Binding of porcine plasma ficolin-alpha and mannose-binding lectin A to biofilm cultures of *Actinobacillus pleuropneumoniae*

by

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ABSTRACT

BINDING OF PORCINE PLASMA FICOLIN-ALPHA AND MANNOSE-BINDING LECTIN A TO BIOFILM CULTURES OF *ACTINOBACILLUS PLEUROPNEUMONIAE*

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Mannose-binding lectin (MBL) and ficolins are complement-activating proteins, and both play an important role in innate immunity by recognizing specific carbohydrate moieties on the surface of wide range of microorganisms. Previous studies have shown that porcine ficolin-α and MBL-A bind to surface polysaccharides of bacteria cultured in suspension, but their interactions with bacteria in biofilm culture have not been studied. The objectives of this thesis were to determine whether porcine plasma ficolin and MBL bind to *Actinobacillus pleuropneumoniae* in biofilm cultures. APP serotype 5a (APP5a) was used because it produced pronounced biofilm in plastic culture dishes, in comparison with APP5b that was previously reported to bind ficolin in suspension cultures. N-acetylglucosamine (GlcNAc) in the biofilm produced by APP5a was stained with wheat germ agglutinin conjugated with Alexa Fluor-555 and identified by confocal laser scanning microscopy (CLSM). Dispersin B prevented APP5a biofilm formation indicating the requirement of poly N-acetylglucosamine (PNAG) for bacterial cohesion. Bound purified ficolin or ficolin in plasma both were eluted with GlcNAc from APP5a biofilm cultures. To address preferential binding of ficolin-α to biofilm matrix, ficolin-α

was eluted with GlcNAc from extracellular polymeric substances (EPS) in supernatant after pelleting the bacteria. Biotinylated-ficolin that retained GlcNAc-binding activity for APP5b planktonic cultures was shown to bind strongly to APP5a biofilm, as detected by fluorescent NeutrAvidin staining and CLSM, but not in the presence of GlcNAc. Further, MBL-A in ficolin-depleted porcine plasma also bound to APP5a biofilm and was eluted with a sugar solution containing GlcNAc, galactose, mannose and glucose. These studies demonstrate that both porcine ficolin-α and MBL-A bind to biofilm cultures of APP5a in a carbohydrate-dependent manner, and suggest that the production of PNAG in biofilm is a binding target for ficolin.

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Declaration of Work Performed

All work presented in this thesis was performed by me, with the following exceptions:

Porcine plasma ficolin was purified by Dr. Jutta Hammermueller.

The confocal laser scanning microscopy images were taken by Dr. Basuvaraj Mahendran

In-gel tryptic digestion and mass spectrometry identification of MBL-A protein was completed by the Advanced Protein Technology Center at the Hospital for Sick Children, Toronto, ON, Canada.

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List of Abbreviations

BHI	brain heart infusion
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
C1q	complement component C1q
C2	complement component C2
C3	complement component C3
C3b	complement component C3b
C4	complement component C4
C4bC2a	complement component C4bC2a
C5a	complement component C5a
CER	cation exchange resin
CLSM	confocal laser scanning microscopy
CRD	carbohydrate recognition domain
CRP	C-reactive protein
DispB	dispersin B
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EPS	extracellular polymeric substances
FBG	fibrinogen-like domain
FLP	ficolin-like proteins
g	grams
GalNAc	N-acetylgalactosamine
GlcNAc	N-acetylglucosamine
HIV-1	human immunodeficiency virus 1
hr	hours
kV	kilovolts
LC-MS/MS	liquid chromatography tandem mass spectrometry
LPS	lipopolysaccharide
LTA	lipoteichoic acid
M	molar
MAC	membrane attach complex
ManNAc	N-acetylmannosamine
MASP	MBL-associated serine proteases
MBL	mannose-binding lectin
mg	milligrams
mM	millimolar
MS	mutant strain
mRNA	messenger RNA
min	minute
NAD	nicotinamide adenine dinucleotide
NMR	nuclear magnetic resonance
PAG	polyacrylamide gel

PAMPs	pathogen associated molecular patterns
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PGA	poly-glucosamine
PIA	polysaccharide intercellular adhesin
PNAG	poly-N-acetylglucosamine
PRRs	pattern recognition receptors
RNA	ribonucleic acid
ROS	reactive oxygen species
RS	reference strain
s	second
SDS	sodium dodecyl sulfate
sMAP	small mannose-binding associated protein
TBS	Tris-buffered saline
TGF-β	transforming growth factor-beta
TR	Texas Red
Tris	tris(hydroxymethyl)aminomethane
V	volts
w/v	weight/volume
WGA	Wheat Germ Agglutinin

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Chapter 1. Introduction and Review of the Literature

Ficolins

Ficolins are complement-activating innate immune lectins (Matsushita et al., 2000; Taira et al., 2000) that bind to various N-acetylated surface polysaccharides, especially N-acetylglucosamine (GlcNAc) (Brooks et al., 2003a; Frederiksen et al., 2005; Matsushita et al., 1996). Initially, ficolins were identified incidentally in porcine uterus membrane as transforming growth factor-β (TGF-β)-binding proteins during a search for TGF-β receptors (Ichijo et al., 1993; Ichijo et al., 1991). In addition to TGF-β, ficolins have also been determined to bind to cortisol, elastin, zymogen, heparin, fibronectin and capsular polysaccharides of wide a range of microorganisms in a GlcNac-dependent manner (Brooks et al., 2003a; Lu and Le, 1998; Lu et al., 2002; Matsushita, 2010; Matsushita et al., 2002). Various types of ficolins and collagenous lectins have been described in humans (Endo et al., 1996; Harumiya et al., 1995; Lu et al., 1996; Matsushita et al., 1996), pigs (Brooks et al., 2003c; Ichijo et al., 1993), rodents (Fujimori et al., 1998; Ohashi and Erickson, 1998), hedgehogs (Omori-Satoh et al., 2000), *Xenopus* frogs (Kakinuma et al., 2003) and invertebrates (Kenjo et al., 2001; Wu et al., 2011). Although the binding specificities for carbohydrates vary among ficolins, the majority of ficolins identified to date in vertebrates and invertebrates bind to GlcNAc (Matsushita, 2010).

Molecular structure of ficolins

Ficolin proteins are comprised of several subunits (Matsushita et al., 1996) with each subunit consisting of three exactly similar polypeptide chains. Each polypeptide chain has

a short N-terminal region of ~13 amino acids with one or two cysteine residues, a collagen-like domain, which is constituted by 11 to 19 glycine-X-Y triplets (where X and Y denote any amino acid), a link domain of nine amino acids, and a fibrinogen-like domain at the C-terminus, which consists of ~209 amino acids (Ichijo et al., 1993). Unlike other collagenous lectins, the coiled neck region is not present in human L- and M-ficolins; however, active oligomers are formed by joining four subunits at the N-terminus (Garlatti et al., 2007). Like complement protein C1q and mannose-binding lectin (MBL), the polypeptide chains at the N-terminal regions reassemble to form a parachute–like structure (Garlatti et al., 2009; Ichijo et al., 1993; Matsushita et al., 1996; Ohashi and Erickson, 1997, 1998; Yae et al., 1991). The fibrinogen domain of ficolin is responsible for carbohydrate binding specificity (Matsushita et al., 1996), and consists of 200-250 residues, of which 40 are highly conserved and 24 are variant, mostly hydrophilic amino acids including 4 cysteines (Runza et al., 2006). The binding sites on ficolins vary between species. Detailed examination of the crystal structure of horseshoe crab tachylectin 5A (TL5A), a hemolymph plasma lectin of *Tachypleus tridentatus*, indicates that ligand binding forms a funnel-like structure made by four aromatic side chains of TL5A with the methyl group of GlcNAc at the base (Kairies et al., 2001). However, structural analysis of ligand-binding sites of recombinant human H- and L-ficolins showed some common features with TL5A, but considerable differences are noted in the orientations of the side chains (Garlatti et al., 2007). This suggests that binding sites of various ficolin forms have different carbohydrate binding specificity. For example, the S1 binding site of L-ficolin, which is homologous to the binding site of TL5A, did not reveal carbohydrate binding (Garlatti et al., 2007). In addition to S1, three

other binding sites are identified in human L-ficolins. These binding sites are classified into S1-S4; binding sites S2 and S3 mainly binds to N-acetylated compounds, S1 interacts with GlcNAc disaccharide and S4 recognizes 1,3-β-D-glucans (Garlatti et al., 2007).

Ficolin expression

Humans have three structurally distinct forms of ficolin, L, H and M. L-ficolin, also called ficolin/P35 or elastin-binding protein-37, is mainly synthesized in hepatocytes and present in the circulation (Matsushita and Fujita, 2001). The median concentration of L-ficolin in the circulation is 3.37 μg/ml (Sallenbach et al., 2011). H-ficolin, also called Hakata antigen, is synthesized in lungs (ciliated bronchial epithelial cells and Type II alveolar epithelial cells) and liver (hepatocytes and bile duct epithelial cells) (Akaiwa et al., 1999). The average serum concentration of H-ficolin is 1.85 μg/ml (Sallenbach et al., 2011). M-ficolin, also called ficolin-1, is a secretory protein present in the cytoplasmic granules of neutrophils and type II pneumocytes of the lung (Endo et al., 1996; Liu et al., 2005). The average mean serum concentration from is 1.07 μg/ml (Wittenborn et al., 2010).

There are two types of ficolins in pigs, ficolin-α and ficolin-β, and they have high (~83%) amino acid homology. Originally, they were identified as TGF-β-binding proteins within the uterus (Ichijo et al., 1993). Ficolin-α mRNA is mainly expressed in the lung, liver, spleen, and bone marrow, and at low levels within the uterus, while low level expression of ficolin-β mRNA is found in bone marrow and neutrophils, and is absent in uterus

(Brooks et al., 2003c; Ichijo et al., 1993; Ohashi and Erickson, 1998). The major plasma ficolin is ficolin-α, which consists of two multimers composed of subunits that migrate in reduced sodium dodecyl sufate-polyacrylamide gels (SDS-PAG) as 38, 40 and 42 kDa forms, whereas ficolin-β (39 kDa) is primarily synthesized, stored, and secreted by porcine neutrophils (Brooks et al., 2003c). Ficolin-α (pI 5.2-5.8) is distinguishable from ficolin-β (pI 6.7-7.4) by 2-dimentional (2D)-PAG.

There are two active forms of ficolin (ficolin-A and -B) in mice and rats. In the mouse, the human-H ficolin gene homolog is a pseudogene (Endo et al., 2004). The mouse ficolin-A gene was first isolated from a liver cDNA library (Fujimori et al., 1998). Ficolin-A is a 35 kDa protein predominately expressed in the liver and spleen (Fujimori et al., 1998), whereas ficolin-B is primarily expressed in bone marrow and weakly expressed in spleen (Ohashi and Erickson, 1998). Ficolin has also been isolated from the skeletal muscle of the European hedgehog (*Erinaceus europeaus*) (Omori-Satoh et al., 2000), hemolymph of the solitary ascidian *Halacynthia roretzi* (Kenjo et al., 2001), *Xenopus laevis* (Kakinuma et al., 2003) and in freshwater crayfish, *Pacifastacus leniusculus* (Wu et al., 2011). The variation in the tissue expression of ficolins within and between the species indicates that these proteins may have distinct tissue-specific physiological roles (Garred et al., 2009).

Binding properties of ficolins

The different forms of ficolin identified to date in various species are known to bind GlcNAc (Matsushita, 2010). Originally, affinity chromatography studies suggested that

ficolins were TGF-β- (Ichijo et al., 1991), elastin- (Harumiya et al., 1995), and corticosteroid-binding proteins (Edgar, 1995). However, the physiologic function and the nature of association of porcine ficolin with TGF-β1 were not described and subsequent studies determined that recombinant porcine ficolin-α and -β did not bind TFG-β (Ichijo et al., 1993). Characterization of ficolin-binding properties by affinity chromatography alone in these previous studies, is difficult to interpret because epoxy-activated chromatography matrices were routinely used, which recognized ficolin independent of specific ligand adducts (Brooks et al., 2003b).

Later studies characterized the sugar binding properties of plasma ficolins (Brooks et al., 2003b; Fujimori et al., 1998; Garlatti et al., 2007; Harumiya et al., 1995; Krarup et al., 2004; Le et al., 1998; Matsushita et al., 1996; Ohashi and Erickson, 1997; Sugimoto et al., 1998). Initial evidence of ficolin binding to GlcNAc came from a study using human L-ficolin, in which L-ficolin was seen to bind to GlcNAc and N-acetylgalactosamine (GalNAc) and did not bind to precursor sugars (Le et al., 1998). L-ficolin binds to GlcNAc in GlcNAc-bovine serum albumin (BSA) conjugate, but not to BSA conjugated with mannose, glucose and galactose, or cellobiose, indicating strong binding specificity for GlcNAc (Matsushita et al., 1996). General N-acetyl recognition properties of ficolin were first reported by Brooks and co-workers who demonstrated that the addition of N-acetylated compounds, such as acetate, acetamide, or GlcNAc, could dissociate porcine ficolin-α bound to *Actinobacillus pleuropneumoniae* (APP) or an N-acetylated matrix (Brooks et al., 2003b). Further, these findings were supported by inhibition of L-ficolin binding to *Streptococcus pneumoniae* 11F by N-acetylated compounds such as GlcNAc,

GalNAc, N-acetylmannosamine (ManNAc), N-acetylcysteine, N-acetylglycine, and acetylcholine (Krarup et al., 2004). *In vivo*, it is unclear to what extent acetyl group recognition by ficolins is relevant, because ficolin binding is affected by neutralizing the charge on the sugar amine without acetyl substitution in the presence of an N-acetyl group (Runza et al., 2008). Elongated carbohydrates such as 1,3-β-D-glucans are present on the surfaces of microbes and apoptotic bodies and are recognized by L-ficolin (Garlatti et al., 2007). Also, lipoteichoic acid (LTA) present on the capsular surfaces of Gram-positive bacteria is recognized by L-ficolin, but not by H-ficolin or MBL (Lynch et al., 2004). H-ficolin recognizes GlcNAc and GalNAc moieties, but not mannose and lactose (Sugimoto et al., 1998) and GlcNAc, GalNAc, and fucose inhibit H-ficolin induced agglutination of human erythrocytes coated with lipopolysaccharide (LPS) from *Escherichia coli* (O111), *Salmonella* Minnesota and *Salmonella* Typhimurium, (Endo et al., 2011). Neoglycoproteins bearing GlcNAc, GalNAc, and sialic acid residues are recognized by M-ficolin (Liu et al., 2005). A recent study demonstrated that M-ficolin also binds to encapsulated strains of *Streptococcus agalactiae* and that binding is inhibited by sialidase and GlcNAc. This suggests that both sialic acid and GlcNAc are ligands for M-ficolin (Kjaer et al., 2011). The differences in binding properties among ficolins and other collagenous lectins emphasize their likely distinct roles in innate immune defense.

Recent glycan array studies indicate that L-ficolin binding affinity is dependent on the conformation of oligosaccharide structures and it strongly bind to oligosaccharides containing acetylated monosaccharides (Krarup et al., 2008). In addition, L-ficolin failed

to bind single GlcNAc residue on a glycan array, therefore, L-ficolin may require multiple GlcNAc residues for strong binding (Krarup et al., 2008). The crystal structure of L-ficolin suggests that more than one site per fibrinogen-like binding domain can interact with N-acetyl groups, which further supports the above findings (Garlatti et al., 2007).

In a recent study, Zhang and co-workers reported that local inflammatory conditions including mild acidosis and reduced calcium levels enhanced the interaction between L-ficolin and C-reactive protein (CRP)-mediated complement activity against *Pseudomonas aeruginosa*. This suggests that L-ficolin binding activity may be enhanced at reduced pH (pH 6.5, calcium 2 mM) (Zhang et al., 2009). In another study, it has been shown that the C-terminus of M-ficolin fibrinogen-like domain undergoes marked conformational change with pH and calcium perturbations. Further, this work demonstrated that under conditions simulating inflammation (pH 6.5, 2.0 mM calcium), the fibrinogen-like domain of ficolin and CRP interactions occur more strongly than binding seen under physiologic conditions (pH 7.4 and 2.5 mM calcium) (Zhang et al., 2011). It is unclear because the structure of binding sites on L-ficolin or H-ficolin are not altered by an acidic pH alone (Garlatti et al., 2009).

Because of ficolin's structural similarity to other calcium-dependent collagenous lectins, previous studies have indicated that ficolin is Ca^{2+}-dependent (Endo et al., 2006; Matsushita et al., 1996). A similar Ca^{2+}-dependent binding dependency was reported for tachylectin 5A from horseshoe crab (Gokudan et al., 1999). However, other laboratories

have reported calcium-independent binding properties for both human and porcine ficolins to acetylated sugars and bacteria (Brooks et al., 2003b; Harumiya et al., 1995; Le et al., 1998; Le et al., 1997; Ohashi and Erickson, 1997). Studies using citrated plasma from human, mouse and pig sources have also shown that samples retain GlcNAc-binding, further suggesting that ficolin binding is not calcium-dependent (Harumiya et al., 1995; Le et al., 1997; Ohashi and Erickson, 1997). X-ray crystallography study of human ficolins indicate that calcium ions in ficolins do not directly interact with ligands (Garlatti et al., 2009). The sensitivity to ethylenediaminetetraacetic acid (EDTA) in some ficolin binding experiments may be due to reduced stabilization of the S1 binding site, which is close to the calcium ion (Garlatti et al., 2009).

Plasma ficolins from both humans and pigs are known to bind to bacterial surface polysaccharides in an N-acetylated manner, although the exact specificity differs between protein subtypes. Human L-ficolin binds to purified LTA from *S. agalactiae* and *Streptococcus pyogenes* (Lynch et al., 2004) to encapsulated strains of *Staphylococcus aureus* and *S. pneumoniae* (Runza et al., 2008), as well as to *Salmonella* Typhimurium TV119 (Matsushita et al., 1996) and *E. coli* (Lu and Le, 1998). Also, L-ficolin recognizes the 1,3-β-D-glucan structure present on yeast and fungal cell walls (Ma et al., 2004). M-ficolin has been shown to bind *S. aureus* and *Salmonella* Typhimurium LT2 (Liu et al., 2005). A recent study has identified an additional specific ligand for H-ficolin: O-specific polysaccharides, in the LPS of four strains (23, PCM 1200, PCM 1203, PCM 1205) of *Hafnia alvei* (Swierzko et al., 2011).

Porcine ficolin-α binds to APP serotype 5b (Brooks et al., 2003a), LPS extracts from Gram-negative bacteria, such as *E. coli, P. aeruginosa, Salmonella* Abortus-equi*, Salmonella* Enteriditis, *Salmonella* Typhimurium, *Serratia marcescens* and *Shigella flexeneri ,* as well as LTA extracts of gram-positive bacteria, such as *Bacillus subtilis*, *S. aureus, S. pyogenes, Streptococcus sanguis* (Nahid and Sugii, 2006). Ficolin-α and L-ficolin have also been demonstrated to bind to porcine reproductive and respiratory syndrome virus (Keirstead et al., 2008) and hepatitis C virus (Liu et al., 2009), respectively. In addition to antimicrobial functions, ficolins also play a role in tissue homeostasis. Soluble innate immune pattern-recognition proteins (sPRPs), including ficolins, participate in clearance of dying cells and cellular material by promoting phagocytosis (Litvack and Palaniyar, 2010). Human L- and H-ficolin are able to recognize DNA on late apoptotic cells and necrotic cells and to promote their uptake by macrophages (Honore et al., 2007; Jensen et al., 2007a).

Antimicrobial functions of ficolins

Some soluble pattern-recognition molecules, including ficolins and MBL, share functional similarities with antibodies and participate in innate immunity processes (Palaniyar, 2010). Ficolins are able to recognize N-acetylated sugars, especially GlcNAc of bacterial and fungal cell walls. Upon binding to a microbial surface, in complex with MBL-associated serine proteases (MASPs), ficolins activate the lectin pathway of the complement cascade (Endo et al., 2011). Ficolins have been identified in plasma complexed with various MASPs, but only MASP-2 complexes can activate the lectin complement pathway (Matsushita et al., 2000; Vousden and Lu, 2002). Ficolin:MASP-2

complex formation leads to autoactivation of MASP-2, which cleaves complement proteins C4 and C2 to generate C3 convertase, C4bC2a. Further, C3 convertase activates the C3 to generate C3b complement fragments, which facilitates opsonization, phagocytosis, and the membrane attack complex (MAC) assembly helps direct microbial killing (Endo et al., 2011; Lynch et al., 2004; Matsushita et al., 2000; Poon et al., 2010; Thiel and Gadjeva, 2009). Some protein fragments of the complement cascade, such as C3a and C5a, also induce inflammation.

Accumulating evidence suggests that all human ficolins when binding to their specific carbohydrate ligands on pathogens activate the complement pathway. Ficolins activate complement pathway upon binding to Gram-negative bacteria, *S.* Typhimurium TV119 (Matsushita et al., 2000), Gram-positive bacteria, *S. aureus* (Lynch et al., 2004), fungal capsular polysaccharides, 1,3-β-D-glucan (Ma et al., 2004) and viral envelope glycoproteins (Liu et al., 2009). Although both ficolins of mouse (ficolin-A and –B) recognize GlcNAc and GalNAc moieties on pathogens, only ficolin-A has been shown to interact with MASP-2 and sMAP, leading to activation of lectin pathway. This suggests that ficolin-A has a similar function to human L-ficolin (Endo et al., 2005). Complement activation by porcine ficolin-α has not yet been investigated.

Mannose-binding lectin and antimicrobial functions

MBL, an innate immune protein, belongs to the collagenous lectin family, and possess both collagen-like regions and carbohydrate recognition domains (CRD). Each MBL subunit is a trimer composed of three identical polypeptide chains of 32 kDa. Each chain

has four distinct regions. A cysteine-rich N-terminus; a collagenous region, which contains 19 Gly-Xaa-Xaa triplets; C-terminal calcium-dependent CRD; and a short neck region (Dommett et al., 2006). The α-helical collagenous-like regions orient the CRD into polarized "bundle of tulip" (sertiform) or cruciform arrays (Holmskov et al., 2003; Ohashi and Erickson, 1998; van de Wetering et al., 2004). MBL is present in serum as an oligomer of two to six subunits and effective activation of the complement cascade occurs when higher order oligomers (tetramers or larger) are present (Dommett et al., 2006). The CRD of MBL is responsible for carbohydrate-binding. Upon binding of CRD, the calcium ion enables interaction with the 3- and 4-hydroxyl groups of sugars such as mannose, GlcNAc, ManNAc, fucose, and glucose (Sheriff et al., 1994; Weis and Drickamer, 1994; Weis et al., 1991). The binding affinity for any CRD to one monosaccharide is weak; therefore, for high-avidity interaction to occur, sugars must be clustered and presented at a nonreducing end. Because higher order oligomers of MBL present multiple CRD, they are able to bind simultaneously to multiple monosaccharides with high avidity (Holmskov et al., 2003; Iobst and Drickamer, 1994).

MBL is primarily produced by hepatocytes and is present in serum; however, it is also expressed in extra-hepatic tissues. For example mouse MBL-C and –A are expressed in the small intestine and kidney, respectively (Uemura et al., 2002; Wagner et al., 2003). MBL synthesis is developmentally and hormonally regulated, but its production may be influenced by inflammatory conditions (Holmskov et al., 2003). Two MBL isoforms (MBL-A and -C) have been identified in pigs, rats, mice, rabbit and rhesus monkey, whereas humans have only one (MBL-C) (Hansen and Holmskov, 1998; Lillie et al.,

2006). The human MBL-A gene is a pseudogene (Guo et al., 1998). Although MBL isoforms share some similarities in amino acid sequence, they are not identical in terms of pathogen recognition. Both MBL-A and –C bind mannan-sepharose beads, but only MBL-A has been shown to bind bacteria incubated with pig plasma (Lillie et al., 2006). MBL-C probably binds to bacteria, but was not recognized in these studies. This could be possibly due to low expression of MBL-C in some pigs which has been associated with polymorphisms in the *MBL2* promoter (Lillie et al., 2007). MBL-A plasma concentration also varies among pigs due to single nucleotide polymorphisms (Juul-Madsen et al., 2011; Juul-Madsen et al., 2006; Lillie et al., 2007). Similarly, mouse MBL-C, but not MBL-A binds *P. aeruginosa* (Phaneuf et al., 2007), further emphasizing variability in MBL isoform bacterial binding functions.

Numerous studies have demonstrated carbohydrate-dependent binding of MBL to a wide range of microorganisms (reviewed in Dommet et al., 2006). Like ficolins, upon recognition of pathogen associated molecular patterns (PAMPs) on microbial surfaces, MBL, in association with MASP, initiates the complement pathway. MBL-mediated complement activation was first discovered in 1987 (Ikeda et al., 1987). Subsequently, MASPs were identified and the concept of a separate lectin-mediated activation pathway was described (Thiel, 1992). Like ficolins, binding of MBL to microbes leads to autoactivation of MASP-2, cleaving complement proteins C4 and C2 to generate C3 convertase C4bC2a. Complement activation can lead to deposition of C4b and C3b onto microbial surfaces and subsequent opsonization, as well as destruction of microorganism through formation of a MAC (Thiel et al., 1997).

Bacterial biofilms

Biofilm is a structured community of bacterial cells encased within a self-developed extracellular polymeric substance (EPS) that is adherent to both living and inert surfaces (Costerton et al., 1999). In specific environmental conditions, basically any bacteria can produce biofilm that affords protection against adverse environmental conditions (Jacques et al., 2010). Biofilm formation has been described as a four-step process involving, a) bacterial adherence to biotic or abiotic surfaces, b) accumulation or formation of microcolonies, primarily mediated by poly-N-acetylglucosamine (PNAG) synthesis (Mack et al., 1996), c) communication of microorganisms during maturation via quorum sensing, and d) detachment and dispersal of bacteria from the biofilm, leading to release of planktonic cells into the surrounding tissue or blood stream. For example, adhesion involving specific proteins such as bifunctional adhesions and autolysins (*AtlE* and *Aae*) that mediate initial adherence to the surface in *Staphylococcus epidermidis* (Heilmann et al., 1997; Heilmann et al., 2003). For quorum sensing, biofilm bacteria produce diffusible chemical signals that transmit amongst their members to monitor the population density and behavior (Deep et al., 2011). In addition, microorganisms within a biofilm exhibit altered physiology and gene expression patterns to adopt to a particular local environmental conditions compared to planktonic bacteria (Stewart and Franklin, 2008)

Diverse microorganisms form biofilm on biotic surfaces (Wagner and Iglewski, 2008) and indwelling medical devices (Donlan, 2001). The clinical importance of biofilm formation was first demonstrated in medical device-related infections (Costerton et al., 1999; Donlan and Costerton, 2002; Hall-Stoodley et al., 2004). Since then, biofilm-

mediated virulence of various pathogenic microorganisms in humans (Donlan and Costerton, 2002) and animals (Jacques et al., 2010) have been described. Some commonly studied biofilm-related bacteria include; *Actinobacillus*, *Bacillus*, *Enterococcus*, *Escherichia*, *Fusobacterium*, *Klebsiella, Mycobacterium*, *Pseudomonas Rhizobia*, *Staphylococcus* and *Streptococcus* spp. (Bhinu, 2005).

Treating biofilm-associated infections is highly challenging as biofilm formation protect bacteria against antibiotics (Roberts and Stewart, 2004); (Izano et al., 2007), immune evasion (Bylund et al., 2006; Matz, 2007), ultraviolet irradiation (Elasri and Miller, 1999), biocidal agents (de Carvalho, 2007) and disinfectants (Marin et al., 2009). Such biofilm resistance is due to the gel-like properties of biofilm, in which reactive agents such as sodium hypochlorite, superoxides, metals or antibacterial agents, are neutralized or bound by EPS (Costerton et al., 1999; Hall-Stoodley et al., 2004). Reduced nutrition availability and decreased growth rate of biofilm bacteria (Roberts and Stewart, 2004) also accounts for biofilm resistance because slow-growing organisms are less susceptible to antimicrobials (Costerton et al., 1999). The other mechanism for biofilm resistance includes existence of distinct biofilm-protected phenotypes, which are biologically resistant subpopulations within biofilms (Costerton et al., 1999).

Actinobacillus pleuropneumoniae (APP) and biofilms

APP is an obligate Gram-negative bacterium that infects the lungs of pigs, causing a severe pleuropneumonia in susceptible animals. These bacteria especially colonize the respiratory tract by binding to mucus, proteins and host cells (Chiers et al., 2010). The

ability to form biofilm is recently implicated in colonization of the respiratory mucosa and also in virulence of various serotypes (Izano et al., 2007; Kaplan and Mulks, 2005). Other virulence factors such as exotoxins, capsular polysaccharides, LPS and urease that also contribute to acute disease have been well characterized (Bosse et al., 2002), but the factors that contribute to chronic infection are less well known. Biofilm formation by strains of APP is thought to play a role in chronic infections by facilitating colonization and resistance to antibiotics (Izano et al., 2007; Kaplan and Mulks, 2005). The biofilm formation is a highly complex and dynamic process, which is regulated by multiple genes. Several tested field isolates of APP *in vitro* produce a thick biofilm (Table 1.1) that is primarily composed of PNAG, a hexosamine-containing polysaccharide. Production of PNAG in APP biofilm mediates biofilm attachment and dispersal (Kaplan et al., 2004). Biosynthesis of PNAG is mediated by *pgaABCD* operon in APP. The *pgaABCD* mutants did not form biofilm *in vitro* (Izano et al., 2007) but increased biofilm formation was observed in *pgaABCD* over expression strains. Recent studies showed increased biofilm formation in *hns* mutants and HN-S can independently regulate biofilm formation via repression of *pga* operon in APP (Bosse et al., 2010; Dalai et al., 2009). Sigma factor (σ^E) can independently regulate *pga* operon and its positive regulation indicates biofilm formation is a part of the extracytoplasmic stress response (Bosse et al., 2010). Grasteau and co-workers (2011) identified 16 unique genes that are associated with abnormal biofilm formation in AAP serotype 1 (S4074) mutants. Further, a *luxS* mutant was shown to exhibit enhanced biofilm formation and attachment independent of autoinducer-2 (AI-2), while AI-2 also can regulate biofilm formation independently (Li et al., 2011; Li et al., 2008). LuxS is an enzyme involved in the activated methyl cycle

required for synthesis of AI-2 that acts as a quorum-sensing molecule. PNAG production in APP biofilm has been shown to impede fluid convection and transport of cetylpyridinium chloride (CPC), suggesting protection of bacteria from CPC (Ganeshnarayan et al., 2009). Further, dispersal of biofilm using DispB increases the antibiotic mediated killing of bacteria (Izano et al., 2007). These findings suggest that production of PNAG by most strains of APP may play a key role in pathogenesis of biofilm infections.

Table 1.1: Biofilm formation by strains of *Actinobacillus pleuropneumoniae*

Strains	Serotype	Biofilm formation	References
S4074 (RS)	1	+	(Labrie et al., 2009)
97-1 (FI)	1	+	(Kaplan and Mulks, 2005)
95-1 (FI)	1	+	(Kaplan and Mulks, 2005)
94-29 (FI)	1	-	(Kaplan and Mulks, 2005)
27088 (RS)	1A	-	(Kaplan and Mulks, 2005)
ISU158 (RS)	1B	-	(Kaplan and Mulks, 2005)
IA1 (FI)	1	+	(Kaplan et al., 2004)
IA5 (FI)	5	+	(Kaplan et al., 2004)
ISU178 (RS)	5	-	(Kaplan and Mulks, 2005)
L20 (RS)	5B	+	(Kaplan and Mulks, 2005)
E11001 (MS)	IA5N	+	(Izano et al., 2007)
E11005 (MS)	IA5N	+	(Izano et al., 2007)
98-1 (FI)	7	+	(Kaplan and Mulks, 2005)
93-6 (FI)	7	+	(Kaplan and Mulks, 2005)
ISU53 (RS)	7	-	(Kaplan and Mulks, 2005)
86-1 (FI)	7	-	(Kaplan and Mulks, 2005)
56513 (RS)	11	+	(Kaplan and Mulks, 2005)

RS- reference strains, FI- field isolates, MS- mutant strains

Poly-N-acetylglucosamine (PNAG)

Biofilm EPS contains various polysaccharides, proteins, lipids, surfactants, DNA, glycolipids, nucleic acids, membrane vesicles, and ions (Ca^{2+}) (Jacques et al., 2010) and their composition varies with culture conditions, age and type of organism (Mayer et al., 1999). Amongst biofilm matrix components, polysaccharides are a major component and have been extensively studied (Sutherland, 2001). These polysaccharides, variously known as PNAG, poly-glucosamine (PGA) or PIA (polysaccharide intercellular adhesin), consist of linear chains of N-acetylglucosamine residues in β-1,6 linkage (Joyce et al., 2003; Mack et al., 1996; Maira-Litran et al., 2002). Biosynthesis of PNAG is encoded by the *icaABCD* operon in *S. epidermidis* (Heilmann et al., 1996). Although expression of *icaA* gene alone encodes the enzyme needed for the synthesis of the GlcNAc polymer, its co-expression with *icaD* induces increased production of PNAG (Gerke et al., 1998). The *icaC* gene encodes a membrane protein, which produces oligomers of PNAG when expressed with *icaA* and *icaD* (Gerke et al., 1998). The *icaB* gene encodes a secreted protein, which is similar to other polysaccharide N-deacetylases such as chitin deacetylase and peptidoglycan GlcNAc deacetylase (Kaplan et al., 2004). Similarly, production of PNAG in *E. coli* is encoded by the *pgaABCD* operon, which may be functionally related to the *icaABCD* operon of *S. epidermidis* (Wang et al., 2004).

PNAG plays a critical role in biofilm attachment and dispersal in diverse microorganisms such as *E. coli*, *Pseudomonas fluorescens,* S. *epidermidis*, *Yersinia pestis*, (Itoh et al., 2005; Wang et al., 2004) and field isolates of APP serotype 1 and 5 (Izano et al., 2007). PNAG produced by *Bordetella* spp. mediates surface attachment to respiratory tract epithelium (Sloan et al., 2007). Accumulating data indicate that PNAG not only plays an

important role in biofilm formation but also in immune system evasion and microbial pathogenicity for several bacterial species including *S. aureus* (Cerca et al., 2007; Kropec et al., 2005), *S. epidermidis* (Cerca et al., 2006), *E. coli* (Wang et al., 2004) and APP species (Izano et al., 2007; Kaplan et al., 2004). In contrast, PNAG does not appear to be a major component of the extracellular matrix of *Staphylococcus lugdunensis* biofilms (Frank and Patel, 2007) and some PNAG mutant strains produce biofilms (O'Gara, 2007; Rohde et al., 2007). This suggests a possible role for other biofilm matrix components in surface attachment.

Host immune response to biofilm infections

Innate as well as adaptive immune responses can be produced against biofilm infections, similar to non-biofilm producing bacterial infections. However, biofilm infections are comparatively more resistant to the host immune response and contribute to impaired clearance of pathogens, persistent infection and collateral tissue damage (Brady et al., 2008; Jensen et al., 2010; Schaudinn et al., 2009).

Infections by biofilm-producing bacteria are resistant to the host immune response. The biofilm matrix acts as a physical barrier, providing protection from leukocytes and their various cytotoxic end products, such as oxygen radicals and antibacterial peptides. Further, by "glueing" individual cells together, EPS aids in the formation of microcolonies, which can evade phagocytosis because they are far bigger than individual bacteria (Matz, 2007). In addition, it has been demonstrated that EPS isolated from *Burkholderia cenocepacia* and *P. aeruginosa* may inhibit neutrophil chemotaxis and

scavenge neutrophil-derived reactive oxygen species (ROS) (Bylund et al., 2006; Simpson et al., 1989). Biofilm-producing bacteria may also be less conspicuous to the immune system because the antigens and ligands typically used by professional phagocytes are hidden within the biofilm matrix (Matz, 2007). *P. aeruginosa*-derived biofilm contains the exopolysaccharide alginate, which protects the bacteria through a process called "frustrated phagocytosis", in which neutrophils and macrophages encounter but cannot engulf bacteria in biofilm. Alginate reduces the efficiency of phagocytosis by neutrophils and macrophages by scavenging hypochlorite (Leid, 2009). Also, biofilm bacteria are able to protect through global response regulators and a quorum sensing mechanism in which biofilm bacteria communicate by producing small signaling molecules to regulate specific target genes in response to fluctuations in cell density (Leid, 2009; March and Bentley, 2004).

Adaptive immunity plays a significant role in eliminating wide range of microorganism from the body. In the case of biofilm infections, adaptive immune response may be initiated simultaneously with innate immune response or after stimulation of the innate response. Persistent biofilm infections may resist released antibodies, enzymes, and activated phagocytes, but the surrounding tissue will be exposed to the deleterious effects of oxygen radicals (Jensen et al., 2010). Also, surface molecules on immune cells may undergo degradation as a result of proteases and exoenzymes released from the host cells, which further contributes to impaired immune function (Kharazmi and Nielsen, 1991; McCormick et al., 1997). *S. epidermidis* biofilm has been shown to resist host immune response by impairing C3b and IgG deposition on the bacterium and increasing

the resistance to phagocyte-mediated killing (Kristian et al., 2008). These findings suggest that host immune responses to mature biofilm infections are often ineffective and, instead may contribute to extensive collateral tissue damage.

Singh and co-workers demonstrated that lactoferrin, when provided at a concentration below that needed to kill or inhibit bacterial growth, blocks biofilm development by *P. aeruginosa*. In their study, they showed that lactoferrin chelates iron and stimulates bacterial 'twitching', which causes bacteria to wander across the surface of a culture plate instead of forming cell clusters and surface adherence (Singh et al., 2002). No information is available about the interactions of soluble PRR such as ficolin and MBL with biofilm infections. Previous investigations of the antibacterial functions of ficolins and MBLs have focused on their ability to bind various carbohydrate moieties on bacterial cell walls or purified components of LPS. Plasma ficolin and MBL recognition of PNAG within bacterial biofilm is unknown.

The current research exploiting N-acetyl binding properties of porcine plasma ficolin and MBL is a new approach to study the interactions of ficolins and MBLs with PNAG residues in biofilm. These studies may enable us to understand and further characterize the potential roles of ficolins and MBLs in the 1) dispersal of biofilm-producing bacteria in the initial stages of bacterial colonization and infection in tissues via opsonization and complement activation or 2) lectin interactions with biofilm after colonization and maturation, which may contribute to collateral tissue damage through excessive

complement activation. It is also possible that PNAG in biofilms can bind ficolin or MBL in a manner that shields bacteria from lectin-mediated complement attack.

Project rationale, hypothesis and objectives

Ficolins and MBL are collagenous lectins that have similar but distinct structural and functional properties. Both are able to recognize various PAMPs on microbial surfaces and trigger the activation of complement via lectin pathway in association with MASPs. Ficolins and MBLs can also act as opsonins by promoting phagocytosis of various bacteria to which they bind. These findings suggest a role for plasma lectins in resistance to bacterial, and possibly viral infection. Recent reports suggest that N-acetylated polysaccharides, especially PNAG, are a major constituent in many bacterial biofilms, including those produced by several strains of APP. These N-acetylated polysaccharides can mediate antibiotic resistance and tolerance to host defenses. Previous studies of the antibacterial functions of ficolins have focused on their ability to bind various N-acetylated saccharides in bacterial capsules or purified components of cell wall such as LPS. However, the ability of ficolin and MBL to bind to PNAG within bacterial biofilm is unknown. Therefore, this research will test the hypothesis that PNAG in bacterial biofilm is an N-acetylated binding target for ficolin and MBL. Such binding might play a role in dispersal of biofilm bacteria during the early stage of biofilm formation by the activation of the complement cascade and opsonization. This approach may also enable identification of other pig plasma proteins that bind to PNAG in an N-acetylglucosamine-dependent manner.

The major objectives of this research were to determine: 1) Whether porcine plasma ficolin binds to bacterial biofilm in an N-acetyl-dependent manner, and
2) if there are other lectins in porcine plasma that bind to bacterial biofilm.

Chapter 2. Binding of porcine plasma ficolin-α to bacterial biofilms

Abstract

Ficolins are complement-activating collagenous lectins that bind to N-acetylated surface polysaccharides on various bacteria. Previous studies have demonstrated that porcine plasma ficolin-α binds to surface polysaccharides of *Actinobacillus pleuropneumoniae* serotype 5b (APP5b) grown in planktonic cultures. Many bacterial pathogens grow as biofilms with a self-developed extracellular polymeric matrix that can protect them from host immune defenses. In the present study, we compared binding of porcine ficolin-α, both purified and in whole plasma, to APP5a biofilm or planktonic cultures. N-acetylglucosamine (GlcNAc), most likely in the form of poly-N-acetylglucosamine (PNAG), in the biofilm matrix was stained with wheat germ agglutinin conjugated with Alexa Fluor-555 and visualized by confocal laser scanning microscopy (CLSM). Dispersin B prevented APP5a biofilm formation indicating the requirement of PNAG for bacterial cohesion. Bound ficolin was eluted with GlcNAc from APP5a biofilm cultures and identified by anti-ficolin antibody. To address preferential binding of ficolin-α to biofilm matrix, ficolin-α was also eluted with GlcNAc from extracellular polymeric substances (EPS) in supernatant after pelleting the bacteria. Biotinylated-ficolin that retained GlcNAc-binding activity for APP5b was shown to bind strongly to APP5a biofilm, as detected by fluorescent streptavidin labeling and CLSM. These results demonstrate that porcine ficolin-α binds in a GlcNAc-dependent manner to biofilm or planktonic cultures of APP5a. These studies suggest that PNAG in biofilms of many bacteria including APP5a might be an important binding target for ficolin.

1. Introduction

Among the collagenous lectins, ficolins and mannose-binding lectins (MBL) have similar structural and functional properties (Matsushita et al., 2000). Ficolins have a fibrinogen-like carbohydrate recognition domain whereas MBL has a C-lectin domain (Matsushita et al., 1996). Ficolins recognize N-acetylglucosamine (GlcNAc) residues on the surface of diverse microorganisms (Matsushita, 2010). Various binding targets have been reported for ficolins of humans (Endo et al., 2011; Matsushita et al., 1996), pigs (Brooks et al., 2003c; Keirstead et al., 2008), rodents (Ohashi and Erickson, 1998) and recently, for freshwater crayfish (Wu et al., 2011). Humans have three ficolins (L-ficolin, H-ficolin and M-ficolin) (Akaiwa et al., 1999; Endo et al., 1996; Matsushita, 1996), and all three ficolins can activate the lectin pathway of complement, despite exhibiting differences in tissue expression and bacteria-binding (Endo et al., 2011). Upon binding to microbial surfaces, autoactivation of MBL-associated serine proteases (MASP)-2 cleaves C4 and C2 and generate C3 convertase, C4bC2a. The result of complement activation is opsonization via deposition of C4b and C3b on microbes and killing of microorganisms through formation of membrane attach complex (MAC) (Endo et al., 2011; Matsushita et al., 1996).

Mouse ficolins A and B recognize GlcNAc and N-acetylgalactosamine (GalNAc) moieties on pathogens; however, only ficolin-A has been shown to interact similarly to human L-ficolin, participating in complement activation (Endo et al., 2005). In contrast,

the functional significance of porcine ficolins and microbial binding specificity are relatively unknown (Brooks et al., 2003b; Keirstead et al., 2008; Nahid and Sugii, 2006), although it is known that GlcNAc is an important binding target for porcine ficolins (Brooks et al., 2003b; Keirstead et al., 2008). Previous studies have demonstrated that porcine plasma ficolin-α binds to surface polysaccharides of *Actinobacillus pleuropneumoniae* serotype 5b (APP5b) grown in planktonic cultures (Brooks et al., 2003b), but this has not been in biofilm cultures.

Biofilm-producing bacterial infections are a major concern in human as well as veterinary medicine because of biofilm-mediated immune evasion and antibiotic resistance (Izano et al., 2007; Jacques et al., 2010). A wide range of microorganisms produces biofilm, which masks surface ligands, preventing host immune system recognition (Matz, 2007). Previous studies of the antibacterial functions of ficolins have focused on their ability to bind various carbohydrate moieties on bacterial cell walls or extracted lipopolysaccharide (LPS) components, but not biofilm. To investigate the binding of ficolins to bacterial biofilm, we studied the interaction between porcine ficolin-α and biofilm produced by *A. pleuropneumoniae* serotype 5a (APP5a).

2. Materials and Methods

Porcine ficolin and plasma

Porcine plasma ficolin was purified as described by Brooks et al. (2003b). Lyophilized ficolin fractions were dissolved in milliQ water (100 µl) to ~0.7 µg/µl for use in binding assays. To examine all plasma proteins that bound to biofilm matrix, previously collected and frozen (–70°C) plasma from healthy pigs were thawed to 4 ºC and dialyzed in 10 volumes of TBS-Ca^{2+} buffer (50 mM Tris, 150 mM NaCl and 10 mM $CaCl_2$) using regenerated cellulose dialysis tubing (12-14 kDa MWCO) (Fisher Scientific, Ottawa, ON, Canada) at 4 ˚C with gentle stirring for 24 h.

Bacterial cultures

Serotype 5a (TF91-1084), 5b (VSB 1104) and 7 (FMV94-1475) strains of *Actinobacillus pleuropneumoniae* (APP) (Brooks et al., 2003b) were grown on trypticase soya agar (TSA) (Animal Health Laboratory, Guelph, ON, Canada) plates pre-coated with 100 µl of 10% (w/v) nicotinamide adenine dinucleotide (NAD) (Roche Diagnostics, Laval, QC) or in brain heart infusion (BHI) (Oxoid Ltd, Basingstoke, Hampshire, UK) broth supplemented with 50 µg/mL NAD (BHI-N). A single colony was transferred into 3 mL BHI broth in a glass tube and incubated at 37 ˚C overnight with agitation at 200 rpm. For biofilm production, the broth was discarded from the overnight cultures and the adherent biofilm was gently washed with fresh broth. The biofilm was then removed by scraping, mixed with fresh broth, and vortexed for 10-15 s. The resultant cell suspension (>1 x 10^8 CFU/mL) was used for the biofilm culture (Izano et al., 2007). For some studies, APP5a

bacteria were harvested (>1 x 10^8 CFU/mL) from overnight broth cultures, supplemented with or without 20 µg/mL dispersin B (DispB) (Kane Biotech Inc, Winnipeg, MB, Canada), by centrifugation (10,000 x g 10 min at 4 ˚C), and washed once and resuspended in 500 µl of PBS pH 7.4. Planktonic cultures were fixed in 10% formaldehyde for 1 h, washed again three times in PBS (pH 7.4) and stored at –20 ˚C (Brooks et al., 2003c).

Biofilm assay

A static biofilm assay in microtiter plates was performed as described by Labrie et al. (2010). Briefly, a sterile 96-well microtiter plate (Costar® 3596, Corning, NY, USA) was inoculated in sextuplicate with an overnight bacterial cultures (APP5a, 5b and 7) diluted in (1/100) 100 µL of BHI-N and incubated at 37 ˚C for 24 h. Control wells were filled with bacterial inoculated broth and DspB (20 µg/mL). After incubation, the effect of DispB on bacterial growth was determined by measuring the optical density (OD) of suspension bacteria was measured at 630 nm using an ELISA plate reader (Biotech, VT, USA). The plate was washed by immersion in PBS pH 7.4 to remove non-adherent bacteria and excess PBS was removed by gently tapping the inverted plate on paper towel. All wells were filled with 100 µL of 0.1% crystal violet and incubated the plate at room temperature for 2-3 min. After washing in PBS pH 7.4, the plate was dried in an incubator at 37 ˚C for 1 h. To measure the optical density (OD) of the adherent biofilm, all wells were filled with 100 µL of 70% ethanol and absorbance (630 nm) was recorded by using an ELISA plate reader.

Polysaccharide characterization

For biofilm preparations, Petri dishes (8.8 cm^2, Nunclon^{TM}, Denmark) were inoculated with an overnight broth cultures (APP5a, 5b) diluted in (1/100) BHI-N and incubated at 37 ˚C for 24 h. Control dishes were contained uninoculated broth and or cultures supplemented with 20 µg/mL DispB. Following incubation the biofilms were washed gently twice with PBS pH 7.4 and approximately 1 mL of working solutions of Wheat Germ Agglutinin (WGA) conjugated with Alexa Fluor (AF)-555 (Ex_{555}, Em_{567}) was added to stain extracellular GlcNAc; Syto 62 (Ex_{652}, Em_{676}) was added to stain DNA and RNA of live and dead bacteria (Invitrogen, Burlington, Canada). The plates were incubated at room temperature for 30 min in the dark then the biofilm was washed gently three times with PBS pH 7.4 to remove unbound fluorescent stain and observed immediately under an upright confocal laser scanning microscope (CLSM) (Leica DM RE microscope connected to a Leica TCS SP2, Leica, Germany). Images were processed using Leica Confocal Software (LCS, version 2.61).

Ficolin binding

Tissue culture flasks (75 cm^2) (Corning, Corning, NY, USA) were inoculated with overnight broth cultures (APP5a) diluted (1:100) in BHI-N and incubated at 37 ˚C for 72 h. Cultures grown with 20 µg/mL of DispB served as negative controls and broth from these flasks were discarded after 24 h and filled with fresh BHI-N. After 72 h, broth was discarded, adherent biofilms were gently washed twice with TBS-Ca^{2+} buffer (25 mM Tris, 145 mM NaCl, 2.5 mM $CaCl_2$ and pH 7.4). Purified porcine plasma ficolin (~70 µg) diluted in TBS-Ca^{2+} buffer or dialyzed whole plasma (10 mL) were added and further

incubated on ice with gentle constant rotation for 4 hr. Unbound fractions of purified ficolin or plasma were collected and the cultures were gently washed with TBS-Ca^{2+} buffer (three times for purified ficolin, five times for plasma) for 5 min with gentle rotation on ice and the final wash fractions were collected. Biofilm bound proteins were eluted by incubation with 400 mM GlcNAc at 4 ºC for 1 h with gentle rotation. The remaining biofilm-bound proteins were then stripped by incubation in 50 mM acetic acid (NaOAc; pH 3.5) at room temperature for 12 min. The final wash and GlcNAc and NaOAc elution fractions were concentrated to an equal volume (500 µL) (Amicon concentrators-10 kDa cutoff, Millipore Corporation, Bedford, MA, USA) and the protein fractions in the eluate were resolved on 12% one-dimensional (1D) SDS-polyacrylamide gel (PAG) and ficolin binding was identified in Western blotting using rabbit anti-porcine ficolin-α antiserum as described previously (Brooks et al., 2003c).

To examine binding of ficolin to the biofilm matrix in the absence of bacteria, biofilm matrix (EPS) including PNAG, other glycans, nucleic acids and proteins was extracted from an APP5a biofilm as described previously (Lin et al., 2009) with some modifications. After discarding the broth, biofilms in flasks were rinsed twice in PBS pH 7.4, and then scraped from the surface and diluted in 5 mL of PBS. The EPS was extracted on a cation exchange resin (CER) (Dowex™ Marathon™ C, Na^+ form, Sigma-Aldrich, Bellefonte, PA) (Lin et al., 2009). Biofilm in PBS was mixed with 5 g of CER, vortexed for 1 h at 4 ˚C, and the supernatant containing EPS was harvested by centrifugation at 4000 x g for 20 min. For binding assays, EPS (0.5 mL) was coated onto 8.8 cm^2 Nunclon™ dishes and incubated at 37 ˚C for 4 h. After complete drying of

coated EPS, dishes were gently washed with TBS-Ca^{2+} buffer twice and incubated with purified ficolin (~ 15 μg) at room temperature for 1 h. Dishes inoculated with purified ficolin alone were used as negative controls. Unbound fractions were collected, gently washed with TBS-Ca^{2+} buffer three times, and bound ficolin was eluted by incubating with 400 mM GlcNAc at room temperature for 30 min. Eluted protein fractions were concentrated and characterized by 1D SDS-PAG and Western blotting as described above.

To examine ficolin binding directly, we also prepared biotinylated purified porcine ficolin-α using a Pierce EZ-Link Micro Sulfo-NHS-LC-Biotinylation Kit (Pierce, Rockford, IL). Purified ficolin (0.7 μg/μL) was mixed with 10 μl of 10 mM Sulfo-NHS-LC-Biotin reagent and incubated on ice for 2 hrs. After completion of protein labeling, excess biotin was removed using a Pierce Zeba Desalt Spin Column, according to the manufacturer's instructions (Pierce, Rockford, IL). Biotinylation of purified ficolin was confirmed by using a modified Western blot with horseradish peroxidase-conjugated streptavidin.

Biotinylated ficolin that retained APP5b planktonic culture-binding activity or non-biotinylated purified ficolin (negative control) were incubated with APP5a biofilm, cultured on Petri dishes (8.8 cm^2) as described above, for 1 h. In addition, DspB (20 μg/mL), which degrades PNAG of biofilm was used as a negative control. To demonstrate GlcNAc-dependence, 100 mM GlcNAc was added to compete with binding of the biotinylated ficolin. Biofilm was then gently washed three times with PBS pH 7.4

to remove unbound ficolin and bound ficolin was detected by addition of NeutrAvidin conjugated with Texas Red (Ex_{595}, Em_{615}) (Invitrogen, Canada), incubated at room temperature for 30 min in the dark. Bacteria in the biofilm were counterstained with fluorescent dye Syto 9 (Ex_{485}, Em_{500}) (Invitrogen, Canada). The biofilm was gently washed three times with PBS pH 7.4 and observed immediately under an upright CLSM (Leica DM RE microscope connected to a Leica TCS SP2). Images were processed using Leica Confocal Software.

Protein characterization

Eluted protein fractions from biofilm and planktonic bacteria binding assays were characterized by reducing 1D SDS-PAG using the Bio-Rad modular Mini-Protean II system (Bio-Rad Laboratories, Hercules, CA, USA) with pre-stained molecular weight markers (range 6 - 202 kDa) (Bio-Rad Laboratories). Electrophoresis was performed at 120 V and resolved protein fractions were visualized with silver nitrate (Blum et al., 1987) or Bio-Safe Coomassie Stain (Bio Rad Laboratories). Western blotting was carried out as described by Brooks et al. (2003b) and membranes were developed using ECL Western blotting detection reagents (Amersham). Images were taken using ChemiDoc™ XRS+ system with Image Lab™ Software (Bio-Rad Laboratories).

Statistical analysis

The significance ($p \leq 0.05$) of difference in biofilm production by strains of APP and effect of DispB on biofilm disruption and bacterial growth were determined (mean OD

values) by t-test using GraphPad Prism version 4.0 (GraphPad Software, San Diego, CA, USA).

1. Results

In the present study we conducted a series of experiments to determine if porcine plasma ficolin-α binds to biofilm cultures in a GlcNAc-dependent manner. APP5a (STF91-1084), but not APP5b (VSB 1104) or APP7 (FMV94-1475) produced a pronounced crystal-violet-staining biofilm (Figure 2.1 A, B, C) at the liquid-air interface in glass tubes or on 96-well microtiter plates, Petri dishes or tissue culture flasks. Consistently, APP5a grown in planktonic cultures tended to clump whereas APP5b bacteria did not, suggesting that some sticky matrix was present in APP5a planktonic cultures. Biofilm formation or clumping of APP5a bacteria was not seen when DspB (20 µg/mL) was included in the medium (Figure 2.1 D). Further, addition of DspB to the culture medium did not affect the growth of APP5a as demonstrated by measuring the OD of the suspension bacteria in cultures (Figure 2.1 E).

With the conditions used APP5a produced an abundant crystal violet staining matrix within 24 h. The biofilm matrix stained strongly with WGA/AF-555 and bacteria were counterstained using Syto 62 (Figure 2.2 A, B, C). There was no crystal violet or WGA/AF-555 fluorescence staining observed with APP5b cultures indicating no biofilm was formed (data not shown).

Next we evaluated GlcNAc-dependent binding of purified porcine plasma ficolin-α to APP5a cultured as a biofilm. Under reducing conditions, GlcNAc consistently eluted a single ~40 kDa protein that was immunoreactive with rabbit anti-porcine ficolin-α

antiserum (Figure 2.3). The porcine ficolin preparation used in these binding experiments contained multiple ficolin forms and two minor bands at ~ 85 kDa and ~25 kDa, consistent with heavy and light chains of IgM, as described previously (Brooks et al., 2003b). A similar ficolin band was eluted by GlcNAc from APP5a biofilm incubated with porcine plasma (Figure 2.4 A, B). To determine if the ficolin bound to extracellular matrix rather than to bacterial cell walls, we extracted EPS (Lin et al., 2009) and coated onto the Petri dishes. Purified ficolin-α bound in a GlcNAc-elutable manner to EPS extracted from APP5a biofilm cultures (Figure 2.5).

We compared binding of purified porcine ficolin-α to APP5a biofilm or planktonic cultures. We used an APP5a strain because previously ficolin-α was found not to bind to APP5a planktonic cultures unlike APP5b (Brooks et al., 2003a). However, in the present, some ficolin-α binding was observed in APP5a planktonic cultures when cells were grown in the presence of DispB which degrades PNAG (Figure 2.6).

Purified biotinylated ficolin-α that retained APP5b bacteria binding (Figure 2.7 B) selectively bound to APP5a biofilm matrix, as detected by CLSM using Texas-Red conjugated Neutravidin (Figure 2.2 D, E, F). No ficolin specific fluorescence staining was observed in biofilm cultures incubated in presence of DispB or with non-biotinylated ficolin. Further, biofilm cultures incubated with biotinylated ficolin and GlcNAc, did not show ficolin specific fluorescence staining and bacteria in the biofilm showed positive fluorescence staining for Syto 9 (Figure 2.2 G, H, I).

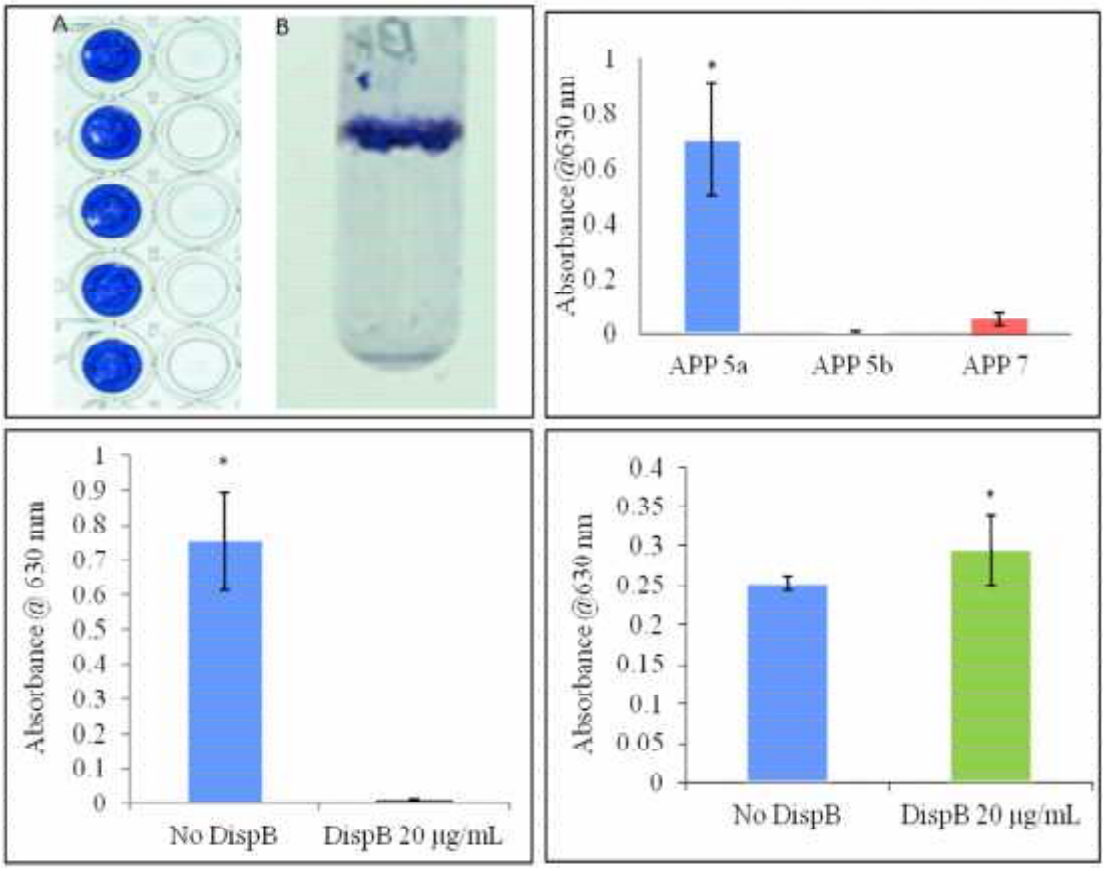

Figure 2.1. Biofilm formation by strains of APP and its removal by dispersin B (DispB) (20 µg/mL) as detected by crystal violet staining in static biofilm assays. (A) Crystal violet stained APP5a biofilm in 96-well microtiter plate (left column) and no adherent biofilm containing in DispB wells (right column). (B) Crystal violet stained biofilm of APP5a at liquid-air interface in glass tube. (C, D) Biofilm formation was measured in 96-well microtiter plates by staining with crystal violet, the optical density (OD) was measured at 630 nm after dissolving the crystal violet in 70% ethanol. (C) APP5a formed pronounced biofilm ($p \leq 0.05$) compared to APP5b cultures, and addition of DispB to the biofilm cultures resulted in complete removal of biofilm ($p \leq 0.05$) (D). (E) Effect of DispB on bacterial growth was determined by measuring the OD (630 nm) of the bacterial suspension in 96-well microtiter plates. Significantly high ($p \leq 0.05$) OD value in DispB added cultures indicates a greater number of bacteria. These results were confirmed by two independent experiments.

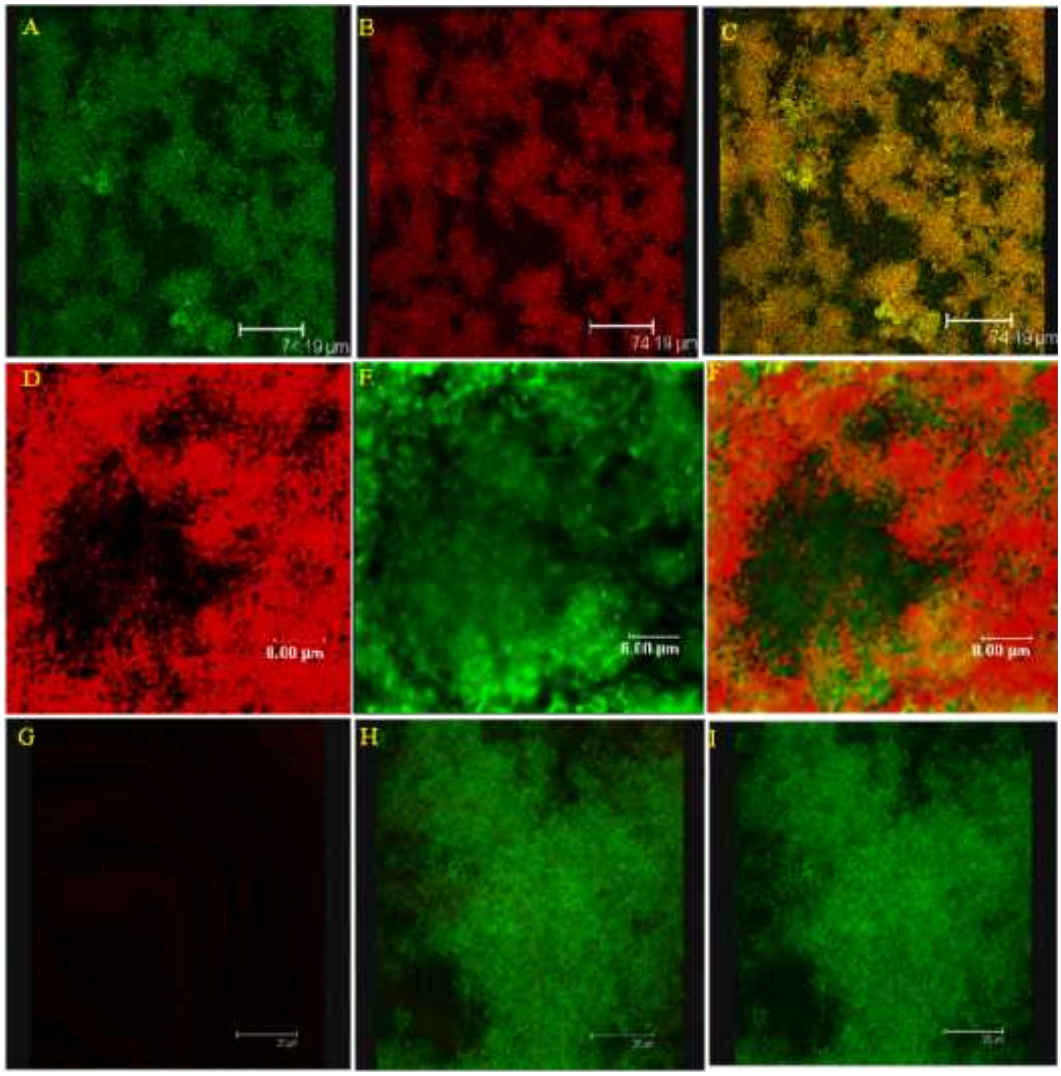

Figure 2.2. Fluorescence staining of GlcNAc residues in biofilms and visualization of biotinylated ficolin-α binding to APP5a biofilm using confocal laser scanning microscopy (CLSM). (A) GlcNAc residues in biofilm were stained with a fluorescent probe, WGA/AF-555 (Ex_{555}, Em_{567}) and (B) bacteria were counterstained using Syto 62 (Ex652, Em_{676}), specific to DNA and RNA of both live and dead bacteria. (C) Two images (A and B) merged; yellow indicate dual staining for both biofilm and bacteria. (D) Biotinylated ficolin-α binding to APP5a biofilm as detected by NeutrAvidin/Texas Red (Ex_{595}, Em_{695}) and bacteria (E) were counterstained by Syto 9 (Ex_{485}, Em_{500}), specific to DNA and RNA of live and dead bacteria. (F) Two image (D and E) images merged; yellow indicate dual staining of biofilm matrix and bacteria. Red only staining indicates pronounced biofilm binding of biotinylated ficolin. (G) Biotinylated ficolin did not bind to APP5a biofilm in presence of 100 mM GlcNAc and only bacteria were stained (H). (I) Two merged images (G and H) did not show biotinylated ficolin-specific staining. These results were confirmed by two independent experiments.

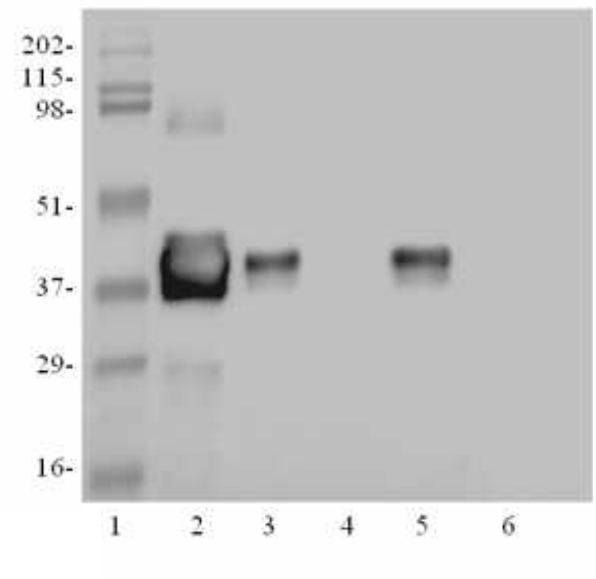

Figure 2.3. Western blot illustrating purified porcine ficolin-α binding to APP5a biofilm. GlcNAc eluted ficolin from APP5a biofilm incubated with purified porcine plasma ficolin was immunoreactive with rabbit anti-porcine ficolin-α antiserum (Brooks et al., 2003a). Lanes 1: MW markers. 2: Purified porcine ficolin-α. 3: Unbound fraction containing some ficolin. 4: Final wash fraction containing no remaining unbound ficolin. 5: 400 mM GlcNAc eluate containing biofilm-bound ficolin. 6: Negative control, 400 mM GlcNAc eluate from APP5a biofilm incubated with 20 µg/mL DispB containing no non-specific ficolin binding. These results were confirmed by three independent experiments.

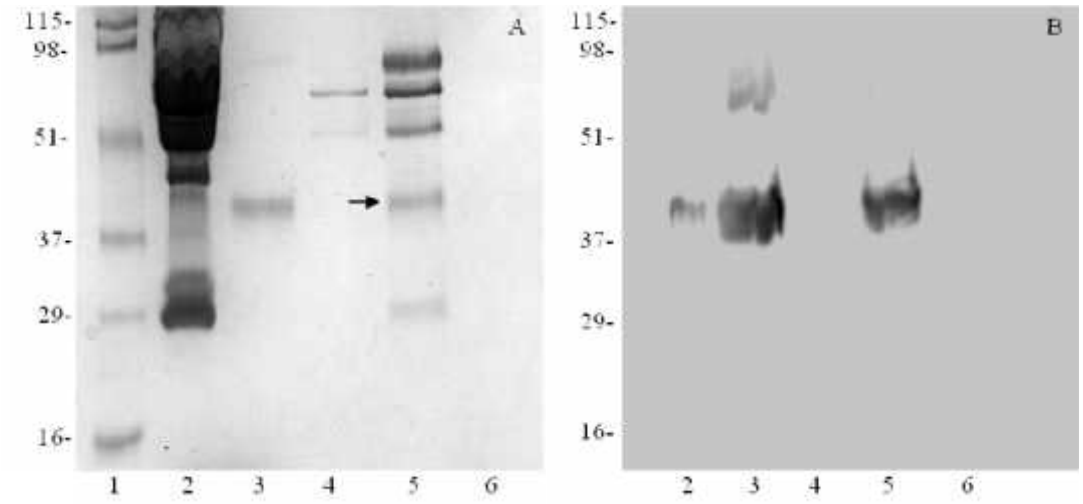

Figure 2.4. Coomassie blue stained reducing SDS-PAG (A) and corresponding Western blot (B) illustrating binding of porcine plasma ficolin to APP5a biofilm cultures. GlcNAc eluted biofilm bound plasma ficolin migrates as a single distinct band at ~40 kDa subunit that is immunoreactive with rabbit anti-porcine ficolin-α antiserum. Lanes 1: MW marker. 2: Unbound plasma after incubation with APP5a biofilm contained some ficolin. 3: Purified porcine ficolin. 4: Final wash containing no unbound ficolin. 5: 400 mM GlcNAc eluate containing ficolin (arrow) and other plasma proteins identified as immunoglobulin subunits. 6: 400 mM GlcNAc eluate from plasma incubated with APP5a biofilm + 20 µg/mL DispB (negative control). (B) Western blot corresponding to Fig 4A. These results were confirmed by two independent experiments.

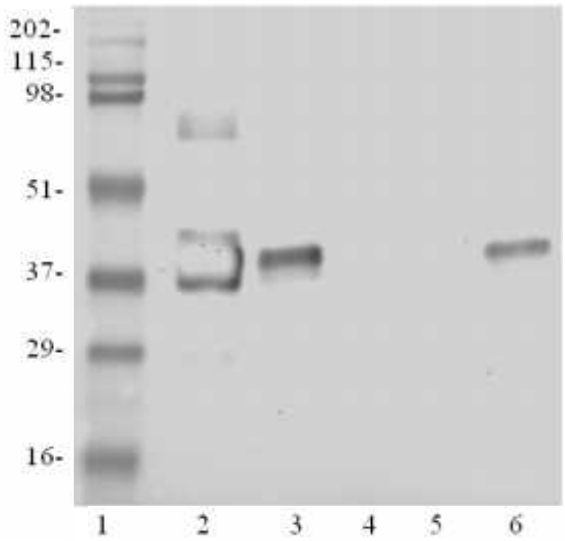

Figure 2.5. Western blot illustrating purified porcine plasma ficolin-α binding to APP5a EPS (after removal of bacteria). GlcNAc eluate of EPS bound ficolin was immunoreactive with rabbit anti-porcine ficolin-α antiserum. Lanes 1: MW marker. 2: Purified porcine ficolin-α. 3: Unbound fraction of ficolin after incubation with APP5a biofilm. 4: Final wash fraction containing no remaining unbound ficolin. 5: Negative control, 400 mM GlcNAc eluate from empty flask containing no nonspecific bound ficolin. 6: 400 mM GlcNAc elute containing biofilm-bound ficolin. These results were confirmed by two independent experiments.

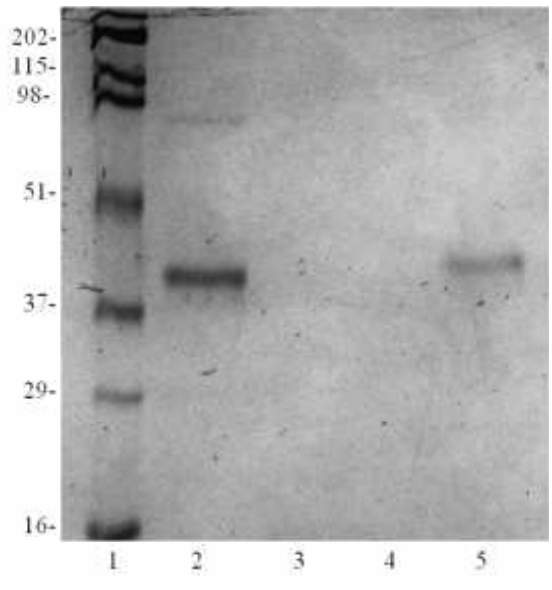

Figure 2.6. Coomassie blue stained reducing SDS-PAG of ficolin-α eluted with GlcNAc from APP5a planktonic cultures in presence of 20 µg/mL DispB. APP5a bacteria bound porcine purified ficolin-α migrates as a single distinct band at ~40 kDa. Lanes 1: Marker. 2: Positive control, porcine purified ficolin-α. 3: Bacteria unbound fraction containing no ficolin. 4: Final buffer wash fraction containing no remaining unbound ficolin. 5: 400 mM GlcNAc elute containing ficolin. These results were confirmed by three independent experiments.

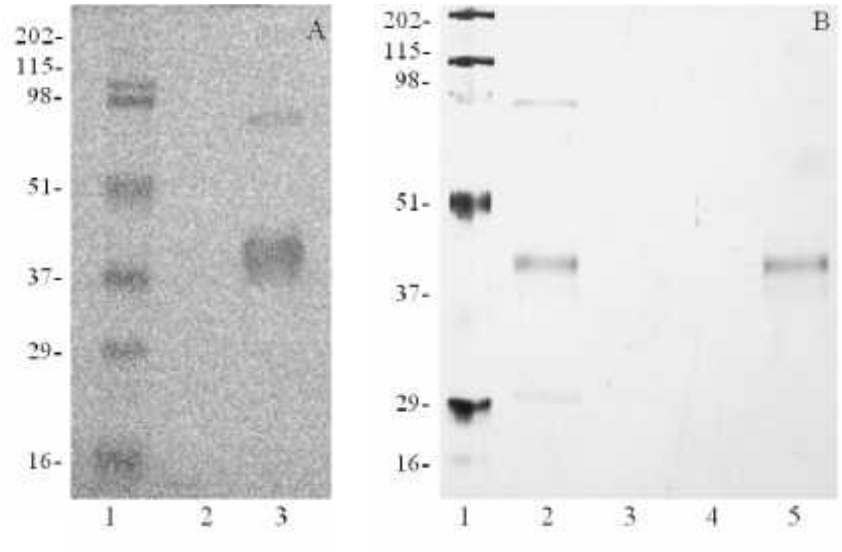

Figure 2.7. Binding of biotinylated purified porcine plasma ficolin-α to APP5b planktonic cultures. (A) Modified Western blot using horseradish peroxidase-conjugated with streptavidin of biotinylated porcine purified plasma ficolin-α. Lanes 1: MW marker. 2: Non-biotinylated purified porcine plasma ficolin. 3: Biotinylated purified porcine plasma ficolin.
(B) Silver-stained reducing SDS-PAG of biotinylated ficolin that bound to APP5b planktonic cultures. Lanes 1: Marker. 2: Positive control, porcine purified ficolin-α. 3: Unbound fraction containing no visible ficolin band. 4: Final buffer wash fraction containing no remaining unbound ficolin. 5: 400 mM GlcNAc eluate containing APP5b planktonic bacteria-bound biotinylated ficolin. These results were confirmed by two independent experiments.

4. Discussion

These experiments provide initial evidence that porcine plasma ficolin-α binds to GlcNAc –positive biofilm produced by APP5a (STF 91-1084) in a GlcNAc-dependent manner. This supports the hypothesis that PNAG in biofilm matrix is a ficolin binding target. In agreement with previous studies (Brooks et al., 2003a), APP5a grown in planktonic cultures was less effective in binding ficolin than was APP5b. The observed differences in ficolin binding to APP5a and APP5b cultures could not be clearly explained by the structures of their LPS and capsular polysaccharides, both of which contain GlcNAc (Altman et al., 1987; Altman et al., 1990; Brooks et al., 2003a). In the present experiments, we observed increased ficolin binding to APP5a grown in planktonic cultures in presence of DispB. The likely explanation for this unexpected finding is that APP5a grown in planktonic cultures produced some loose PNAG and bound ficolin is lost as the suspended bacteria are washed and pelleted repeatedly. However, if PNAG is removed with DispB before the addition of ficolin-α, then APP5a and APP5b are similarly able to bind ficolin to their cell wall components.

Although several strains of APP are reported to produce PNAG- rich biofilms (Izano et al., 2007; Kropec et al., 2005; Labrie et al., 2010), it was essential to confirm the production of PNAG in a given biofilm phenotype because many PNAG-deficient bacterial strains also produce biofilm (O'Gara, 2007; Rohde et al., 2007). We demonstrated the abundance of extracellular GlcNAc residues, most likely in the form of PNAG, in APP5a biofilm using CLSM with a fluorescent probe, WGA/AF-555. Further, DispB which specifically hydrolyses the β-1,6 linkage of PNAG, completely removed the

biofilm from surface attachment without affecting the growth of bacteria, suggesting the presence of PNAG in biofilm as well as its possible role in surface attachment of these strains.

Although ficolin-α bound to both APP5a biofilm and planktonic cultures in our experiments, biofilm-specific binding was addressed by the EPS-binding assay, where purified porcine ficolin-α bound to extracellular GlcNAc residues after removal of bacteria. This study is the first to demonstrate GlcNAc-dependent binding of biotinylated porcine ficolin-α to bacterial biofilm by direct observation using CLSM. Biotinylated porcine ficolin-α binding to biofilm was not observed in presence of 100 mM GlcNAc, which suggests preferential binding of ficolin-α via a GlcNAc-dependent mechanism (Figure 2.2 G, H, I).

The antimicrobial functions of porcine ficolin-α have not been determined and the functional significance of biofilm binding is largely unknown, but the recognition of N-acetylated sugars and participation in innate immunity is an expected property based on other known functions of human MBL and ficolin (Matsushita, 2010; Thiel and Gadjeva, 2009). Biofilm infections are highly resistant to host immune responses. This resistance is thought to be primarily attributable to physicochemical properties of the biofilm matrix, which prevents penetration of leukocytes and their end products, such as free oxygen radicals and antibacterial peptides (Matz, 2007). By glueing individual cells together, EPS within the biofilm supports the formation of microcolonies, which escape phagocytosis because of their large size (Matz, 2007). *Staphylococcus epidermidis*

biofilm-producing infection has been shown to resist host immune response by impairing complement activation (Kristian et al., 2008). Further, reduced nutrition availability and decreased growth rate of biofilm bacteria also accounts for antimicrobial resistance (Roberts and Stewart, 2004). The biofilm resistance accounts for impaired clearance of microorganisms, persistent infections in the long term and collateral tissue damage by host immune response (Alhede et al., 2009; Jensen et al., 2007b; Jensen et al., 2010; Jensen et al., 2004; Van Gennip et al., 2009).

This study demonstrated that porcine plasma ficolin-α binds to APP5a biofilm as well as planktonic bacteria in a GlcNAc-dependent manner. The presence of GlcNAc inhibited ficolin binding to biofilm. PNAG in biofilm matrix may act as a binding target for collagenous lectins, such as ficolin and MBL. This is the first evidence of ficolin binding to biofilm produced by APP5a. While the binding significance is still unknown, our results indirectly suggest that ficolin may have beneficial role by preventing the early stages of biofilm formation or delaying the onset of biofilm-associated infections. However, collateral tissue damage due to uncontrolled complement activation to mature biofilm infections may also be possible. Further research is required to clarify the potential role of ficolin in biofilm infections.

Chapter 3. Binding of porcine plasma mannose-binding lectin A to biofilm cultures of *Actinobacillus pleuropneumoniae*

Abstract

Mannose-binding lectin (MBL) plays an important role in innate immunity by recognizing specific carbohydrate moieties on the surface of wide range of microorganisms. Interactions with bacterial biofilm have not been described. This study investigated binding of porcine plasma MBL to *Actinobacillus pleuropneumoniae* serotype 5a (APP5a) biofilms and planktonic cells. N-acetylglucosamine (GlcNAc) in APP5a biofilm was stained with wheat germ agglutinin (WGA) conjugated with Alexa Fluor-555 and identified by confocal laser scanning microscopy (CLSM). MBL-A bound to biofilm and planktonic cultures of APP5a was eluted with a mixed sugar solution containing GlcNAc, galactose, mannose and glucose, and characterized by reducing SDS-PAG and by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) analysis. MBL-A was found to bind to bacterial biofilm in a carbohydrate-dependent manner, suggesting a potential role in some biofilm-positive bacterial infections. Polysaccharides in the biofilm matrix is likely an important binding target for MBL-A.

1. Introduction

Mannose-binding lectin (MBL) and ficolin are similar complement-activating protein lectins that bind to surface carbohydrates of a wide range of microorganisms, and participate in innate immunity processes (Fujita et al., 2004; Matsushita, 2010; Thiel and Gadjeva, 2009). These lectins are assembled as trimers at the collagen-like domains that mediate complement activation and phagocytosis. The carbohydrate recognition domain (CRD) of MBL is a C-lectin that binds a calcium ion, thereby modifying the structure in a manner that allows interaction with the 3- and 4-hydroxyl groups of various saccharides, including mannose, N-acetylglucosamine (GlcNAc), N-acetyl-mannosamine (ManNAc), fucose and glucose (Sheriff et al., 1994; Weis and Drickamer, 1994; Weis et al., 1991). MBL oligomers of 2 to 6 trimers can preferentially bind to microbial surface saccharide patterns composed of these saccharides (Dommett et al., 2006). Upon recognition of pathogen associated molecular patterns (PAMPs) on microbial surfaces, MBL initiates complement activation, with or without involvement of MBL-associated serine proteases (MASP), which may resulting in opsonization and destruction of the microorganism (Matsushita, 2010; Thiel and Gadjeva, 2009). MBL has also been shown to bind apoptotic cells and play a role in reduction of inflammatory responses (Palaniyar et al., 2004; Stuart et al., 2006).

In pigs, two MBL forms, MBL-A and –C, have been identified (Lillie et al., 2006). MBL proteins are similar in most mammals, but humans express only MBL-C because human MBL-A gene is a pseudogene (Guo et al., 1998). Although MBL forms share some

similarities in amino acid sequence, they are not identical in terms of recognition of PAMPs. Porcine MBL-A and -C both bind to yeast mannan, but only MBL-A has been shown to bind bacteria including some pathogenic isolates of *Actinobacillus pleuropneumoniae* (APP), *Actinobacillus suis* and *Haemophilus parasuis,* (Lillie et al., 2006). On the other hand, mouse MBL-C, but not MBL-A binds to *P. aeruginosa* (Phaneuf et al., 2007), further emphasizing variability in MBL isoform bacterial binding functions.

Previous reports of MBL binding to bacterial cell walls or purified components of cell walls, such as lipopolysaccharide have been done with bacteria grown in planktonic cultures. Many bacterial species form biofilms *in vivo* as a form of protection from hostile environments, such as the host immune response or antibiotics. APP is an obligate Gram-negative bacterium that causes necrotizing fibrinous pleuropneumonia in swine. Most field isolates of APP form biofilms, and this is thought to contribute to the pathogenicity of the organism (Izano et al., 2007). The present study investigated the binding of porcine plasma MBL to *A. pleuropneumoniae* serotype 5a (APP5a) cultured under biofilm and non-biofilm conditions.

2. Material and Methods

Planktonic and biofilm cultures of APP5a were grown and PNAG in biofilm was characterized as described in chapter 2. Binding of porcine MBL to APP5a biofilm and planktonic cultures was detected after incubating with pooled ficolin-depleted pig plasma. Ficolin-depleted pig plasma from citrated blood was prepared as described (Brooks et al, 2003). Previously frozen (–70°C) plasma was thawed to 4 ºC and dialyzed in 10 volumes of TBS-Ca^{2+} buffer (50 mM Tris, 150 mM NaCl and 10 mM $CaCl_2$) using regenerated cellulose dialysis tubing (12-14 kDa MWCO) (Fisher Scientific, Ottawa, Canada) at 4 ˚C with gentle stirring for 24 h.

Tissue culture flasks (75 cm^2) (Corning, Corning, NY, USA) were inoculated with APP5a overnight cultures (1/100) diluted in 30 mL of BHI-N and incubated for 72 h at 37˚C. Flasks containing uninoculated BHI-N and BHI-N containing 20 µg/mL DispB were used as negative controls and the media from these flasks were discarded after 24 h and replaced with fresh BHI-N. After 72 h, the broth was discarded, adherent biofilm was gently washed twice with TBS-Ca^{2+} buffer (25 mM Tris, 145 mM NaCl, 2.5 mM $CaCl_2$ and pH7.4) and incubated with dialyzed ficolin-depleted plasma (10 mL) on ice with gentle constant rotation for 4 hr. Unbound plasma was collected and biofilm was gently washed with TBS-Ca^{2+} buffer five times of 5 min each with gentle rotation on ice. Final wash fractions were collected and carbohydrate-dependent bound plasma proteins were eluted by incubating in 10 mL of sugar mix solution (100 mM GlcNAc, 100 mM galactose, 100 mM ManNAc and 100 mM glucose in TBS-Ca^{2+}) for 1 h at 4 ºC. Proteins that bound in a calcium-dependent manner were then stripped with 50 mM

ethylenediaminetetraacetic acid (EDTA) by incubating at room temperature for 12 min. The final wash, sugar mix elution, and EDTA elution fractions were concentrated to equal volume (500 µL) (Amicon concentrators-10 kDa cutoff, Millipore Corporation, Bedford, MA, USA) and characterized by one-dimensional (1-D) SDS-polyacrylamide gel (PAG) and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) analysis.

Previous studies have shown that MBL-A binds to strains of APP1 and 5a, *A. suis* and *H. parasuis* grown as planktonic cultures (Lillie et al., 2006). To determine if MBL can bind to planktonic bacteria (APP5a), we performed the following assay. A single colony was transferred into BHI-N broth supplemented with 20 µg/mL DispB and incubated at 37 ºC overnight with agitation. Bacteria (>1 x 10^8 CFU/mL) were harvested by centrifugation (10,000 x g 10 min at 4 ˚C), washed once in PBS pH 7.4, fixed in 10% formaldehyde for 1 h, washed again three times in PBS pH 7.4 and stored at -20 ˚C for bacterial binding assays.

The MBL-A binding assays were conducted as described previously (Lillie et al., 2006). Briefly, formalin-fixed bacteria (APP5a) were thawed and washed with PBS pH 7.4 and incubated with dialyzed ficolin-depleted plasma for 4 h at 4°C with slow constant rotation. Unbound ficolin-depleted plasma in the supernatant was collected following centrifugation at 4000 x g for 15 min at 4 ºC and then the bacterial pellet was washed 10 times in PBS pH 7.4 at 16, 000 x g for 5 min each at 4 ºC. The bacterial pellet was incubated with a sugar mix solution for 1 h at 4 ºC with slow constant rotation. Bacteria

were pelleted by centrifugation at 16,000 x g for 5 min and sugar mix eluted fractions were collected.

Sugar mix and EDTA eluted proteins from biofilm and planktonic bacteria binding assays were characterized by reducing SDS-PAG using the Bio-Rad modular Mini-Protean II system (Bio-Rad Laboratories, Hercules, CA, USA) with pre-stained molecular weight markers (range 6 - 202 kDa) (Bio-Rad). Electrophoresis was performed at 120 V and resolved protein fractions were visualized with Bio-Safe Coomassie Stain (Bio Rad Laboratories). Several distinct protein bands from the sugar mix and EDTA eluate were excised with sterile blades and stored in 1% acetic acid at -20 ˚C. These and the original eluates (~20 µg of protein) were analysed by trypsin digestion and LC-MS/MS (Advanced Protein Technology Center, Hospital for Sick Children, Toronto, ON, Canada). MS/MS-based peptide and protein identifications were done using Scaffold Software version 3.1.2 (Proteome Software Inc., Portland, OR, USA).

3. Results and Discussion

APP5a (STF91-1084), a field isolate, produced abundant biofilm formation at the liquid-air interface of glass tubes, on the bottom of 96-well microtiter plates, and on the surfaces of Petri dishes and tissue culture flasks that could be visualized when stained with 0.1% crystal violet (Figure 2.1 A, B). The biofilm matrix was strongly stained with WGA/AF-555 (Figure 2.2 A, C), indicating abundance of extracellular GlcNAc residues, most likely in the form of PNAG. Similarly, a recent study demonstrated PNAG-rich biofilm formation by strains of APP serotype 1 (S4074) and APP5b (L20) using CLSM with a fluorescent probe, WGA-Oregon Green, to PNAG (Labrie et al., 2010). The chemical structure of PNAG of APP biofilm is almost identical to biofilm matrix polysaccharides produced by *Escherichia coli*, *Staphylococcus aureus* and *Staphylococcus epidermidis* (Izano et al., 2007) and in diverse bacterial species PNAG has been shown to be responsible for biofilm cohesion (Cramton et al., 1999; Drace and Darby, 2008; Itoh et al., 2005; Kaplan et al., 2004; Mack et al., 1994). This suggests that PNAG within biofilm produced by a wide variety of bacteria may act as a binding target for collagenous lectins such as ficolins and MBLs that bind GlcNAc. Biofilm cultures incubated with 20 µg/mL DspB demonstrated complete biofilm disruption without affecting the growth of bacteria, indicating the presence of PNAG as well as its critical role in biofilm formation. These results are consistent with a previous study showing that DspB specifically hydrolyses glycosidic linkages of poly-ß-1, 6-N-acetylglucosamine of biofilm without affecting the growth of bacteria (Izano et al. 2007).

The mixed saccharides eluted several proteins from APP5a biofilm incubated with ficolin-depleted pig plasma (Figure 3.3). These included a reduced ~32 kDa protein consistent with MBL, which has been previously shown to bind to field isolates of APP5a and 1, *Actinobacillus suis* and *Haemophilus parasuis* (Lillie et al., 2006). These results were reproducible in two independently conducted experiments. The ~32 kDa band was digested with trypsin, characterized by LC-MS/MS analysis, and MBL-A was found to be the highest ranked protein (24 identified peptides). The sugar mix elution also contained four other prominent bands at ~ 85 kDa, ~70 kDa, 50 kDa and 25 kDa, which were subsequently identified by trypsin LC/MS as heavy chain of IgM, albumin, immunoglobulin gamma chain and immunoglobulin lambda chain, respectively. The EDTA elution followed by sugar mix elution did not show prominent MBL-A band on reducing SDS-PAG, however, MBL-A protein was found in EDTA elution fraction as detected by LC-MS/MS analysis. MBL-A was not detected in plasma ficolin binding studies (Chapter 2). This could be due to the low calcium concentration used in these binding assays. Alternatively, ficolin and MBL may compete for similar saccharides in biofilm.

Even though MBL-A has been shown to bind capsular surface structures of APP5a, binding observed on an adherent biofilm produced by APP5a was most likely biofilm polysaccharide-specific, because bacterial surface ligands within the biofilm are likely masked by EPS (Matz, 2007). One of the potential mechanisms of biofilm bacteria protection is via embedding of bacteria in the polymeric matrix, which inhibits binding of phagocyte-receptor, antibody or complement on bacterial capsular surface (Matz, 2007).

Previous studies showed that porcine MBL-A binds to some strains of APP5a and 1 and did not bind to some strains of APP 1 and 7 planktonic cultures(Lillie et al., 2006), but the reasons for differences in MBL-A binding were not established. The current study followed similar binding protocols and initially could not detect MBL-binding to a field strain of APP5a, (STF91-1084), grown as planktonic cultures (Figure 3.4 A). However, when bacteria were grown in planktonic cultures in presence of 20 µg/mL DispB, MBL-A binding to bacteria was detected (Figure 3.4 B). APP5a planktonic cultures produce a sticky matrix that causes it to clump, but clumping of APP5a cells were not seen when DspB (20 µg/mL) was included in the cultures. These observations suggest that some PNAG is produced in planktonic cultures of APP5a and that biofilm materials may have interfered with MBL-A binding by masking surface ligands exposed by DispB treatment. It is also possible that MBL bound to loosely associated surface PNAG which was lost during repeated washing of broth cultured organisms.

Pigs have two forms of MBL, MBL-A and -C, which are the products of the *MBL1* and *MBL2* genes, respectively. The present study identified only MBL-A but not MBL-C as bacterial or biofilm-binding protein. This finding is consistent with a previous report (Lillie et al. 2006) that porcine MBL-A binds to strains of APP1 and 5a, *A. suis* and *H. parasuis* planktonic bacteria. The failure to detect MBL-C in the present and previous studies may suggests that MBL-C has different binding targets or its binding was not detected because of low expression levels in some pigs associated with polymorphisms in the *MBL2* promoter (Lillie et al., 2007).

The functional significance of MBL-A binding to biofilm cultures is largely unknown, but recognition of PAMPs and participation in innate immunity is an expected property of MBLs based on known functions of human MBL (Neth et al., 2000). Since MBLs have binding targets on bacterial capsular surfaces as well as biofilm, it is assumed that MBL interaction during the early stage of biofilm colonization may stimulate the complement pathway and possibly play a role in opsonization and dispersal of biofilm bacteria. No other reports on biofilm binding functions of MBL have been published to date. Indirect support for our findings comes from a recent meta-analysis, which indicates that MBL deficiency in humans is associated with earlier acquisition of *Pseudomonas aeruginosa* infection (Chalmers et al., 2011), a classical biofilm-producing bacterial infection in humans. This result indirectly suggests a possible beneficial role of collagenous lectins in preventing early onset of biofilm infections or colonization of biofilm-producing bacteria.

Biofilm infections are highly resistant to host immune response, which could be primarily attributable to the basic physicochemical properties of biofilm matrix, which prevents penetration of leukocytes, their products, such as free oxygen radicals, and antibacterial peptides. Additionally, by gluing individual cells together, extracellular polymeric substance (EPS) forms microcolonies, which escape phagocytosis since they are far bigger than individual bacteria (Matz, 2007). *S. epidermidis* biofilm infection was shown to resist host immune response by impairing complement system (Kristian et al., 2008). Further, reduced nutrition availability and decreased growth rate of biofilm bacteria also

accounts for antimicrobial resistance (Roberts and Stewart, 2004). The biofilm resistance accounts for impaired clearance of microorganisms, persistent infections in the long term and collateral tissue damage by host immune response (Alhede et al., 2009; Jensen et al., 2007b; Jensen et al., 2010; Jensen et al., 2004; Van Gennip et al., 2009). On the other hand, it is possible that collagenous lectins that bind to the biofilm colonies activate complement and provoke a greater inflammatory response that might be harmful in some septic diseases. Further research is required to demonstrate circumstances in which MBL binds bacterial colonies *in vivo*, and how this is related to the pathogenesis of disease and immune response.

This study demonstrated only one of the two forms of porcine MBL, MBL-A, binds to APP5a biofilm and planktonic bacteria in a carbohydrate-dependent manner. However, in these studies, MBL-A eluted with various other proteins from plasma from which ficolin had been removed. Thus it is not yet known if MBL-A binds directly to PNAG within the biofilm matrix, or indirectly via other proteins that bound in these studies. However, it is proposed that PNAG may act as a binding target for both ficolin and MBL. On the other hand, the observed binding after Dispersin B treatment to planktonic cultures suggests that PNAG is not required for binding of MBL-A. However, it is possible that repeated washing of suspension cultures might remove some lectins bound to loosely attached polysaccharides, and expose attached saccharides in LPS that also serve as ligands for MBL. To our knowledge, this is the first report of MBL binding to bacterial biofilm cultures in any species but the significance of MBL binding to biofilm is

still unknown. Thus, further research with purified MBLs similar to what was done for ficolin A are required to clarify the potential role of MBL in biofilm infections.

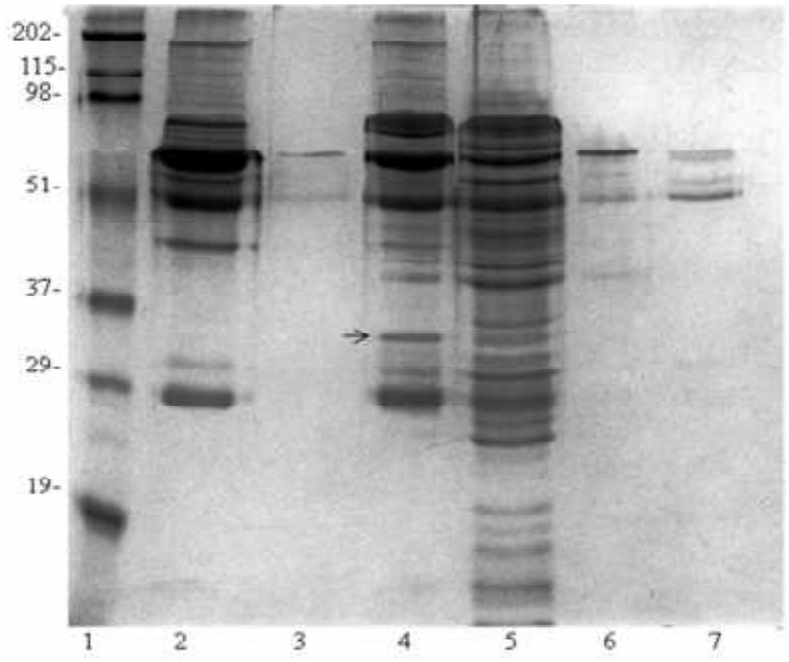

Figure 3.1. Coomassie blue stained reducing SDS-PAG illustrating MBL-A eluted with a monosaccharide cocktail from APP5a biofilm cultures incubated with ficolin-depleted pig plasma. A single distinct band at ~32 kDa (arrow) was excised and identified as MBL-A by LC-MS/MS analysis. Lanes 1: Marker. 2: Biofilm unbound fraction of ficolin-depleted plasma that contained no visible MBL band. 3: Final wash containing no unbound plasma fractions. 4: Sugar cocktail eluate containing biofilm-bound MBL (arrow) and also plasma immunoglobulins and various bacterial proteins. 5: EDTA eluate containing several plasma and bacterial proteins, and less amount of MBL-A. 6: Negative control, sugar cocktail eluate from biofilm-removed flasks containing no non-specific bound MBL. 7: Negative control, sugar cocktail eluate from uninoculated dish containing no nonspecifically bound MBL. These results were confirmed by two independent experiments.

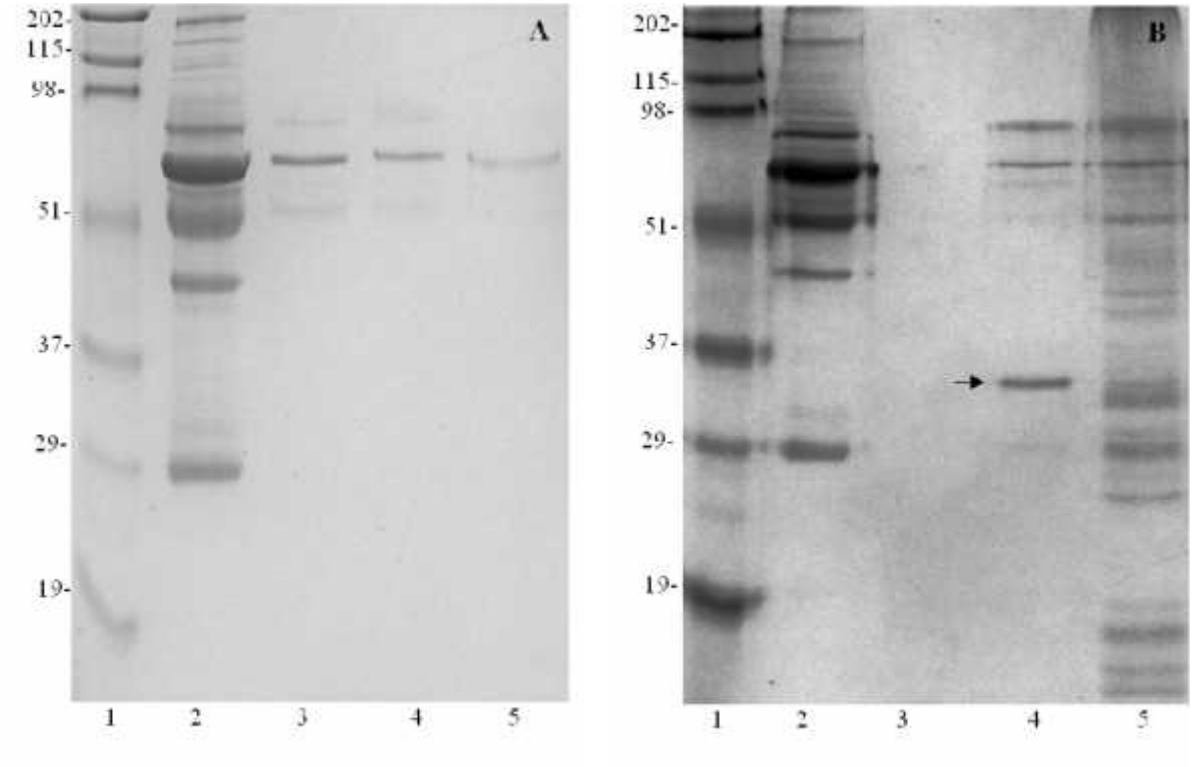

Figure 3.2. Coomassie blue stained reducing SDS-PAG illustrating binding of porcine plasma MBL-A to APP5a planktonic bacteria in presence of 20 µg/mL DispB. Ficolin-depleted plasma was incubated with APP5a planktonic cultures with or without DispB. (A) MBL binding was not observed in planktonic cultures without DispB. Lanes 1: Marker. 2: Bacteria unbound fraction of ficolin-depleted plasma that contained no visible MBL band. 3: Final wash fraction containing some albumin. 4: Sugar mix eluate containing no visible MBL protein band. 5: EDTA eluate containing no MBL protein band.
(B) MBL-A was eluted with mixture of sugars from APP5a planktonic bacteria treated with DispB. Lanes 1: Marker. 2: Bacteria unbound fraction of ficolin-depleted plasma that contained no visible MBL band. 3: Final wash containing no unbound plasma proteins. 4: Sugar mix eluate containing MBL protein at ~32 kDa (arrow). 5: EDTA eluate containing many plasma and bacterial proteins, but less protein in the region MBL-A. These results were confirmed by two independent experiments.

Chapter 4. General Discussion

The objectives of this work were to determine if porcine plasma ficolin-α binds to bacterial biofilm and also to identify other biofilm-binding lectin such as MBL in porcine plasma. Biofilm was of interest because this growth form is increasingly considered to be relevant to how many pathogenic bacteria survive in tissues. Ficolin and MBL were of primary interest because of their well known roles in innate immunity (Thiel and Gadjeva, 2009), and because, in previous studies in pigs and mice, ficolins (Brooks et al., 2003b; Keirstead et al., 2008) and MBLs (Lillie et al., 2006; Phaneuf et al., 2007) were found to be the most abundant bacterial binding plasma proteins other than immunoglobulins. In these earlier studies, binding of plasma proteins to planktonic bacteria was demonstrated, but it was not known if these lectins also interact with bacteria grown as biofilm or cohesive colonies *in vivo*. No previous reports of interactions between ficolins or MBLs of any species with bacterial biofilm cultures were found in the available literature, so the work in this thesis is the first to demonstration that ficolin and MBL can bind to bacterial biofilms *in vitro*.

The present research demonstrated that porcine ficolin-α (Chapter 2) and MBL-A (Chapter 3) bind to APP5a biofilm in a carbohydrate-dependent manner, similar to the manner by which they bind to some strains of APP planktonic bacteria (Brooks et al., 2003b; Keirstead et al., 2008); (Lillie et al., 2006; Phaneuf et al., 2007). Further, these studies demonstrated that biofilm cultures of APP5a have an abundance of extracellular GlcNAc residues most likely in the form of PNAG, which consists of linear chains of

GlcNAc residues in β-1,6 linkage (Cerca et al., 2006; Izano et al., 2007; Kropec et al., 2005). This interpretation was based on dense labeling of APP5a biofilm with WGA conjugated with AF-555, which indicates high GlcNAc content, and dispersion of biofilm by DispB, which degrades the GlcNAc β-1,6 linkage, consistent with other studies of biofilms of APP (Izano et al., 2007). Although it has been previously reported that several strains of APP produce PNAG-rich biofilm (Izano et al., 2007; Kropec et al., 2005; Labrie et al., 2010), the amounts produced vary with strain and culture conditions. Abundance of PNAG in biofilm together with the general GlcNAc recognition properties of both ficolin and MBL supports the hypothesis that biofilm PNAG is a binding target for some forms of porcine ficolin and MBL, but the functional significance of this is still unknown. Possibly, these lectins have some role in preventing or disrupting biofilm. On the other hand, biofilm matrix enables bacteria to withstand various host defence factors so it is possible that lectin binding might occur where it does not harm the organisms within the colony.

It was apparent from this study that ficolin bound to the extracellular matrix of APP5a in a GlcNAc-dependent manner. We were able to prepare a form of biotinylated ficolin that retained the ability to bind to APP5b in a GlcNAc-dependent manner, and then demonstrated using NeutrAvidin-TR and CLSM that biotinylated porcine ficolin-α bound to bacterial biofilm. Fluorescence staining specific to ficolin binding was diffusely distributed, suggesting strong binding of ficolin-α to biofilm matrix. In the presence of 100 mM GlcNAc, biotinylated ficolin did not bind to biofilm, indicating that ficolin binding likely interacts with these residues in the matrix.

To identify other porcine plasma lectins that bind to biofilm (Chapter 3), ficolin-depleted plasma was dialyzed and incubated with biofilm in the presence of Ca^{2+}. Because there are various potential lectin targets in the biofilm matrix, we used a cocktail of various monosaccharides to elute, similar to what has been successfully used in previous studies (Lillie et al., 2006). The monosaccharide cocktail eluate showed a distinct band under reducing conditions at ~32 kDa, which was consistent with previous identification of the bacterial binding protein, MBL-A, in pig plasma (Lillie et al., 2006). MBL-A was not detected in plasma ficolin binding studies (Chapter 2). This could be due to the low calcium concentration used in these binding assays. Alternatively, ficolin and MBL may compete for similar saccharides in biofilm.

Pigs have two forms of MBL, MBL-A and -C, which are products of genes *MBL1* and *MBL2* genes, respectively. However, some pigs produce low amounts of MBL-C due to a promoter polymorphisms (Lillie et al., 2007), and plasma levels of MBL-A are genetically variable (Juul-Madsen et al., 2011; Juul-Madsen et al., 2006). The present study identified only MBL-A, as a bacterial- or biofilm-binding protein. This finding was consistent with the previous report of Lillie et al. (2006), who first demonstrated that porcine MBL-A but not MBL-C binds to strains of APP5a and 1, *A. suis* and *H. parasuis* grown in planktonic cultures. The failure to detect MBL-C in the present and previous studies suggests that MBL-C has different binding targets than MBL-A or binding was not detected because of very low concentration in plasma of some pigs. It is still largely unknown how the presence of MBL-A contributes to innate immunity because humans do not express MBL-A. Thus all the known MBL-binding organisms demonstrated with

human MBL actually bind MBL-C, so our demonstration of MBL-A, but not MBL-C binding to biofilm cannot yet be extrapolated into an expected function for humans.

Previous studies have shown that both porcine ficolin-α (Brooks et al., 2003a) and MBL-A (Lillie et al., 2006) bind to only a few strains of APP. The present study also demonstrated binding of both porcine ficolin-α and MBL-A to the planktonic cultures of APP5a bacteria when cultured in presence of DispB, which degrades PNAG. However, our bacterial binding studies did not detect either ficolin or MBL binding to planktonic cultures of APP5a, under similar experimental conditions to as those described previously (Brooks et al., 2003a; Lillie et al., 2006). These findings likely relate to differences in the methods used to assess binding to organisms in planktonic state or attached as biofilms. The presence of some sticky matrix substances was apparent in APP5a planktonic cultures because they tended to clump. However, when APP5a bacteria were grown in planktonic cultures in the presence of DispB which removes biofilm, APP5a in planktonic cultures was then able to bind to ficolin-α and MBL-A. One possibility is that biofilm on the surfaces of APP5a grown in planktonic cultures may interfere in binding assays by masking surface pathogen-associated molecular patterns (Matz, 2007). Another possibility is that DispB might increase ficolin and MBL binding by altering the structure of surface polysaccharides. However, the most likely explanation is the removal of loosely associated biofilm matrix material including PNAG from the outer surfaces of APP5a during the many washing needed to recover suspended bacteria. Both APP5a and APP5b have capsular and cell wall oligosaccharides that contain GlcNAc (Altman et al.,

1992) that could be exposed after removal of the unattached glycans during the repeated washing steps.

Other conditions that affect binding of these collagenous lectins are calcium concentration and pH. MBL binding is calcium-dependent and the protein binds to a wide range of sugar moieties including mannose, GlcNAc, N-acetyl-mannosamine (ManNAc), fucose, and glucose (Sheriff et al., 1994; Weis and Drickamer, 1994; Weis et al., 1991). Ficolin also generally recognizes N-acetylated glycan patterns and initially it was thought that calcium was required for ficolin binding because of the presence of a calcium-binding motif in the fibrinogen-like domain, as well as molecular similarity to other calcium-dependent collagenous lectins (Endo et al., 2006; Gokudan et al., 1999; Matsushita et al., 1996). However, many studies, including the present, have since demonstrated calcium-independent binding of ficolins to acetylated sugars and bacteria (Brooks et al., 2003b; Harumiya et al., 1995; Le et al., 1998; Le et al., 1997; Ohashi and Erickson, 1997). A recent study by Zhang and co-workers showed that local inflammatory conditions inducing mild acidosis and reduced calcium levels enhance the interaction between L-ficolin and CRP, inducing complement-mediated activity against *P. aeruginosa*. This also suggested that L-ficolin binding may be increased at lower pH (pH 6.5, calcium 2 mM) (Zhang et al., 2009). It is unclear how this occurs because X-ray crystallography has not revealed an acidic pH-associated structure change of the binding pockets of L-ficolin or H-ficolin (Garlatti et al., 2009). In the present study, when we carried ficolin-binding assays at neutral pH with reduced or no calcium, no significant differences in ficolin binding were revealed.

The antimicrobial functions of porcine ficolin-α and MBL-A have not been determined. The functional significance of biofilm binding is largely unknown, but recognition of N-acetylated sugars and participation in innate immunity is an expected property of collagenous lectins based on other known functions of human MBL and ficolin (Matsushita, 2010; Neth et al., 2000; Thiel and Gadjeva, 2009). Currently there are no direct evidences to address functional significance of ficolin or MBL binding to bacterial biofilm. Indirect support is present in a recent meta-analysis study, which indicated that MBL insufficiency in humans was associated with earlier acquisition of *P. aeruginosa* infection, a classical biofilm infection of humans (Chalmers et al., 2011). On the other hand, accumulating data indicates that biofilm infections are highly resistant to the host immune response. This could be attributable to physicochemical properties of biofilm matrix that prevents penetration of leukocytes and their products, such as free oxygen radicals and antibacterial peptides, into the biofilm (Matz, 2007). By glueing an individual cells together, EPS forms microcolonies, which escape phagocytosis since they are far bigger than individual bacteria (Matz, 2007). *S. epidermidis* biofilm infections have been shown to resist host immune response by impairing complement activity (Kristian et al., 2008). The biofilm resistance accounts for impaired clearance of microorganisms, persistent infections in the long term and collateral tissue damage by host immune response (Alhede et al., 2009; Jensen et al., 2007b; Jensen et al., 2010; Jensen et al., 2004; Van Gennip et al., 2009). Considering both aspects of complement activation, at this stage, it is difficult to attribute the significance to biofilm binding by porcine MBL-A or ficolin. Further research is required to clarify how collagenous lectins interfere with biofilm colonization and dispersal.

The identification of ficolin and mannose-binding lectin (MBL) in tissue lesions of biofilm infections should be examined in future research. It will also be interesting to investigate what saccharides are present on bacteria in lesions since saccharides composition may vary with the growth conditions. Further, ficolin or MBL interactions with purified PNAG can be directly investigated by coupling PNAG onto the amine-activated Toyopearl matrix that does not bind ficolin (Brooks et al., 2003b). Similarly, ficolin or MBL binding experiments can be conducted to identify other lectins targets in biofilm by coupling extracted EPS onto the amine-activated Toyopearl matrix.

The present study showed that ficolin and MBL bind to biofilm in a carbohydrate-dependent manner. While such binding significance is still unknown, these interactions can be viewed as either threat or an opportunity for biofilm infections. Therefore, it is also worth investigating opsonization and complement activation roles of porcine plasma ficolin and MBL.

Summary and Conclusions

1. Under some culture conditions *Actinobacillus pleuropneumoniae* serotype 5a strain, STF 91-1084, (APP5a) can produce abundant biofilm.

2. APP5a biofilms contain large amounts of N-acetylglucosamine (GlcNAc) most likely in the form of poly-N-acetylglucosamine (PNAG) that is degraded by dispersin B (DispB), an enzyme that disrupts the polysaccharide linkage of poly β(1-6)-GlcNAc.

3. Porcine plasma ficolin-α, both purified and in plasma, binds to APP5a biofilms in a GlcNAc-dependent manner.

4. Sulfo-NHS-LC biotin that links to lysine and terminal amino residues can be used to label purified porcine ficolin-α in a way that it preserves its ability to bind to APP bacteria in a GlcNAc-dependent manner.

5. Porcine MBL-A, but not MBL-C, in plasma binds to APP5a biofilm and planktonic cultures in a carbohydrate-dependent manner.

6. Planktonic cultures of APP5a become less sticky after incubation with DispB, suggesting that some biofilm matrix is produced in planktonic cultures. When this

matrix is removed, APP5a resembles APP5b in its ability to bind ficolin and MBL, suggesting that biofilm matrix covers cell-wall targets for these lectins.

7. While porcine ficolin-α and MBL-A can bind to biofilms or planktonic bacteria, the functional significance of these interactions is still unknown. However, collateral tissue damage due to excessive complement activation against mature biofilm infections might also occur.

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Appendix A: Supplemental LC-MS/MS data analysis

Table A-1: LC-MS/MS analysis of sugar mix elute from biofilm incubated with porcine ficolin-depleted plasma.

	Identified proteins	Accession Number	Molecular weight	A	B
1	albumin [Sus scrofa]	gi|833798	69 kDa	24	1
2	ch4 and secrete domains of swine IgM [Sus scrofa]	gi|1236646	49 kDa	13	7
3	mannose-binding protein A [Sus scrofa]	gi|55741579	26 kDa	24	37
4	immunoglobulin lambda-chain [Sus scrofa]	gi|164511	19 kDa	4	2
5	immunoglobulin gamma-chain [Sus scrofa]	gi|164507	51 kDa	5	1
6	RecName: Full=Serotransferrin	gi|136192	77 kDa	4	0
7	IgG heavy chain [Sus scrofa]	gi|47523192	52 kDa	8	0
8	PREDICTED: LOW QUALITY PROTEIN: alpha-2-macroglobulin	gi|311256213	164 kDa	6	0
9	apolipoprotein A-I [Sus scrofa]	gi|164359	30 kDa	0	3
10	PREDICTED: keratin, type I cytoskeletal 10-like [Sus scrofa]	gi|311267330	63 kDa	9	2
11	apolipoprotein A-I [Sus scrofa]	gi|147780441	192 kDa	13	2
12	PREDICTED: keratin, type I cytoskeletal 10-like [Sus scrofa]	gi|311272367	182 kDa	4	0
13	PREDICTED: keratin, type II cytoskeletal 6A-like [Sus scrofa]	gi|311255389	64 kDa	4	4
14	PREDICTED: keratin, type II cytoskeletal 1b-like [Sus scrofa]	gi|194037336	64 kDa	3	1
15	immunoglobulin heavy chain variable region [Sus scrofa]	gi|54888402	13 kDa	3	0
16	immunoglobulin heavy chain variable region [Sus scrofa]	gi|119662933	13 kDa	3	0
17	inter-alpha-trypsin inhibitor heavy chain H4 [Sus scrofa]	gi|48374067	102 kDa	4	0
18	immunoglobulin heavy chain variable region [Sus scrofa]	gi|54888464	13 kDa	3	0

A: Sugar mix eluate from APP5a biofilm incubated with porcine ficolin-depleted plasma contained ~ 32 kDa protein band, in reducing SDS-PAG, was excised and analyzed by LC/MS-MS.

B: Sugar mix eluate from APP5a planktonic cultures with 20 µg/mL DispB incubated with porcine ficolin-depleted plasma contained ~ 32 kDa protein band, in reducing SDS-PAG, was excised and analyzed by LC/MS-MS