGCATCACCGATTT</td>
</tr>
<tr>
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<td>TTCTTTCTGACGGTTTCGGTTTCTT</td>
</tr>
<tr>
<td>ASU2-rho-R1</td>
<td>AACGCGGGATTGCTTGTG</td>
</tr>
<tr>
<td>ASU2-rpoB-F1</td>
<td>AACCGCAACAAGATCACCTCAAGGTT</td>
</tr>
<tr>
<td>ASU2-rpoB-R1</td>
<td>ATTTGACGACGAAACCCTAAGTAAA</td>
</tr>
</tbody>
</table>
Figure 4.1. Growth rates of *A. suis* H91-0380 in BHI in aerobic and anoxic static conditions

Cultures were grown aerobically with (Epinephrine) and without (Aerobic) 50 µM epinephrine in the growth medium, and under anoxic static (Anoxic) growth conditions. Error bars represent the standard error of the mean (SEM). At least three biological replicates were done for each growth curve.
Table 4.3. Comprehensive stability rankings of epinephrine study reference genes from early exponential phase samples

<table>
<thead>
<tr>
<th></th>
<th>Aerobic</th>
<th>Epinephrine</th>
<th>Combined</th>
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<tbody>
<tr>
<td>ackA</td>
<td>1.86</td>
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</tr>
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<td>pyk</td>
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<td>16S rRNA</td>
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<tr>
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<td>rho</td>
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<tr>
<td>glyA</td>
<td>6.44</td>
<td>ackA</td>
<td>6.74</td>
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</tbody>
</table>

Note: Smaller numbers represent more stably expressed genes
Table 4.4. Comprehensive stability rankings of anoxic study reference genes from early exponential phase samples

<table>
<thead>
<tr>
<th></th>
<th>Aerobic</th>
<th>Anoxic</th>
<th>Combined</th>
</tr>
</thead>
<tbody>
<tr>
<td>gyrA</td>
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<td>16S rRNA</td>
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<td>proS</td>
<td>2.21</td>
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<td>gyrA</td>
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<td>rpoB</td>
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<td>5.24</td>
<td>pyk</td>
<td>4.23</td>
</tr>
<tr>
<td>ackA</td>
<td>5.69</td>
<td>glyA</td>
<td>6.48</td>
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<td>ackA</td>
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<tr>
<td>rpoB</td>
<td>6.96</td>
<td>rho</td>
<td>7.44</td>
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</tbody>
</table>

Note: Smaller numbers represent more stably expressed genes
Table 4.5. Comprehensive stability rankings of epinephrine study reference genes from early stationary phase samples

<table>
<thead>
<tr>
<th>Aerobic</th>
<th>Epinephrine</th>
<th>Combined</th>
</tr>
</thead>
<tbody>
<tr>
<td>pyk</td>
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<tr>
<td>glyA</td>
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<td>5.23</td>
</tr>
<tr>
<td>ackA</td>
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<td>8.00</td>
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</tbody>
</table>

Note: Smaller numbers represent more stably expressed genes
Table 4.6. Comprehensive stability rankings of anoxic study reference genes from early stationary phase samples

<table>
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<tr>
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<th>Aerobic</th>
<th>Anoxic</th>
<th>Combined</th>
</tr>
</thead>
<tbody>
<tr>
<td>rpoB</td>
<td>1.41</td>
<td>1.41</td>
<td>pyk</td>
</tr>
<tr>
<td>pyk</td>
<td>1.57</td>
<td>gyrA</td>
<td>proS</td>
</tr>
<tr>
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<td>3.41</td>
<td>proS</td>
<td>rpoB</td>
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<tr>
<td>gyrA</td>
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<td>gyrA</td>
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<td>ackA</td>
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</tr>
<tr>
<td>glyA</td>
<td>6.48</td>
<td>rpoB</td>
<td>ackA</td>
</tr>
<tr>
<td>ackA</td>
<td>8.00</td>
<td>pyk</td>
<td>rho</td>
</tr>
</tbody>
</table>

*Note: Smaller numbers represent more stably expressed genes*
Table 4.7. Comprehensive stability rankings of epinephrine study reference genes from exponential and stationary growth phase samples

<table>
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<tr>
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<th>Aerobic</th>
<th>Epinephrine</th>
<th>Combined</th>
</tr>
</thead>
<tbody>
<tr>
<td>proS</td>
<td>1.68</td>
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<td>1.57</td>
</tr>
<tr>
<td>pyk</td>
<td>1.68</td>
<td>2.06</td>
<td>2.06</td>
</tr>
<tr>
<td>glyA</td>
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</tr>
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<tr>
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<tr>
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<td>5.44</td>
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<td>5.73</td>
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</table>

Note: Smaller numbers represent more stably expressed genes.
Table 4.8. Comprehensive stability rankings of anoxic study reference genes from exponential and stationary growth phase samples

<table>
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<th>Anoxic</th>
<th>Combined</th>
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</thead>
<tbody>
<tr>
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<td>gyrA</td>
<td>proS</td>
</tr>
<tr>
<td>glyA</td>
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<td>proS</td>
<td>2.06</td>
</tr>
<tr>
<td>pyk</td>
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<td>pyk</td>
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<tr>
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<td>16S rRNA</td>
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<td>ackA</td>
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<td>rho</td>
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<tr>
<td>rho</td>
<td>7.24</td>
<td>rpoB</td>
<td>8.00</td>
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</tbody>
</table>

Note: Smaller numbers represent more stably expressed genes
Table 4.9. Comprehensive stability rankings of genes from epinephrine and anoxic studies at exponential and stationary phase

<table>
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<th>Exponential</th>
<th>Stationary</th>
<th>Combined</th>
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<tbody>
<tr>
<td>16S rRNA</td>
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<td>rpoB</td>
<td>pyk</td>
</tr>
<tr>
<td>gyrA</td>
<td>2.06</td>
<td>pyk</td>
<td>glyA</td>
</tr>
<tr>
<td>ackA</td>
<td>2.59</td>
<td>gyrA</td>
<td>gyrA</td>
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<tr>
<td>pyk</td>
<td>3.81</td>
<td>glyA</td>
<td>proS</td>
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<tr>
<td>rpoB</td>
<td>3.94</td>
<td>proS</td>
<td>rpoB</td>
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<tr>
<td>glyA</td>
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<td>ackA</td>
<td>ackA</td>
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<td>proS</td>
<td>5.60</td>
<td>16S rRNA</td>
<td>16S rRNA</td>
</tr>
<tr>
<td>rho</td>
<td>8.00</td>
<td>rho</td>
<td>rho</td>
</tr>
</tbody>
</table>

Note: Smaller numbers represent more stably expressed genes
Chapter 5: Differential expression of putative adhesin genes of

*Actinobacillus suis* grown in *in vivo*-like conditions

This chapter is based on a manuscript of the same title that will be submitted to the journal Veterinary Microbiology with the authors: Adina R. Bujold, Josée Labrie, Mario Jacques, and Janet I. MacInnes
5.1 Abstract

*Actinobacillus suis* is an opportunistic pathogen that resides in the tonsils of the soft palate of swine. Unknown factors can cause this organism to invade the host, leading to septicaemia and various sequelae including death. To better understand the pathogenesis of *A. suis*, the expression of selected adhesin genes was measured by RT-qPCR grown in conditions that mimicked the host environment during different stages of interaction with the host including with different nutrient and oxygen levels and in the presence of the stress hormone epinephrine. The presence of 50 µM epinephrine in the growth medium did not affect the growth rate or the expression of *A. suis* adhesin genes, but there was a significant growth phase effect for many genes. All genes were differentially expressed in exponential or stationary growth. In addition, most adhesin genes were also differentially expressed during anoxic static growth or aerobic growth. Using the time*treatment interactions observed in the anoxic study, a model of persistence of *A. suis* in the host environment in both biofilm and planktonic states is proposed. Biofilm formation studies using diluted overnight cultures as well as exponential and stationary cultures of wild type *A. suis* and isogenic mutants of the type IVb pilin (∆*flp1*), the outer membrane protein (∆*ompA*), and the putative fibronectin-binding (∆*comE1*) suggested that disruption of these adhesin genes impacts the early stages of biofilm formation, but that in most cases, the biofilm formation by the mutant strains is similar to wild type at the later stages of development. We postulate that this may be due to compensatory mechanisms by other adhesins resulting from redundancies and duplications in adhesive functions.
5.2 Introduction

Tonsils play a significant role in the host immune response of many animals, acting as a first defence against antigens entering the upper respiratory tract (Horter et al., 2003). They are also an important site of colonisation of commensal organisms and can act as a portal of entry for a number of important bacterial pathogens (Horter et al., 2003). *Actinobacillus suis* is a gram-negative facultative anaerobe that is a common commensal of the tonsils of the soft palate of swine (Kernaghan et al., 2012). While it typically resides harmlessly at this site, in response to unknown factors, *A. suis* can invade and cause septicaemia and sequelae such as meningitis, pleuritis, and arthritis (MacInnes and Desrosiers, 1999).

Colonisation of the host, an important early step in the pathogenesis of many bacteria, is mediated by adhesins. Previous studies have shown that *A. suis* is genetically similar to other members of the family *Pasteurellaceae*, including the primary swine pathogen *A. pleuropneumoniae* and the opportunistic pathogens *A. ureae* and *A. capsulatus* (Bujold and MacInnes, 2015a). It has been further shown that *A. suis* shares many of the same adhesin genes with these organisms, including the tight adherence (*tad*) locus, a type IV pilus operon, a filamentous haemagglutinin (FHA), six autotransporters (ATs), and 12 outer membrane proteins (OMPs) (Bujold and MacInnes, 2015a). It is tempting to speculate that each adhesin plays a different role in colonisation and disease, but at present, nothing is known about the role or regulation of expression of the adhesins of *A. suis*.

It is generally thought that microbes persist at body sites such as the tonsils as part of complex biofilms (Diaz et al., 2011; Jacques et al., 2010). The first step of biofilm formation is
attachment of planktonic cells to surfaces such as host tissues, followed by maturation of the biofilm, dispersion of cells from the mature biofilm, a return to the planktonic state, and the development of biofilms at new sites (Monroe, 2007). Nutrient and oxygen availability is thought to be different at the various stages of biofilm development. In the biofilm form, cells are likely in a low oxygen, low nutrient environment, and in the stationary phase of growth. Over time, cells shed from biofilms entering the planktonic stage grow exponentially in a higher nutrient and oxygen environment.

Little is known about the host factors that control the expression of genes involved in the different states of biofilm formation. However, one possible host signal—catecholamines—has been shown to directly affect the growth and expression of virulence-associated genes in a number of pathogens (Lyte, 2014). For example, epinephrine and norepinephrine affect the growth of some, but not all, human periodontal pathogens in vitro (Roberts et al., 2002). These hormones have also been shown to increase biofilm formation and up-regulate genes that lead to the increased attachment of enterohaemorrhagic *E. coli* to epithelial cells in vitro (Bansal et al., 2007). In contrast, *A. pleuropneumoniae* biofilm formation and the ability to adhere to epithelial cells in vitro is not affected by either of these two hormones, at least under the conditions used in studies by Li et al. (2012a).

Many of the homologues of the putative adhesins identified in *A. suis* have described roles in biofilm formation and host cell attachment in other organisms (Table 5.1; (Bujold and MacInnes, 2015a). For example, in other members of the family *Pasteurellaceae* and some other gram-negative bacteria, type IV pili (PilA) contribute to biofilm formation and attachment to epithelial
cells (Craig et al., 2004). Similarly, homologues of genes encoding type IVb pili (encoded within the tight adherence (tad) locus—flp1-flp2-tadV-rcpC-rcpA-rcpB-tadZ-tadA-tadB-tadC-tadD-tadE-tadF-tadG) are important for the development of microcolonies and persistent biofilms in the oral pathogen *Aggregatibacter actinomycetemcomitans*, *Haemophilus ducreyi*, and other species (Spinola et al., 2003; Tomich et al., 2007). Strains of *A. pleuropneumoniae* with type IVb pili are better able to adhere to porcine lung epithelial cells than nonpiliated strains (Li et al., 2012b). Fine-tangled pili (FtpA), belonging to the DNA protection during starvation (DPS) protein family, have been shown to contribute to adhesion to host cells and auto-aggregation in *Campylobacter jejuni*, *Haemophilus influenzae*, and other species (Haikarainen and Papageorgiou, 2010; Pang et al., 2012; Theoret et al., 2012). A *comE1* gene homologue, *comE1*, whose product has been associated with attachment to the extracellular matrix (ECM) component fibronectin in several members of the family *Pasteurellaceae* (Mullen et al., 2008a) is also present in *A. suis* (Bujold and MacInnes, 2015a). Filamentous haemagglutinin (encoded by *fhaCB*) contributes to attachment and host colonisation as well as biofilm formation in *A. suis* and several other species such as *Bordetella* spp., *Histophilus somni*, *Pasteurella multocida*, and *Acinetobacter baumannii* (Darvish Alipour Astaneh et al., 2014; Nicholson et al., 2009; Ojha et al., 2005; Sandal et al., 2009). The OmpA protein in *A. suis* has a demonstrated role in attachment to swine tonsil explants and porcine brain microvascular endothelial cells (Ojha et al., 2010; Ojha et al., 2005). An OmpP2 homologue has also been identified in *A. suis*. In nontypeable *Haemophilus influenzae*, the OmpP2 porin is involved in attachment to the host through mucin (Reddy et al., 1996). Finally, classical autotransporters, such as YcgV, and trimeric autotransporter adhesins (TAAs) have been implicated in biofilm formation and
adherence to host epithelial cells and ECM components in *Aggregatibacter actinomycetemcomitans* (reviewed by Douglas et al. (2014)), *A. pleuropneumoniae* (Qin et al., 2015), and other organisms (Cotter et al., 2005).

In this study, gene expression for twelve adhesin genes was measured under different growth conditions and during different growth phases. The genes chosen for this work encode the various classes of adhesins, including fimbriae, autotransporters, outer membrane proteins (OMPs), and miscellaneous other afimbrial adhesins. We hypothesised that *A. suis* will express different adhesins in environments predicted to mimic those encountered during biofilm and planktonic growth phases and that an unknown trigger, perhaps a stress signal such as the catecholamine epinephrine, could cause *A. suis* to assume an invasive phenotype wherein it would express a different complement of adhesins.

### 5.3 Methods

#### 5.3.1 Bacterial strains and growth conditions

The strains and plasmids used in this study are listed in Table 5.1. *Actinobacillus suis* H91-0380, a virulent O2:K2 clinical isolate (MacInnes et al., 2012), and isogenic mutants of this strains were grown in brain heart infusion (BHI) (BD, Sparks, MD) as previously described (Bujold and MacInnes, 2015b). *E. coli* β2155 was routinely cultured in Luria-Bertani (LB) medium supplemented with 1 mM diaminopimelic acid (DAP; Sigma-Aldrich, St. Louis, MO). Chloramphenicol (Sigma-Aldrich, St. Louis, MO) was also added at 25 μg/mL or 5 μg/mL, as required.
5.3.2 RNA extraction and cDNA synthesis

RNA was extracted from ~1 x 10^8 CFU collected from aerobic cultures at 60 and 180 minutes post-inoculation (mpi), and from anoxic cultures at 60 and 210 mpi (representing exponential and stationary phases, respectively), as previously described (Bujold and MacInnes, 2015b). Four independent biological replicates of each culture were done at each time point.

cDNA was synthesised as per the manufacturer’s instructions from 500 ng of total cellular RNA by random priming using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) in the presence of RNase inhibitor.

5.3.3 Semi-quantitative real-time PCR

Primers were designed using Primer3 as previously described (Thornton and Basu, 2011), and are listed in Supplemental table 5.1S. The amplification efficiencies of all primer pairs were between 95.0 and 116.9%. RT-qPCR was carried out in a StepOnePlus Thermocycler (Applied Biosystems, Foster City, CA) as previously described (Bujold and MacInnes, 2015b), with three technical replicates per sample, and cDNA diluted 1:15. Stepwise melt curves were done at the end of each run to confirm that only one template was amplified.

Expression data were analysed using the Pfaffl method and normalised to 3 validated reference genes (glyA, gyrA, and pyk; Table 5.1) as previously described (Bujold and MacInnes, 2015b).

5.3.4 Mutant generation

Unmarked isogenic mutants were generated for the flp1, ompA, and comEl genes of A. suis H91-0380. The flp1 gene was chosen as it is the pilin gene in the tight adherence (tad) locus.
which encodes type IVb pili that have been previously implicated in biofilm formation and autoaggregation (Tomich et al., 2007). The ompA gene was selected as the OmpA outer membrane protein was previously shown to attach to tonsil explants (Ojha et al., 2010), and we postulated that it may also participate in biofilm formation. The comE1 gene was chosen because it encodes a fibronectin-binding protein thought to be important in attachment to the host extracellular matrix (Mullen et al., 2008a).

Based on the genome sequence (MacInnes et al., 2012), primers were designed containing restriction enzyme (RE) cut sites to amplify the 5′ (flanked by Sail and XhoI RE sites) and 3′ (flanked by XhoI and NotI RE sites) portions of each gene. PCR products were digested with appropriate REs, purified, and ligated into the pEMOC2 plasmid that was linearised with Sail and NotI REs. The resultant plasmid was electroporated into E. coli β2155 and mobilised into A. suis H91-0380 using a modification of the filter mating technique previously described by Lone et al. (2009). Briefly, cells from overnight cultures of E. coli β2155 carrying the modified pEMOC2 plasmids grown in LB broth with 25 ug/mL chloramphenicol and A. suis H91-0380 grown in BHI broth were pelleted at 6000 X g for 3 minutes, washed with TNM buffer (1 mM Tris-HCl, pH 7.2; 10 mM MgSO₄; 100 mM NaCl), and re-suspended in TNM buffer to a final OD₆₀₀nm of 1.0. One hundred µL of the E. coli suspension and 10 µL of the A. suis suspension were combined, and the volume was adjusted to 1 mL with TNM buffer. Twenty-five microlitres of the cell suspension were spotted onto a 0.45 µm nitrocellulose mating filter (25 mm diameter) placed on a BHI agar plate containing 1 mM DAP and 10 mM MgSO₄. The plate was inverted and incubated at 37°C in an atmosphere of 5% CO₂ for 16-24 h. The cells were washed off the filter with 2 mL BHI broth, pelleted by centrifugation at 6000 X g for 3 minutes, and re-
suspended in 500 µL sterile PBS. Serial dilutions of the re-suspended cells were plated on BHI agar containing 5 µg/mL chloramphenicol and incubated at 37°C in an atmosphere of 5% CO₂ for 24-48 hours. When required, cells were subcultured to obtain single colony isolates. Trans-conjugants (A. suis isolates that had successfully integrated the modified pEMOC2 plasmid into the genome) were verified by colony PCR amplification.

Overnight cultures of trans-conjugants were grown in 5 mL Mueller-Hinton (MH) broth and used to seed fresh MH broth, which was grown for an additional 2 h. Cells were pelleted and re-suspended in 100 µL MH broth and the entire volume was spread onto MH agar containing 10% sucrose. Plates were incubated at 30°C for up to 48 hours and subcultured onto fresh plates to obtain single colonies. Mutants were confirmed by colony PCR and sequencing. A summary of this procedure is shown in supplementary figure 5.1S.

5.3.5 Biofilm formation assays

For the first set of experiments, overnight cultures of A. suis H91-0380 wild type, Δflp1, ΔompA, and ΔcomE1 were diluted 1/100 with BHI and added in technical triplicate to the wells of a sterile 96-well microtitre plate (Costar 3599, Corning, NY, USA). After incubation for 6 or 24 h at 37°C in an atmosphere of 5% CO₂, wells were washed and stained with crystal violet (0.1%), and absorbance was measured, as previously described (Labrie et al., 2010). The experiment was repeated in biological triplicate.

For the second set of experiments, each strain was grown to exponential or stationary phase as for the RNA extractions, then cultures were added to the wells of a microtitre plate and
incubated for 6 and 24 hours as in the first biofilm experiment. The wells were stained and quantified as above. Three biological and three technical replicates were done.

5.3.6 Statistical analysis

Real-time PCR data were analysed using PROC MIXED of SAS 9.2 (SAS Institute), using the model:

\[ Y = \mu + D_i + T_j + D*T_{ij} + \varepsilon_{ijk} \]

Where:

\( Y \) = The dependant variable

\( \mu \) = The variable mean

\( D_i \) = The fixed effect of treatment

\( T_j \) = The fixed effect of time

\( D*T_{ij} \) = The interaction of time and treatment

\( \varepsilon_{ijk} \) = The residual error

Culture within treatment was used as a repeated measure, along with 5 variance/covariance structures. The variance/covariance structure with the lowest AIC and BIC values were used for statistical analysis.

Differences in biofilm phenotypes among strains were determined by a paired-one-tailed \( t \)-test using GraphPad Prism version 4.0 (GraphPad Software, San Diego, CA, USA).
5.4 Results

5.4.1 Effect of epinephrine and growth phase on adhesin gene expression

Adhesin gene expression in aerobic cultures grown in the presence (Epinephrine) and absence (Aerobic) of epinephrine at early exponential and early stationary phases of growth was analysed by RT-qPCR. Consistent with previous data (Bujold and MacInnes, 2015b), the presence of 50 µM epinephrine in the growth medium did not affect the growth rate of *A. suis*, nor did it affect the expression of the adhesin genes tested compared to Aerobic cultures (Figure 5.1). However, several genes were differentially expressed during stationary growth compared to exponential growth (Figure 5.2). Four genes were significantly up-regulated in stationary phase (*ftpA, pilA, fhaB, and ompA*) while four genes were up-regulated in exponential phase (*tadD, tadG, comE1, and ompP2*).

5.4.2 Effect of anoxic static growth on adhesin gene expression

Adhesin gene expression was also measured in anoxic static (Anoxic) cultures at early exponential and early stationary phases of growth and compared to expression in Aerobic cultures. Eight genes were differentially expressed in Anoxic versus Aerobic cultures (Figure 5.3), i.e., *flp1, tadD, pilA, fhaC, and plp4* were up-regulated, while *ftpA, ompA*, and *ycgV* were down-regulated. Additionally, all of the adhesin genes evaluated were differentially expressed in one growth phase (Figure 4): *tadD, tadG, pilA, comE1, plp4*, and *ompP2* were up-regulated in exponential phase, while *flp1, ftpA, fhaB, fhaC, ompA*, and *ycgV* were up-regulated in stationary phase.
5.4.3 Time*treatment effects

In addition to treatment and time effects, time*treatment effects were also observed for 8 of the genes (Table 5.2, Figure 5.5). The fimbriae-associated *flp1* gene from the *tad* locus was up-regulated in anoxic conditions during both exponential and stationary growth, but more so in stationary phase. Another member of the *tad* locus, *tadD*, was up-regulated in anoxic conditions during exponential phase. The fine-tangled pili gene, *ftpA*, was up-regulated in anoxic conditions during exponential phase, but more substantially up-regulated in aerobic conditions during stationary phase. The type IV pilin gene, *pilA*, was highly up-regulated during anoxic exponential growth. Likewise, the filamentous haemagglutinin gene, *fhaB*, was significantly up-regulated during anoxic exponential growth; however, its transporter gene, *fhaC*, was up-regulated during anoxic stationary phase. The porin *ompA* was also up-regulated in aerobic culture, particularly during stationary phase while another porin, *plp4*, was up-regulated during anoxic exponential growth. The conventional autotransporter, *ycgV* was significantly up-regulated in the anoxic exponential phase, and even more highly up-regulated during aerobic stationary growth.

5.4.4 Biofilm formation

The ability to form biofilms was evaluated for the *A. suis* H91-0380 wild type, Δ*flp1*, Δ*ompA*, and Δ*comE1* strains (Figure 5.6). Despite the fact that the Δ*ompA* strain grew more slowly than the other mutants the ability of diluted overnight cultures of all three isogenic mutant strains to form biofilms was significantly reduced compared to wild type after 6 h of incubation, (Figure 5.6 a). After 24 h of incubation of the diluted overnight cultures, only the Δ*ompA* strain
had a reduced ability to form biofilm compared to wild type, despite a comparable level of growth observed for all strains at this time (Figure 5.6 b).

The ability of exponential cultures of the $\Delta$ompA mutant to form biofilms after 6 h of incubation was significantly reduced compared to wild type, but no significant difference was observed with the other mutants (Figure 5.7 a), nor was there any significant difference in the ability of any of the exponential cultures of the adhesin mutants tested to form biofilms after 24 h of incubation (Figure 5.7 b). The ability of $\Delta$flp1 and the $\Delta$ompA mutants in stationary culture to form biofilms after 6 h of incubation was less than wild type (Figure 5.7 c), but no significant difference was observed in biofilm formation after 24 h of incubation (Figure 5.7 d).

5.5 Discussion

5.5.1 Effect of epinephrine on adhesin gene expression

The relative expression of adhesin genes was measured in aerobic cultures grown in the presence of 50 µM epinephrine in the growth medium (Epinephrine) compared to cultures grown without epinephrine (Aerobic). Consistent with the results of a previous study of the effect of epinephrine on $A.\ pleuropneumoniae$ (Li et al., 2012a), but in contrast to studies in $E.\ coli$ (Bansal et al., 2007), the presence of 50 µM epinephrine did not significantly affect the expression of any of the adhesin genes examined. However, most of the genes tested here were up-regulated in one growth phase. This finding is consistent with the notion that some adhesins are involved in attachment/colonisation only during specific stages of growth. To gain a better
understanding of the effects of growth phase on differential expression, other treatments were examined, namely, anoxic static growth.

5.5.2 Effect of anoxic static growth on adhesin gene expression

It is generally thought that when bacteria colonise a host, they exist as part of a biofilm during at least some part of their lifecycle. In the current study, growth conditions were selected to simulate the oxygen and nutrient availability expected during the various phases of biofilm development, and the expression of selected putative adhesin-associated genes was characterised by RT-qPCR (Figure 5.8). Exponentially growing Anoxic cultures with abundant but ever-decreasing nutrient and oxygen availability as the cells deplete the medium over time were used to represent the microcolony stage of early biofilm development. Anoxic cultures during stationary phase were used to represent the environment of a more mature biofilm with its depleted nutrients and oxygen. Samples from Aerobic cultures during the stationary phase were posited to represent the initial stages of dispersion with cells in a planktonic state but still in stationary phase of growth immediately after breaking away from the mature biofilm. Finally, samples taken from Aerobic cultures during exponential phase were used to represent planktonic cells with their readily available nutrients and higher levels of oxygen.

Microcolony formation

In the samples representing the early microcolony formation stage (anoxic exponential), 8 genes were significantly differentially expressed. Of particular note, flp1, tadD, pilA, and plp4 were up-regulated while ompA was down-regulated. Previous studies suggest that the genes of the tad locus from flp1 to tadD are co-transcribed (Haase, 2003), so the coordinated expression
of these genes was not unexpected. These results are also consistent with the findings of Kachlany et al. (2000), wherein the authors showed that in *Aggregatibacter actinomycetemcomitans*, type IVb (Flp) pili play an important role in the non-specific attachment and aggregation of the bacterium to a variety of surfaces, including host and bacterial cell membranes and ECM components. Our findings are also consistent with the report of Auger et al. (2009) that two genes within the potentially co-transcribed region of the Tad locus (*tadB* and *rcpA*) are up-regulated during the adherence of *A. pleuropneumoniae* to epithelial cells.

The expression of the type IV pilin gene, *pilA*, was up-regulated in the conditions mimicking early biofilm formation. A previous study by Boekema et al. (2004) found that type IV pili were not present on the surface of *A. pleuropneumoniae* when it was grown in rich media, but could be detected when the organism was grown in chemically defined medium under microaerophilic conditions. This result suggests that the limited oxygen rather than the composition of the growth medium may have been responsible for the apparent down-regulation of the type IV pili in this study. However, further characterisation of other genes in the *pilABCD* operon would be helpful to determine if they demonstrate an expression profile similar to that of *pilA* during anoxic growth. Deslandes et al. (2010) also observed that the *A. pleuropneumoniae* type IV pili genes *apfB* and *apfC* are up-regulated *in vivo* during natural infection, and Boekema et al. (2004) further observed that the *apf* promoter is active *in vitro* during attachment to epithelial cells and *in vivo* during the early stages of infection. Together with the results of the current study, it seems reasonable to postulate that *pilA* could be involved in early attachment and microcolony formation of *A. suis*. 

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Given their different and multifunctional roles in the cell, the opposite expression of the *plp4* and *ompA* porin genes was not surprising (Galdiero et al., 2012). The previously described role of *ompA* in attachment to swine tonsil explants (Ojha et al., 2010) might lead to the hypothesis that this gene would be up-regulated in these samples, but it is difficult to explain this result without further studies.

**Maturation**

In conditions representing those in the mature biofilm (anoxic stationary), 5 genes were significantly differentially expressed. Of particular note was the up-regulation of *flp1* and *fhaC*. The *flp1* gene was up-regulated during microcolony formation, but its expression further increased during the maturation phase of biofilm formation. These results are consistent with previous findings of the important role of type IVb pili in biofilm formation (reviewed by (Tomich et al., 2007). The expression of the *fhaC* gene, thought to encode the transporter for the FHA, was also up-regulated more than in other growth conditions. In one study of *A. pleuropneumoniae* (Deslandes et al., 2010), FHA was found to be up-regulated during the acute phase of infection, and proposed to be important in establishing disease, but in other *in vitro* expression studies by these researchers, differential expression of this gene was not detected (Tremblay et al., 2013). In the current studies, the up-regulation of the transporter is consistent with an increase in the transport of the FHA adhesin to the surface of the cell. The ability of some species to adhere to eukaryotic cells is enhanced by secreted FHA (Ward et al., 1998), so differential expression of the transporter might be sufficient to increase the presence of FHA on
the surface of the cell or secretion into the extracellular space, but further studies would be needed to demonstrate this.

**Dispersion**

In samples representing the dispersion step (aerobic stationary), 5 genes were differentially expressed. Of note, *ftpA*, *ompA*, and *ycgV* were all significantly up-regulated in these samples. These findings are not consistent with *in vitro* studies by Auger et al. (2009) who found that the encoding fine-tangled pilus in *A. pleuropneumoniae* (*ftpA*) was down-regulated during attachment to epithelial cells, but up-regulated during planktonic growth over epithelial cells. However, up-regulation of the *A. pleuropneumoniae* *ftpA* gene during natural infection (Deslandes et al., 2010) and in growth media favouring biofilm formation (Labrie et al., 2010) has been demonstrated. Up-regulation of *ftpA* in samples representing the dispersion step of the biofilm cycle is consistent with this adhesin being important primarily in the transition from a biofilm to a planktonic lifestyle, but more detailed studies are necessary.

The putative classical autotransporter *ycgV* was also up-regulated during aerobic stationary growth in this study. In *Bordetella* species and *E. coli*, this gene encodes a pertactin-like adhesin with described roles in auto-aggregation and adherence to epithelial cells among other functions (Cotter et al., 2005; Nicholson et al., 2009; Sherlock et al., 2005). Based on the proposed role of this gene in early attachment and biofilm formation, up-regulation of this gene in these conditions was not anticipated, but *ycgV* might have other functions that are not yet described.
Planktonic

In samples representing the planktonic stage (aerobic exponential), 8 genes were differentially expressed, including ftpA, fhaB, and ycgV, all of which were down-regulated. The expression of the ftpA gene was lowest in these conditions compared to all others. This finding is in contrast to the *in vitro* expression studies of *A. pleuropneumoniae* (Auger et al., 2009), but the authors report that their attachment results were strain-dependent, so it would not be surprising that gene expression results were, as well. The expression of the ftpA gene may also be associated with its other roles in DNA protection during stress (Haikarainen and Papageorgiou, 2010) in response to unmeasured variables in this study; however, further experiments need to be done to demonstrate the role(s) of this gene in planktonic and/or biofilm states. The down-regulation of fhaB expression during planktonic growth seems consistent with the role of FHA in attachment and biofilm formation described in other organisms (Darvish Alipour Astaneh et al., 2014; Nicholson et al., 2009; Sandal et al., 2009).

### 5.5.3 Biofilm formation of *A. suis* wild type and mutants

The biofilm assays conducted in this work were under static growth conditions in an anoxic environment. In all instances where reduced biofilm formation was observed, the greatest effects were found at the earlier incubation time (6 h). In most cases, the phenotypes of the mutant strains at the later incubation time (24 h) were not significantly different than wild type. The only exception to this was the biofilm formation of the ∆ompA strain from diluted overnight culture. Given the described functions of *flp1* in autoaggregation and biofilm formation, and the up-regulation of this gene in anoxic static growth, the results of the biofilm assays are consistent
with previous findings. Considering the up-regulation of \textit{comE1} in exponential phase, which we hypothesise to be equivalent to planktonic growth and the microcolony formation stage of biofilm formation, the abrogated biofilm formation of this mutant in the earlier incubation of the biofilm assay also supports our findings. Furthermore, given that ComE1 attaches to the host glycoprotein fibronectin, which can be found in both the ECM underlying epithelial cells and in soluble form in plasma (Theocharis et al., 2015), it seems reasonable to postulate that these types of interactions would be important during the early stages of host attachment, though this remains to be verified. The significantly reduced biofilm formation of the diluted overnight cultures of the \textit{\Delta ompA} mutant after 24 h of incubation are more difficult to explain, since they appear to be in contrast to the expression results above. However, the difference may be attributed to the slower growth rate of this strain compared to the other strains tested, or perhaps to off-target effects on the cell caused by the other roles \textit{ompA} plays in cell wall integrity and perhaps even nutrient acquisition (Galdiero et al., 2012). But, as iterated above, further experiments into characterising the role of \textit{ompA} in attachment and biofilm formation are clearly warranted.

The biofilm assays conducted using cultures grown to exponential and stationary phases were undertaken to better match the growth conditions used in the expression component of this study. Overall, the findings of these assays were that the early stages of biofilm establishment (in this case, the 6 h post-incubation samples) were when the mutant phenotypes for impeded biofilm formation, if any, were most apparent. This may suggest that at these early stages in biofilm formation, the effects of the adhesins that participate in this process are more marked, whereas by the later stages in biofilm formation (e.g., 24 h post-incubation), other adhesins are better able
to compensate for the lacking adhesins, a finding that would be consistent with previous suggestions of redundancies and overlaps in adhesive structures. Further examination of the adhesins at play in the later stages of biofilm would be valuable to clarify this.

5.6 Conclusion

In this work, we examined the expression of adhesin genes of the opportunistic swine pathogen *A. suis* H91-0380 in conditions that mimic the host environment present in the tonsils of the soft palate. These included different oxygen and nutrient levels, as well as the presence of the host stress hormone epinephrine. Epinephrine added to the growth medium at 50 µM did not affect the growth rate or expression of the adhesin genes tested, but phase of growth (exponential or stationary) and anoxic static growth conditions had a significant effect on the expression of several adhesin genes. From these results, we present a possible model for the persistence of *A. suis* in the tonsils of the soft palate of swine in both biofilm and planktonic states.

We also generated isogenic mutants of three important adhesin genes, *flp1*, *ompA*, and *comE1*, that are involved in autoaggregation and biofilm formation, maintenance of cell wall integrity and attachment to tonsil explants, and fibronectin-binding, respectively. With these mutants, we examined the ability of *A. suis* to form biofilms in a static system inoculated with diluted overnight cultures or with exponential or stationary phase cultures. Overall, we observed that when biofilm formation was impeded in the mutant strains, the most marked effects were observed in the earlier stages of biofilm, but that the wild type phenotype was usually recovered in the samples that were incubated for a longer period of time. We postulate that this is the result
of a compensatory mechanism by other adhesins with overlapping or redundant functions that allow the bacterium to better adapt to its environment.
Table 5.1. Strains and genes used to characterise the expression of *A. suis* adhesin genes in different growth conditions and growth phases

<table>
<thead>
<tr>
<th>Bacterial strain or genes</th>
<th>Characteristic(s)</th>
<th>Reference or locus tag</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Actinobacillus suis</em> H91-0380</td>
<td>O2:K2 clinical isolate</td>
<td>(MacInnes et al., 2012; Van Ostaaijen et al., 1997)</td>
</tr>
<tr>
<td><em>E. coli</em> β2155</td>
<td><em>thrB1004 pro thi hsdS lacZΔM15 (F’ lacZΔM15 lacI1 traD36 proA+ proB+</em>) ∆dap: :erm (Ermr)</td>
<td>(Baltes et al., 2003)</td>
</tr>
<tr>
<td><em>Actinobacillus suis</em> Δ<em>flp1</em></td>
<td>Δ<em>flp1</em></td>
<td>This study</td>
</tr>
<tr>
<td><em>Actinobacillus suis</em> Δ<em>ompA</em></td>
<td>Δ<em>ompA</em></td>
<td>This study</td>
</tr>
<tr>
<td><em>Actinobacillus suis</em> Δ<em>comE1</em></td>
<td>Δ<em>comE1</em></td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pEMOC2</td>
<td>Transconjugation vector based on pBluescript SK with mobRP4, polycloning site, Cm and transcriptional fusion of the <em>omlA</em> promoter with the <em>sacB</em> gene</td>
<td>(Baltes et al., 2003)</td>
</tr>
<tr>
<td>pEMOC2-<em>flp1</em>&lt;sup&gt;mut&lt;/sup&gt;</td>
<td>pEMOC2 vector with ligated 5’ and 3’ fragments of <em>flp1</em> and flanking sequence in region of insertion</td>
<td>This study</td>
</tr>
<tr>
<td>pEMOC2-<em>ompA</em>&lt;sup&gt;mut&lt;/sup&gt;</td>
<td>pEMOC2 vector with ligated 5’ and 3’ fragments of <em>ompA</em> and flanking sequence in region of insertion</td>
<td>This study</td>
</tr>
<tr>
<td>pEMOC2-<em>comE1</em>&lt;sup&gt;mut&lt;/sup&gt;</td>
<td>pEMOC2 vector with ligated 5’ and 3’ fragments of <em>comE1</em> and flanking sequence in region of insertion</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Reference Genes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>16S rRNA</em></td>
<td>16S ribosomal subunit</td>
<td>ASU2_r11471, ASU2_r11473, ASU2_r11475, ASU2_r11477, ASU2_r11469, ASU2_r11479</td>
</tr>
<tr>
<td><em>ackA</em></td>
<td>Acetate kinase A</td>
<td>ASU2_03825</td>
</tr>
<tr>
<td><em>glyA</em></td>
<td>Glycine/serine hydroxymethyltransferase</td>
<td>ASU2_01625</td>
</tr>
<tr>
<td><em>gyrA</em></td>
<td>DNA gyrase subunit A</td>
<td>ASU2_01490</td>
</tr>
<tr>
<td>Gene</td>
<td>Description</td>
<td>Accession</td>
</tr>
<tr>
<td>-------</td>
<td>-------------------------------------------------------</td>
<td>------------</td>
</tr>
<tr>
<td>proS</td>
<td>Prolyl-tRNA synthetase</td>
<td>ASU2_08190</td>
</tr>
<tr>
<td>pyk</td>
<td>Pyruvate kinase</td>
<td>ASU2_06045</td>
</tr>
<tr>
<td>rho</td>
<td>Transcription termination factor Rho</td>
<td>ASU2_01275</td>
</tr>
<tr>
<td>rpoB</td>
<td>DNA-directed RNA polymerase subunit β</td>
<td>ASU2_09775</td>
</tr>
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</table>

**Putative adhesin genes**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>Accession</th>
</tr>
</thead>
<tbody>
<tr>
<td>comE1</td>
<td>Fibronectin-binding protein homologue</td>
<td>ASU2_10345</td>
</tr>
<tr>
<td>fhaB</td>
<td>Filamentous haemagglutinin transporter</td>
<td>ASU2_06635</td>
</tr>
<tr>
<td>fhaC</td>
<td>Filamentous haemagglutinin</td>
<td>ASU2_06640</td>
</tr>
<tr>
<td>fip1</td>
<td>Tight adherence locus; pilin</td>
<td>ASU2_04295</td>
</tr>
<tr>
<td>ftpA</td>
<td>Fine-tangled pili</td>
<td>ASU2_09130</td>
</tr>
<tr>
<td>ompA</td>
<td>Outer membrane protein A homologue</td>
<td>ASU2_09940</td>
</tr>
<tr>
<td>ompP2</td>
<td>Outer membrane protein P2 homologue</td>
<td>ASU2_00030</td>
</tr>
<tr>
<td>pilA</td>
<td>Type IV pilus pilin</td>
<td>ASU2_05045</td>
</tr>
<tr>
<td>plp4</td>
<td>Outer membrane protein A homologue</td>
<td>ASU2_11270</td>
</tr>
<tr>
<td>tadD</td>
<td>Tight adherence locus</td>
<td>ASU2_04345</td>
</tr>
<tr>
<td>tadG</td>
<td>Tight adherence locus</td>
<td>ASU2_04360</td>
</tr>
<tr>
<td>ycgV</td>
<td>Adhesin/invasion autotransporter</td>
<td>ASU2_07665</td>
</tr>
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</table>
Figure 5.1. The treatment effect of 50 μM epinephrine on the expression of *A. suis* adhesin genes, relative to aerobic cultures grown without epinephrine

Aerobic cultures are shown with white bars, Epinephrine cultures with black bars. Relative expression is normalised to the geometric mean of three validated reference genes. Error bars represent the standard error of the mean (SEM).
Figure 5.2. The time effect of growth phase on adhesin gene expression

Exponential phase cultures are shown with white bars, stationary phase with black bars. Relative expression is normalised to the geometric mean of three validated reference genes. Error bars represent the standard error of the mean (SEM). Statistical significance is indicated as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. 
**Figure 5.3.** The treatment effect of Anoxic growth on the expression of *A. suis* adhesin genes, relative to Aerobic growth

Aerobic cultures are shown with white bars, Anoxic with black bars. Relative expression is normalised to the geometric mean of three validated reference genes. Error bars represent the standard error of the mean (SEM). Statistical significance is indicated as follows: * p < 0.05, ** p < 0.01, *** p < 0.001.
Figure 5.4. The time effect of growth phase on the expression of *A. suis* adhesin genes during Aerobic and Anoxic growth

Exponential phase cultures are shown with white bars, stationary phase with black bars. Relative expression is normalised to the geometric mean of three validated reference genes. Error bars represent the standard error of the mean (SEM). Statistical significance is indicated as follows: * p < 0.05, ** p < 0.01, *** p < 0.001.
Table 5.2. Analysis of variance (ANOVA) of treatment, time, and time*treatment effects of Aerobic and Anoxic culture adhesin gene expression

<table>
<thead>
<tr>
<th>Gene</th>
<th>Treatment</th>
<th>Time</th>
<th>Time*Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>flp1</td>
<td>&lt;0.0001</td>
<td>0.003</td>
<td>0.001</td>
</tr>
<tr>
<td>tadD</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.0471</td>
</tr>
<tr>
<td>tadG</td>
<td>0.1133</td>
<td>0.002</td>
<td>0.7538</td>
</tr>
<tr>
<td>ftpA</td>
<td>0.01</td>
<td>0.001</td>
<td>0.003</td>
</tr>
<tr>
<td>pilA</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>comE1</td>
<td>0.12</td>
<td>0.003</td>
<td>0.52</td>
</tr>
<tr>
<td>fhaB</td>
<td>0.18</td>
<td>0.005</td>
<td>0.94</td>
</tr>
<tr>
<td>fhaC</td>
<td>0.0008</td>
<td>0.003</td>
<td>0.12</td>
</tr>
<tr>
<td>ompA</td>
<td>0.0006</td>
<td>0.0007</td>
<td>0.03</td>
</tr>
<tr>
<td>plp4</td>
<td>0.008</td>
<td>0.002</td>
<td>0.009</td>
</tr>
<tr>
<td>ompP2</td>
<td>0.61</td>
<td>&lt;0.0001</td>
<td>0.01</td>
</tr>
<tr>
<td>ycgV</td>
<td>0.004</td>
<td>0.003</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Note: Numbers represent p values
Figure 5.5. Time*treatment interactions of adhesin gene expression for Aerobic and Anoxic cultures

Aerobic exponential cultures are shown with white bars, Anoxic exponential with light grey, Aerobic stationary with dark grey, and Anoxic stationary with black bars. Relative expression is normalised to the geometric mean of three validated reference genes. Error bars represent the standard error of the mean (SEM). Statistical significance is indicated as follows: * p < 0.05, ** p < 0.01, *** p < 0.001.
Figure 5.6. Biofilm formation by *A. suis* H91-0390 wild type, ∆*flp*, ∆*ompA*, and ∆*comE1* strains from overnight cultures after 6 (a) and 24 h (b) of incubation.

Error bars represent the standard error of the mean (SEM). Statistical significance is indicated as follows: * p < 0.05, ** p < 0.01, *** p < 0.001.
Figure 5.7. Biofilm formation by *A. suis* H91-0390 wild type, Δ*fpl1*, Δ*ompA*, and Δ*comE1* strains grown to exponential (a and b) and stationary (c and d) phase, after 6 (a and c) and 24 h (b and d) of incubation.

Error bars represent the standard error of the mean (SEM). Statistical significance is indicated as follows: * p < 0.05, ** p < 0.01, *** p < 0.001. (* p < 0.05, ** p < 0.01, *** p < 0.001)
Figure 5.8. Model of *A. suis* attachment and persistence in the tonsils, and adhesin genes involved in each step

It is postulated that planktonic bacteria (Aerobic exponential) will attach to the host during initial attachment (Anoxic exponential) and microcolony formation. Over time, the biofilm will mature (Anoxic stationary), and eventually cells will detach from the biofilm and disperse into the extracellular space (Aerobic stationary). The dispersed cells will give rise to planktonic cells that go on to establish new biofilms or perhaps to invade the host environment. Up-regulated genes are shown by green arrows (↑), and down-regulated by red arrows (↓). Parenthesis indicate time effects rather than time*treatment* interactions.
Supplementary table 5.1S. Primers used to characterise the expression of *A. suis* adhesin
genes in different growth conditions and growth phases

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5′-3′)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASU2-16srRNA-F1</td>
<td>GTGTAACGGTGAAATGCGTAGAG</td>
<td>(Bujold and MacInnes, 2015b)</td>
</tr>
<tr>
<td>ASU2-16srRNA-R1</td>
<td>ACATGAGCGTCACTACATTTCCA</td>
<td>(Bujold and MacInnes, 2015b)</td>
</tr>
<tr>
<td>ASU2-ackA-F1</td>
<td>AGGCACCTATTCTACATCTACAAAA</td>
<td>(Bujold and MacInnes, 2015b)</td>
</tr>
<tr>
<td>ASU2-ackA-R1</td>
<td>TACGAAACACAGATACAGAACACC</td>
<td>(Bujold and MacInnes, 2015b)</td>
</tr>
<tr>
<td>ASU2-thaB-F1</td>
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<td>(Bujold and MacInnes, 2015a)</td>
</tr>
<tr>
<td>ASU2-thaB-R1</td>
<td>ACATGAGCGTCACTACATTTCCA</td>
<td>(Bujold and MacInnes, 2015a)</td>
</tr>
<tr>
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<td>(Bujold and MacInnes, 2015a)</td>
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Supplementary figure 5.1S. Experimental design for generating unmarked isogenic mutants of A. suis H91-0380

Mutated adhesin gene Δadh was cloned into the pEMOC2 vector (pEMOC2Δadh). Wild type A. suis H91-0380 was mated with E. coli carrying pEMOC2Δadh and single crossover events yielded trans-conjugants with pEMOC2Δadh integrated into the A. suis genome. Trans-conjugants were selected for chloramphenicol resistance (Cm\(^\text{r}\)). Sucrose counter-selection for second single crossover events was used to identify isolates that excised the plasmid, leaving Δadh in place of adh\(^{\text{wt}}\). (Adapted from Oswald et al. (1999))
Chapter 6: Attachment of *Actinobacillus suis* H91-0380 and its isogenic adhesin mutants to extracellular matrix components of the tonsils of the soft palate of swine

6.1 Abstract

The tonsils of the soft palate of swine are important secondary lymphoid organs that conduct immune surveillance of antigens entering the upper respiratory tract. In spite of their immunological function, they can also act as a site of persistence and invasion of bacterial and viral pathogens. *Actinobacillus suis* is a common resident of the tonsils of the soft palate in pigs of all ages, but under certain circumstances, *A. suis* is able to invade, resulting in septicaemia and related sequelae. Twenty-four putative adhesins are predicted to be encoded by the *A. suis* genome, but to date, very little is known about how these adhesins might be involved in colonisation or invasion. To better understand these processes, lysates of the tonsils of the soft palate of swine were characterised by mass spectrometry. Fifty-nine extracellular matrix (ECM) proteins were identified including collagen I, collagen IV, fibronectin, laminin, vitronectin, small-leucine-rich proteoglycans, integrins, and other cell-surface receptors. In addition, the ability of wild type and 3 adhesin mutants to bind collagen I, collagen IV, fibronectin, laminin, and vitronectin was evaluated. Exponential cultures of wild type *A. suis* adhered significantly more than stationary cultures to all of the ECM components tested except collagen I. The Δ*flp1* strain attached less than wild type to collagen IV during exponential growth, while the Δ*ompA* strain had reduced attachment to all ECMs during exponential growth. The Δ*comE1* mutant had
less attachment to collagen IV, fibronectin, and vitronectin during exponential growth. Furthermore, ∆comE1 exhibited differential attachment to collagen I over short adherence time points during exponential growth. These results suggest that all of the adhesin genes tested are important during the early but not the later stages of attachment to ECM components found in the tonsils. This latter result is consistent with the notion that other adhesins may have compensatory effects during the later stages of attachment. To our knowledge, this is the first report of the proteome of porcine tonsils and one of only a small number of investigations into the interaction of a member of the family Pasteurellaceae with extracellular matrix components in swine.

6.2 Introduction

Tonsils are secondary lymphoid organs that are an important part of the host immune response against antigens entering the body through the mouth and nose. Pigs have 5 sets of tonsils located around the oropharyngeal cavity in the so-called Waldeyer’s ring, the largest being the tonsils of the soft palate (Casteleyn et al., 2011a). The tonsils of the soft palate of swine have as many as 200 branching crypts that end blindly within the lymphoid tissue beneath (Belz and Heath, 1996). The lumina of the crypts are covered by a layer of non-keratinised squamous epithelium that is continuous with the epithelium of the oral cavity, while lymphoepithelium lines the deeper areas of the crypts (Kernaghan et al., 2012). Lymphoepithelium has a thinner epithelial lining interspersed with goblet cells and M cells, the latter of which can be used by some pathogens to invade the tonsil (Belz and Heath, 1996). The lymphoepithelium also allows passage of lymphocytes, plasma cells, and macrophages (Horter et
The epithelium of the crypts overlays a basement membrane (sometimes fragmented), which is in turn supported by an interstitial extracellular matrix (ECM) of varying thickness, depending on the location in the tonsil (Belz and Heath, 1996). Despite studies of the ultrastructure and fine structure of the tonsils of swine (Belz and Heath, 1996; Casteleyn et al., 2011a) and studies into the ECM components and host cell receptors present in tonsils of other species (Castaños-Velez et al., 1995; Curran et al., 2015; Maatta et al., 2004; Ruco et al., 1995), little has been done to characterise these components in the tonsils of the soft palate of swine.

Components of the ECM have important functions in the host, providing both structure to tissues and an anchor to the overlying epithelium (Theocharis et al., 2015). Cell signalling also occurs between the epithelial cells and the ECM components via integrins and other receptors expressed on the basolateral side of the cell surface that can affect the morphology of the cytoskeleton (Theocharis et al., 2015). The ECM components, including glycoproteins, proteoglycans, and other molecules, are present in different regions of the connective tissue. The specific composition of the ECM can vary depending on the body site. For example, laminin and collagen IV form independent networks in the basement membrane and are interconnected by nidogens and perlecan (Theocharis et al., 2015). Fibronectin and collagen I are ubiquitous ECM components in the connective tissues of the body and are mainly found in the interstitial matrix (Theocharis et al., 2015), though fibronectin can also be found in a soluble form in plasma (To and Midwood, 2011). Another adhesive glycoprotein, vitronectin, is also abundant in blood plasma and in the ECM, where it participates in adhesion and tissue repair, assists in formation of the membrane attack complex, and promotes neutrophil infiltration during infection (Singh et al., 2010). There are also extracellular proteoglycans called small leucine-rich proteoglycans
(SLRPs) that serve the role of “tissue organisers” during development, attachment, and wound repair by interacting with fibrillar collagens, growth factors, and host receptors (Iozzo et al., 2011). Other important components of the ECM include hyaluronan, numerous types of collagen, elastin and its associated proteins, proteases such as matrix metalloproteases and plasmin(ogen), growth factors, and many other molecules (Mecham, 2011).

In addition to their role in host tissue integrity and structure, the components of the ECM are also used by many bacteria as a site of attachment, colonisation, and sometimes invasion (Horter et al., 2003). It should be noted that in order for bacteria to gain access to the ECM, they must first breach the barrier of the epithelium. This can be accomplished when damage occurs to the epithelium that exposes the ECM, by breaking the tight junctions between epithelial cells, by invading the epithelial or M cells and emerging on the basolateral side, or by being taken across the epithelial barrier by leucocytes (Belz and Heath, 1996; Bosch et al., 2013; Horter et al., 2003; Kraehenbuhl and Neutra, 2000).

The processes of attachment and colonisation are mediated primarily by fimbrial and afimbrial adhesins and various host cell receptors including ECM components. A limited number of studies have been done to identify host receptors in the oral cavity of swine, but among members of the family Pasteurellaceae, the trimeric autotransporter adhesin DsrA of Haemophilus ducreyi binds to fibronectin and vitronectin in vitro (Leduc et al., 2009), and the EmaA autotransporter of Aggregatibacter actinomycetemcomitans binds to collagen V (Tang et al., 2007). The purified lipoprotein e (P4) of nontypeable Haemophilus influenzae binds with high affinity to laminin, fibronectin, and vitronectin (Su et al., 2016), while the OmpA outer
membrane protein (OMP) of *Pasteurella multocida* adheres to cell surface fibronectin (Katoch et al., 2014), as does OmpA of *Mannheimia haemolytica* (Lo and Sorensen, 2007). The ComE1 protein, which is present in several members of the family *Pasteurellaceae*, also attaches to fibronectin (Mullen et al. (2008a).

*Actinobacillus suis*, a member of the family *Pasteurellaceae*, is a gram-negative facultative anaerobe that is a common resident of the tonsils of the soft palate of swine (MacInnes et al., 2008). Typically, it resides harmlessly at this site, but unknown factors can cause this organism to invade the bloodstream resulting in often fatal septicaemia and sequelae such as meningitis, pleuritis, and arthritis (MacInnes and Desrosiers, 1999). Little is known about the pathogenesis of *A. suis*, including how it attaches to and colonises the tonsils, where it typically resides, or by what mode it enters the bloodstream. An OmpA homologue may be important for *A. suis* infection (Ojha et al., 2005) and crossing the blood-brain barrier (Ojha et al., 2010), but nothing is known about the host receptor for OmpA or anything about the receptors for the other 23 putative adhesins identified by characterising the genome of *A. suis* (Bujold and MacInnes, 2015a). Therefore, the first objective of this study was to identify ECM components in the tonsils of the soft palate of swine and the second objective was to investigate the possible interaction of 3 putative adhesins with some of these molecules. The adherence of exponentially growing and stationary phase cultures of isogenic mutants of *flp1, comE1, and ompA* to selected ECM components was compared to that of the wild type. The *flp1* gene encodes the pilin of the type IVb pilus of the tight adherence (*tad*) locus, which is involved in biofilm formation in many members of the family *Pasteurellaceae* and other gram-negative organisms (Tomich et al., 2007). The *comE1* gene encodes a putative fibronectin-binding protein previously described by
Mullen et al. (2008a) in other members of the Pasteurellaceae. The A. suis ompA gene encodes an outer membrane protein (OMP) that attaches to swine tonsil explants and brain microvascular epithelial cells (Ojha et al., 2010).

6.3 Methods

6.3.1 Bacterial strains and growth conditions

Strains and plasmids used in this study are listed in Table 6.1. *Actinobacillus suis* H91-0380, a virulent O2:K2 clinical isolate (MacInnes et al., 2012), and unmarked isogenic mutants (Δflp1, ΔompA, and ΔcomE1) generated from this strain (Chapter 5) were grown in brain heart infusion (BHI; BD, Sparks, MD) as previously described (Bujold and MacInnes, 2015b).

6.3.2 Tonsil preparation for mass spectrometry

Approximately one gram of frozen tonsil tissue, collected from pigs exhibiting no signs of clinical disease, was minced with sterile scissors and a scalpel blade and added to 3 mL ice-cold phosphate-buffered saline (PBS). The sample was fully homogenised using a Bio-Gen PRO200® homogeniser (Diamed, Mississauga, ON) in five 15-second intervals at a speed ramping from 5 000 to 35 000 rpm. The homogenate was freeze-thawed twice at -70°C and spun at 10 000 X g at 4°C for 20 minutes to remove cellular debris. Total protein concentration was quantified by BCA assay (Pierce, Thermo Fisher Scientific, Waltham, MA) and diluted to a final concentration of 1 mg/mL prior to trypsinisation and analysis by mass spectrometry.

The procedure described above was conducted a second time using Tris-buffered saline (TBS) instead of PBS. TBS homogenate was further processed using the Anionic Cell Lysis Kit
SP810 (Protea Biosciences, Inc., Morgantown, WV) according to manufacturer’s instructions, including the degradation of the Anionic Acid Labile Surfactant (AALS) II. This AALS tonsil lysate was trypsinised and fractionated by gradual elution by strong cation exchange chromatography from SCX SpinTips (Protea Biosciences, Inc., Morgantown, WV) using increasing concentrations of ammonium formate (20 mM to 500 mM) in 10% acetonitrile.

The crude lysates in PBS and TBS and the AALS processed lysates were submitted to the SPARC BioCentre at the Hospital for Sick Children (Toronto, ON); the fractionated AALS lysate samples were submitted to the University of Guelph Advanced Analysis Centre (Guelph, ON), for analysis by mass spectrometry.

6.3.3 Mass spectrometry

Samples analysed at the SPARC BioCentre were trypsinised and the peptides were loaded onto a 150 μm ID pre-column (Magic C18, Michrom Biosciences, Bruker Co., Billerica, MA) at 4 μL/min and separated over a 75 μm ID analytical column packed into an emitter tip containing the same packing material. The peptides were eluted over 60 minutes at 300 nL/min. using a 0 to 40% acetonitrile gradient in 0.1% formic acid with an EASY n-LC nano-chromatography pump (Proxeon Biosystems, Odense, Denmark). The peptides were eluted into a LTQ-Orbitrap hybrid mass spectrometer (Thermo-Fisher, Bremen, Germany) operated in a data dependent mode. MS was acquired at 60 000 FWHM resolutions in the FTMS and MS/MS was carried out in the linear ion trap. Six MS/MS scans were obtained per MS cycle. The raw data were searched using Mascot 2.3.02 (Matrix Sciences, London, UK) and search results were analysed using Scaffold 3.4.3 (Proteome Software Inc., Portland, OR), with SwissProt, UniProt
(http://www.uniprot.org/), and Ensembl (http://www.ensembl.org/) databases queried. Protein and peptide thresholds were set to 95% with a minimum of two peptides detected for positive protein identification.

The fractionated samples analysed at the Advanced Analysis Centre were trypsinised and loaded on a 1200 HPLC liquid chromatograph (Agilent Technologies Co. Ltd., Santa Clara, CA) interfaced with a UHD 6530 Q-TOF mass spectrometer (Agilent Technologies Co. Ltd., Santa Clara, CA). A C18 column (Agilent AdvanceBio Peptide Map, 100 mm x 2.1 mm 2.7 µm) was used for chromatographic separation with the solvents water with 0.1% formic acid for solvent A and acetonitrile with 0.1 % formic acid for solvent B. The mobile phase gradient was as follows: initial conditions, 2% B increasing to 45% B in 40 minutes and then to 55% B in 10 minutes, followed by column wash at 95% B and 10 minute re-equilibration. The first two and last five minutes of gradient were sent to waste rather than the spectrometer. The flow rate was maintained at 0.2 mL/min, the mass spectrometer electrospray capillary voltage at 4.0 kV, and the drying gas temperature at 350°C with a flow rate of 13 L/min. Nebuliser pressure was 40 psi and the fragmentor was set to 150 psi. Nitrogen was used as both nebulising and drying gas and collision-induced gas. The mass-to-charge ratio was scanned across the m/z range of 300-2000 m/z in 4 GHz extended dynamic range positive-ion auto MS/MS mode. Three precursor ions per cycle were selected for fragmentation. The instrument was externally calibrated with the ESI TuneMix (Agilent Technologies Co. Ltd., Santa Clara, CA). The sample injection volume was 100 µL. Raw data files were loaded directly into PEAKS 7 software (Bioinformatics Solutions Inc., Waterloo, ON) where the data were refined and subjected to de novo sequence identification and database searching. Methionine oxidation modifications were considered
within the search parameters. The tolerance values used were 10 ppm for parent ions and 0.5 Da for fragment ions.

6.3.4 Mutant generation

Unmarked isogenic mutants were generated for the flp1, ompA, and comE1 genes of A. suis H91-0380, as previously described (Chapter 5).

6.3.5 Attachment assays

Single colonies of A. suis H91-0380 wild type, Δflp1, ΔompA, and ΔcomE1 grown on Columbia agar plates containing 5% sheep were used to inoculate 3 mL of brain heart infusion (BHI) and incubated overnight at 37°C with 200 rpm shaking. A 500 µL inoculum was added to 25 mL pre-warmed BHI and incubated as before. Culture was removed at 60 minutes post-inoculation (mpi; representing exponential phase) and 180 mpi (representing stationary phase) for all strains, except for ΔompA, where the stationary phase culture was taken at 210 mpi to compensate for a slower growth rate, as determined in growth curve studies (data not shown). The number of CFU/mL was determined by plating 10-fold dilutions on BAPs. For attachment assays, 100 µL exponential and stationary phase cultures of each strain were added to Stripwell plates pre-coated with one of the purified human extracellular matrix components: collagen I, collagen IV, fibronectin, laminin, and vitronectin (EMD Millipore, Billerica, MA). In each experiment, one ECM-coated well inoculated with cell-free BHI and one BSA-coated well inoculated with bacterial culture were used as controls. After addition of the culture, wells were sealed with adhesive film (Fisher Scientific, Waltham, MA) centrifuged at 1500 rpm for 10 minutes, and then incubated at 37°C in the presence of 5% CO₂. At 0, 15, 30, 45, 60, and 120
minutes post-attachment (mpa), culture was removed from wells for each ECM component and washed with phosphate-buffered saline (PBS) three times. The negative control wells were incubated for 120 mpa before washing.

When the assays were complete, cells were heat-fixed in the wells at 55°C for 20 minutes. Ninety-five microlitres crystal violet (1%) were then added to each well and the strips were incubated at room temperature for 45 minutes. Following incubation with crystal violet, the wells were washed 5 times with sterile deionised water, air dried for 30 minutes at room temperature, then de-stained with 100 µL 95% ethanol for 15 minutes. A plate reader (Beckman Coulter Inc, Brea, CA) was used to measure absorbance at 595 nm, blanked with the absorbance from the culture in the BSA-coated well. Blanked absorbance measurements were standardised to $A_{595nm}/input\ CFU$ based on plate counts.

6.3.6 Statistical analysis

Wild type profile data were analysed using PROC MIXED repeated measures analysis with a Tukey post-hoc test in SAS (v. 9.4; SAS Institute Inc., Cary, NC). The statistical model was $Y = \mu + \text{Culture}_j + \text{Time}_j + \text{Culture}^*\text{Time}_{ij} + \epsilon_{ijk}$, where time was the repeated measure. Variance/covariance structures were tested, and the variance/covariance structure with the lowest AIC/BIC value was used for analysis. Wild type post-hoc planned contrasts among the Culture*Time$_{ij}$ interactions were done to compare:

1) Different time points among the same growth phase

2) Same time points between the exponential and stationary growth phases.
Mutant data were analysed using PROC MIXED repeated measures analysis with a Tukey post-hoc test in SAS. The statistical model was

\[ Y = \mu + \text{Strain}_i + \text{Culture}_j + \text{Time}_k + \text{Strain}^*\text{Culture}_{ij} + \text{Strain}^*\text{Culture}^*\text{Time}_{ijk} + \epsilon_{ijkl}, \]

where time was the repeated measure. Variance/covariance structures were tested, and the variance/covariance structure with the lowest AIC/BIC value was used for analysis. Post-hoc planned contrasts among the \text{Strain}^*\text{Culture}^*\text{Time}_{ijk} interactions were done to compare:

1) Different time points among the same strain and same growth phase

2) Same time points among the same strain and between the 2 growth phases

3) Same time points among the different strains, within the same growth phase.

### 6.4 Results

#### 6.4.1 Proteins in pig tonsils

A total of 1677 proteins were found in the tonsil samples analysed by MS (Table 6.2). Among these, 59 proteins of interest were identified: 35 ECM components, 5 members of the small leucine-rich proteoglycan (SLRP) family, 7 receptors, and 12 additional proteins. The ECM components included fibronectin, laminin, vitronectin, collagen I, and collagen IV.

#### 6.4.2 Attachment of *A. suis* H91-0380 wild type to ECM components

To determine whether *A. suis* H91-0380 wild type adhered to the common ECM components collagen I, collagen IV, fibronectin, laminin, and vitronectin, attachment was measured for exponential and stationary growth phase cultures incubated for 0, 15, 30, 45, 60, and 120 mpa.
A significant reduction in attachment was observed in stationary phase compared to exponential phase cultures with collagen IV, fibronectin, laminin, and vitronectin. No significant effects were observed for wild type attachment at different times to any ECM component tested.

Attachment of A. suis mutants to ECM components

The attachment of three A. suis H91-0380 knockout mutants (Δflp1, ΔompA, and ΔcomE1) to various purified ECM components was also measured and compared to wild type. The average attachment of Δflp1 to collagen IV during exponential phase was significantly less compared to wild type (Figure 6.2). There was also no apparent attachment of Δflp1 to collagen I during exponential phase compared to background (BSA-coated well) signal; however, this result was not significant due to variations between biological replicates.

There was significantly less attachment of the ΔompA mutant during exponential growth compared to wild type with all ECMs tested (Figure 6.3). In the case of collagen I and collagen IV, there was no measurable attachment during exponential phase relative to background signal.

Compared to wild type, there was significantly less attachment of the ΔcomE1 strain during exponential growth to collagen IV, fibronectin, and vitronectin (Figure 6.4). Furthermore, differential attachment over time (strain*culture*time effects) was observed for collagen I (Figure 6.5), where differences in attachment between wild type and ΔcomE1 during exponential phase were observed at 0, 30, 45, and 120 mpa.
6.5 Discussion

In this study, we sought to identify ECM components and host receptors present in the tonsils of the soft palate of swine. We further set out to characterise the attachment of a virulent clinical isolate of A. suis and three isogenic adhesin gene mutants to purified components of the ECM. Tonsils from healthy pigs were analysed by mass spectrometry to identify ECM components and host cell receptors that might be used by bacteria for host-pathogen interactions. Among the larger and more common ECM components, collagen types I, III, IV, V, VI, VII, XII, XIV, XV, and XVIII were identified in this study, as were fibronectin, several laminin isoforms, and vitronectin. To our knowledge, there have been no other reports on the components of the ECM in swine tonsils in the literature. Collagen types I, II, III, IV, VII, and XVIII have been found in human tonsils, as have fibronectin, laminin, vitronectin, tenascin, and thrombospondin (Castaños-Velez et al., 1995; Maatta et al., 2004). Maatta et al. (2004) also detected collagen type XVII in the crypt epithelium of human palatine tonsils, but this type of collagen was not identified in the current study; thrombospondin was also not detected. This finding was unexpected given its reported presence in the basement membrane and vessels of the extrafollicular areas of human tonsils (Castaños-Velez et al., 1995). Of the other ECM components identified by MS in this study, the SLRP lumican has been detected in low levels in human tonsil (Botella et al., 2004), Some of the proteins detected (Table 6.2) such as fibrinogen, are important in wound healing, and therefore it seems reasonable to assume that it would be present in tonsils to aid in tissue repair.
Due to their abundance in host connective tissues, and the sequence similarity between humans and pigs, purified human collagen I, collagen IV, fibronectin, laminin, and vitronectin were examined to determine whether \textit{A. suis} was able to attach to them. As might have been predicted from earlier expression studies (Chapter 5), there was significantly greater attachment of \textit{A. suis} H91-0380 wild type to collagen IV, fibronectin, laminin, and vitronectin during exponential phase relative to stationary phase (Figure 6.1).

In the isogenic $\Delta$\textit{flp}1 mutant, the pilin gene of the tight adherence (\textit{tad}) locus was disrupted. Previous studies in the genera \textit{Actinobacillus}, \textit{Aggregatibacter}, \textit{Haemophilus}, \textit{Pasteurella}, \textit{Yersinia}, and others have demonstrated that the type IVb pili encoded by the \textit{tad} locus are involved in biofilm formation and host colonisation (Saito et al., 2010; Tomich et al., 2007). To date, no host receptor has been described for these pili, and it has been proposed that the type IVb pili engage in non-specific attachment through autoaggregation (Clock et al., 2008). In this work, there was no difference in attachment of the $\Delta$\textit{flp}1 mutant to ECM components compared to wild type with most of the ECM components tested, with the exception of collagen IV attachment, which was significantly reduced in exponential cultures (Figure 6.2). Furthermore, there appeared to be little attachment of the $\Delta$\textit{flp}1 mutant to collagen I, but due to variations between biological replicates, no significant difference between the $\Delta$\textit{flp}1 mutant and wild type cultures at exponential phase was detected. These results suggest that the type IVb pili encoded by the \textit{tad} locus could contribute to more than just autoaggregation and that these pili may attach to collagen IV, and perhaps collagen I, though further work remains to be done to confirm the latter interaction. This finding is noteworthy because collagen IV is a component of the basement
membrane, which would suggest that the *tad* locus would aid organisms in attaching at this site after having breached the epithelial barrier.

We next examined the attachment of the Δ*ompA* mutant of *A. suis* to ECM components. The outer membrane protein OmpA has been previously shown to confer cell viability, serum resistance, and to participate in attachment of *A. suis* to tonsil explants (Ojha et al., 2010), and in attachment of *Mannheimia haemolytica* to epithelial cells (Hounsome et al., 2011). In the current study we observed significantly less attachment of the Δ*ompA* mutant (versus wild type) during exponential phase to all ECM components tested even when corrected for slower growth rate (Figure 6.3). It might also be noted that in previous studies (Chapter 5), the Δ*ompA* mutant produced significantly less biofilm than the wild type or other mutant strains. In our previous studies, we found the expression of *ompA* to be up-regulated in aerobic growth, particularly during stationary phase (Chapter 5). Therefore, it is somewhat surprising that the attachment of the Δ*ompA* mutant was not also reduced compared to the wild type strain during stationary phase. This may be a result of other adhesins participating in attachment to ECM components during stationary phase, thus compensating for the effects of Δ*ompA* attachment at this time point, though further studies are needed to evaluate this hypothesis.

Homologues of *comE1* in other members of the family *Pasteurellaceae* have been shown to bind to fibronectin, and in *Pasteurella multocida*, this interaction has been shown to involve the FnIII9-10 region of fibronectin (Mullen et al., 2008a; Mullen et al., 2008b). With the *A. suis* Δ*comE1* mutant, there was reduced attachment of exponential cultures to collagen IV, fibronectin, and vitronectin compared to the wild type strain (Figure 6.4). In addition to these
findings, there was differential binding of exponential cultures of \( \Delta comE1 \) to collagen I over time (Figure 6.5), whereby attachment at 0, 30, 45, and 120 mpa was attenuated compared to the corresponding wild type cultures. These results suggest that binding of \( A. suis \) to ECM components changes over short periods of time, though the factors that contribute to this phenotype are not yet clear. It also appears that, in addition to previous findings by Mullen et al. (2008a), the \( comE1 \) homologue of \( A. suis \) participates in more than just fibronectin-binding. The arginyl-glycyl-aspartic acid (RGD) motif is potentially also important for interactions of \( comE1 \) with these ECM components and perhaps other proteins not examined here. This hypothesis is supported by the fact that collagen IV, fibronectin, laminin, vitronectin, and over 100 other proteins contain RGD motifs (Mecham, 2011). The importance of RGD motifs in cell adhesion and their recognition by many integrins (D'Souza et al., 1991) suggests that more specific examination of the interactions of these motifs with \( A. suis \) is warranted.

### 6.6 Conclusions

Though it is widely accepted that initial attachment and colonisation of the host is an important early step in the pathogenesis of many organisms, little is known about these mechanisms for \( A. suis \) in the tonsils of the soft palate of swine. Here, we have for the first time identified several ECM components, including glycoproteins, proteoglycans, and other proteins present in these tonsils. We have further demonstrated that \( A. suis \) can attach to purified collagen IV, fibronectin, laminin, and vitronectin during exponential growth and to a lesser degree during the stationary phase. Using knockout mutants, we have demonstrated that the biofilm-associated Flp1 pilin encoded by the \( tad \) locus is required for optimal binding of \( A. suis \) to collagen IV.
contrast, previous work has failed to identify a receptor (Tomich et al., 2007). In addition, we found that disrupting the gene encoding the outer membrane protein OmpA resulted in reduced attachment to all ECM components tested. Finally, we found that disrupting comE1, a homologue of a previously described fibronectin-binding protein, resulted in reduced attachment of A. suis to collagen IV, fibronectin, and vitronectin, as well as reduced attachment to collagen I in a time-dependent manner. Taken together, these findings suggest that ECM proteins present in the tonsils of the soft palate of swine may play a role in the colonisation of bacterial pathogens. The characterisation of A. suis attachment to the selected ECM components provides a basis for further studies into the mechanisms of attachment, and perhaps even invasion, of A. suis and other members of the family Pasteurellaceae via the tonsils.
Table 6.1. Strains, plasmids, and genes used to characterise differential attachment to ECM components

<table>
<thead>
<tr>
<th>Bacterial strain or genes</th>
<th>Characteristic(s)</th>
<th>Reference or locus tag</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Actinobacillus suis</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H91-0380</td>
<td>O2:K2 clinical isolate</td>
<td>(MacInnes et al., 2012; Van Ostaaijen et al., 1997)</td>
</tr>
<tr>
<td><em>E. coli</em> β2155</td>
<td><em>thrB1004 pro thi hsdS lacZΔM15 (F’ lacZΔM15 lacI’ traD36 proA</em> proB*) Δdap: :erm (Erm')</td>
<td>(Baltes et al., 2003)</td>
</tr>
<tr>
<td><em>Actinobacillus suis</em> Δflp1</td>
<td>Δflp1</td>
<td>Chapter 5</td>
</tr>
<tr>
<td>ΔompA</td>
<td>ΔompA</td>
<td>Chapter 5</td>
</tr>
<tr>
<td>ΔcomE1</td>
<td>ΔcomE1</td>
<td>Chapter 5</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pEMOC2</td>
<td>Transconjugation vector based on pBluescript SK with <em>mob</em>RP4, polycloning site, Cm', and transcriptional fusion of the <em>omlA</em> promoter with the <em>sacB</em> gene</td>
<td>(Baltes et al., 2003)</td>
</tr>
<tr>
<td>pEMOC2-flp1mut</td>
<td>pEMOC2 vector with ligated 5’ and 3’ fragments of <em>flp1</em> and flanking sequence in region of insertion</td>
<td>Chapter 5</td>
</tr>
<tr>
<td>pEMOC2-ompAmut</td>
<td>pEMOC2 vector with ligated 5’ and 3’ fragments of <em>ompA</em> and flanking sequence in region of insertion</td>
<td>Chapter 5</td>
</tr>
<tr>
<td>pEMOC2-comE1mut</td>
<td>pEMOC2 vector with ligated 5’ and 3’ fragments of <em>comE1</em> and flanking sequence in region of insertion</td>
<td>Chapter 5</td>
</tr>
<tr>
<td><strong>Genes</strong></td>
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<tr>
<td><em>comE1</em></td>
<td>Fibronectin-binding protein homologue</td>
<td>ASU2_10345</td>
</tr>
<tr>
<td><em>flp1</em></td>
<td>Tight adherence locus; pilin</td>
<td>ASU2_04295</td>
</tr>
<tr>
<td><em>ompA</em></td>
<td>Outer membrane protein A homologue</td>
<td>ASU2_09940</td>
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Table 6.2. Extracellular matrix proteins identified in tonsils of the soft palate of swine by mass spectrometry and bioinformatic identification

<table>
<thead>
<tr>
<th>(Putative) Protein Name</th>
<th>Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ECM components</strong></td>
<td></td>
</tr>
<tr>
<td>Collagen, type VI, subunit alpha-3</td>
<td>I3LUR7_PIG</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>F1SS24_PIG</td>
</tr>
<tr>
<td>(Collagen, type VI, subunit alpha-2)</td>
<td>I3LQ84_PIG</td>
</tr>
<tr>
<td>Collagen, type XIV, subunit alpha-1</td>
<td>F1S285_PIG</td>
</tr>
<tr>
<td>(Collagen, type XII, subunit alpha-1)</td>
<td>F1RQI0_PIG</td>
</tr>
<tr>
<td>Collagen, type VI, subunit alpha-1</td>
<td>I3LS72_PIG</td>
</tr>
<tr>
<td>Laminin, subunit gamma-1</td>
<td>F1S663_PIG</td>
</tr>
<tr>
<td>Nidogen-2</td>
<td>F1SFF3_PIG</td>
</tr>
<tr>
<td>Biglycan</td>
<td>F1S2B6_PIG (+1)</td>
</tr>
<tr>
<td>Collagen, type I, subunit alpha-2</td>
<td>F1SFA7_PIG</td>
</tr>
<tr>
<td>(Laminin, subunit alpha-3)</td>
<td>I3LBH9_PIG</td>
</tr>
<tr>
<td>Dermatopontin</td>
<td>DERM_PIG (+1)</td>
</tr>
<tr>
<td>Laminin, subunit alpha-2</td>
<td>F1SPT5_PIG</td>
</tr>
<tr>
<td>Tenascin</td>
<td>TENA_PIG</td>
</tr>
<tr>
<td>Collagen, type IV, subunit alpha-1</td>
<td>F1RLM1_PIG (+1)</td>
</tr>
<tr>
<td>Laminin, subunit alpha-4</td>
<td>F1RZM4_PIG</td>
</tr>
<tr>
<td>Collagen, type V, subunit alpha-2</td>
<td>Q59IP2_PIG</td>
</tr>
<tr>
<td>Laminin, subunit beta-3</td>
<td>F1SF45_PIG (+1)</td>
</tr>
<tr>
<td>Vitronectin</td>
<td>VTNC_PIG</td>
</tr>
<tr>
<td>Fibrillin-1</td>
<td>F1SN67_PIG</td>
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<tr>
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<tr>
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<td>F1SD87_PIG</td>
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<tr>
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<tr>
<td>Laminin, subunit alpha-3</td>
<td>I3LV27_PIG</td>
</tr>
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<td>Collagen, type IV, subunit alpha-2</td>
<td>F1RLI9_PIG</td>
</tr>
<tr>
<td>Laminin, subunit gamma-2</td>
<td>F1S662_PIG (+1)</td>
</tr>
<tr>
<td>Collagen, type XV, subunit alpha-1</td>
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<td>Collagen, type VII, subunit alpha-1</td>
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<td>Laminin, subunit beta-1</td>
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<td>(Collagen preprotein, type I, subunit alpha-1)</td>
<td>I3LJX2_PIG</td>
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<tr>
<td>Collagen, type I, subunit alpha-1</td>
<td>F1RT61_PIG (+1)</td>
</tr>
<tr>
<td>Collagen, type V, subunit alpha-1</td>
<td>F1S021_PIG (+1)</td>
</tr>
<tr>
<td>Periostin</td>
<td>F1RS37_PIG</td>
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<tr>
<td>Collagen, type III, subunit alpha-1</td>
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<tr>
<td>Fibulin-1</td>
<td>F1SM61_PIG</td>
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<tr>
<td>Protein Name</td>
<td>Accession</td>
</tr>
<tr>
<td>----------------------</td>
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</tr>
<tr>
<td>Fibulin-2</td>
<td>F1SPG5_PIG</td>
</tr>
<tr>
<td>Small leucine-rich proteoglycan (SLRP) family</td>
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</tr>
<tr>
<td>Decorin</td>
<td>PGS2_PIG</td>
</tr>
<tr>
<td>Prolargin</td>
<td>F1S6B4_PIG</td>
</tr>
<tr>
<td>Lumican</td>
<td>F1SQ09_PIG</td>
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<td>Asporin</td>
<td>F1SUE4_PIG</td>
</tr>
<tr>
<td>Fibromodulin</td>
<td>F1S6B5_PIG</td>
</tr>
<tr>
<td>Receptors</td>
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<tr>
<td>Integrin alpha-L</td>
<td>A8DNU5_PIG (+3)</td>
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<tr>
<td>Integrin beta</td>
<td>F1RVE7_PIG</td>
</tr>
<tr>
<td>CD97</td>
<td>F1SD42_PIG (+2)</td>
</tr>
<tr>
<td>CD48</td>
<td>A0A0B8S0A6_PIG (+1)</td>
</tr>
<tr>
<td>CD44</td>
<td>F1SGT4_PIG (+6)</td>
</tr>
<tr>
<td>Basigin</td>
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</tr>
<tr>
<td>Integrin beta</td>
<td>K7GP66_PIG</td>
</tr>
<tr>
<td>Other</td>
<td></td>
</tr>
<tr>
<td>Fibrinogen beta chain</td>
<td>F1RX37_PIG</td>
</tr>
<tr>
<td>(Fibrinogen gamma chain)</td>
<td>F1RX35_PIG</td>
</tr>
<tr>
<td>Perlecan</td>
<td>F1SU03_PIG</td>
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<tr>
<td>(Fibrinogen alpha chain)</td>
<td>F1RX36_PIG</td>
</tr>
<tr>
<td>Galectin</td>
<td>A3EX84_PIG</td>
</tr>
<tr>
<td>(Perlecan)</td>
<td>I3LAA4_PIG</td>
</tr>
<tr>
<td>Galectin</td>
<td>B7U2G7_PIG</td>
</tr>
<tr>
<td>(Fibrinogen A alpha chain)</td>
<td>Q28936_PIG</td>
</tr>
<tr>
<td>Lactadherin</td>
<td>B2CZF8_PIG (+1)</td>
</tr>
<tr>
<td>Galectin</td>
<td>A0A0B8RT17_PIG (+4)</td>
</tr>
<tr>
<td>(Perlecan)</td>
<td>F1SU00_PIG</td>
</tr>
</tbody>
</table>

( ) indicates proteins where name was assigned based on blastp homologues
Figure 6.1. Attachment of *A. suis* H91-0380 to purified ECM components during exponential (exp) and stationary (sta) growth

Attachment results have been corrected for non-specific binding by subtracting the average signal from BSA-coated wells from each experimental sample. Error bars represent standard error of the mean (SEM). Statistical significance is expressed with: * p < 0.05, ** p < 0.01, *** p < 0.001. Each data point is an average of three biological replicates.
Figure 6.2. Attachment of *A. suis* H91-0380 wild type and Δflp1 to ECM components during exponential (exp) and stationary (sta) growth

Attachment results have been corrected for non-specific binding by subtracting the average signal from BSA-coated wells from each experimental sample. Error bars represent standard error of the mean (SEM). Statistical significance is expressed with: * p < 0.05, ** p < 0.01, *** p < 0.001. Each data point is an average of three biological replicates.
Figure 6.3. Attachment of *A. suis* H91-0380 wild type and ΔompA to ECM components during exponential (exp) and stationary (sta) growth

Attachment results have been corrected for non-specific binding by subtracting the average signal from BSA-coated wells from each experimental sample. Error bars represent standard error of the mean (SEM). Statistical significance is expressed with: * p < 0.05, ** p < 0.01, *** p < 0.001. Each data point is an average of three biological replicates.
Figure 6.4. Attachment of *A. suis* H91-0380 wild type and ΔcomE1 to ECM components during exponential (exp) and stationary (sta) growth

Attachment results have been corrected for non-specific binding by subtracting the average signal from BSA-coated wells from each experimental sample. Error bars represent standard error of the mean (SEM). Statistical significance is expressed with: * p < 0.05, ** p < 0.01, *** p < 0.001. Each data point is an average of three biological replicates.
Figure 6.5. Attachment of A. suis H91-0380 wild type and ΔcomE1 during exponential (exp) and stationary (sta) phase to collagen I over different incubation times

Attachment results have been corrected for non-specific binding by subtracting the average signal from BSA-coated wells from each experimental sample. Error bars represent standard error of the mean (SEM). Statistical significance is expressed with: * p < 0.05, ** p < 0.01, *** p < 0.001. Each data point is an average of three biological replicates.
Chapter 7: General Discussion

Tonsils are one of the first lines of defence against pathogens entering the upper respiratory tract. Paradoxically, they are also an important site of colonisation and can be a portal of entry for many pathogens. Despite their significant role, virtually nothing is known about how bacteria attach to or the factors that control invasion of the tonsils. To address these questions, the gram-negative facultative anaerobe, *Actinobacillus suis*, was used as a model pathogen. *A. suis* is a common resident of swine tonsils, but it sometimes invades the bloodstream, leading to systemic disease and death. *A. suis* and the primary pathogen *A. pleuropneumoniae* share many genetic similarities in adhesins and other virulence factors, suggesting that the differences in tissue tropism and disease manifestation of these two organisms might be the result of relatively small genetic differences and/or different levels of expression. The finding that many of the closest homologues of the *A. suis* adhesin genes are found in other opportunistic pathogens in the genus *Actinobacillus* that cause systemic infections in species other than swine is consistent with the notion that adhesins may be important determinants of the type of disease caused by an organism, perhaps even more so than host range, but much more work is needed to test this hypothesis.

7.1 Research summary

The onset of disease caused by *A. suis* is often swift and can result in high mortality, particularly in high health status herds (MacInnes and Desrosiers, 1999). It is reasonable to surmise that the rapid course of disease caused by *A. suis* contributes to the fact that so little is known about the early stages of disease. Additionally, the high degree of genome similarity
between *A. suis* and *A. pleuropneumoniae* (genetic rearrangements notwithstanding) raises the question of why these organisms have such different courses of disease.

With these observations in mind, the objectives of this thesis were to identify and characterise the adhesins of the opportunistic pathogen *Actinobacillus suis* and to begin to examine the manner in which this organism interacts with the tonsils of the soft palate. To meet these objectives, we focused on the first steps of attachment and colonisation in the host. Putative adhesins were identified using several bioinformatic approaches, and then their expression was analysed under various growth conditions designed to mimic *in vivo* conditions. Putative host receptors and extracellular matrix (ECM) components were also identified by mass spectrometry. From there, the role of select adhesins in attachment to the ECM was investigated. Together, these studies aimed to identify and characterise bacterial adhesins, then to determine the conditions and host molecules needed for optimal success of this pathogen.

In the first study, we conducted a bioinformatic analysis of two complete genomes and three draft genomes of *A. suis* strains and revealed 47 putative adhesin-associated genes that were predicted to encode 24 adhesins. Among these were 6 autotransporter-associated genes, 25 fimbriae-associated genes encoding 3 adhesins, 12 outer membrane protein-encoding genes, and 4 additional genes encoding 3 adhesins. All of the putative adhesins identified had homologues in the genetically similar primary pathogen *A. pleuropneumoniae*, with the exception of two autotransporter-encoding genes (*aidA* and *ycgV*). These two classical autotransporters both have described roles in virulence in other species, where they contribute to bacterial attachment and invasion of the host (Henderson et al., 2004). The majority of the closest homologues of the
putative *A. suis* adhesin genes identified in this study were also found in *A. capsulatus* and *A. ureae* — species that cause systemic infection in other animals. This suggests that the different diseases, tissue tropism, and host range of *A. suis* and *A. pleuropneumoniae* are a result of subtle genetic differences, and possibly differential expression of virulence factors during infection.

Furthermore, the finding that many putative adhesins of *A. suis* share the greatest homology with other systemic pathogens within the family *Pasteurellaceae* may indicate that these organisms employ similar strategies of host invasion, but this hypothesis remains to be tested.

In the second and third studies, we investigated the expression of 12 putative adhesin genes of the virulent clinical isolate *A. suis* H91-0380 in growth conditions and growth phases that mimic the oxygen and nutrient levels hypothesised to exist in the tonsils of the soft palate, and in the presence of the stress hormone epinephrine. In the third study, we chose 12 putative adhesin genes from the various classes of adhesins identified in the first study. In the first part of the study, we found that 50 µM epinephrine did not significantly affect the expression of any of the adhesin genes tested, but that there was a significant growth phase effect observed for many genes. In the next part of the study, we found that most adhesin genes were differentially expressed under anoxic static growth conditions compared to aerobic cultures, and that all genes exhibited differential expression during exponential or stationary growth. We further observed that different adhesin genes appear to be important during the various stages of persistence in the tonsils. Based on these findings, we proposed a model (Figure 5.8) in which different adhesins contributed to persistence in the host during the initial attachment and microcolony formation, biofilm maturation, dispersion, or planktonic phases.
To test this model, we examined the involvement of several adhesins identified in studies 1, 2, and 3 in biofilm formation using a static system. We employed isogenic mutants of three adhesin genes: the autoaggregation-associated flp1 gene from the tight adherence locus, the outer membrane protein-encoding gene ompA, and the fibronectin-binding comE1 gene. We found that disrupting these genes produced less biofilm during earlier incubation times, suggesting that the adhesins encoded by these genes were important during the initial stages of biofilm formation. We postulated that few effects were observed during the later incubation times of biofilm formation due to compensatory effects by other adhesins that duplicate adhesive functions. This study suggested that early attachment is a nuanced process that requires an organism to establish robust interactions to persist and grow at the site of adherence.

Having identified genes involved in biofilm formation, we then investigated the role these genes play in attachment to extracellular matrix (ECM) components. In the fourth study, our aim was to identify ECM proteins in the tonsils of the soft palate of swine that may interact with A. suis and other microbes. We further aimed to measure the attachment of A. suis wild type and isogenic adhesin gene mutants to the ECM components collagen I, collagen IV, fibronectin, laminin, and vitronectin. Using mass spectrometry, we identified 59 proteins of interest in the tonsils of the soft palate of swine, many of which had not been previously identified at this site. Among these were several different types of collagen, numerous laminin isoforms, fibronectin, and vitronectin. A subset of these proteins was used to determine interaction with A. suis adhesins identified in studies 1, 2, and 3.
Using an ECM attachment assay, we measured the role of three adhesins in adhering to five common ECM proteins. We found that the Δflp1 strain had reduced attachment to collagen IV during exponential phase, in contrast to previous studies that suggest that the type IVb pili encoded by the tad locus adhere to host cells mainly through autoaggregation (Clock et al., 2008). The ΔompA strain had reduced attachment during exponential phase to all ECM components tested, despite findings in the third study that this gene is up-regulated during growth conditions hypothesised to be similar to the dispersal stage of biofilm formation. However, these conflicting results may be attributed to the complex process of attachment, whereby multiple adhesins possess overlapping functions that play a compensatory role when one adhesin is disrupted or absent. Finally, the ΔcomE1 strain demonstrated reduced attachment of exponential cultures to collagen IV, fibronectin, and vitronectin. Moreover, there was differential binding of exponential cultures of ΔcomE1 over time, where attachment at 0, 30, 45, and 120 minutes post-attachment was diminished compared to the corresponding wild type cultures. These in vitro results indicated that comE1 in A. suis does not only participate in fibronectin binding, as previously suggested by other studies (Mullen et al., 2008a), but is able to adhere to other ECM components, as well.

In summary, these results support the hypothesis that attachment is a dynamic process that is subject to change over short increments of time, though the factors contributing to this phenotype are not yet elucidated. It is also interesting to note that of the ECM components tested, all but collagen I possess RGD motifs, which are recognised by many integrins and are important in cell adhesion in the host (D'Souza et al., 1991). A. suis and other organisms may in fact employ more
generalised modes of attachment through the ubiquitous RGD motif in order to allow for more diverse opportunities for host interaction.

7.2 Major themes

The work on identifying *A. suis* adhesins, and investigating their role in biofilm formation and ECM attachment revealed two important themes. First, the process of attachment seems to include many redundancies, likely in order to maximise the odds of success in colonising the host. Second, these redundancies in *A. suis* attachment and colonisation provide evidence of providing compensatory mechanisms that enable the organism to persist once it has established itself at a body site, even in the absence of some adhesins.

First, *A. suis* appears to contain many redundancies in its attachment and colonisation processes. In this thesis, the differential expression of adhesin genes during different growth phases and nutrient/oxygen levels suggested that the encoded adhesins participate in attachment and colonisation during different phases of interaction with the host environment and with other members of the microbial community. Furthermore, the attachment of *A. suis* to multiple ECM components, and the attenuation of attachment to several of the same ECM components by the three isogenic adhesin gene mutants, suggests that there is an overlap in adhesins responsible for attachment to multiple sites to enhance the success of colonisation.

Second, these redundancies may contribute to a compensatory and self-correcting phenotype during attachment and colonisation. When disrupting selected adhesin genes, the impact on biofilm formation was primarily observed during the early stages, but the wild type phenotype was often recovered by the later stages. This supports the notion that there are many functional
redundancies among adhesins that could allow the organism to avoid dependence on just one mechanism for persistence in the host milieu.

7.3 Future research

In the future, research should measure the interplay of host-pathogen gene products during different stages of the infection process. To begin to examine these host-pathogen interactions, expanding this research to organ cultures and transwell or other cell culture systems coupled with RNA-seq or proteomics might shed light on how pathogens such as *A. suis* affect host cells during attachment. Animal infection studies with different adhesin mutants might be helpful to determine adhesins that are essential for the invasion process, will help improve overall understanding of bacterial pathogenesis under conditions that more closely align with those occurring *in vivo*, and may confirm some of the findings and refined hypotheses generated in the present work. Through these methods, we can better investigate what makes *A. suis*, unlike some of its close relatives, an opportunistic pathogen capable of invasive disease.

Furthermore, making use of tonsil sections to co-localise both the ECM components and the site(s) of colonisation and persistence of *A. suis* would provide useful hypotheses for determining the mode of invasion of this and other opportunistic pathogens known to colonise tonsils. Studies to determine whether and/or how these organisms reach the ECM would also be helpful. For example, examining whether these organisms are actively crossing the epithelium to reach the connective tissue or simply exploiting epithelia that have been damaged through other means (e.g., mechanical, viral, etc.) would provide critical information for elucidating the virulence factors important during the infection process. In addition, given previous reports that *A. suis* can
adhere to PBMECs (Ojha et al., 2010), it would be useful to determine which adhesins, and indeed other virulence factors, are expressed in the blood stream compared to the tonsils. Furthermore, it would be interesting to engage in longer term studies to examine the effects of microbiome composition, particularly in high health status herds that can be considered largely immunologically naïve, and determining if altering the microbial composition to include more benign members of the family Pasteurellaceae could contribute to reduced severity of A. suis outbreaks and improved overall health outcomes. In short, comparing A. suis to closely related primary pathogens and commensal colonisers of the tonsils could also provide insight into the nature of opportunistic pathogenesis and effective methods of developing preventative measures.
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