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Evaluation of engineered bacterial transferrin binding proteins as vaccine antigens

by

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## Abstract

Many important Gram-negative pathogens that cause disease in humans and food production animals have specifically evolved to colonize and reside in the mucosal surfaces of their hosts. A key enabling factor in the survival and the pathogenesis of these bacteria is the bacterial transferrin receptor, which is used by these organisms to acquire iron, an essential micronutrient, from their host environment.

The bacterial transferrin receptor is composed of the transferrin binding protein A and B (TbpA and TbpB), which bind exclusively to host protein transferrin and thus, limits the niche for these bacteria exclusively to their host species. TbpA is an outer, transmembrane TonB-dependent transporter, which enables the internalization of iron into the periplasm. TbpB is a surface exposed lipoprotein anchored to the bacterial membrane and aids in capturing iron loaded transferrin in the extracellular milieu. The bacterial transferrin receptor has been considered as a prospective vaccine antigen against these pathogens hence the objective of this thesis is to evaluate these proteins as an antigen.

Previous studies have shown that when a TbpB mutant from porcine pathogen *Glaesserella parasuis* engineered to be defective in binding to porcine transferrin (Y167A TbpB) was used as a vaccine antigen, superior protection was observed in a swine model compared to the wildtype TbpB control. For the first aim, findings from this thesis demonstrated that a murine model of sepsis can be used to evaluate different TbpB mutants, providing a tool to screen different mutants prior to evaluating in a swine model.

For the second aim, new TbpB mutants defective in binding to porcine transferrin were generated and evaluated as vaccine antigens. For the third aim, the cross-reactivity of immunizations with either single or multiple TbpB antigens is explored. A combined vaccine formulation with two antigens elicited a broadly cross-reactive response raising the possibility that a final TbpB based vaccine limited to these two antigens may be sufficient in providing protection against the entire repertoire of TbpB variants. Finally, a method for generating chimeric/hybrid antigens using TbpB based scaffolds to display extracellular loops from membrane proteins like TbpA in order to take advantage of

TbpA as a vaccine antigen is discussed. Overall, the findings of this thesis adds to our understanding of the bacterial transferrin receptor as a vaccine antigen and brings us closer to the final, optimal vaccine composition for eliciting efficacious and broadly cross-protective coverage.

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## LIST OF SYMBOLS, ABBREVIATIONS AND NOMENCLATURE

<b>Symbol</b>	<b>Definition</b>
Å	Angstrom
a.a.	Amino acid
ABC transporter	ATP-binding cassette transporters
AmpR	Ampicillin resistance
ANOVA	Analysis of variance
Apo-Tf	Transferrin without iron bound
AV	AddaVax
AsH57	<i>Actinobacillus suis</i> strain H57
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
CFU	Colony forming units
C-lobe	C-terminal lobe
ddH <sub>2</sub> O	Double distilled water
DNase I	Deoxyribonuclease I
DPBS	Dulbecco's phosphate buffered saline
ELISA	Enzyme linked immunosorbent assay
FbpA	Ferric binding protein A
FbpB	Ferric binding protein B
FbpC	Ferric binding protein C
FHbp	Factor H binding protein
Fe <sup>2+</sup>	Ferrous iron
Fe <sup>3+</sup>	Ferric iron
FPLC	Fast-purification liquid chromatography
HBMT	Polyhistidine-biotinylation sequence-maltose binding protein-tobacco etch virus cleavage site fusion construct
HMW	High molecular weight
HRP	Horseradish peroxidase
hTf	Human transferrin
IMAC	Immobilized metal affinity chromatography
IgG	Immunoglobulin G

K <sub>aff</sub>	Affinity constant
Kb	Kilobases
K <sub>D</sub>	Dissociation constant
kDa	Kilodaltons
LAL	Limulus amoebocyte lysate
LB	Luria
LbpA	Lactoferrin binding protein A
LbpB	Lactoferrin binding protein B
LCL	Loopless C-lobe
LPS	Lipopolysaccharide
MAFFT	Multiple alignment using fast fourier transform
MBP	Maltose binding protein
MGv	Montanide Gel 01 + poly I:C
MWCO	molecular weight cutoff
N-lobe	N-terminal lobe
NAD	Nicotinamide adenine dinucleotide
Ni-NTA	Nickel-nitrilotriacetic acid
NT	Non-typeable
OD	Optical density
OD <sub>450</sub>	Optical density at 450nm
OD <sub>600</sub>	Optical density at 600nm
OMV	Outer membrane vesicle
OMP	Outer membrane protein
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline + Tween
PDB	Protein database
PPLO	Pleuropneumoniae like organisms
pTf	Porcine transferrin
rLCL	Recombinant loopless C-lobe
RT	Room temperature
RTX	Repeats in toxin

s.c.	Subcutaneous
SBA	Serum bactericidal activity
SBP	Streptavidin binding peptide
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SLAM	Surface lipoprotein assembly modulator
SLP	Surface lipoprotein
SOE-PCR	Splicing by overlap extension polymerase chain reaction
TBDT	TonB-dependent transporter
Tbp	Transferrin binding protein
TbpA	Transferrin binding protein A
TbpB	Transferrin binding protein B
TBS	Tris buffered saline
TEV	Tobacco etch virus
Tf	Transferrin
TFR1	Transferrin receptor 1
TLR	Toll-like receptor
TMB	3, 3',5,5'-tetramethylbenzidine
v/v	Volume/volume
VIDO	Vaccine and Infectious Disease Organization
WAG	Whelan and Goldman
WT	Wildtype
ZnuD	Zinc acquisition protein

## CHAPTER 1: INTRODUCTION

### 1.1 Porcine pathogens and disease pathogenesis

#### 1.1.1 *Glaesserella parasuis* and Glässer's disease

*Glaesserella parasuis*, a member of the *Pasteurellaceae* family, is a Gram-negative bacterium endemic in the global swine population (Aragon, Segalés, & Tucker, 2019). Recently renamed from *Haemophilus parasuis* (Dickerman, Bandara, & Inzana, 2020)), *G. parasuis* is considered one of the most important bacterial pathogens affecting swine health, and subsequently, the global pig industry. Although, *G. parasuis* exists as a commensal inhabitant of the swine respiratory microbiota, it can also act as an opportunistic pathogen and is the etiological agent of Glässer's disease (GD) in pigs. GD is a severe, systemic affliction commonly characterized by polyserositis, pericarditis, meningitis, arthritis, and septicemia and is the leading cause of mortality in nursery herds, though older animals like growers (weaned pigs) and sows (female pigs after first litter) can also be affected (Aragon et al., 2019).

*G. parasuis* is an early colonizer of piglets, a process posited to be mediated through contact with sows and transmitted through direct contact between infected and susceptible animals (Aragon et al., 2019). Despite detection of strains from the nasal cavities of piglets as soon as 2 days after birth (M Cerdà-Cuéllar et al., 2010), disease does not occur until they are older (4 -8 weeks of age), and only on occasion (Costa-Hurtado, Barba-Vidal, Maldonado, & Aragon, 2020). It is unclear as to why certain animals are susceptible to infection and succumb to disease, but risk factors include diminishing maternal immunity, exposure to different strains through mixing of litters, and compromised immunity caused by the presence of other pathogens and co-infections. Additionally, environmental conditions such as stress and management practices are also believed to play a role in triggering disease, however, direct correlations have not yet been established.

*G. parasuis* strains have been classified into 15 serovars (Kielstein & Rapp-Gabrielson, 1992), though the increasing prevalence of non-typable strains have been observed (Espindola et al., 2019). While these serovars have been classified as either

virulent or non-virulent (Kielstein & Rapp-Gabrielson, 1992), there is a growing consensus that serovar type does not correlate with virulence as classical non-virulent serovars are increasingly implicated in disease outbreaks (Castilla et al., 2012; Dazzi et al., 2020; Zhang et al., 2012). However, studies comparing virulent and non-virulent strains have aided in the understanding of virulence factors that play a role in the pathogenesis of GD.

Both virulent and non-virulent strains are detected in the swine upper-respiratory tract where they colonize by adhering to the mucosal layer and underlying epithelium (Bernardo Bello-Orti, Costa-Hurtado, Martinez-Moliner, Segalés, & Aragon, 2014; Rafael Frandoloso, Martínez-Martínez, Gutiérrez-Martín, & Rodríguez-Ferri, 2012). However, non-virulent strains are unable to progress into the lower respiratory tract as they are efficiently cleared by host defense mechanisms, predominantly phagocytosis by porcine alveolar macrophages (B. Bello-Orti, Costa-Hurtado, Martinez-Moliner, Segales, & Aragon, 2014).

In contrast, virulent strains are able to resist clearance by the host and persist in the lower respiratory tract (A. Olvera, Ballester, Nofrarias, Sibila, & Aragon, 2009). The main virulence factor attributed with increased resistance to phagocytosis is the expression of a capsule by strains, which is known to disrupt complement deposition (Sarma & Ward, 2011). Virulent strains also exhibit immunoglobulin (IgA) protease activity, which is essential for protection from host mucosal defenses (Mullins, Register, Bayles, & Butler, 2011) and enables the bacteria to invade endothelial cells (R. Frandoloso et al., 2013). These virulence factors are thought to aid *G. parasuis* strains invasion into the bloodstream, where survival is likely enhanced by the ability of virulent strains to resist serum complement bactericidal activity (Marta Cerdà-Cuellar & Aragon, 2008) allowing for systemic spread and results in fibrinous polyserositis (A. Olvera, Segales, & Aragon, 2007).

Control measures to prevent *G. parasuis* infections are strict husbandry practices, extensive usage of antimicrobials, and vaccination campaigns. Increases in the prevalence on antimicrobial resistance, partly due to overuse in agricultural settings, is a major

concern for both human and pig health (Costa-Hurtado et al., 2020). Thus, preventative measures like more efficacious vaccines are needed.

### *1.1.2 Actinobacillus pleuropneumoniae and porcine pleuropneumonia*

*Actinobacillus pleuropneumoniae*, a Gram-negative bacterium belonging to the *Pasteurellaceae* family, is the etiological agent of porcine pleuropneumonia (Gottschalk & Broes, 2019; Sassu et al., 2018). Porcine pleuropneumonia is a severe, contagious respiratory disease associated with significantly impaired animal welfare and high mortality, resulting in economic losses in swine herds globally ((Bosse et al., 2002; Klitgaard, Friis, Angen, & Boye, 2010). Symptoms associated with porcine pleuropneumonia are clinically presented differently depending on the form of disease occurring, ranging from the peracute form to the subclinical form.

Characterized by high mortality, the peracute form of porcine pleuropneumonia progresses rapidly, resulting in vomiting and severe respiratory distress (Bosse et al., 2002). Acute form of the disease usually results in high to intermediate mortality and is characterized by shortness of breath, coughing, and mouth breathing. Both the peracute and acute forms of disease cause lung lesions that are observed at slaughter. Chronic cases are associated with low mortality and few, less severe symptoms like intermittent coughing (Nahar, Turni, Tram, Blackall, & Atack, 2021; Sassu et al., 2018). Subclinical disease does not cause any clinical symptoms and often no lung lesions are observed at slaughter. During chronic or subclinical forms of porcine pleuropneumonia, pigs can be asymptotically infected with *A. pleuropneumoniae*, leading to the long-term presence of highly virulent strains in a herd and potentially resulting in transmission to susceptible animals (Nahar et al., 2021; Tobias et al., 2014)

Domesticated and wild pigs are the only known natural host for *A. pleuropneumoniae* (Sassu et al., 2018; Vengust, Valencak, & Bidovec, 2006) however naturally occurring disease in other hosts have been reported, although rarely (Perez Marquez et al., 2014). *A. pleuropneumoniae* colonizes the porcine upper respiratory tract (Pohl, Bertschinger, Frederiksen, & Mannheim, 1983) and has been isolated from the tonsils and nasal cavities (Chiers, Haesebrouck, van Overbeke, Charlier, & Ducatelle, 1999). Transmission to naive pigs occur via direct contact (Tobias et al., 2014) and/or by

aerosol spread from infected animals, however the latter mode was found to be ten times less efficient (Tobias et al., 2013). Translocation to the lower respiratory tract and binding to the mucosal surface is associated with disease development (Belanger, Dubreuil, Harel, Girard, & Jacques, 1990; Belanger, Rioux, Foiry, & Jacques, 1992).

*A. pleuropneumoniae* is classified into two biovars; biovar 1 strains (typical) depend on nicotinamide adenine dinucleotide (NAD or V factor) for growth while biovar 2 strains (atypical) are NAD-independent (Pohl et al., 1983). Isolates are further differentiated into serovars where variations within the capsular polysaccharide serve as the basis for typing (Bosse et al., 2018). Currently, there are 18 serovars of *A. pleuropneumoniae* although serovar 19 has been recently proposed (Stringer et al., 2021). Additionally, the existence of non-typeable strains have been also reported (Sassu et al., 2018; To et al., 2020). Although all serovars of *A. pleuropneumoniae* can cause disease, the severity of infection varies remarkably, with some serovars exhibiting high mortality, while others produce no symptoms (Frey, 1995).

The pathogenesis of porcine pleuropneumonia can be attributed to multiple virulence factors, especially those associated with adhesion and colonization, nutrient acquisition, immune clearance, and tissue damage (Bosse et al., 2002; Haesebrouck, Chiers, Van Overbeke, & Ducatelle, 1997). Severe lung tissue damage is predominantly caused by four secreted exotoxins with haemolytic and/or cytolytic activities; Apx I, II, III, and IV, belonging to the RTX (repeats in toxin) family of toxins (Linhartova et al., 2010). Expression of the toxins is variable across serovars as different strains encode different toxin profiles (Yee, Blackall, & Turni, 2018), partially explaining some of the variation in virulence observed among isolates. It has been noted that the most virulent serovars of *A. pleuropneumoniae* typically produce both the ApxI and ApxII toxins (Frey, 1995). The fourth toxin, ApxIV, which is required for full virulence and is only expressed during *in vivo* conditions, is encoded in all strains (Nahar et al., 2021; Sassu et al., 2018).

Controlling and preventing infections by *A. pleuropneumoniae* is mainly through a combination of strict husbandry practices, antibiotic treatments, and vaccines (Gottschalk & Broes, 2019). As with *G. parasuis*, the emergence of antimicrobial

resistance is an emerging concern (Michael et al., 2018) and there is a need for new preventative strategies.

### *1.1.3 A. suis and disease*

*Actinobacillus suis* is a Gram-negative, opportunistic pathogen also belonging to the *Pasteurellaceae* family. *A. suis* is considered an emerging disease of concern in North America and other countries as an important pathogen of swine herds (MacInnes et al., 2008). Compared to the other two organisms discussed in this thesis, the least is known about *A. suis* pathogenesis. *A. suis* resides asymptotically in the tonsils of the soft palate of swine (Kernaghan, Bujold, & MacInnes, 2012). Factors resulting in *A. suis* shifting from a commensal to a pathogen have yet to be elucidated however it is known that infection with the organism can result in septicaemia, especially in neonatal piglets, and sudden death (J. I. MacInnes & R. Desrosiers, 1999).

Infections can occur in pigs of all ages and are characterized by a variety of clinical conditions, including but not limited to pneumonia, arthritis, enteritis, and abortion. Genomic and transcriptomic analyses have revealed that *A. suis* has a number of putative common virulence factors including iron-regulated outer membrane proteins, capsule, and lipopolysaccharide (Bujold & MacInnes, 2015; Bujold, Shure, Liu, Kropinski, & MacInnes, 2019; Ojha, Lacouture, Gottschalk, & MacInnes, 2010; Ojha, Sirois, & MacInnes, 2005). In addition, *A. suis* has several established virulence factors in common with *A. pleuropneumoniae*, including iron-uptake mechanisms, urease, and the Apx toxins (Bahrami, Ekins, & Niven, 2003; Bujold & MacInnes, 2015; Kamp, Vermeulen, Smits, & Haagsma, 1994; Van Ostaaijen, Frey, Rosendal, & MacInnes, 1997).

## *1.2 Vaccines against G. parasuis, A. pleuropneumoniae, and A. suis and vaccine development*

### *1.2.1 The immune system*

The purpose of the immune system is to protect the host from foreign pathogenic microbes (as reviewed in (Chaplin, 2010; Owen, Punt, Stranford, Jones, & Kuby, 2013)). This highly adaptable process can defend the host from a diverse group of pathogens

ranging from intracellular viruses to extracellular parasitic worms. Thus the ability to mobilize an efficient and effective response against these pathogens require a large repertoire of recognition and elimination strategies in order to best fight off the different types of invaders. Critical to this process is the ability of the immune system to distinguish self from non-self. To successfully detect and destroy invading pathogens, vertebrates have evolved a complex and dynamic network of cells, molecules, and pathways encompassing the innate and adaptive arms of the immune system.

The innate immune system provides a non-specific, immediate response against all invading pathogens. These defense mechanisms are encoded in their mature functional forms in the germline of the host and include a variety of strategies. Physical barriers that directly prevent entry are often the first line of defense. The skin, which is composed of epithelial cell layers expressing cell-to-cell connecting tight junctions, is one such defense mechanism. The secreted mucosal layer overlaying the epithelium lining of the respiratory, gastrointestinal, and genitourinary tract, as well as the epithelial cilia that sweep away the mucosal layer contaminated with pathogens and inhaled particles are important physical barriers in the mucosal spaces of the host.

The innate system defense mechanisms also include secreted molecules and proteins such as anti-microbial peptides on the skin and lysozymes in the saliva which aid to destroy invading pathogens. Lastly, the innate immune system relies on membrane bound and cytoplasmic proteins expressed by both immune and non-immune cells that are able to recognise and bind to specific molecular patterns expressed by different groups of pathogens, known as pathogen associated molecular patterns (PAMPs), and trigger an immune response designed to eliminate that group of pathogens. A core characteristic of the innate immune response is the lack of memory such that for every repeat invasion by the same pathogen, the response initiated by the host is the same.

In contrast, the adaptive immune response is defined by its ability to manifest a highly specific response against its target, a specific epitope associated with a foreign antigen. This arm of the immune response is predominantly mediated by B- (humoral) and T- (cellular) lymphocytes. These lymphocytes express membrane bound epitope specific receptors are known as T-cell receptor (TCR) and B-cell receptor (BCR), where

each lymphocyte only expresses receptors for the same epitope. The ability of the different BCRs and TCRs on different B- and T- cells to recognize an immense number of epitopes is the product of somatic rearrangement of a limited number of germline encoded BCR/TCR genes, resulting in billions of unique combinations.

Interactions between a BCR/TCR and a specific epitope results in the clonal expansion of that particular B- and T-cell, followed by differentiation of those cells into various subsets with different functionalities. After clonal expansion, some of the B-cells differentiate into plasma cells, responsible for secreting large quantities of epitope specific antibodies, initially immunoglobulin M (IgM). The remaining B-cells form germinal centers in the lymph nodes where they undergo class switching to immunoglobulin G (IgG), which is associated with complement mediated killing and opsonophagocytosis. This is a critical process as the functionality of the antibodies is largely mediated by the class type (IgM vs IgG etc.). IgGs are responsible for complement mediated killing and opsonophagocytosis, which are often the most effective methods of destroying extracellular bacterial pathogens such as those described in this thesis.

Another critical process occurring within the germinal centre is somatic hypermutation, where the epitope binding region of the BCR is subjected to a high rate of mutation, followed by affinity maturation, where these new BCRs are selected for their higher affinity to the epitope. The result of these germinal centre processes is the selection and survival of B-cells that can secrete high affinity IgG with greater epitope specificity. These processes occur with the help of epitope specific T-cells, specifically CD4<sup>+</sup> or helper T-cells. These cells are able to secrete soluble mediators, called cytokines, that modulate the immune response generated. While this cellular immunity is absolutely essential for triggering a robust humoral response, evaluation of cellular immunity is beyond the scope of this thesis.

A defining characteristic of the adaptive immune response is the generation of memory B- and T-cells. Unlike the immediate nature of the innate immune response, the adaptive immune response is delayed the first time the host encounters a specific pathogen. However, due to the generation of memory, any subsequent exposure by the

host to the same pathogen yields a more rapid, specific response by the adaptive arm. This memory response is the foundation behind the development of vaccines.

### *1.2.2 Vaccines and immune responses*

The goal of a vaccine is to induce an immune response that provides protection against a specific infection or disease such that upon exposure to the pathogen in question, a rapid, specific, and protective response is mounted (reviewed in (Pollard & Bijker, 2021)). In order to accomplish this task, a vaccine must contain antigens derived from the pathogen itself for stimulating an adaptive immune response and eliciting high functional antibody titres, mediated by B-cells with support from T-cells, against the intended target. This humoral immunity is considered important for the pathogens discussed in this thesis.

A key aspect of the adaptive immune response is immune memory through the formation of long-lasting memory B-cells that during exposure to the pathogen is able to generate plasma B-cells in order to mount a protective response. While long term memory is not being evaluated in this thesis, it is important to determine the duration of immunity conferred by vaccines. Vaccines generally have the ability to prevent disease, however some vaccines are able to elicit sterilizing immunity resulting in protection from colonization thereby decreasing or preventing transmission and leading to herd immunity.

Mechanisms of protection against these pathogens are still under investigation, with more understood about the humoral arm of adaptive immunity than the cellular arm. Pigs exposed to live cultures of *G. parasuis* or inactivated whole cells (bacterins) generate a transient immunoglobulin M (IgM) response followed by an increasing immunoglobulin G (IgG) response (Martín de la Fuente et al., 2009). Elevated antibody titres in pigs has been associated with protection while low titres leave the animal susceptible to succumbing to GD (Rapp-Gabrielson, Kocur, Clark, & Muir, 1997; Riising, 1981). Decreases in circulating maternal antibodies in piglets is also associated with GD (Aragon et al., 2019). Furthermore, passive immunization with anti-*G. parasuis* antibodies have reportedly conferred protection against a lethal challenge by *G. parasuis* (Nedbalcova et al., 2011). These antibodies are considered to play a role in the opsonization of the infecting strain for facilitating efficient phagocytosis and killing (A.

Olvera et al., 2009). While these studies have predominantly looked at whole cell-based vaccines, levels of antibody titres to protein antigens from a subunit vaccine have also correlated with the levels of protection observed in immunized and challenged pigs (Martinez-Martinez et al., 2016) Therefore, vaccines able to elicit high, functional antibody titres are considered important for mounting an effective response against *G. parasuis*.

The importance of antibodies has also been investigated for its role in preventing disease by *A. pleuropneumoniae*. The decline in maternal antibodies is associated with increased replication of the organisms in the tonsils and subsequent increase in susceptibility for disease development (Chiers, De Waele, Pasmans, Ducatelle, & Haesebrouck, 2010; Chiers et al., 1999). Antibodies are thought to play a role in neutralizing the Apx toxins, which are responsible for many of the clinical symptoms, and increasing phagocytic activity through opsonization (Devenish, Rosendal, & Bossé, 1990). Taken together, the development of vaccines that are able to elicit strong antibody responses is also vital against disease by *A. pleuropneumoniae*.

### *1.2.3 Available vaccines against the porcine pathogens*

The predominant approach in the prevention of infections by *G. parasuis*, *A. pleuropneumoniae*, and *A. suis* has been the widespread usage of inactivated whole cell vaccines (bacterins), which tend to induce a largely strain/serovar specific protective response against the extracellular polysaccharide thus little cross-protection is observed (Bak & Riising, 2002; Takahashi et al., 2001). Several such commercial bacterins exist for *G. parasuis* (Liu, Xue, Zeng, & Zhao, 2016). Autogenous vaccines, which are killed bacterial vaccines using strains from specific herds suffering from an outbreak, can be used to control infections by the aforementioned pathogens (Costa-Hurtado et al., 2020; Lapointe, D'Allaire, Lacouture, & Gottschalk, 2001; McOrist, Bowles, & Blackall, 2009; Sassu et al., 2018). A major disadvantage of this strategy is the inability to accurately identify and isolate the organism behind the outbreak due to a lack of resources leading to variability in efficacy. Thus, autogenous vaccines are generally not approved for widespread use by regulatory authorities (Costa-Hurtado et al., 2020) limiting their

utility. Additionally, it can be costly to develop multiple autogenous vaccines in comparison to a single commercial vaccine.

Subunit vaccines have been investigated against both *G. parasuis* and *A. pleuropneumoniae* to overcome the limitations of whole vaccines. Various recombinant protein antigens have been shown to protect mice against lethal challenge with *G. parasuis* (Guo et al., 2017; Wen et al., 2016; Zheng et al., 2017) and have elicited variable protection in pig infection models against homologous challenge (Shulin Fu et al., 2013; Macedo, Oliveira, Torremorell, & Rovira, 2016; Alex Olvera et al., 2011). Further investigations into the cross-reactive and cross-protective properties of these responses are needed.

In addition to bacterins, subunit vaccines consisting of the *A. pleuropneumoniae* Apx toxins have been used widely. Multiple studies investigating vaccine efficacy of Porcilis App, one such formulation, have reported that while the vaccine decreased the prevalence of pleuritis and pneumoniae associated with *A. pleuropneumoniae*, it did not prevent infection or transmission (Del Pozo Sacristan, Michiels, Martens, Haesebrouck, & Maes, 2014; Sjolund & Wallgren, 2010). Overall, while these existing vaccines have been able to decrease disease burden in the pig industry, outbreaks occur frequently (Espindola et al., 2019) suggesting that new vaccines are needed.

### *1.3 Bacterial iron acquisition*

#### *1.3.1 Iron- an essential micronutrient*

Iron is a micronutrient essential for most forms of life. Iron is critical for various physiological functions including but not limited to serving as a cofactor in various enzymatic reactions, energy metabolism, oxygen transport, and gene regulation (Sheftel, Mason, & Ponka, 2012). Iron is also vital in many redox reactions as ferrous iron ( $\text{Fe}^{2+}$ ) can act as an electron donor and ferric iron ( $\text{Fe}^{3+}$ ) can act as an electron acceptor. The versatility of iron as an element coupled with the high abundance of iron in the Earth's crust is likely why iron has been so prolifically integrated with biological processes during evolution of early life (Andrews, Robinson, & Rodriguez-Quinones, 2003).

After the oxygenation of Earth, iron became insoluble thereby becoming scarce and growth-limiting. Additionally, interactions between oxygen or reactive oxygen species and iron may potentially generate toxic hydroxyl radicals causing damage to biological systems and structures (Kehrer, 2000). Thus, iron is considered both limited and toxic in aerobic conditions and as such, processes have evolved in organisms to enable efficient scavenging of iron from the surrounding environment and careful regulation iron transport and storage (Andrews et al., 2003; Touati, 2000). In mammals, iron is chelated by host iron binding proteins therefore, the level of free extracellular iron is incredibly low, estimated to be around  $10^{-18}$  M (Bullen, Rogers, & Griffiths, 1978). These levels are insufficient in supporting bacterial survival and growth hence in mammalian host environments bacteria must possess strategies for acquiring iron from the host in order to meet their own iron requirements (Hood & Skaar, 2012a).

### *1.3.2 Transferrin and role in mammalian iron homeostasis*

A critical mammalian iron host binding protein is transferrin (Tf), an 80 kDa plasma glycoprotein. When iron is acquired through the diet, it is quickly bound by Tf, whose main role is to transport iron through the bloodstream in a soluble form to tissues in need of the micronutrient. Within cells iron is mainly stored in ferritin, an intracellular protein, which releases iron in a controlled manner as needed for cellular functions (MacKenzie, Iwasaki, & Tsuji, 2008).

Tf is a bi-lobed protein (Aisen, Leibman, & Zweier, 1978), both of which contain a ferric binding domain (Bailey et al., 1988; Hall et al., 2002; Schryvers & Stojiljkovic, 1999) that binds iron tightly with an association constant of  $10^{20}$  M<sup>-1</sup> at physiological pH and is synthesized in the liver. Holo-transferrin (Tf bound to iron) binds to the human Tf receptor 1 (TFR1) and TFR2. TFR1 is expressed almost universally by all cells whereas TFR2 is found expressed by the liver, duodenum, and erythroid cells (Lambert, 2012). Upon binding to TFR1/TFR2 on the cell surface, Tf is internalized via receptor mediated endocytosis (Chung, 1984). After the iron is removed through acidification of the endosome, fusion of the vesicle to the cytoplasmic membrane allows recycling of apo-transferrin (Tf not bound to iron) back to the cell surface where Tf dissociates from its receptor (Aisen, Enns, & Wessling-Resnick, 2001; Morgenthau, Pogoutse, Adamiak,

Moraes, & Schryvers, 2013b). Holo-Tf represents only a third of circulating serum transferrin as higher concentrations of Apo-Tf in the blood allows rapid uptake of free iron.

Tf also mediates nutritional immunity due to its iron-sequestration function and keeping free iron levels in the plasma lower than what is needed to sustain bacterial growth (Cassat & Skaar, 2013; Weinberg, 1971). Diseases causing iron concentration to exceed the total ability of the body to bind and chelate free iron are often associated with the increased prevalence of bacterial infections, thus highlighting the role of transferrin in innate and nutritional immunity (Johnson & Wessling-Resnick, 2012). The role of Tf in this innate immune mechanism has not been well elucidated in mucosal spaces.

Taken together, mechanisms involved in mammalian iron homeostasis result in tight iron regulation and rapid uptake of any free iron thereby limiting free iron availability to sustain growth of organisms like the aforementioned porcine pathogens. Therefore, these bacteria have evolved various strategies for acquiring iron from the host environment in order to survive.

### *1.3.3. Bacterial iron acquisition from Tf*

The primary method of bacterial iron acquisition is siderophore chelation; secretion of high affinity of iron chelators that sequester iron and are then taken up via correlating siderophore receptor (Koster, 2001; Noinaj, Easley, et al., 2012). Although this method of iron acquisition is most commonly used by bacterial organisms, these pathogens rely on cell surface receptors to acquire iron directly from host iron binding proteins (Bahrami et al., 2003; Baltes, Tonpitak, Hennig-Pauka, Gruber, & Gerlach, 2003; Pogoutse & Moraes, 2017). It has been suggested that this may be a survival tactic aimed at avoiding ‘cheater’ bacteria that express siderophore receptors but do not produce and secrete their own siderophores, enabling them to harvest the micronutrient at the expense of other organisms residing within their niche (Barber & Elde, 2015). One such mechanism of binding host proteins for iron acquisition is through the expression of the bipartite bacterial transferrin receptor consisting of a surface anchored lipoprotein called transferrin binding protein B (TbpB) and integral outer membrane embedded transferrin binding protein A (TbpA) (Gray-Owen & Schryvers, 1996; Schryvers &

Morris, 1988), which is shared by members of the *Pasteurellaceae*, *Neisseriaceae*, and *Moraxellaceae* families (Pogoutse & Moraes, 2017). However, each organism expressing the bacterial transferrin receptor has evolved such that their receptor selectively binds to transferrin of their host species.

One component of the bipartite transferrin receptor, TbpB, is a surface-exposed lipoprotein (SLP) anchored to the bacterial outer membrane and consists of two structurally similar lobes, the N-terminal and C-terminal lobes (Calmettes, Alcantara, Yu, Schryvers, & Moraes, 2012). Outer membrane protein Slam is responsible for translocating SLPs like TbpB to the outer membrane (Hooda et al., 2016; Hooda, Lai, & Moraes, 2017). The other component of the transferrin receptor is TbpA, a TonB-dependent transporter (TBDT) embedded in the outer membrane of Gram-negative bacteria belonging to the aforementioned families. TbpA contains a 22-stranded beta-barrel surrounding a globular plug domain and is involved in mediating the process of iron removal from holo-transferrin and enabling the internalization of iron into the periplasm (Gray-Owen & Schryvers, 1996; Noinaj, Buchanan, & Cornelissen, 2012; Yadav et al., 2019).

Genes encoding *tbpA* and *tbpB* are found in an operon (Gonzalez, Yu, Rosteck, & Schryvers, 1995; Ogunnariwo, Woo, Lo, Gonzalez, & Schryvers, 1997), the expression of which is regulated by intracellular iron concentrations via the Fur repressor such that under iron limiting conditions expression of TbpA and TbpB is maximized (Bagg & Neilands, 1987; Hasan, Holland, Smith, & Williams, 1997).

Solved structures of the bacterial transferrin receptor over the past 12 years have yielded numerous insights into the functional mechanism behind interactions with Tf and subsequent iron extraction. The first structure solved of TbpB was from *A. pleuropneumoniae* (Moraes, Yu, Strynadka, & Schryvers, 2009), which was followed by additional structures of TbpBs expressed by the porcine pathogens (Calmettes et al., 2011; R. Frandoloso et al., 2015). Subsequent studies exploring binding interactions revealed that the N-lobe of TbpB binds to conserved domain on the C-terminal lobe of transferrin (Silva et al., 2011) to initiate iron acquisition (Calmettes et al., 2011; Moraes et al., 2009; Silva et al., 2011). Additionally, the function of the anchor peptide was

revealed to aid in the formation of a ternary complex between TbpA, TbpB and Tf during iron acquisition (Yang, Yu, Calmettes, Moraes, & Schryvers, 2011).

Crystal structures of TbpBs from *N. meningitidis* strains have also been solved since then (Adamiak, Calmettes, Moraes, & Schryvers, 2015; Calmettes, Alcantara, Schryvers, & Moraes, 2012; Noinaj, Easley, et al., 2012) and one TbpB structure has been solved from *Mannheimia haemolytica* (unpublished). Comparisons between the various crystal structures demonstrate structural similarity with the overall architecture highly conserved despite the sequence identity between multiple TbpB orthologues of different bacterial species being low.

The recently solved structure of TbpA from the human pathogen *N. meningitidis* in complex human transferrin has similarly allowed insights into the mechanism of TbpA-Tf interactions, specifically how TbpA is able to extract iron from Tf (Noinaj, Easley, et al., 2012). TbpA possess long extracellular loops, some that extend as far as 60Å from the  $\beta$ -barrel sheets, which play an important role in binding to host Tf. Specifically, loop 3 contains an alpha helix domain that inserts into the iron binding cleft in the C-terminal of transferrin to enable release of the iron. (Cash, Noinaj, Buchanan, & Cornelissen, 2015).

The current understanding of iron acquisition by the bipartite receptor is as follows; TbpB is believed to extend away from the bacterial surface with the help of its long, N-terminal anchor peptide, which tethers the protein to the outer membrane and allows it to capture transferrin in the extracellular milieu via TbpB N-lobe and Tf C-lobe interactions. The anchor peptide also aids in the formation of the ternary complex between TbpB, TbpA, and Tf, which increases the local concentration of holo-Tf near TbpA and allows for iron acquisition and internalization by TbpA (Yang et al., 2011).

There are numerous studies characterising the importance of these transferrin binding proteins in human and porcine pathogens. In the porcine infection model with *A. pleuropneumoniae*, mutants lacking either, or both, of the transferrin binding proteins were incapable of causing infection and clinical disease highlighting their necessity (Baltes, Hennig-Pauka, & Gerlach, 2002). These receptors have also been shown to be essential for colonization *in vivo* by *N. gonorrhoeae*, causative agent of gonorrhoea, in a

human male infection model (Cornelissen et al., 1998). Both *tbpA* and *tbpB* in *N. meningitidis* have been shown to be upregulated in the presence human blood (Echenique-Rivera et al., 2011). In *N. meningitidis*, mutants lacking TbpA were incapable of iron uptake from transferrin *in vitro* (Irwin et al., 1993). Taken together these observations strongly posit that the bacterial transferrin receptor is essential for survival and pathogenesis of the aforementioned pathogens, therefore vaccines targeting the receptor is unlikely to result in vaccine escape making them attractive vaccine targets.

#### *1.3.4 Diversity analysis of the transferrin receptor*

Phylogenetic analyses of the bacterial transferrin receptor have been performed for the porcine pathogens as well as *N. meningitidis* (Adamiak et al., 2015; Curran et al., 2015) in order to evaluate the diversity between the homologues, referred to as variants in this thesis. In both analyses, TbpB exhibits considerable sequence diversity, most of which is localized to the N-lobe. In contrast, TbpA displays high sequence conservation. This presence or absence of high sequence diversity correlates with the breadth of the immune response observed. In a mouse experimental model, sera against rTbpA displayed high cross-reactivity against TbpA from heterologous strains whereas sera against rTbpB displayed low cross-reactivity (West et al., 2001). Immunizations with porcine pathogen derived TbpB could only elicit heterologous cross-reactivity against TbpB variants of high protein identity (Curran et al., 2015; J.E. Fegan, Yu, Islam, & Schryvers, 2021b). However, immunizations with TbpA resulted in cross-protection in guinea pigs against bacterial challenge with different serovars of *G. parasuis* (Huang et al., 2013).

While the property of high sequence conservation paints TbpA as a compelling vaccine target, challenges arising during protein expression and isolation have resulted in the focus being predominantly on TbpB as a putative vaccine antigen. However, a novel method for generating antigens expressing loops of TbpA is described in chapter 4, manuscript 3 to overcome the challenges noted.

#### *1.4 The transferrin receptor as a vaccine antigen*

TbpB-based vaccines had previously been under development for the bacterial pathogen *Neisseria meningitidis*, the causative agent of meningitis in humans – an

endeavour that was later abandoned as initial promising results from a TbpB-based vaccine evaluated in mice and rabbits (Danve et al., 1993) were not replicated in Phase 1 of a clinical trial in humans (Danve et al., 1998). The opportunity to explore some of the reasons for the poor performance in the human Phase I trials arose when the first structure of a TbpB protein was determined (Moraes et al., 2009), and since it was from a pathogen in pigs, it provided the ability to readily test in the natural host.

Based on the hypothesis that the host protein may be interfering with development of an optimal protective response, a mutant TbpB protein was generated and tested in a swine immunization and challenge model (R. Frandoloso et al., 2015). It was found that immunizing pigs with Y167A TbpB, a TbpB mutant with defective binding to porcine transferrin, resulted in a more protective response than immunizing with the wildtype TbpB upon challenge by *G. parasuis* Nagasaki strain, providing strong support for this strategy of generating improved TbpB antigens (R. Frandoloso et al., 2015).

In a follow up study, it was demonstrated that while immunization of pigs with Y167A TbpB provided substantial protection against challenge with *G. parasuis* strain 174, none of the pigs immunized with a different binding-defective TbpB mutant (W176A TbpB) survived till the end of the challenge experiment (Guizzo et al., 2018). A key takeaway from this study was that not all binding-defective TbpB mutants are equally efficacious vaccine candidates and that prospective mutant TbpB antigens had to be evaluated using immunization and challenge experiments in pigs. Recently, immunizations of piglets with Y167A TbpB formulated with different adjuvants also resulted in protection against a lethal challenge and prevention of natural colonization by *G. parasuis* (R. Frandoloso, Chaudhuri, Frandoloso, Yu, & Schryvers, 2020).

Taken together, these studies show that an engineered TbpB antigen defective in binding to porcine transferrin is a superior antigen and immunizations with this antigen elicit robust protection in multiple swine immunization and challenge studies resulting in both homologous and heterologous protection against *G. parasuis*. Lastly, the observation that immunization with Y167A TbpB can prevent colonization by *G. parasuis* is a promising indication that sterilizing immunity can be achieved with these antigens.

### 1.5 Thesis objective

The objective of this thesis is to further explore and evaluate the potential of transferrin binding proteins as prospective vaccine candidates against key bacterial pathogens. There are two main hypotheses that will be evaluated and discussed over three manuscripts.

The first main hypothesis being evaluated in this thesis is that a single vaccine composition with a limited number of representative transferrin non-binding TbpB mutants can provide broad spectrum coverage response against all three porcine pathogens. In order to address this hypothesis, the following aims will be pursued:

*Aim 1: Evaluate binding defective TbpB mutants as vaccine candidates in a murine sepsis model*

As discussed above, a key takeaway from a recent study evaluating two different TbpB binding defective mutants (Y167A TbpB and W176A TbpB) against invasive disease by *G. parasuis* in pigs found that not all TbpB mutants are equally efficacious vaccine antigens. Although it was unclear as to the reasoning behind the success of one TbpB mutant as a vaccine antigen over another, the authors noted that evaluating these antigens in a swine immunization and challenge model served as the most effective method for discerning an effective vaccine antigen from a poor one. The purpose of this aim is to determine whether a murine immunization and sepsis model can replicate the findings observed in the swine model thus serving as a potential screening method for evaluating binding defective TbpB mutants from other strains. In chapter 2 (manuscript 1), mice will be immunized with the same antigens used in the prior study (Guizzo et al., 2018) and challenged with a lethal bacterial dose. Due to difficulties in establishing a murine sepsis model with *G. parasuis*, *A. pleuropneumoniae* will be utilized as the challenge strain, thereby enabling evaluation of the ability of a TbpB based vaccine antigen to elicit a cross-protective response.

*Aim 2: Design and evaluate novel TbpB binding defective mutants*

Immunizations with Y167A TbpB, a cluster 3 variant, have resulted in robust protection from challenges with both homologous and heterologous *G. parasuis* strains

expressing TbpBs belonging in cluster 3. However, analysis of porcine Y167A TbpB antiserum has shown that the breadth of reactivity elicited is limited to TbpB variants within cluster 3. It is evident that TbpB based antigens from different clusters are required for providing broader coverage. Therefore, the purpose of this aim is to design and evaluate novel porcine transferrin binding defective TbpB mutants from an *A. pleuropneumoniae* strain expressing a cluster 1 TbpB. In chapter 3 (manuscript 2) we generate novel TbpB binding defective mutants and evaluate them in the murine sepsis model in order to identify the most efficacious binding defective TbpB antigen for future studies.

*Aim 3: Characterize the cross-reactive response elicited by a one- and two-TbpB antigen-based vaccine against a repertoire of TbpB variants representing the existing diversity*

Diversity analysis of TbpB proteins have shown that these iron acquisition proteins are found in all isolates of *G. parasuis*, *A. pleuropneumoniae*, and *A. suis* tested thus far and that they group into three clusters, independent of species (Curran et al., 2015; Guizzo et al., 2018), suggesting that it may be possible to target all three pathogens with a rationally designed TbpB based vaccine. Due to the high sequence diversity of the TbpBs between clusters it is important to evaluate whether the elicited immune response against TbpB is able to provide broad coverage against all variants in order to prevent the possibility of vaccine escape. This aim, discussed in both chapter 2 (manuscript 1) and chapter 3 (manuscript 2), looks at determining whether a TbpB based vaccine consisting of one or two antigens can elicit a broadly cross-reactive response. Mice will be immunized with either one or two separate TbpB based antigens and the resulting antiserum will be used to assess the breadth of cross-reactivity achieved.

The second hypothesis of this study evaluates a novel method for producing antigens that target TBDTs like TbpA. Chapter 4 (manuscript 3) is a methods manuscript exploring how a Tbp/SLP based scaffold can serve to display epitopes on outer membrane proteins to generate novel chimeric vaccine antigens. This is another strategy for developing engineered antigens based on the bacterial transferrin receptor that can be utilized alone or in combination with engineered TbpB based antigens described above to

develop broadly protective vaccines. As noted previously, outer membrane proteins like TbpA make attractive vaccine candidates, however, due to challenges with protein production, tend not to be used in commercial vaccines. The chapter details the design of these hybrid/chimeric antigens, production of these antigens, and the evaluation of antisera raised against the hybrid antigens in a novel TBDT assay.

Overall, the findings of this thesis will lead to a better understanding of the bacterial transferrin receptor as a vaccine antigen and brings us closer to the final, optimal vaccine composition for eliciting efficacious and broadly cross-protective coverage.

## CHAPTER 2: MANUSCRIPT 1

**Immunization of mice with a TbpB-based vaccine against *Glaesserella parasuis*  
exhibits partial cross-protection against lethal challenge by *Actinobacillus*  
*pleuropneumoniae***

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*Actinobacillus pleuropneumoniae*, *Actinobacillus suis*

## 2.1 Abstract

*Glaesserella parasuis*, *Actinobacillus pleuropneumoniae*, and *Actinobacillus suis* are bacterial pathogens that reside in the upper respiratory tract of pigs and are responsible for causing a significant proportion of porcine disease globally. Vaccination remains an important strategy for disease prevention; however, current vaccines provide inadequate protection. Recently, an engineered transferrin binding protein B (TbpB) mutant, defective in binding to porcine transferrin, was able to elicit a protective immune response against *G. parasuis* infection in pigs, however, a separate binding defective TbpB mutant was unable replicate that protection. The authors suggested that prospective mutant TbpB vaccine candidates have to be evaluated using a physiologically relevant animal model, in this case pigs, in order to verify the efficacy of the vaccine.

This study was initiated to determine whether a murine model of sepsis could be used to evaluate and screen different binding defective TbpB mutants as potential vaccine candidates. We demonstrated through our immunization and challenge experiments that we are able to recapitulate the “real-world” protection levels observed in the native porcine host in our murine model. Additionally, we demonstrated that immunization of mice with the TbpB mutant resulted in a cross-protective response against an *A. pleuropneumoniae* challenge which we believe is the first observation of this kind.

## 2.2 Introduction

Key pathogens responsible for causing disease in humans and important food production animals have evolved to colonize in specific mucosal niches within their host (Morgenthau, Pogoutse, Adamiak, Moraes, & Schryvers, 2013a; Ostan, Morgenthau, Yu, Gray-Owen, & Schryvers, 2017). These include Gram-negative pathogenic bacteria belonging to the *Pasteurellaceae*, *Neisseriaceae*, and *Moraxellaceae* families, all of which share a common evolutionary adaptation of acquiring iron from host proteins present on mucosal surfaces. This iron acquisition system is the bacterial surface receptor transferrin binding protein A and B (TbpA and TbpB) (Schryvers & Gray-Owen, 1992;

Schryvers & Lee, 1993; Schryvers & Morris, 1988), which binds exclusively to the host iron-binding glycoprotein transferrin, thus limiting the niche for these bacteria exclusively to their host species (Gray-Owen & Schryvers, 1996).

*Glaesserella parasuis*, *Actinobacillus pleuropneumoniae*, and *Actinobacillus suis* are bacterial pathogens inhabiting the upper respiratory tract of pigs that share this mechanism of iron acquisition (Bahrami et al., 2003; Gonzalez, Caamano, & Schryvers, 1990; Morton & Williams, 1989; Ricard, Archibald, & Niven, 1991) and depend upon TbpB and TbpA for survival and disease causation (Baltes et al., 2002). Early immunization and challenge experiments in pigs demonstrated that TbpB is able to generate a protective immune response against *A. pleuropneumoniae* infection (Rossi-Campos et al., 1992). More recently, it was demonstrated that immunizing pigs with a TbpB mutant (Y167A TbpB) with a substantially reduced ability to bind to porcine transferrin resulted in a more protective response than immunizing with the wild-type (WT) TbpB during challenge by *G. parasuis* Nagasaki strain (R. Frandoloso et al., 2015). This study demonstrated the importance of using a binding-defective TbpB mutant as a vaccine antigen to avoid binding of the vaccine antigen by host transferrin during immunization and hiding epitopes in the binding interface, thereby hampering the immune response.

In a follow up study, it was demonstrated that while immunization of pigs with Y167A TbpB provided substantial protection against challenge with *G. parasuis* strain 174, none of the pigs immunized with a different binding-defective TbpB mutant (W176A TbpB) survived till the end of the challenge experiment (Guizzo et al., 2018). A key takeaway from this study was that not all binding-defective TbpB mutants are equally efficacious vaccine candidates and that prospective mutant TbpB antigens needed to be evaluated using immunization and challenge experiments in pigs.

This study was initiated to determine whether mice would be a good model to evaluate and screen different TbpB mutants as vaccine candidates. Mice were immunized with either WT TbpB, Y167A TbpB, W176A TbpB, or two bacterins and subsequently challenged. We demonstrated that immunizations with Y167A TbpB resulted in partial protection whereas W176A TbpB resulted in little to no protection – a similar pattern to

what was previously observed with *G. parasuis* immunization and challenge experiments in pigs (Guizzo et al., 2018), suggesting that we are able to recapitulate the “real-world” protection levels observed in the native porcine host using our mouse model.

Due to difficulties in establishing a murine sepsis model with *G. parasuis*, *A. pleuropneumoniae* was used as the challenge strain, thereby enabling us to also evaluate the ability of a TbpB based vaccine antigen to elicit a cross-protective response. Herein, we demonstrate that immunization with Y167A TbpB exhibits partial cross-protection against *A. pleuropneumoniae* serovar 1, and we observe elevated levels of systemic IgG antibody titres against both homologous and heterologous WT TbpBs from *G. parasuis* strain 174 and *A. pleuropneumoniae* serovar 1, respectively. Lastly, we assessed the cross-reactivity of anti-Y167A TbpB sera against other WT TbpB variants and demonstrated that there is substantial cross-reactivity against different species within the same cluster however, gaps in coverage also exist. This data adds to the existing literature exploring TbpB based vaccines against *G. parasuis*, *A. pleuropneumoniae*, and *A. suis*.

### 2.3 Materials and methods

#### *Recombinant antigen production*

The wildtype TbpB (WT TbpB), transferrin binding-defective mutant Y167A TbpB (Y167A TbpB), and transferrin binding-defective mutant W176A TbpB (W176A TbpB) were produced for vaccine formulation as described previously (R. Frandoloso et al., 2015; Guizzo et al., 2018). Briefly, genes encoding the mature sequence of the TbpB proteins were cloned into a T7 expression vector with an N-terminal polyhistidine fusion tag. The resulting vectors were transformed into competent *Escherichia coli* ER2566 cells and proteins were expressed by growth in autoinduction media at 37°C overnight in a shaking incubator. Cells were harvested the following day by centrifugation and lysed with a cell disruptor. The crude lysate was separated from the cell debris by centrifugation and then subjected to Ni-NTA chromatography and eluted off the column with an elution buffer containing 300 mM imidazole. If needed, an additional purification step of anion exchange with a Q-Sepharose column was performed. Fractions were analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to determine the purity of the sample. Eluted fractions were pooled, dialyzed against

Dulbecco's phosphate-buffered saline (DPBS), and concentrated. Protein samples were filter sterilized (0.22 µm filter), quantified, and stored in aliquots at -20°C until use.

#### *Formalin treated bacterin formulation*

Formalin treated bacterins were formulated as described previously (Hau, Eberle, & Brockmeier, 2021). *G. parasuis* strain 174 was grown on a PC55 chocolate agar plate (Dalynn Biological, Canada) overnight in a 37°C incubator with 5% CO<sub>2</sub>. The following day, the bacteria were grown in 200 mL pleuropneumoniae like organisms broth (PPO broth, Difco, USA) supplemented with 2.5 mg/mL D-glucose (Sigma-Aldrich, USA) and 60 µg/mL nicotinamide adenine dinucleotide (β-NAD, Sigma-Aldrich, USA) from a starting optical density at 600 nm (OD<sub>600</sub>) of 0.1. The bacteria were grown for 3 hours or until the OD<sub>600</sub> reached 0.6, indicating the log phase of bacterial growth. 1 mL of the culture was removed to count colony-forming units (CFU) by performing ten-fold dilutions and plating the bacteria. 1 ml of 37.5% formalin (Sigma-Aldrich, USA) was then added to the culture to a final dilution of 0.5%. The culture was incubated at 37°C overnight with gentle rotation at 80 rpm followed with a second incubation at 4°C for 24 hours. The cells were then harvested and washed three times with sterile phosphate buffered saline (PBS), resuspended in 10 mL of sterile Dulbecco's PBS (DPBS), and subsequently stored at 4°C until use. A 100-µL sample was plated on chocolate agar to ensure no viable bacteria were present. A formalin treated bacterin was formulated with *A. pleuropneumoniae* serovar 1 using the same protocol.

#### *Vaccine formulation*

Vaccines for the immunization and challenge study were formulated as previously described (R. Frandoloso et al., 2020). Briefly, 1.25 mg of antigen and 50 µg of poly I:C (HMW, Invivogen, USA) was mixed with 1 mL (20% v/v) of Montanide Gel 01 adjuvant (Seppic, France) and brought to a final volume of 5 mL with sterile DPBS. The bacterin was prepared by mixing 5 X 10<sup>9</sup> CFU of formalin-treated *G. parasuis* strain 174 and 50 µg poly I:C with 1 mL (20% v/v) of Montanide Gel 01 adjuvant, made up to a final volume of 5 mL with sterile DPBS. The adjuvant-only control group was formulated in the same manner but with Montanide Gel 01, poly I:C, and DPBS only. The mixtures, formulated for 50 dosages (25 µg of antigen per dose/ 1 X 10<sup>8</sup> CFU per dose), were

gently rotated using a tabletop rotary shaker at room temperature overnight and then stored at 4°C in 1.6-mL aliquots until use.

For the cross-reactivity study, vaccines were formulated with either the Montanide Gel 01 and poly I:C mixture or AddaVax (Invivogen, USA), a research-grade squalene-based adjuvant similar to MF59. The Montanide Gel 01 and poly I:C preparations were formulated as described above. The AddaVax-adjuvanted vaccines were prepared according to the manufacturer's instructions (<https://www.invivogen.com/addavax#specifications>). 400 µg of the antigen were mixed with 800 µL of AddaVax (50% v/v) and brought to a final volume of 1.6 mL with sterile DPBS. This mixture, formulated for 16 dosages (25 µg of antigen per dose), were prepared the day off for each immunization timepoint.

#### *Mice immunizations*

For the immunization and challenge study, groups of 8 mice (6-week-old C57BL/6, n=4 female, n=4 male) were subcutaneously immunized with a 100-µL dose of the vaccine. Vaccine groups are listed in Table 2.1. Mice were immunized with a prime and two boosters where each dose was given 21 days apart on Day 0, Day 21, and Day 42 of the experiment. 100 µL of blood were collected from the tail vein from each mouse at all three timepoints as well as on Day 49, 7 days after the last immunization, for assessing systemic antibody titres.

For the cross-reactivity immunization study, groups of 8 mice (6-week-old C57/B6, all male) were subcutaneously immunized with a 100-µL dose of either the Montanide Gel 01 plus poly I:C or AddaVax-adjuvanted Y167A TbpB formulations, as listed in Table 2.3. Mice were immunized on Day 0, Day 21, and Day 42 as described above. 14 days after the last immunization, mice were euthanatized using CO<sub>2</sub> asphyxiation, and intracardiac bleeds were performed.

**Table 2.1.** Groups for immunization and challenge study. Table contains initial number of mice per group and final number of mice per group as some mice had to be removed from study due to ulcerative wounds at injection site.

Group – Adjuvanted with Montanide + poly I:C	Initial # of mice/group	Final # of mice/group
WT TbpB	n = 8	n = 4
Y167A TbpB	n = 8	n = 8
W176A TbpB	n = 8	n = 8
<i>Gps</i> bacterin	n = 8	n = 6
<i>App</i> bacterin	n = 8	n = 5
Adjuvant	n = 8	n = 8

**Table 2.2.** Groups for cross-reactivity immunization study (n=8/group)

Adjuvant used		
Montanide Gel 01 + poly I:C	Y167A TbpB	Adjuvant only
AddaVax	Y167A TbpB	Adjuvant only

*Heterologous bacterial challenge of immunized mice with A. pleuropneumoniae*

To prepare the challenge dose, *A. pleuropneumoniae* serovar 1 was grown on a PC55 chocolate agar plate overnight in a 37°C incubator with 5% CO<sub>2</sub>. The following day, the bacteria were grown in 15 mL PPLO broth supplemented with 2.5 mg/mL D-glucose and 120 µg/mL β-NAD from a starting OD<sub>600</sub> of 0.1. Filter-sterilized deferoxamine mesylate (Desferal, Sigma-Aldrich, USA) was added to the inoculated

broth to a final concentration of 100  $\mu$ M to iron-starve the bacteria and trigger the expression of the transferrin receptor (not shown). The bacteria were grown for 3 hours or until the OD<sub>600</sub> reached 0.6, indicating the log phase of bacterial growth. The cells were harvested and washed three times with sterile PBS and diluted to an OD<sub>600</sub> of 0.2 in DPBS from which 100- $\mu$ L doses consisting of 1 X 10<sup>6</sup> CFU of *A. pleuropneumoniae* serovar 1 were prepared.

Immunized mice were challenged on Day 56, 14 days after the 3<sup>rd</sup> immunization, with an intraperitoneal injection. Filter sterilized iron supplement, prepared in 100- $\mu$ L doses each containing 4 mg of iron loaded pTf (Life Technologies-Gibco, USA) in DPBS, were also injected intraperitoneally in order to provide an iron source for the bacteria during challenge. Mice were monitored for 48 hours for clinical symptoms such as rough fur coat and low activity. Mice were euthanized using CO<sub>2</sub> asphyxiation upon reaching either the clinical (i.e. if symptoms became too severe to humanely continue the experiment) or experimental (i.e. after 48 hours of monitoring) endpoint, and intracardiac bleeds were performed. Blood samples were plated on chocolate agar plates overnight in a 37°C incubator with 5% CO<sub>2</sub> to determine if bacteremia was present.

*Recombinant protein production for indirect enzyme-linked immunosorbent assays (ELISAs)*

WT TbpB variants (V1-V8 – Table 3) were produced using protocol described previously (J.E. Fegan et al., 2021b). Briefly, the mature sequence of each WT gene (V1 TbpB<sup>6-574</sup>, V2 TbpB<sup>2-528</sup>, V3 TbpB<sup>20-537</sup>, V4 TbpB<sup>6-528</sup>, V5 TbpB<sup>6-526</sup>, V6 TbpB<sup>6-575</sup>, V7 TbpB<sup>2-574</sup>, V8 TbpB<sup>20-528</sup>) was cloned into a custom T7 expression vector containing an N-terminal fusion construct consisting of a polyhistidine tag, biotinylation sequence, maltose binding protein, and tobacco etch virus protease cleavage site. Resulting vectors were transformed into competent *E. coli* ER2566 cells for small-scale protein expression. Transformed cells were directly grown in 200 mL of autoinduction media overnight in 37°C with shaking at 200 rpm. Cells were harvested the following day and lysed by mechanical disruption. Cell debris was pelleted by centrifugation and the resulting crude lysate supernatant containing biotinylated antigens was diluted 5-fold in cold 1X PBST (PBS with 0.05% Tween 20, pH 7.4).

### *Indirect ELISA IgG titre analysis*

Antisera collected from blood samples harvested at Day 0 (D0), Day 21 (D21), Day 42 (D42), and Day 49 (D49) were used to quantify elicited antibody titres against immobilized WT TbpBs (*G. parasuis* strain 174 and *A. pleuropneumoniae* serovar 1) as described previously (J.E. Fegan et al., 2021b). In brief, 100  $\mu$ L of crude cell lysate in PBST preparations were added to wells pre-coated with streptavidin (ThermoFisher, USA) for protein capture and immobilization. Coated plates were incubated at room temperature (RT) for 1 hour (hr) and then washed 3 times with 250  $\mu$ L of PBST. All wash steps mentioned henceforth were performed 3 times with 250  $\mu$ L of PBST. Wells were blocked with 250  $\mu$ L of 5% skim milk in PBST for 1 hr at RT and washed. 100  $\mu$ L of antisera diluted in 2.5% skim milk in PBST was added in 2-fold dilutions starting at 1 in 200 and incubated for 1 hr at RT and washed. 100  $\mu$ L of goat anti-mouse IgG secondary antibody [conjugated to horseradish peroxidase (HRP)] (Sigma Aldrich, USA) diluted 7,500-fold in 2.5% PBST was added to each well, incubated for 1hr at RT, and washed. Plates were developed using 50  $\mu$ L of 3,3',5,5'-tetramethylbenzidine (TMB) reagent (Sigma Aldrich, USA) for 20 minutes in the dark. Development was quenched with 25  $\mu$ L of 4 N HCl and OD<sub>450</sub> readings taken using a 96-well plate reader.

The results were described as mean endpoint antibody titres, which was defined as the reciprocal of the highest dilution giving a positive OD<sub>450</sub> reading (R. Frandoloso et al., 2020). A reading was considered positive if it was at least two times greater than the OD<sub>450</sub> values of the negative control (lysate only and lysate plus secondary antibody). Samples for D0, D21, and D42 were assessed once while samples from D49 were assessed in independent duplicates.

### *Indirect ELISA Tf blocking analysis*

Serum samples from D49 were used to assess whether the elicited antibodies could bind to *G. parasuis* strain 174 WT TbpB and block binding by porcine transferrin (pTf) as a mechanism of protection in the natural host. Plates were prepared, blocked, and washed as described above. 100  $\mu$ L of antiserum samples diluted 4-fold in 2.5% skim milk in PBST were added to wells, incubated for 1 hr at RT, and washed. 100  $\mu$ L of porcine transferrin conjugated to HRP (pTf-HRP; produced in-house (J.E. Fegan et al.,

2021b)) diluted 1:1000 in 2.5% skim milk in PBST were added to wells and incubated for 1 hr at RT, washed, and developed as described above. Results were described as comparisons between OD<sub>450</sub> readings with the serum samples and pTf-HRP on its own (i.e. no serum added). Samples were assessed in duplicates.

#### *Indirect ELISA cross-reactivity analysis*

Cross-reactivity of elicited antibodies was assessed against WT TbpBs from *G. parasuis*, *A. pleuropneumoniae*, and *A. suis* (V1-V8, Table 2.3). Mice were immunized with Y167A TbpB (adjuvanted with either Montanide Gel 01 and poly I:C, AddaVax) or adjuvants only as a negative control three times, 21 days apart, and were euthanized 14 days after the last dose. Intracardiac bleeds were performed after euthanasia and serum samples were collected to assess sera for cross-reactivity.

Seven variant WT TbpBs from the three clusters as well as the homologous WT TbpB (8 TbpB variants total, Table 3) were selected in order to represent the total diversity among TbpB variants expressed by the three aforementioned porcine pathogens. Streptavidin-coated ELISA plates were coated with WT TbpB lysates as shown in Figure 2.1, produced as described above. Plates were washed, blocked, and 100 µL of antiserum samples diluted 10,000-fold in 2.5% skim milk in PBST were added to wells, incubated for 1 hr at RT, and washed. Secondary antibody solution was prepared and added, and plates were developed as described above. For each variant of WT TbpB, 100 µL of pTf-HRP diluted 1000-fold in 2.5% skim milk in PBST was added to two wells to normalize OD<sub>450</sub> readings to pTf binding. The latter analysis was performed as different variants bind to pTf with different affinities and we wanted to examine if that was a variable. The mean OD<sub>450</sub> of the pTf-HRP readings for each variant was used to normalize each OD<sub>450</sub> reading from a serum sample for that variant, which was then plotted in addition to raw OD<sub>450</sub> values. Samples were assessed in duplicate.

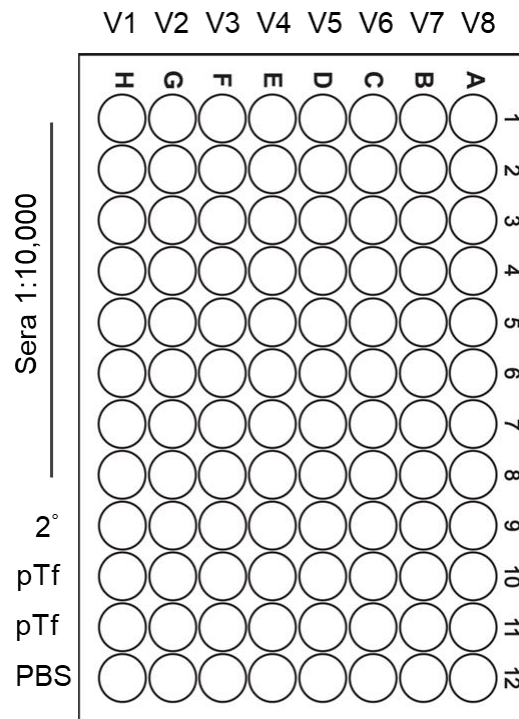
**Table 2.3.** Summary of cross-reactivity to variants analysis. Pattern exists for both Montanide Gel 01 plus poly I:C and AddaVax adjuvanted Y167A TbpB (n=8/adjuvant). Cross reactivity is labelled as high if over raw OD of 1.

#	Species/strain	Cluster	% Identity to Y167A	Cross-reactivity	Cross-protection
V1	<i>A. pleuropneumoniae</i> SV 1 (VIDO)	1	52.2	High	Yes*
V2	<i>A. pleuropneumoniae</i> h87 Unk	2	60.2	Low	N/A
V3	<i>G. parasuis</i> 174 SV 7	3	100	High	N/A
V4	<i>A. pleuropneumoniae</i> h80 Unk	2	60.2	Low	N/A
V5	<i>G. parasuis</i> h410 SV 2	3	76.8	High	N/A
V6	<i>G. parasuis</i> h412 SV 4	1	52.1	Low	N/A
V7	<i>A. suis</i> h57 Unk	1	52.1	Low	N/A
V8	<i>A. pleuropneumoniae</i> h49 SV 7	3	81.5	High	N/A
n/a	<i>G. parasuis</i> Nagasaki SV 5	3	74.5	N/A	Yes**

\* This study

\*\*From Frandoloso *et al.* 2015 (R. Frandoloso et al., 2015). Immunization and challenge experiments performed in pigs

SV – serovar, Unk – serovar unknown



**Figure 2.1.** Schematic of cross-reactivity indirect ELISA. Each serum sample was diluted 1:10,000 and added across the 8 well row. Each well in the row is coated with a different TbpB variant (8 variants total). For each TbpB variant binding to porcine transferrin HRP was tested.

### *Statistical Analysis*

All data were analyzed using GraphPad Prism™ V9 (GraphPad Software, San Diego, California, USA). The comparative survival analysis was performed using the log rank Mantel Cox curve analysis and Bonferroni method. The mean immunoglobulin titres from ELISAs were examined for significance using the Brown Forsythe and Welch one way ANOVA followed by Dunnett's T3 multiple comparisons test. Mean cross-reactive immunoglobulin titres elicited by different antigen/adjuvant combinations were compared using two-way ANOVA followed by Sidak's multiple comparisons test. Serological results are reported as means  $\pm$  SD, and p-values  $< 0.05$  were considered significant.

### *Statement of Institutional Animal Care*

All animal experiments were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals as indicated by the Canadian Council on Animal Care and the University of Calgary (protocol #: AC18-0210).

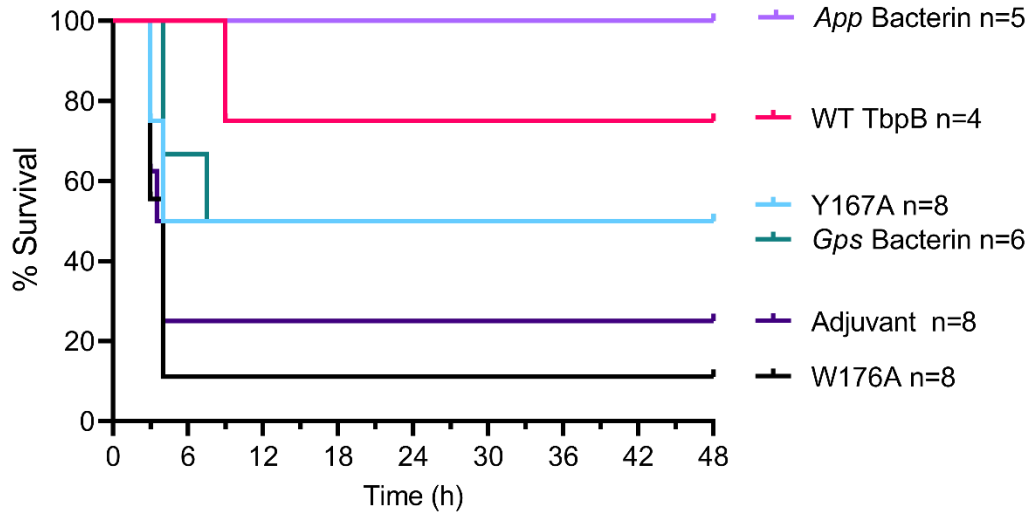
### *2.4 Results*

#### *Immunization with TbpB antigens from G. parasuis protected mice from a challenge by A. pleuropneumoniae*

This study was initiated to determine whether it was possible to evaluate binding defective TbpB mutants in mice. Initial attempts at utilizing *G. parasuis* strain 174 as the challenge strain were abandoned after encountering difficulties in developing a murine infection model (Dazzi *et al.*, 2020). Such challenges in developing a murine model with *G. parasuis* have been reported by other groups (de la Fuente, Gutiérrez-Martín, Martínez-Martínez, Frandoloso, & Rodríguez-Ferri, 2007). Thus, an *A. pleuropneumoniae* serovar 1 strain initially isolated from a porcine lung lesion was used, enabling us to also evaluate cross-protection.

Mice were immunized with either WT TbpB, Y167A TbpB, or W176A TbpB and challenged with a lethal dose of *A. pleuropneumoniae* in order to observe if survival trends were similar to those seen in prior pig experiments (Guizzo *et al.*, 2018). Groups of mice were immunized with formalin treated bacterins derived from either *G. parasuis* strain 174 (*Gps* bacterin) or *A. pleuropneumoniae* serovar 1 strain (*App* bacterin) as controls. An adjuvant only group lacking any antigens was also included as a control. Mice that received the bacterin derived from the challenge strain, *App* bacterin, were completely protected (100% survival, n = 5) from the challenge, as expected (Figure 2.2). Also expected was the lack of protection observed in the adjuvant only group (25% survival, n = 8). Mice that received W176A TbpB succumbed to the challenge (11% survival, n = 8) whereas mice that received Y167A TbpB or WT TbpB had higher survival rates at 50% (n = 8) and 75% (n = 4), respectively. Lastly, mice immunized with the bacterin derived from *G. parasuis* strain 174 had 50% survival rate (n = 6). In this study, we have identified three formulations (Y167A TbpB, WT TbpB, and *Gps* bacterin) that exhibit cross-protective properties. We believe this is the first instance where

immunizations with a *G. parasuis*-derived subunit vaccine resulted in cross-protection against challenge by an *A. pleuropneumoniae* strain.



**Figure 2.2.** Survival curve of immunized mice challenged with  $1 \times 10^6$  CFU of *A. pleuropneumoniae* serovar 1. No significance was seen between groups, assessed by Mantel – Cox log rank test.

All mice displayed clinical symptoms of disease (such as low activity and rough hair coat) within the first 2 hours after challenge. When these symptoms worsened, mice were euthanized by  $\text{CO}_2$  asphyxiation, usually within the 3 to 9 hour mark. The remaining mice improved clinically after 9 hours post-challenge and displayed normal behaviour by 18 hours post-challenge (data not shown). After mice were euthanized, intracardiac bleeds were performed, and blood samples were plated on chocolate agar to test for bacteremia. Bacteria were recovered from all euthanized mice indicating that those mice had severe bacteremia (data not shown), which likely contributed to symptoms of disease observed.

Release of hemolytic and cytolytic toxins by *A. pleuropneumoniae* serovar 1 strain, which have been shown to be lethal in mice (Seah, Frey, & Kwang, 2002) may have contributed to the severity and rapid spread of the infection in this model. Challenge with a heat-killed bacterial dose did not result in any clinical symptoms (not shown); thus, death due to endotoxin shock was ruled out. In our model, challenged mice likely got sick due to bacteremia and damage by toxins within the first few hours. In groups where the vaccine showed efficacy, the infection was likely brought under control very quickly, allowing mice to survive. A modified murine model using an *A. pleuropneumoniae* challenge strain with the Apx toxins knocked out or with genes encoding inactivated Apx toxins could improve this analysis.

While these results were not significantly different, we believe that trends observed are informative and increasing group numbers will likely aid in seeing significance. Most notably, in this study mice immunized with Y167A TbpB had a higher survival rate compared to mice immunized with W176A TbpB, similar to what was observed in the swine immunization and challenge model (Guizzo et al., 2018). These results suggest that binding defective TbpB-based antigens can be screened in a murine model of sepsis, which can accelerate the generation of new TbpB mutants from other strains.

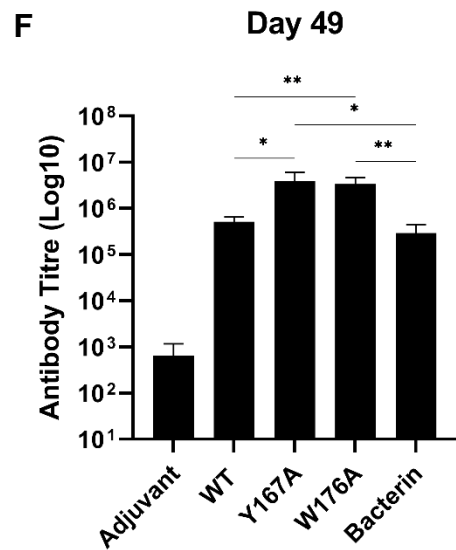
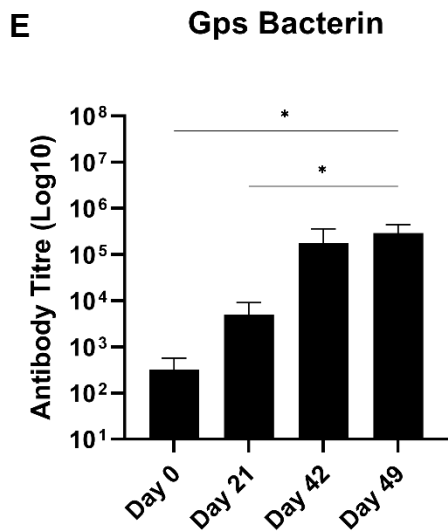
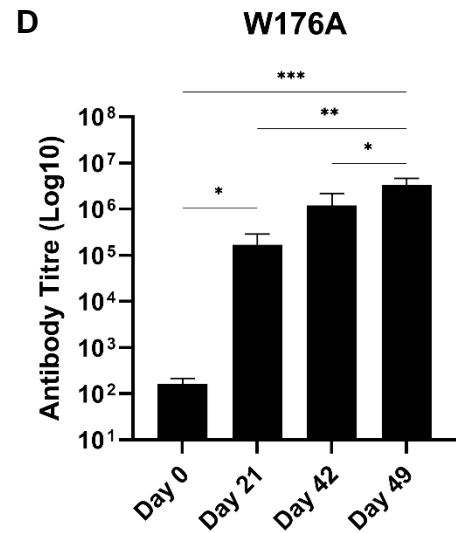
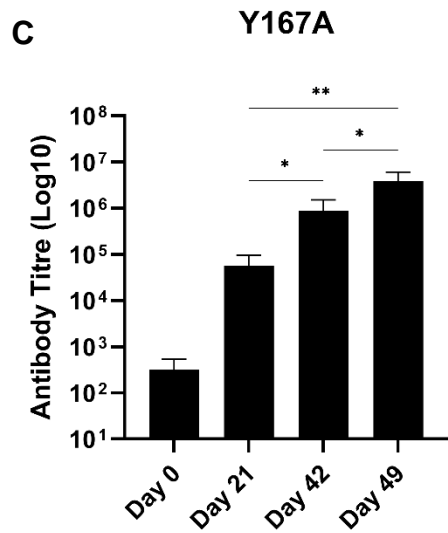
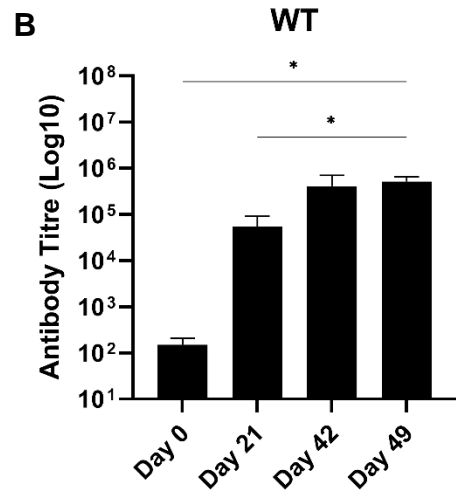
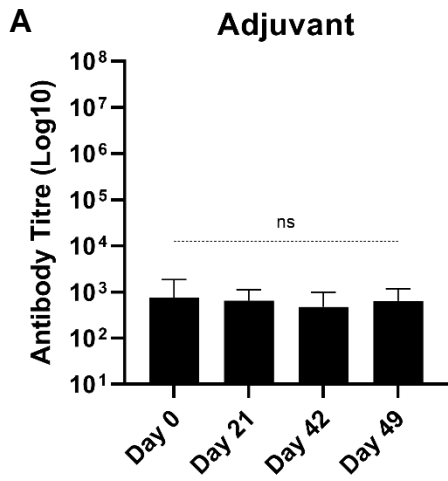
*Immunizations with TbpB antigens from Gps elicit high IgG titres against both homologous and heterologous WT TbpBs*

Prior to challenge, serum samples from immunized and control mice were collected at different timepoints to assess systemic antibody titres generated by different groups against the WT TbpB from *G. parasuis* strain 174. Systemic IgG titres against homologous WT TbpB from *G. parasuis* strain 174 were examined at D0, prior to the prime dose, and in response to immunizations at three timepoints: D21, D42, and D49 (Figure 2.3A-E). The adjuvant group had low-level background IgG titres prior to the prime dose at D0 but did not show any changes throughout the remaining experiment. 21 days after the prime, an increase in IgG titres was observed in all groups immunized with a vaccine formulation. Titres elevated 21 days after the first booster shot was seen in all

vaccine groups, with a further boost in IgG titres in the groups that received the non-binding TbpB mutants a week after the second booster shot (Figure 2.3C-D).

Systemic IgG titres were compared at D49, 7 days prior to challenge, between all groups (Figure 2.3F). As expected, the adjuvant group generated low background IgG titres. Both the WT and the *Gps* bacterin group also elicited low titres. 4/8 mice from the WT group were excluded during the immunization portion of the study as significant ulcerative wounds were found at the injection site. It is thus difficult to determine the relevance of the observation regarding lower IgG titres due to a smaller number of mice tested. It is possible that WT TbpB as an antigen, for reasons unknown, elicits lower IgG titres, however this observation is consistent with those seen previously in pig immunization experiments comparing WT TbpB and Y167A TbpB as vaccine antigens (Martinez-Martinez et al., 2016). The bacterin group was expected to generate lower IgG titres against immobilized WT TbpB (Figure 2.3F). The bacteria were not iron-starved during growth for bacterin preparation; thus, the transferrin receptors would not be maximally expressed. Furthermore, the immune response generated would be against multiple antigens present in the bacterin formulation, which would not be fully captured in a single antigen TbpB-based assay.

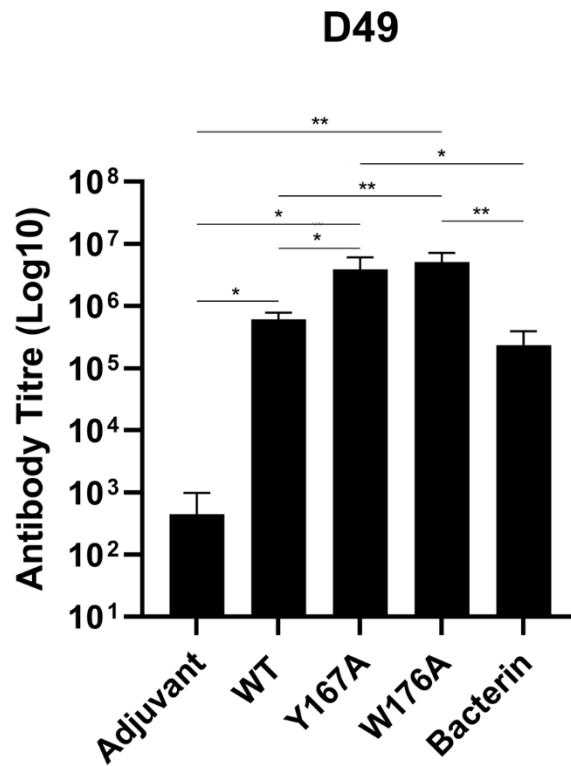
No significant differences in IgG titres between the two mutant TbpB groups were observed, similar to what was noted in the study comparing Y167A TbpB and W176A TbpB in pigs (Guizzo et al., 2018). This further supports the evidence presented in this study supporting the notion that a murine sepsis model is a physiologically relevant infection model that is able to recapitulate trends observed in a previously described porcine infection model and can thus be readily used for evaluating TbpB non-binding mutant vaccine candidates.



**Figure 2.3.** Analysis of systemic IgG antibody titres elicited by immunization with different antigens against homologous WT TbpB from *G. parasuis* strain 174. A-E show kinetics of antibody titres from sera collected from each group at different time points during the immunization schedule. For each group (n= 4-8 mice), sera collected during first three timepoints was assessed in 2-fold dilutions once. Sera collected during the last time point was assessed in duplicate. F compares D49 IgG titres from sera collected one week before challenge. Bacterin refers to *Gps* bacterin. Sera was assessed in duplicate. Significance was determined using the Brown Forsythe and Welch ANOVA followed by a Dunnett's T3 multiple comparisons test comparing each group to each other (\* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ ). For graphs C and F, all groups compared to the adjuvant were significant (not displayed).

Systemic IgG titres from sera collected on D49 were then assessed for reactivity against the WT TbpB from *A. pleuropneumoniae* serovar 1 strain (challenge strain) to determine if there was a correlation to protection from the bacterial challenge (Figure 2.4). High IgG titres were observed against the heterologous WT TbpB, similar to what was noted against the WT homologous TbpB. The WT TbpBs from *A. pleuropneumoniae* serovar 1 strain and *G. parasuis* strain 174 share approximately 50% amino acid identity. Despite the relatively low sequence identity, IgG titres in Figure 3.4 demonstrate a high level of cross-reactivity.

No differences in IgG titres between the two mutant TbpB groups were observed again, suggesting that titre levels are not correlated with protection. While it is important to have high titres of systemic IgG antibodies, the functionality of those antibodies is more likely to correlate with protection. In our rapid model of infection, it is anticipated that cross-reactive functional antibodies that carry out effective opsonophagocytosis and complement-mediated killing are important mechanisms for protection.

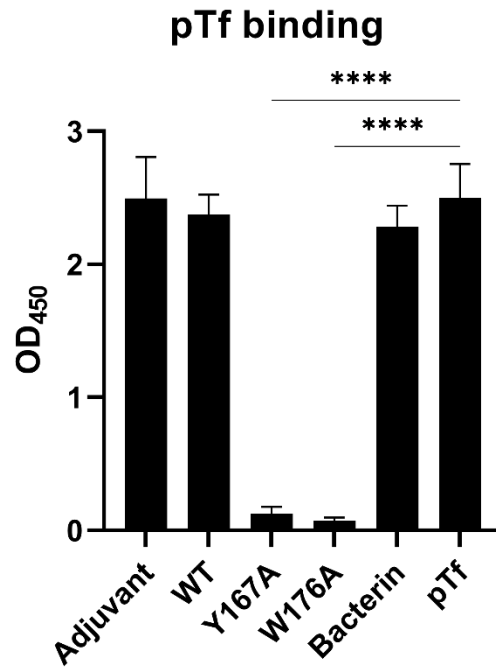


**Figure 2.4.** Analysis of systemic IgG antibody titres elicited by immunization with different antigens against heterologous WT TbpB from *A. pleuropneumoniae* serovar 1. Figure shows comparisons between D49 IgG titres from sera collected one week before challenge. Bacterin refers to *Gps* bacterin. Sera was assessed in duplicate, and significance was determined using the Brown Forsythe and Welch ANOVA followed by a Dunnett's T3 multiple comparisons test comparing each group to each other (\* $p \leq 0.05$ , \*\* $p \leq 0.01$ ).

*Antibodies elicited by TbpB antigens from Gps can prevent binding to pTf*

Antibodies elicited by TbpB-based antigens could have the capacity to bind to TbpB expressed on the cell surface and reduce or prevent binding by host transferrin, thereby resulting in protection against disease via nutritional immunity. We wanted to examine if systemic IgG antibodies generated in all groups in this study could provide protection by nutritional immunity. Nutritional immunity may not be a mechanism of protection in this specific model due to the rapid nature of infection and death; however, it could be a mechanism of protection in the native host. If the antibodies elicited by this antigen could interfere with binding to transferrin, which is present in lower quantities on mucosal surfaces of respiratory tissues, then these antibodies could starve the bacteria by preventing iron acquisition from its main iron source.

Only TbpB mutants defective in binding were able to elicit IgG antibodies that prevented binding by pTf conjugated to HRP (Figure 2.5), and neither the WT TbpB nor the *Gps* bacterin elicited pTf blocking antibodies. A similar observation was noted using sera from pigs immunized with WT TbpB, Y167A TbpB, and W176A TbpB where both anti-mutant antibodies were able to block binding by pTf (Barasuol et al., 2017). In this study, no difference was observed between the mutants in terms of their ability to block pTf binding when compared to the pTf control in the absence of sera (Figure 2.5). These results, in conjunction with prior observations (Barasuol et al., 2017), suggest that this is not a mechanism of protection as both mutants are capable of generating blocking antibodies. However, these results do further highlight our ability to use the murine model to evaluate vaccine candidates as similar findings to those seen in studies in pigs are consistently observed.



**Figure 2.5.** Ability of antibodies to block binding by pTf to WT homologous TbpB from *G. parasuis* strain 174. Sera collected one week before challenge (D49) was added to wells coated with immobilized WT TbpB at 1:4 dilution followed by addition of pTf-HRP at 1:1000 dilution. Significance was determined using the Brown Forsythe and Welch ANOVA followed by a Dunnett's T3 multiple comparisons test comparing each group to the control pTf (\*\*\*\* $p \leq 0.0001$ ). All experiments were performed in duplicate.

*Cross-reactivity of antisera elicited by a TbpB antigen derived from Gps against a representative panel of TbpB variants*

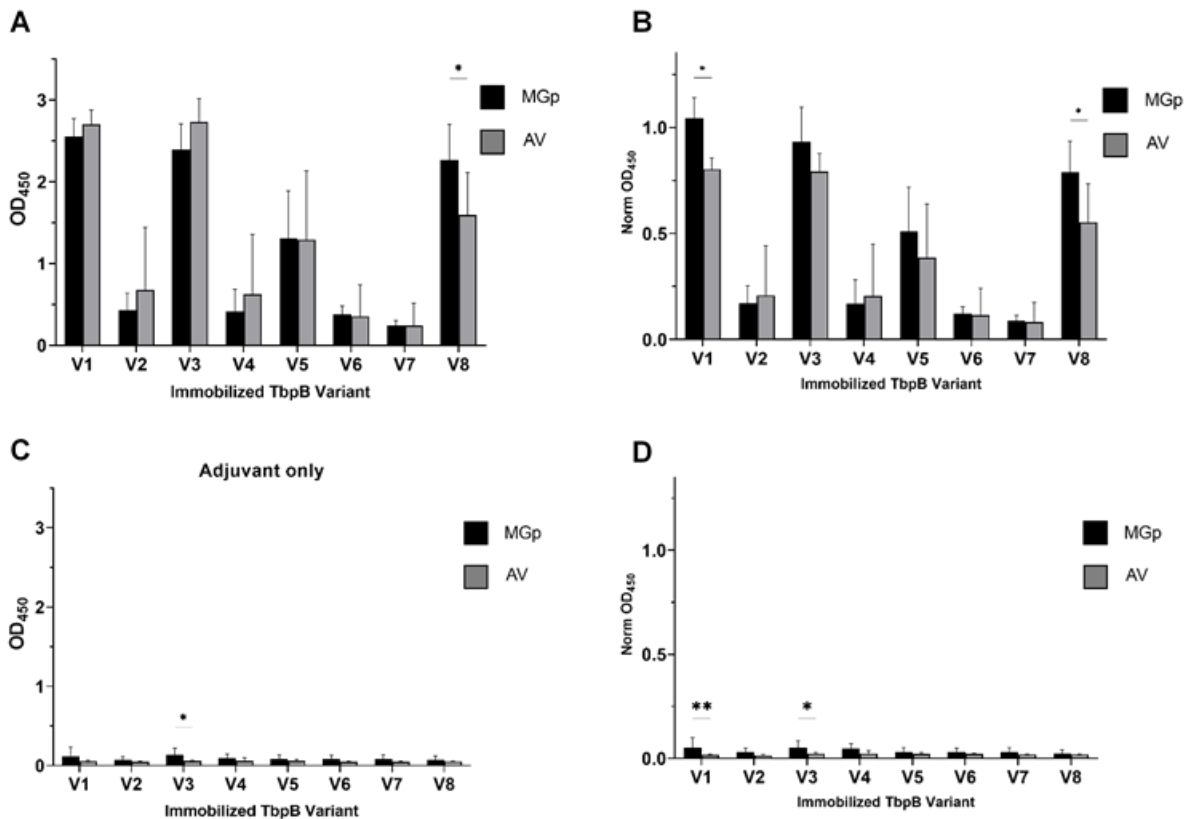
In this study, we demonstrated that immunizations with Y167A TbpB led to cross-reactivity (Figure 2.4) and cross-protection (Figure 2.2) against *A. pleuropneumoniae* serovar 1, a strain expressing cluster 1 TbpB, in mice. We then chose to explore the breadth of cross-reactivity exhibited by anti Y167A TbpB sera against seven variant WT TbpBs representing the existing TbpB diversity as well as the homologous WT TbpB (8 TbpB variants total, Table 3). It was recently demonstrated that different adjuvant formulations of Y167A TbpB led to different levels of protection upon homologous challenge by *G. parasuis* strain 174 in pigs (R. Frandoloso et al., 2020) thus mice were immunized with Y167A TbpB formulated with two different adjuvants, and the resulting sera were tested for their reactivity against multiple TbpB variants.

The overall cross-reactivity pattern was similar between the two adjuvanted vaccines (Figure 2.6A-B). In both groups, we observed anti-Y167A TbpB IgG react strongly against V1, V3, V5, and V8, as shown in Table 2.3. V3 is the homologous WT TbpB (cluster 3) and V1 is the cluster 1 WT TbpB from *A. pleuropneumoniae* serovar 1, our challenge strain from this study. V5 (*G. parasuis*) and V8 (*A. pleuropneumoniae*) are both TbpBs from cluster 3. V2 (*A. pleuropneumoniae*) and V4 (*A. pleuropneumoniae*) are TbpBs from cluster 2 and V6 (*A. suis*) and V7 (*A. suis*) are TbpBs from cluster 1. Our results indicate that the highest level of cross-reactivity is observed within cluster 3 TbpBs, with the exception of TbpB from *A. pleuropneumoniae* serovar 1, which is from cluster 1.

There were no significant differences in the cross-reactivity titres and patterns between the two adjuvanted vaccine groups (Figure 2.6A-B). We observed slightly higher raw IgG titres generated by Montanide Gel 01 plus poly I:C adjuvanted immunizations reacting against V8 as well as slightly higher normalized IgG titres against both V1 and V8. In both cases, the differences were statistically significant. There was a tendency for AddaVax-adjuvanted Y167A TbpB to elicit more variable titres between individual mice in that group. It is unknown at this time how biologically

relevant those differences are. No significant background in either adjuvant-only groups was observed, as expected (Figure 2.6C-D).

Summarized results in Table 2.3 demonstrate that while cross-reactivity can be predicted by percent identity, V1 clearly deviates from that pattern, suggesting that specific epitopes may be important. Figure 2.6 highlights existing gaps in cross-reactivity between different clusters, which may be an indication that additional non-binding TbpB mutants from those clusters need to be included in the vaccine formulation in order to provide broad-spectrum coverage.



**Figure 2.6.** Analysis of anti-Y167A TbpB elicited antibodies to cross-react with variant WT TbpBs representing existing diversity. Sera was diluted 1:10,000 and added to wells coated with variant WT TbpBs as shown in Table 1. **A.** compares cross-reactivity profile of anti-Y167A TbpB antibodies elicited using raw absorbance values at OD<sub>450</sub> between

Montanide Gel 01 + poly I:C and addaVax adjuvanted formulations. **B.** compares cross-reactivity profile of anti-Y167A TbpB antibodies elicited using absorbance values at OD<sub>450</sub> normalized to binding by pTf -HRP between Montanide Gel 01 + poly I:C and addaVax adjuvanted formulations. **C-D** show background level response elicited using the Montanide Gel 01 + poly I:C and addaVax adjuvants alone. **C.** shows the raw OD<sub>450</sub> values. **D.** shows the normalized OD<sub>450</sub> values. Immunoglobulin mean titres between the two adjuvants for each variant was compared using two-way ANOVA followed by Sidak's multiple comparisons test to determine significance (\* $p \leq 0.05$ , \*\* $p \leq 0.01$ ). Sera was assessed in duplicate. MGp – Montanide Gel 01 + poly I:C, AV - AddaVax

## 2.5 Discussion

Currently available commercial vaccines against *A. pleuropneumoniae* are either inactivated whole cell bacterins, subunit/toxoid vaccines, or a combination (Sassu et al., 2018). Whole cell vaccines targeting specific serovars demonstrate limited cross-serovar protection as most of the immune response is directed at the capsule. Commercially available subunit vaccines consisting of combinations of the Apx toxoids and outer membrane proteins are believed to exhibit some degree of cross-protection against most serovars; however, the validity of this claim and applicability to real-world scenarios is difficult to ascertain from existing literature (Loera-Muro & Angulo, 2018). Porcilis App is a commercial vaccine by MSD Animal Health containing inactivated Apx toxins I–III, an outer membrane protein, and the adjuvant alpha-tocopherol acetate, and is widely used in Europe (Sassu et al., 2018). Multiple studies investigating vaccine efficacy in the field have reported that while the vaccine decreased the prevalence of pleuritis and pneumonia associated with *A. pleuropneumoniae*, it did not prevent *A. pleuropneumoniae* natural infection or transmission (Del Pozo Sacristan et al., 2014; Sjolund & Wallgren, 2010). Likewise, other available vaccines against *A. pleuropneumoniae* have been able to decrease clinical symptoms and mortality, to varying extents, but none have been completely successful in preventing development of disease (Loera-Muro & Angulo, 2018), highlighting the need for developing vaccines that can fully protect against pleuropneumonia.

The motivation for targeting the transferrin receptor, used for iron acquisition, for development of a vaccine against *A. pleuropneumoniae* is based on the observation that they are essential for disease causation in a porcine infection model using *A. pleuropneumoniae* (Baltes et al., 2002) and present in all strains tested (Curran et al., 2015). These receptors are also proposed to be essential for survival for two additional swine pathogens, *G. parasuis* and *A. suis*, in their sole ecological niche, the mucosal surface of the porcine respiratory tract.

TbpB-based vaccines have been under development for the important human pathogen *Neisseria meningitidis*. After initial promising results demonstrating protection against meningococcal disease by a TbpB-based vaccine in mice and rabbits (Danve et al., 1993), a Phase 1 clinical trial in humans produced disappointing results (Danve et al., 1998). Studies were subsequently initiated to use a structure-based approach for designing non-binding TbpB mutants to determine whether specific binding by host transferrin to the WT TbpB vaccine antigen during the immunization process interfered with the immune response elicited by the vaccine.

It was demonstrated that immunizing pigs with Y167A TbpB, a cluster 3 non-binding TbpB mutant derived from *G. parasuis* strain 174, resulted in superior protection against disease compared to pigs immunized with the WT TbpB (R. Frandoloso et al., 2015). We later demonstrated that immunization with Y167A TbpB provided substantial protection against challenge with *G. parasuis* however, none of the pigs immunized with W176A TbpB, a different binding defective TbpB mutant, survived till the end of this challenge experiment, demonstrating that not all non-binding TbpB mutants are equally efficacious vaccine candidates (Guizzo et al., 2018). We were unable to identify the mechanism by which one binding defective mutant was a better vaccine candidate than the other and thus proposed that multiple mutants need to be assessed in a pig model to answer that question.

The findings of this study show that immunizations with W176A TbpB did not result in protection, similar to what had been previously observed with *G. parasuis* immunization and challenge experiments in pigs (Guizzo et al., 2018), suggesting that it is possible to recapitulate protection levels previously observed in the native host using

our mouse model. No differences in systemic IgG titres were observed between groups immunized with Y167A TbpB and W176A TbpB – a finding that was consistent with previous observations in pigs (Guizzo et al., 2018). Our results indicate that an immunization and challenge mouse model can be used to assess multiple TbpB mutants as potential vaccine candidates. Experiments with mice are considerably less expensive than pig experiments; thus, it may be beneficial to initially use the mouse model to eliminate less effective vaccine candidates and only assess the most promising candidates in pigs.

In this study, we demonstrated for the first time that mice immunized with subunit vaccine Y167A TbpB, derived from *G. parasuis* strain 174, protected against a lethal challenge by *A. pleuropneumoniae* serovar 1, adding to existing data supporting the efficacy of Y167A TbpB as a cross-protective vaccine antigen. To the best of our knowledge, we believe this is the first evidence of a subunit vaccine that has shown protection against challenge by both *G. parasuis* and *A. pleuropneumoniae*. The TbpBs from *G. parasuis* strain 174 and *A. pleuropneumoniae* serovar 1 share approximately 50% amino acid sequence identity (Table 2.3), suggesting that sequence identity at least partially correlates with cross-protection. Accordingly, we saw high systemic IgG antibody titres against both homologous and heterologous TbpBs.

Although only 50% of mice with Y167A TbpB were protected against challenge, the severity and rapidity of this model makes this protection notable. Mice developed severe symptoms within the first two hours after challenge and succumbed to disease by nine hours post-challenge. Bacteremia was observed in intracardiac bleeds from all deceased mice, indicating disease from systemic bacterial replication. We hypothesized that Y167A TbpB could elicit cross-protective antibodies that target the TbpB expressed by *A. pleuropneumoniae* serovar 1 challenge strain, and our results provide preliminary evidence supporting this hypothesis.

In addition to bacteremia, release of hemolytic and cytolytic toxins are likely to play a role in pathogenesis of disease. *A. pleuropneumoniae* strains are known to secrete combinations of four different RTX (repeats in toxin) exoproteins, ApXI – IV (Frey, 1995). Serovar 1 strains – such as the challenge strain used in this study – are typically

associated with secretion of ApxI, ApxII, and ApxIV. Assessments of virulence of multiple *A. pleuropneumoniae* strains based on their serovar designation found high mortality in mice that were challenged with serovar 1 strains intraperitoneally within 12 hours (Komal & Mittal, 1990). The authors noted that observed virulence patterns between various serovars appeared to correlate with the toxin profile associated with the particular serovar.

Bacteremia and secretion of toxins in infected mice within the first few hours of challenge likely results in a scenario where only a strong and rapid vaccine-induced immune response is able to control the infection, allowing mice to recover. Further analysis investigating the role of antibody-mediated mechanisms of action like opsonophagocytosis and complement-mediated killing could deepen our understanding of the protection observed. These mechanisms are likely relevant in rapidly controlling the spread of bacteria after challenge.

Immunizations with a live attenuated *A. pleuropneumoniae*-based vaccine resulted in 80% and 90% survival when challenged by *G. parasuis* strains MD0322 and SH0165, respectively (S. Fu et al., 2013). Immunizations with a bacterin derived from *G. parasuis* strain 174 was able to protect against challenge by *A. pleuropneumoniae* serovar 1 (50% survival), demonstrating that taken together, some cross-protection is attainable using whole cell-based vaccines. However, in many situations, immunizations with a bacterin have not resulted in cross-serovar protection (R. Frandoloso et al., 2015; Sassu et al., 2018); therefore, while warranting further investigation, it is unlikely that this protection will be broadly cross-protective.

We wanted to assess whether the mechanism of protection of nutritional immunity played a role in protection. Although nutritional immunity may not be a mechanism of protection in this specific model due to the rapidity of infection, it may contribute to protection in the native host. Early experiments investigating the importance of the bacterial transferrin receptor found that *tbpb*-, *tbpA*-, and *tbpba*-deficient *A. pleuropneumoniae* strains were unable to cause disease in pigs (Baltes et al., 2002). Recently, it was shown that immunizations of pigs with Y167A TbpB prevented natural colonization by *G. parasuis* (R. Frandoloso et al., 2020). If antibodies elicited by the

immunizing antigen interfere with binding of TbpB to porcine transferrin, which is likely to be present in low quantities on mucosal surfaces of respiratory tissues, then these antibodies could starve the bacteria of an essential micronutrient by preventing iron acquisition. When we assessed whether elicited antibodies were able to block binding of porcine transferrin conjugated to horseradish peroxidase (HRP) to immobilized WT TbpB, we observed that both mutants were able to generate blocking antibodies. However, the lack of protection observed in pigs immunized with W176A TbpB despite eliciting blocking antibodies suggest that this is not the dominant mechanism of protection.

Cross-reactivity is a critical first step towards and acts as a surrogate for cross-protection. Phylogenetic analysis of sequence diversity of TbpB proteins from *G. parasuis*, *A. pleuropneumoniae*, and *A. suis* demonstrated that these sequences group into three main clusters with cluster 3 containing the most sequences and greatest amount of diversity (Curran et al., 2015; Guizzo et al., 2018). This clustering is independent of species, geographical region, and time of isolation, making it logical to posit that a single, rationally designed TbpB-based vaccine can target all three species. This hypothesis is further supported by the finding that immunizations with wild-type TbpB from *A. pleuropneumoniae* elicited antiserum that demonstrated high cross-reactivity levels against a TbpB variant from the same phylogenetic cluster (Curran et al., 2015). Additionally recent analysis of anti-Y167A TbpB porcine serum also demonstrated cross-reactivity against all three species, however, also limited to cluster 3 TbpBs (J.E. Fegan et al., 2021b).

The cross-reactivity of the response elicited by immunizations with Y167A TbpB formulated with two different adjuvants was assessed in this study. WT TbpBs from the three clusters and the three porcine pathogens were used in this analysis to determine whether there were any gaps in Y167A TbpB-generated coverage. In both adjuvanted formulations, anti-Y167A TbpB IgG antibodies reacted strongly against WT TbpB variants from cluster 3 and the cluster 1 WT TbpB from *A. pleuropneumoniae* serovar 1, the challenge strain used in this study. Less reactivity was observed against other WT TbpBs from cluster 1 as well as cluster 2 WT TbpBs.

Taken together, the cross-reactivity response appears to be independent of species and mostly dependent on protein sequence identity and clustering patterns. Breaking from this pattern, however, is the cluster 1 challenge strain WT TbpB, which shares only 50% identity with Y167A TbpB, and yet, immunization with Y167A TbpB resulted in high levels of cross-reactivity against the challenge WT TbpB. In this particular instance, it is possible that there is a specific dominant epitope shared by both TbpB variants which has yet to be identified and characterized. Adjuvants can play a key role in modulating the immune response; thus, we assessed whether our choice of adjuvant affected the cross-reactivity response. Our analysis did not show any effect of the adjuvant on the cross-reactivity pattern; however, the possibility that increases in cross-reactivity could be triggered by the use of other adjuvants cannot be ruled out.

In summary, our analysis further showcases Y167A TbpB as a promising vaccine candidate and provides preliminary evidence to support investigating this antigen in pig experiments to determine its ability to protect against disease caused by *A. pleuropneumoniae*. Our cross-reactivity analysis shows strong response against TbpBs from cluster 3 and highlights gaps in the other clusters. Additional non-binding mutant TbpBs from clusters 1 and 2 need to be included in the final vaccine formulation to broaden coverage; however, at this point, we are uncertain about the number of antigens required to provide full cross-protection against porcine pathogens expressing TbpBs belonging to all 3 clusters. Our results demonstrate that the mouse model described herein can be used to evaluate non-binding mutant TbpBs as potential vaccine antigens as we observed similar protection trends in our model to those previously observed in the native porcine host (Guizzo et al., 2018). Thus, to address the gaps in cross-protective coverage identified in this study, other non-binding mutants TbpBs from other clusters should be produced and initially assessed in mice to narrow down possible final vaccine compositions, which should then be tested in pigs.

We recently demonstrated that immunizations with two different formulations of Y167A TbpB was able to prevent natural colonization by *G. parasuis* in pigs (R. Frandoloso et al., 2020); however, it has yet to be determined whether immunization of pigs with Y167A TbpB also results in prevention of natural colonization by *A. pleuropneumoniae* serovar 1. Future experiments should therefore also address whether

Y167A TbpB can provide heterologous protection against colonization as well as clinical disease. Taken together, the existing body of work exploring TbpB as a vaccine candidate indicate that it is a potent antigen and provides the exciting potential of a rationally designed TbpB based vaccine capable of eliciting broad cross-protection against all strains of *G. parasuis*, *A. pleuropneumoniae*, and *A. suis*, including prevention of natural colonization.

#### DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

#### ETHICS STATEMENT

The animal study was reviewed and approved by Health Sciences Animal Care Committee, Cumming School of Medicine, University of Calgary.

#### AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: SC and AS. Performed the experiments: SC, LsB, RhY. Wrote the paper: SC, and AS. All authors contributed to the article and approved the submitted version.

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## CHAPTER 3: MANUSCRIPT 2

### **A novel binding-defective TbpB mutant derived from *Actinobacillus pleuropneumoniae* elicits a homologous protective immune response as well as cross-reactive antibodies targeting heterologous variants**

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Key Words: transferrin binding protein, broad-spectrum vaccines, *Glaesserella parasuis*, *Actinobacillus pleuropneumoniae*, *Actinobacillus suis*

### 3.1 Abstract

*Glaesserella parasuis*, *Actinobacillus pleuropneumoniae*, and *Actinobacillus suis* are Gram-negative bacterial pathogens residing in the respiratory tract of pigs. The inadequate protection conferred by existing vaccines has led to disease outbreaks resulting in significant morbidity and mortality within the global pig industry. The bacterial surface lipoprotein transferrin binding protein B (TbpB) has been proposed as an attractive vaccine candidate due to its necessity for pathogen survival and ability to elicit robust protection in animal immunization and challenge experiments. However, as a vaccine antigen, TbpB exhibits considerable sequence diversity across different strains, making it challenging to develop a broadly cross-protective vaccine with a single antigen. Hence, we initiated this study to evaluate the breadth of cross-reactivity elicited with a vaccine formulation composed of a limited number of antigens.

We first designed and evaluated new TbpB mutants, with a reduced ability to bind to porcine transferrin, as prospective vaccine candidates prior to conducting cross-reactivity studies. Surprisingly, Y109A TbpB, which provided the best levels of protection in a murine immunization and challenge model, generated a response that was highly cross-reactive against all TbpB variants tested. Furthermore, a two-antigen vaccine formulation consisting of Y167A TbpB – a highly efficacious vaccine antigen identified in previous studies – and Y109A TbpB elicited even higher titres against the diverse panel of TbpB variants tested than Y109A TbpB alone, overcoming the lack of cross-reactivity previously observed with Y167A TbpB alone. Our results indicate that a vaccine comprised of two mutant TbpBs induces a broadly cross-reactive immune response that could form the basis for a broadly cross-protective vaccine composition.

### 3.2 Introduction

*Glaesserella parasuis*, *Actinobacillus pleuropneumoniae*, and *Actinobacillus suis* are bacterial porcine pathogens responsible for causing infections which lead to significant morbidity and mortality in the global pig industry. *G. parasuis* is the causative agent for Glässer's disease (GD), a systemic disease characterized by polyserositis, polyarthritis, septicemia, and meningitis, and is one of the leading causes of mortality in nursery herds worldwide (del Rio et al., 2006; R. Frandoloso et al., 2011). A.

*pleuropneumoniae* is the causative agent for porcine pleuropneumonia, a contagious respiratory disease associated with impaired animal welfare, high morbidity and mortality, and results in important economic losses in pig production (Klitgaard et al., 2010). *A. suis*, a related pathogen, is an emerging etiological agent of disease that can cause septicemia, pneumonia, and death in healthy herds (Lapointe et al., 2001; J.I. MacInnes & R. Desrosiers, 1999).

While expensive biosecurity measures and vaccines targeting these pathogens have somewhat reduced the overall disease prevalence, the pig industry is still plagued by disease outbreaks. Currently available vaccines simply do not provide complete protection against all variants of these pathogens (Espindola et al., 2018; Lapointe et al., 2001), highlighting the need for more broadly cross-protective vaccines.

These swine pathogens have evolved to reside within the respiratory mucosal space and share a common mechanism for iron acquisition ((Morgenthau et al., 2013a; Ostan et al., 2017). This iron acquisition system is composed of the bipartite bacterial outer membrane surface receptor consisting of transferrin binding proteins A and B (TbpA and TbpB) (Schryvers & Gray-Owen, 1992; Schryvers & Lee, 1993; Schryvers & Morris, 1988), which bind exclusively to porcine transferrin, the host iron-binding protein. This receptor system is essential for pathogen survival and disease causation (Baltes et al., 2002) and thus is present in all disease-causing isolates tested so far (Adamiak et al., 2015; Curran et al., 2015). To overcome the limitations of current vaccines, we investigated TbpB as a potential vaccine candidate.

TbpB-based vaccines had previously been under development for the bacterial pathogen *Neisseria meningitidis*, the causative agent of meningitis in humans – an endeavour that was later abandoned as initial promising results from a TbpB-based vaccine evaluated in mice and rabbits (Danve et al., 1993) were not replicated in Phase 1 of a clinical trial in humans (Danve et al., 1998). The opportunity to explore some of the reasons for the poor performance in the human Phase I trials arose when the first structure of a TbpB protein was determined (Moraes et al., 2009), and since it was from a pathogen in pigs, it provided the ability to readily test in the natural host. Based on the hypothesis that the host protein may be interfering with development of an optimal

protective response, a mutant TbpB protein was generated and tested in a swine immunization and challenge model (R. Frandoloso et al., 2015). It was found that immunizing pigs with Y167A TbpB, a TbpB mutant with defective binding to porcine transferrin, resulted in a more protective response than immunizing with the wildtype TbpB upon challenge by *G. parasuis* Nagasaki strain suggesting that interactions between wildtype TbpB and pTf hampered the immune response, likely by hiding important epitopes from the immune system. These findings also provided strong support for this strategy of generating improved TbpB antigens (R. Frandoloso et al., 2015).

Diversity analysis of the TbpB proteins from *G. parasuis*, *A. pleuropneumoniae*, and *A. suis* revealed that sequences corresponding to each of the three species are interspersed throughout three main clusters, suggesting that the clustering is not correlated with species (Curran et al., 2015; Guizzo et al., 2018) and thus making it highly plausible for a single TbpB-based vaccine formulation to target all three pathogens. It is also suggestive of the occurrence of genetic exchange between the three species, indicating that there may be a reservoir of TbpB variants that have to be targeted all at once in order prevent vaccine escape. We recently demonstrated that anti-Y167A TbpB antibodies display substantial intra-cluster cross-reactivity against TbpBs from the three pathogens; however, we also identified gaps in coverage of the other clusters (unpublished, manuscript 1) suggesting that Y167A TbpB alone is not sufficient in inducing a broadly cross-reactive response. In order to ultimately develop a broadly cross-protective TbpB-based vaccine, additional TbpB binding-defective mutants from other clusters are likely needed.

In this study, we designed and evaluated new TbpB mutants with a reduced ability to bind to porcine transferrin (pTf) derived from a cluster 1 TbpB variant belonging to an *A. pleuropneumoniae* serovar 1 strain as prospective vaccine candidates. We identified one promising TbpB binding defective mutant, Y109A TbpB, which provided partial protection in an immunization and challenge murine model. Unexpectedly, we found that anti-Y109A TbpB sera alone is cross-reactive against all TbpB variants tested, independent of the cluster. Furthermore, we demonstrated that combining Y109A TbpB with Y167A TbpB in a single vaccine formulation elicited a response that was highly cross-reactive against all TbpB variants tested, overcoming the limited cross-reactivity

exhibited by Y167A TbpB alone. Taken together, these results add to an existing body of work investigating TbpB-based vaccines and highlights the exciting possibility of a broad-spectrum vaccine consisting of a limited number of antigens that is capable of targeting all strains of *G. parasuis*, *A. pleuropneumoniae*, and *A. suis*.

### 3.3 Methods

#### *Mutant TbpB design and construction*

The gene sequence encoding the mature TbpB was amplified from *A. pleuropneumoniae* serovar 1 genomic DNA using oligonucleotide primers 4738 and 4739 (Table 3.1) and sequenced. The amino acid sequence of the mature TbpB<sup>(6-574)</sup> protein was found to be 75% identical to the mature sequence of TbpB from *A. suis* h57<sup>(2-574)</sup>, another cluster 1 variant (Curran et al., 2015). Two binding defective TbpB mutants from *A. suis* h57 were previously generated (Calmettes et al., 2011). A pairwise alignment of the *A. suis* h57 TbpB and *A. pleuropneumoniae* serovar 1 TbpB sequences showed that only one of the two amino acids selected for mutagenesis in *A. suis* 57 were conserved in the TbpB from *A. pleuropneumoniae* serovar 1 (Figure 3.1A). Thus, additional amino acids were identified for mutagenesis for generating novel TbpB mutants derived from *A. pleuropneumoniae* serovar 1 that are predicted to be defective in their ability to bind to pTf.

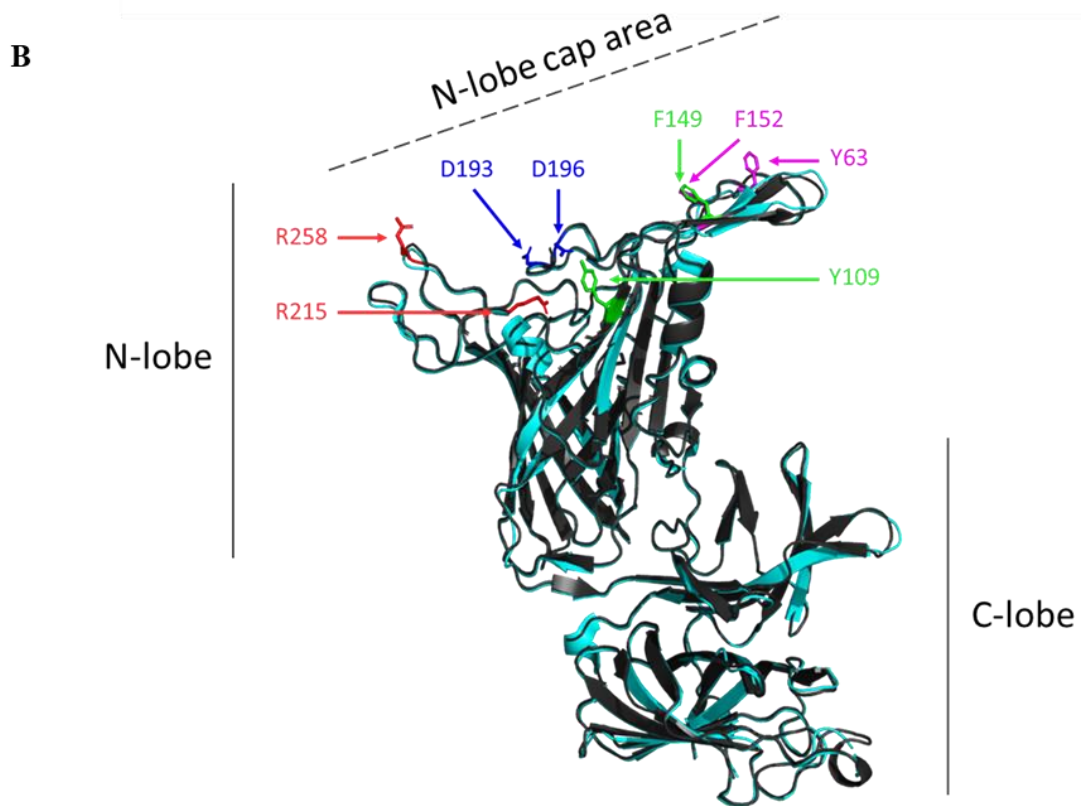
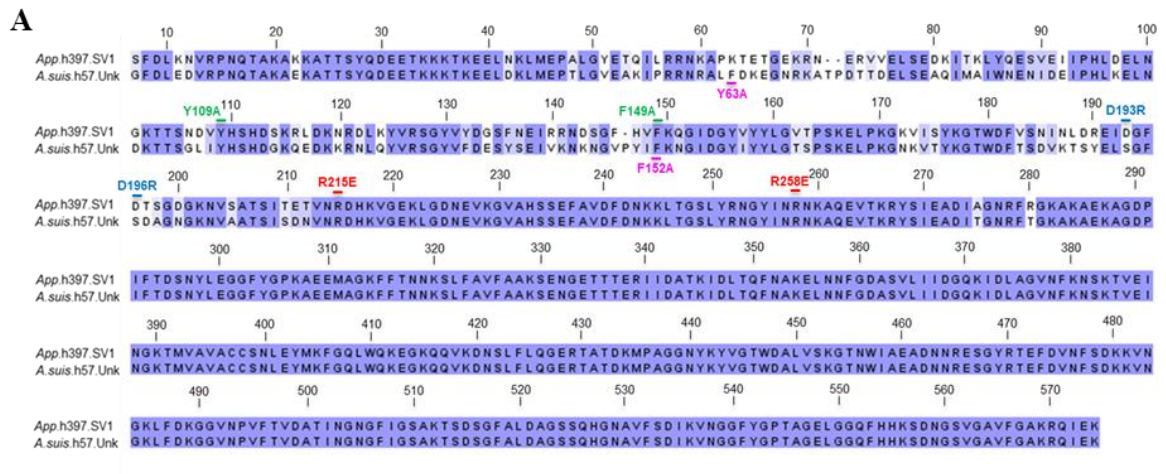
A computational model of *A. pleuropneumoniae* serovar 1 TbpB (Figure 3.1B) was generated based on the crystal structure of TbpB from *A. suis* h57 (PDB 3PQU (Calmettes et al., 2011)) using the online protein structure prediction software RaptorX (Kallberg et al., 2012). Six amino acids (two with aromatic residues, two with positively charged residues, and two with negatively charged residues) from the pTf:TbpB binding interface located in the “cap” area of the N-terminal lobe (N-lobe) were selected for mutagenesis (Figure 3.1B). Oligonucleotide primers (Table 3.1) designed for splicing by overlap extension polymerase chain reaction (SOE-PCR) were used to generate gene fragments with point mutations designed to introduce the desired amino acid substitutions in the recombinant protein. These gene fragments were then cloned into a T7 expression vector containing an N-terminal region encoding a polyhistidine tag, a biotinylation sequence, maltose binding protein, and a tobacco etch virus protease cleavage site

(HBMT). Plasmids were sequenced to confirm the presence of the desired point mutations.

**Table 3.1.** Table of the mutagenic oligonucleotide primers used in this study.

Primer #	Primer Name	Primer Sequence
4738	5'Fw	TCAGGGATCCTTTGATTTAGAAGATGTCCGGCC
4739	3'Rv	GCAGAAGCTTTTATTTTCTATTTGTCGTTTTGCACC
4841	Y109A Fw	AGCAATGATGTT <i><b>gc</b></i> TCATTCTCACGAT
4840	Y109A Rv	ATCGTGAGAATG <i><b>Agc</b></i> AACATCATTGCT
4843	F149A Fw	GGATTCCATGTT <i><b>gc</b></i> TAAACAGGGTATA
4842	F149A Rv	TATACCCTGTTT <i><b>Agc</b></i> AACATGGAATCC
4845	D193R Fw	GATCGTGAAAT <i><b>Acg</b></i> TGGATTTCGACAC
4844	D193R Rv	GTGTCGAATCC <i><b>Acg</b></i> TATTTTCACGATC
4847	D196R Fw	ATAGATGGATT <i><b>Ccg</b></i> CACTTCAGGTGAT
4846	D196R Rv	ATCACCTGAAGT <i><b>Gcg</b></i> GAATCCATCTAT
4849	R215E Fw	GAAACTGTCAAT <i><b>ga</b></i> AGATCATAAAGTT
4848	R215E Rv	AACTTTATGATC <i><b>Ttc</b></i> ATTGACAGTTTC
4851	R258E Fw	GGTTATATCAAC <i><b>ga</b></i> AAATAAAGCGCAA
4850	R258E Rv	TTGCGCTTTATTT <i><b>Ttc</b></i> GTTGATATAACC

\* Mutations that were introduced into the oligonucleotide primers are in bold. Lower caps indicate the change in the nucleotide sequence introduced to produce the change in amino acid and italics indicate the nucleotide sequence of the mutated amino acid.



**Figure 3.1.** Comparison of *A. suis* h57 TbpB and *A. pleuropneumoniae* serovar 1 TbpB for mutant design. **A.** Pairwise alignment of both TbpB protein sequences with relevant amino acids highlighted in colour and labelled as the intended mutation. Pink – original amino acids selected for generating *A. suis* h57 TbpB binding defective mutants (Calmettes et al., 2011). Remaining highlighted amino acids are those identified for mutagenesis of *A. pleuropneumoniae* serovar 1 TbpB (this study). Green – aromatic to

alanine residue change, blue – negative to positive residue charge reversal, and red – positive to negative residue charge reversal. **B.** Structure of *A. suis* h57 TbpB [PDB 3PQU, (Calmettes et al., 2011)] in black and model of *A. pleuropneumoniae* serovar 1 TbpB in cyan superimposed with relevant amino acid residues on the N-lobe cap area highlighted with same colour scheme as above. Y63 and F152 are located on *A. suis* h57 TbpB and Y109, F149, D193, D196, R215, and R258 are located on *A. pleuropneumoniae* serovar 1 TbpB.

#### *Small scale protein expression and purification*

*Escherichia coli* strain ER2566 transformed with the aforementioned expression plasmids was used to inoculate 10 mL of autoinduction media supplemented with ampicillin (100 µg/mL, Sigma Aldrich, USA), and the resulting cultures were grown overnight for 20 hours at 37 °C with shaking at 200 rpm. The cultures were spun down at 4,000 rpm for 10 minutes at 4°C to pellet the cells. The supernatant was discarded, and the pellet was resuspended in 1 mL of nickel-NTA resuspension buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 10 mM imidazole, pH 8.0). Glass beads (0.1 mm in diameter) were added to the cell pellet suspension, and cells were broken by shaking with a cell disruptor. The resulting homogenate was centrifuged at 13,000 rpm for 20 minutes at 4°C to pellet the cell debris and the beads. The resulting supernatant from the crude cell lysate was collected, and 60 µL of Ni-NTA beads in resuspension buffer (1:1) was added to the samples and allowed to incubate for 1 hour at room temperature (RT) with gentle rotation using a tabletop rotary shaker. The Ni-NTA beads were washed twice with wash buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 20 mM imidazole, pH 8.0) and eluted using 50 µL of elution buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 300 mM imidazole, pH 8.0). Samples were analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in order to assess protein expression levels.

#### *Evaluation of TbpB mutants for binding*

To evaluate whether the TbpB mutants were defective in binding to pTf, a solid phase binding assay was used, as previously described (J.E. Fegan et al., 2021b). Briefly, *E. coli* strain ER2566 cultures expressing the various mutants were grown and used for

small scale protein expression and purification using the aforementioned protocol. 3  $\mu$ L of mutant protein eluate was applied to a nitrocellulose membrane, which was then blocked with 1% skim milk in Tris-buffered saline (TBS) for 15 minutes with shaking at RT. The membrane was then treated with horseradish peroxidase (HRP)-labeled pTf (HRP-pTf, made in house and diluted 1000-fold in 1% skim milk in TBS) and the resulting mixture incubated overnight at 4°C with shaking. The following day, the membrane was washed three times in TBS and developed by treating the membrane with a chloronaphthol/hydrogen peroxide substrate mixture (HRP developing reagent, made in house) for 15 minutes, or until a signal was clearly visible on the membrane. TbpB mutants exhibiting defective binding properties were identified from the lack of signal produced upon development and were carried forward for immunization studies.

#### *Large-scale recombinant antigen production*

The wild-type TbpB and the binding-defective TbpB mutants identified in this study were produced as previously described (R. Frandoloso et al., 2015; Guizzo et al., 2018). In brief, gene sequences encoding the mature TbpB proteins alongside an N-terminal polyhistidine fusion tag were cloned into a T7 expression vector and transformed into competent *E. coli* ER2566 cells. Proteins were expressed by growth in autoinduction media supplemented with ampicillin (100  $\mu$ g/mL) overnight at 37°C with shaking. The following day, the culture was centrifuged at 4,000 rpm for 20 minutes at 4°C to collect the cell pellet, which was then lysed using a mechanical cell disruptor. The homogenate was spun down at 30,000 rpm for 90 minutes at 4°C to separate the soluble cell lysate from the insoluble cell debris, and proteins were isolated using Ni-NTA chromatography. Eluted fractions were analyzed by SDS-PAGE to verify purity of the sample. Samples were dialyzed against Dulbecco's phosphate-buffered saline (DPBS). The final protein samples were concentrated, filter sterilized (0.22  $\mu$ m filter), and stored in aliquots at -20°C until use.

#### *Formalin-treated bacterin formulation*

The formalin-treated *App* bacterin was formulated using a protocol previously described (unpublished, manuscript 1). Briefly, *A. pleuropneumoniae* serovar 1 was grown on a chocolate agar plate (PC55, Dalynn Biological, Canada) overnight in a 37°C

incubator supplemented with 5% CO<sub>2</sub>. The cells were then harvested and used to freshly inoculate 200 mL of pleuropneumonia-like organisms broth (PPLO broth, Difco, USA) supplemented with 2.5 mg/mL D-glucose (Sigma-Aldrich, USA) and 60 µg/mL of nicotinamide adenine dinucleotide (β-NAD, Sigma-Aldrich, USA). Bacteria were grown from an adjusted starting optical density at 600 nm (OD<sub>600</sub>) of 0.1 and were allowed to reach an OD<sub>600</sub> of 0.6, indicating log phase of growth. To determine the number of colony-forming units (CFUs), 1 mL of the culture was removed and plated in ten-fold dilutions. 1 mL of 37.5% formalin solution (Sigma-Aldrich, USA) was added to the culture to attain a final dilution of 0.5% and the resulting mixture incubated at 37°C overnight with gentle rotation at 80 rpm, followed by a second incubation overnight at 4°C. Cells were washed with sterile phosphate-buffered saline (PBS), resuspended in 10 mL sterile DPBS, and stored at 4°C until use. To ensure no viable bacteria were present, a 100-µL sample was plated on chocolate agar and incubated overnight at 37°C with 5% CO<sub>2</sub>.

#### *Vaccine formulation*

Vaccines for the immunization and challenge study were formulated as previously described (R. Frandoloso et al., 2020). Briefly, vaccines were formulated in batches consisting of 50 doses. These 50-dose formulations contained 1.25 mg of antigen (25 µg of protein antigen/dose), 50 µg poly I:C (HMW, Invivogen, USA) and 1 mL (20% v/v) Montanide Gel 01 adjuvant (Seppic, France), and were made up to a final volume of 5 mL with sterile DPBS. The *App* bacterin was also prepared for 50 doses by mixing 50 µg poly I:C and 1 mL (20% v/v) Montanide Gel 01 with 5 X 10<sup>9</sup> CFU of formalin-treated *A. pleuropneumoniae* serovar 1 (1 X 10<sup>8</sup> CFU/dose) and was adjusted to a final volume of 5 mL with sterile DPBS. The adjuvant-only control group was formulated in the same manner but in the absence of any antigen. Vaccine formulations were gently rotated using a tabletop rotary shaker at RT overnight and then stored at 4°C until use.

For the cross-reactivity immunization study, vaccines were formulated with either the Montanide Gel 01 and poly I:C adjuvant mixture (as described above) or the adjuvant AddaVax (Invivogen, USA). The AddaVax vaccine preparations were made fresh for each immunization timepoint and were thus formulated in batches consisting of 16 doses.

These 16-dose batches contained 400 µg of antigen and 800 µL of AddaVax (50% v/v) in a final volume of 1.6 mL (adjusted with sterile DPBS).

#### *Mice immunizations*

For the challenge study, groups of 8 mice (6-week-old C57BL/6, n=4 female, n=4 male) were subcutaneously immunized with a 100-µL dose (either of WT TbpB, TbpB mutants, *App* bacterin, or adjuvant alone) three times, spaced 21 days apart, on Day 0 (D0), Day 21 (D21), and Day 42 (D42). At each immunization timepoint and on Day 49 (D49), 7 days after the last immunization, 100 µL of blood was collected via the tail vein from each mouse in order to assess systemic antibody titres.

For the cross-reactivity immunization study, groups of 8 mice (6-week-old C57/B6, male) were subcutaneously immunized with a 100-µL dose of either the Montanide Gel 01 plus poly I:C or AddaVax-adjuvanted Y109A TbpB alone or Y167A TbpB plus Y109A TbpB in combination. Mice were immunized on D0, D21, and D42 as described above. On Day 56 (D56), 14 days after the last immunization, mice were euthanized using CO<sub>2</sub> asphyxiation, and intracardiac bleeds were performed to collect antiserum for cross-reactivity analysis.

#### *Bacterial challenge using A. pleuropneumoniae*

*A. pleuropneumoniae* serovar 1 was first grown on a chocolate agar plate overnight as described above. The cells were harvested and used to inoculate 15 mL of supplemented PPLO broth at a starting OD<sub>600</sub> of 0.1. To induce iron starvation and promote pTf receptor expression (not shown), filter-sterilized deferoxamine mesylate (Desferal, Sigma-Aldrich, USA) was added to a final concentration of 100 µM. The bacterial culture was allowed to reach OD<sub>600</sub> of 0.6, indicating log phase of growth, before cells were washed three times in sterile PBS. Cells were resuspended in sterile DPBS and diluted to an OD<sub>600</sub> of 0.2, from which 100-µL bacterial challenge doses were prepared, each consisting of 1.2 X 10<sup>6</sup> CFU of *A. pleuropneumoniae* serovar 1. 100-µL aliquots consisting of 4 mg of filter-sterilized, iron-loaded pTf (Life Technologies-Gibco, USA) in DPBS were prepared in order to provide an iron source for the bacteria during challenge. Immunized mice were infected via an intraperitoneal injection with a bacterial challenge dose administered alongside a dose of iron-loaded pTf. The mice were

monitored for clinical symptoms (i.e. rough fur, low activity) for 48 hours, after which the experiment was concluded (experimental endpoint). If mice displayed severe symptoms during the monitoring period, they were euthanized using CO<sub>2</sub> asphyxiation (humane endpoint). Blood samples collected from intracardiac bleeds were plated on chocolate agar plates and incubated overnight in 37°C with 5% CO<sub>2</sub> to detect the presence of bacteremia.

#### *Protein production for indirect ELISAs*

Wild-type TbpB variants (V1-V8, unpublished, manuscript 1) were produced using a protocol described previously (J.E. Fegan et al., 2021b). Briefly, eight plasmids encoding WT TbpB variants (V1- V8) alongside an N-terminal HBMF fusion construct were transformed into competent *E. coli* ER2566 cells for small-scale protein expression using the protocol described above, up until the cell lysis step. Crude lysate supernatant containing recombinant proteins overexpressed in the cytoplasm of the expression strain was collected and diluted 5-fold in cold PBST (PBS with 0.05% Tween 20, pH 7.4).

#### *Indirect ELISA - IgG titre analysis*

Antiserum collected from blood samples harvested at D0, D21, D42, and D49 was used to quantify elicited antibody titres against immobilized WT TbpB from *A. pleuropneumoniae* serovar 1, as described previously (J.E. Fegan et al., 2021b). In brief, preparations of 100 µL of crude cell lysate in PBST, containing biotinylated proteins, were added to 96-well ELISA plates pre-coated with streptavidin (ThermoFisher, USA) (J.E. Fegan et al., 2021b). Following the addition of lysate, plates were incubated at RT for 1 hour (hr). Plates were then washed three times with 250 µL of PBST, blocked with 250 µL of 5% skim milk in PBST, incubated for 1 hr at RT, then washed three more times with PBST. 100 µL of antisera in 2.5% skim milk in PBST was added to the wells in 2-fold dilutions (starting dilution: 1/200). The plates were then incubated for 1 hr at RT and subsequently washed. 100 µL of goat anti-mouse IgG secondary antibody (conjugated to HRP) (Sigma Aldrich, USA) diluted 1/7500 in 2.5% skim milk in PBST was added to each well and the plates incubated for 1 hr at RT and subsequently washed. Plates were then developed with 50 µL of 3, 3',5,5'-tetramethylbenzidine (TMB) reagent (Sigma Aldrich, USA) for 20 minutes in the dark. Development was quenched with

25  $\mu$ L of 4 N HCl and the plates analyzed by obtaining absorbance readings at 450 nm (optical density at 450 nm, OD<sub>450</sub>) using a plate reader.

The results were described as mean endpoint antibody titres, defined as the reciprocal of the highest dilution giving a positive absorbance reading (R. Frandoloso et al., 2020). A reading was considered positive if it was at least two times greater than the mean OD<sub>450</sub> values of the negative controls (lysate only and lysate plus secondary antibody). Samples for D0, D21, and D49 were assessed once and samples from D49 were assessed in two independent replicates.

#### *Indirect ELISA - pTf blocking analysis*

Serum samples from D49 were used to assess whether antibodies elicited could bind to epitopes on the *A. pleuropneumoniae* serovar 1 WT TbpB that overlap with the pTf:TbpB binding interface, thereby enabling the antibodies to block binding by pTf, which is hypothesized to be a possible mechanism of protection against infection in the natural host. Plates were prepared with WT TbpB, blocked, and washed as described above. 100  $\mu$ L of antiserum samples diluted 4-fold in 2.5% skim milk in PBST were added to wells and the plates incubated for 1 hr at RT and subsequently washed. 100  $\mu$ L of HRP-labelled pTf diluted 1000-fold in 2.5% skim milk in PBST were added to wells and the plates incubated for 1 hr at RT, washed, and developed as described above. If the interaction between antiserum samples and WT TbpB blocked binding by pTf-HRP, then a lack of signal would be observed. Results were depicted as OD<sub>450</sub> absorbance readings, and samples were assessed in duplicate.

#### *Phylogenetic analysis*

An updated phylogenetic tree depicting the sequence diversity among mature TbpB amino acid sequences from *G. parasuis*, *A. pleuropneumoniae*, and *A. suis* was generated in order to include all the TbpB variants tested in the cross-reactivity analysis. Sequencing of *tbpB* genes was performed as previously described (Curran et al., 2015; Guizzo et al., 2018). Complete TbpB sequences were manually assembled from the forward, internal, and reverse reads, and sequences corresponding to the mature TbpB were identified using SignalP 4.0 (<http://www.cbs.dtu.dk/services/SignalP/>) (Almagro Armenteros et al., 2019). Alignments produced using MAFFT v7

(<https://mafft.cbrc.jp/alignment/server/>) (Katoh, Rozewicki, & Yamada, 2019) were used to generate a maximum likelihood phylogenetic tree using PhyML (Guindon et al., 2010), employing the WAG substitution model (Whelan & Goldman, 2001) with 100 bootstraps to evaluate branch support. Tree visualizations and annotations were performed using FigTree v1.4.2 and Microsoft PowerPoint.

#### *Indirect ELISA - cross-reactivity analysis*

Cross-reactivity of elicited antibodies was assessed against WT TbpBs from *G. parasuis*, *A. pleuropneumoniae*, and *A. suis* (V1-V8). Serum samples were collected from intracardiac bleeds from mice immunized with Y167A TbpB plus Y109A TbpB or just Y109A TbpB alone, adjuvanted with either Montanide Gel 01 plus poly I:C or AddaVax, to investigate the effect of different formulations on cross-reactivity. Streptavidin-coated ELISA plates were coated with WT TbpBs, produced as described above, derived from eight variants (V1-V8). Plates were washed, blocked, and 100  $\mu$ L of antiserum samples diluted 1/10,000 in 2.5% skim milk in PBST was added to wells and the plates incubated for 1 hr at RT and subsequently washed. Secondary antibody solution was prepared and added, and plates were developed as described above. Samples were assessed in duplicate, and the mean OD<sub>450</sub> values were plotted.

#### *Statistical analysis*

All data were analyzed using GraphPad Prism™ V9 (GraphPad Software, San Diego, California, USA). The comparative survival analysis was performed using log rank Mantel Cox curve analysis and Bonferroni method (Figure 3.3). The mean immunoglobulin titres were examined for significance using Brown Forsythe and Welch one-way analysis of variance (ANOVA) followed by Dunnett's T3 multiple comparisons test (Figures 3.4-3.6). Comparisons of mean cross-reactive immunoglobulin titres between different adjuvants and different vaccine groups against each TbpB variant were performed using two-way ANOVA followed by Sidak's multiple comparisons test (Figure 3.8). Serological results are reported as means  $\pm$  SD, and *p*-values < 0.05 were considered significant.

#### *Statement of Institutional Animal Care*

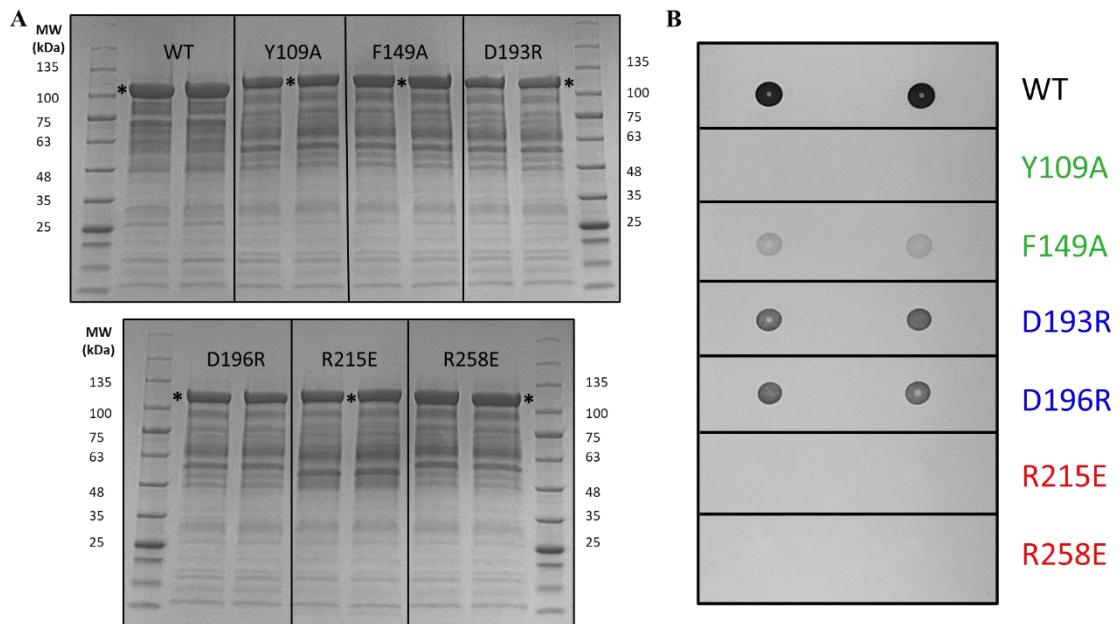
All animal experiments were carried out at the University of Calgary Animal Research centre under approved protocol AC18-0210 and in accordance with all guidelines indicated by the Canadian Council on Animal Care (CCAC).

### *3.4 Results*

#### *Evaluation of binding-defective TbpB mutants*

Amino acids present on the N-lobe cap region, where the transferrin binding interface is localized, were selected for mutagenesis in order to produce prospective binding-defective TbpB mutants (Figure 3.1B). Two such mutations resulted in changing amino acids with aromatic residues to alanine, two mutations resulted in negative to positive residue charge reversals, and two mutations resulted in positive to negative residue charge reversals. These novel TbpB mutants were expressed in the cytoplasm of *E. coli* and analyzed using SDS-PAGE to assess for soluble protein expression, which revealed that the WT and all six mutant TbpBs were expressed as soluble proteins (Figure 3.2A). In order to determine whether any of the mutants exhibited a reduced ability to bind to pTf, a solid-phase binding assay using HRP-labelled pTf was used.

Three of the TbpB mutants, Y109A TbpB, R215E TbpB, and R258E TbpB, produced no signal upon development of the binding assay, suggesting that binding to porcine transferrin was either completely abrogated or reduced significantly (Figure 3.2B). F152A TbpB, another mutant that was evaluated, appeared to have reduced binding, indicated by the faint dot observed. Neither of the negative to positive residue charge reversals had any effect on binding of TbpB to pTf. TbpB mutants (Y109A TbpB, R215E TbpB, and R258E TbpB) that exhibited no detectable binding to pTf were carried forward in the immunization and challenge experiment in mice.



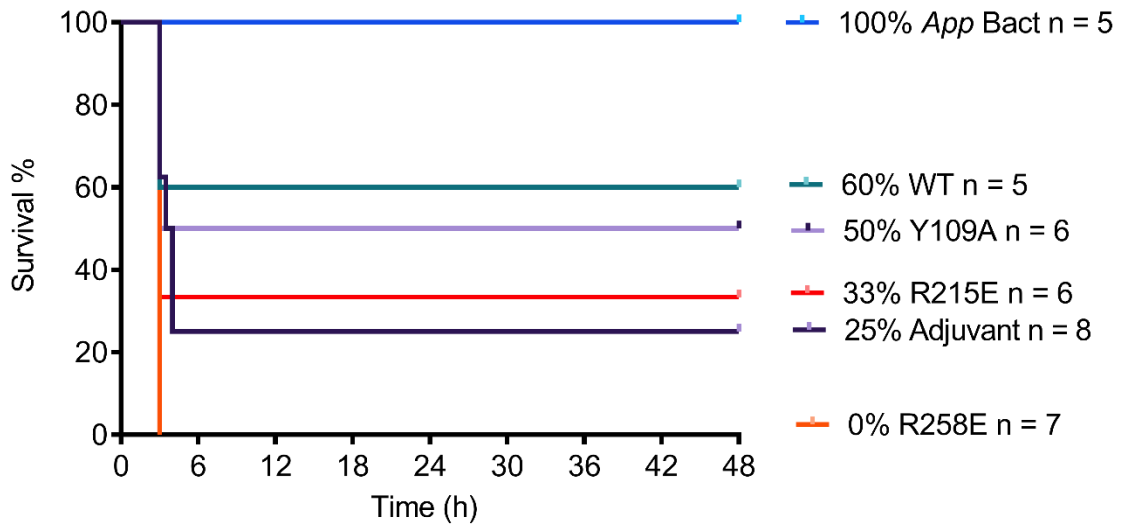
**Figure 3.2.** Evaluation of novel TbpB mutants from *A. pleuropneumoniae* serovar 1. **A.** SDS-PAGE showing protein expression of WT TbpB and six mutant TbpBs (with N-terminal HBMT fusion tag), with each protein sample analyzed in duplicate in adjacent lanes. Asterisks are placed by bands indicating proteins of interest (~112 kDa total, 64 kDa for TbpB and 48 kDa for fusion tag). **B.** Solid-phase binding assay (dot assay) for evaluating binding of WT and mutant TbpB to pTf-HRP was conducted. Each protein sample is analyzed in duplicate in adjacent columns. TbpB mutants are labelled in the colour scheme used above (green – aromatic to alanine, blue – negative to positive, red-positive to negative) to denote the type of residue substitution. The absence of a dot for Y109A, R215E, and R258E TbpBs indicates a complete abrogation of binding properties.

*Immunization with TbpB antigens from A. pleuropneumoniae partially protect mice from a homologous challenge*

To evaluate the binding-defective TbpB mutants identified in this study as prospective vaccine candidates, mice were immunized with either WT TbpB, Y109A TbpB, R215E TbpB, or R258E TbpB and challenged with a lethal dose of *A. pleuropneumoniae* serovar 1. Groups of mice were immunized with either the adjuvant alone as a negative control or the formalin-treated bacterin derived from *A. pleuropneumoniae* serovar 1 strain (*App* bacterin) as a positive control.

The group immunized with WT TbpB had a 60% (3/5) survival rate during challenge while groups immunized with Y109A TbpB, R215E TbpB, and R258E TbpB had 50% (3/6), 33% (2/6), and 0% (0/7) survival rates, respectively. Mice that received the *App* bacterin were completely protected (100% survival, 5/5) whereas the adjuvant-only group had a 25% (2/8) survival rate. While the differences amongst these results were not statistically significant, we believe the trends observed herein are informative for future vaccine design and evaluation studies. In this study, Y109A TbpB was identified as a binding-defective TbpB mutant that provided the best levels of protection in this challenge experiment employing a rapid-onset murine sepsis model.

Mice that displayed severe clinical symptoms and reached the humane endpoint were euthanized using CO<sub>2</sub> asphyxiation. Intracardiac blood samples from those mice plated on chocolate agar resulted in bacterial growth, indicating the presence of bacteremia (data not shown), which likely contributed to the clinical symptoms of disease observed.

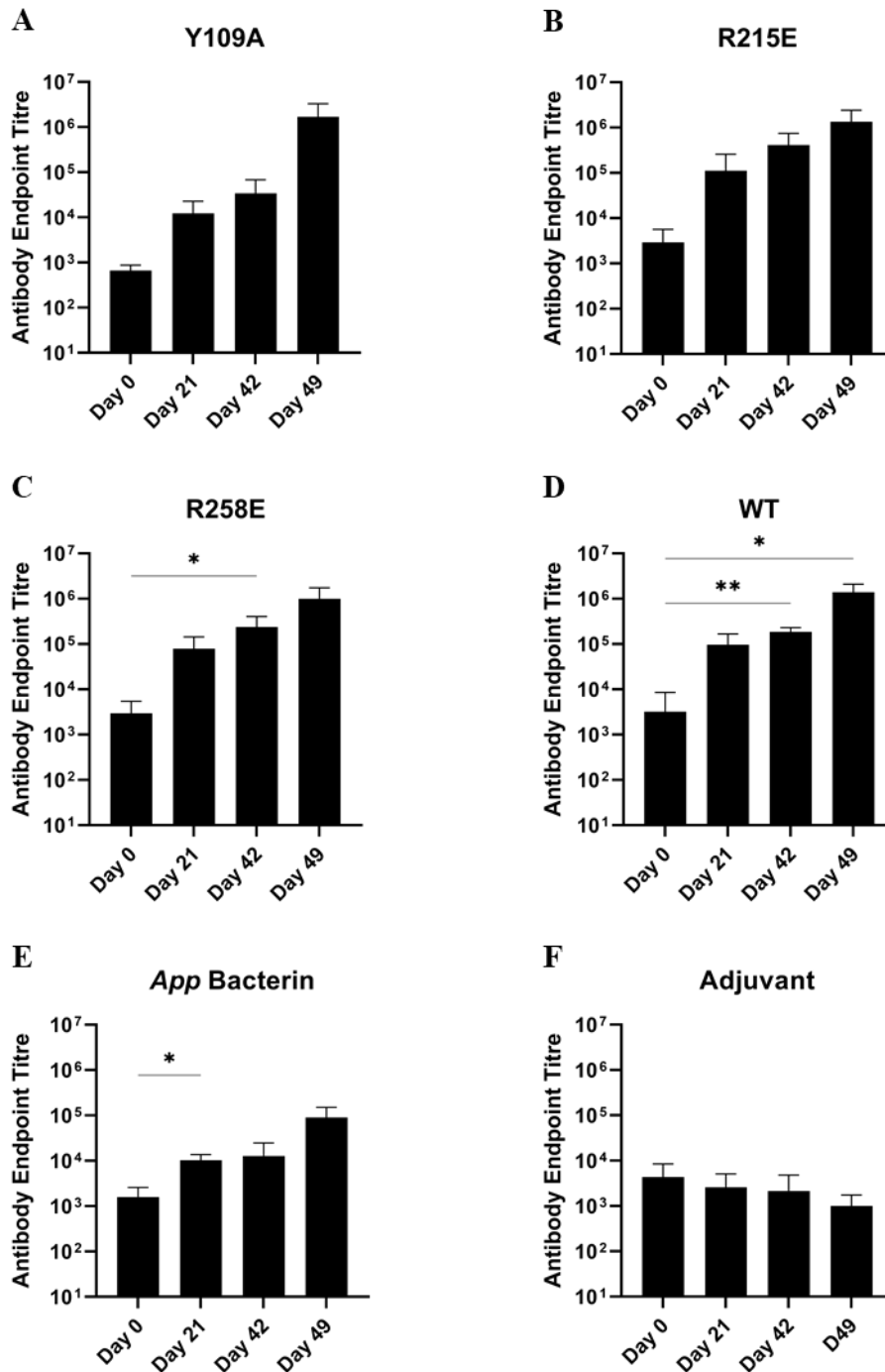


**Figure 3.3.** Survival curve of immunized mice in acute sepsis model. Mice (n= 5-8) were immunized with either WT TbpB, TbpB mutants, *App* bacterin, or adjuvant (Montanide Gel 01 + poly I:C) alone and challenged with  $1.2 \times 10^6$  CFU of iron-starved *A. pleuropneumoniae* serovar 1 alongside a 4-mg dose of iron-loaded pTf supplement and monitored for 48 hours. The comparative survival analysis was performed using log rank Mantel Cox curve analysis and Bonferroni method, and no significance was observed.

### *Immunizations with TbpB antigens elicit high IgG titres*

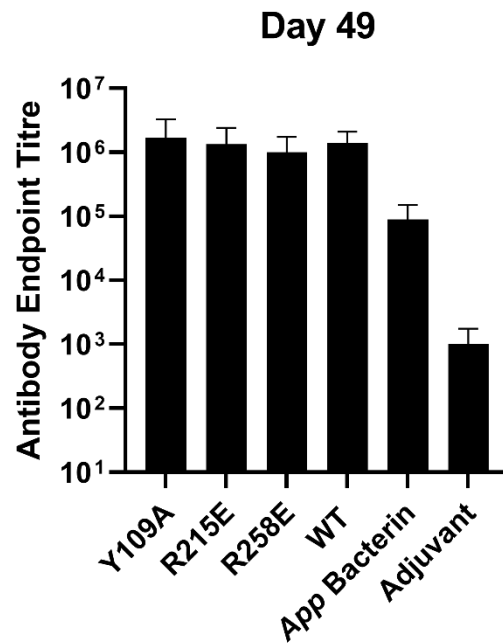
Systemic IgG titres against WT TbpB from *A. pleuropneumoniae* serovar 1 were assessed at D0, prior to the prime dose, and in response to immunizations at three timepoints (D21, D42, D49) (Figure 3.4A-3.4F). Although the adjuvant-only group had low-level background systemic IgG titres prior to immunizations at D0, they remained the same for the duration of the experiment (Figure 3.4F). All other groups also had similarly low levels of background IgG titres at D0. Increases in IgG titres were observed in all groups immunized with the TbpB-based and bacterin vaccine formulations at D21. Further increases in systemic IgG titres against the WT TbpB were observed after each booster shot in all the vaccinated groups (Figure 3.4A-3.4E).

Systemic IgG titres were compared amongst sera sampled at D49, 7 days prior to challenge, to determine if there was a correlation between IgG titres and the protection observed during the bacterial challenge (Figure 3.5). All TbpB-based vaccine groups had similar levels of systemic anti-WT TbpB IgG titres at D49, and the bacterin group had slightly lower IgG titres, which is inconsistent with the pattern of protection observed. It is likely that the functionality of those antibodies and not simply the titre levels is relevant in terms of correlation with protection during challenge.



**Figure 3.4.** Analysis of systemic IgG antibody titres against WT TbpB from *A. pleuropneumoniae* serovar 1 elicited by immunization with different TbpB antigens. A-F show levels of antibody titres from sera collected from each group at different time points during the immunization schedule. For each group (n= 5-8 mice), sera collected during

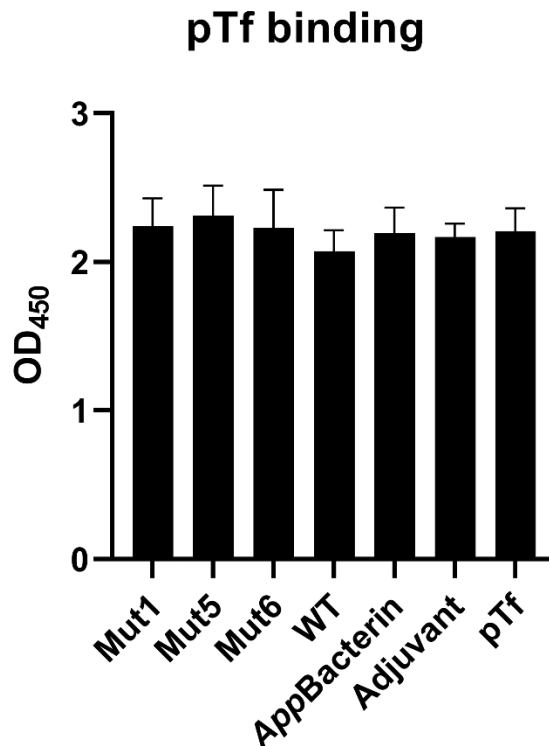
first three timepoints was assessed in 2-fold dilutions once. Sera collected during the last time point (D49) was assessed in duplicate. Significance was determined using the Brown Forsythe and Welch ANOVA followed by a Dunnett's T3 multiple comparisons test comparing each group to each other (\* $p \leq 0.05$ , \*\* $p \leq 0.01$ ).



**Figure 3.5.** Analysis of systemic IgG antibody titres elicited by immunization with different antigens against WT TbpB from *A. pleuropneumoniae* serovar 1 prior to challenge. Figure compares D49 IgG titres from sera collected one week before challenge. Significance was determined using the Brown Forsythe and Welch ANOVA followed by a Dunnett's T3 multiple comparisons test comparing each group to each other.

### *Antibodies elicited by TbpB antigens cannot prevent binding to pTf*

To evaluate whether systemic IgG antibodies elicited by the vaccines had the ability to prevent binding by WT TbpB to pTf, thereby potentially starving the bacteria of iron in the native porcine host, a pTf blocking ELISA was performed. However, none of the formulations were able to elicit IgG antibodies that prevented binding by pTf conjugated to HRP (Figure 3.6), suggesting that the antibodies elicited target epitopes on the N-lobe that do not overlap with the pTf binding interface or they predominantly target epitopes located on the C-terminal lobe (C-lobe) of TbpB. It is also possible that the titre of potential blocking antibodies is not sufficient to overcome the binding of labelled pTf.



**Figure 3.6.** Ability of antibodies to block binding of pTf to WT TbpB from *A. pleuropneumoniae* serovar 1. Sera collected one week before challenge (D49) was diluted 4-fold in a skim milk solution and added to wells coated with immobilized WT TbpB, followed by addition of pTf-HRP at a dilution of 1/1000. Significance was determined using the Brown Forsythe and Welch ANOVA followed by a Dunnett's T3 multiple

comparisons test comparing each group to the control pTf. All experiments were performed in duplicate.

*Anti-Y109A TbpB antibodies are highly cross-reactive*

Mice were immunized with Y109A TbpB formulated with either the Montanide Gel 01 and poly I:C adjuvant combination or AddaVax, and the resulting anti-serum was tested for reactivity against multiple WT TbpB variants. Variants were selected from each phylogenetic cluster to represent the overall diversity of TbpB variants amongst the three porcine pathogen species (Figure 3.7). Unexpectedly, anti-Y109 TbpB sera was strongly cross-reactive against WT TbpBs from all clusters despite there being substantial diversity between the variants (Figure 3.8A). Mice immunized with the Montanide Gel 01 plus poly I:C adjuvant formulation displayed higher reactivity against V1 WT TbpB and V8 WT TbpB compared to the mice immunized with the AddaVax formulation. There were no significant differences observed between the two adjuvanted formulations against the other WT TbpB variants. Interestingly, there appeared to be a trend for the AddaVax-formulated vaccines to result in more variable responses between mice from the same group, consistent with what was observed previously (unpublished, manuscript 1).

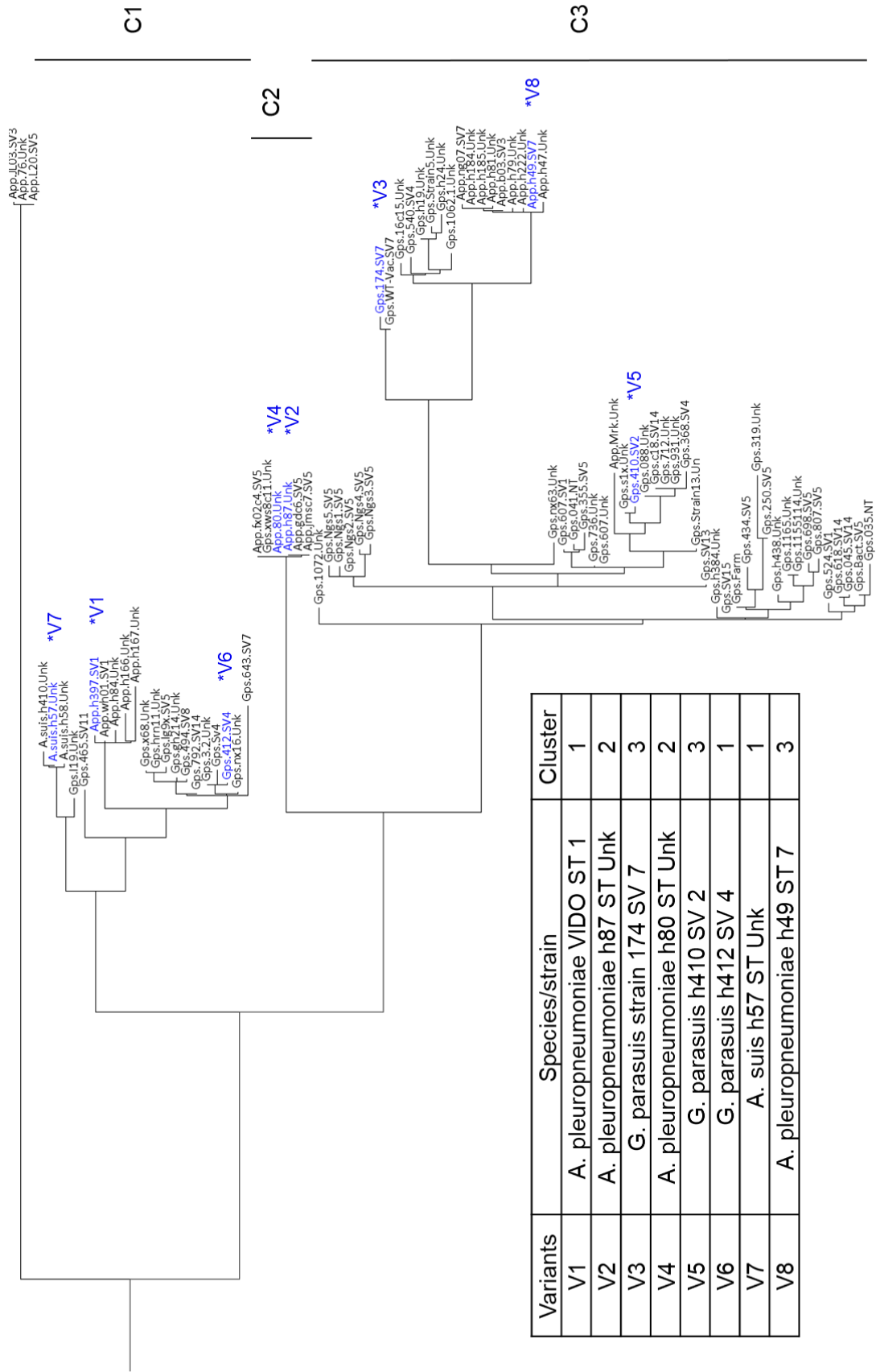
*A combination of anti-Y109A TbpB and anti-Y167A TbpB antibodies is highly cross-reactive*

In a previous study (unpublished, manuscript 1), immunizations with Y167A TbpB generated a cross-reactive response that was mainly restricted to variants belonging to cluster 3. To determine whether the addition of Y109A TbpB to the Y167A TbpB vaccine formulation resulted in eliciting a broader cross-reactive response, mice were immunized with both the Y167A TbpB and Y109A TbpB antigens in a single formulation.

Antiserum from mice immunized with both antigens demonstrated that the addition of Y109A TbpB expanded the cross-reactivity of the immune response compared to what was observed with Y167A TbpB alone (Figure 3.8B, unpublished

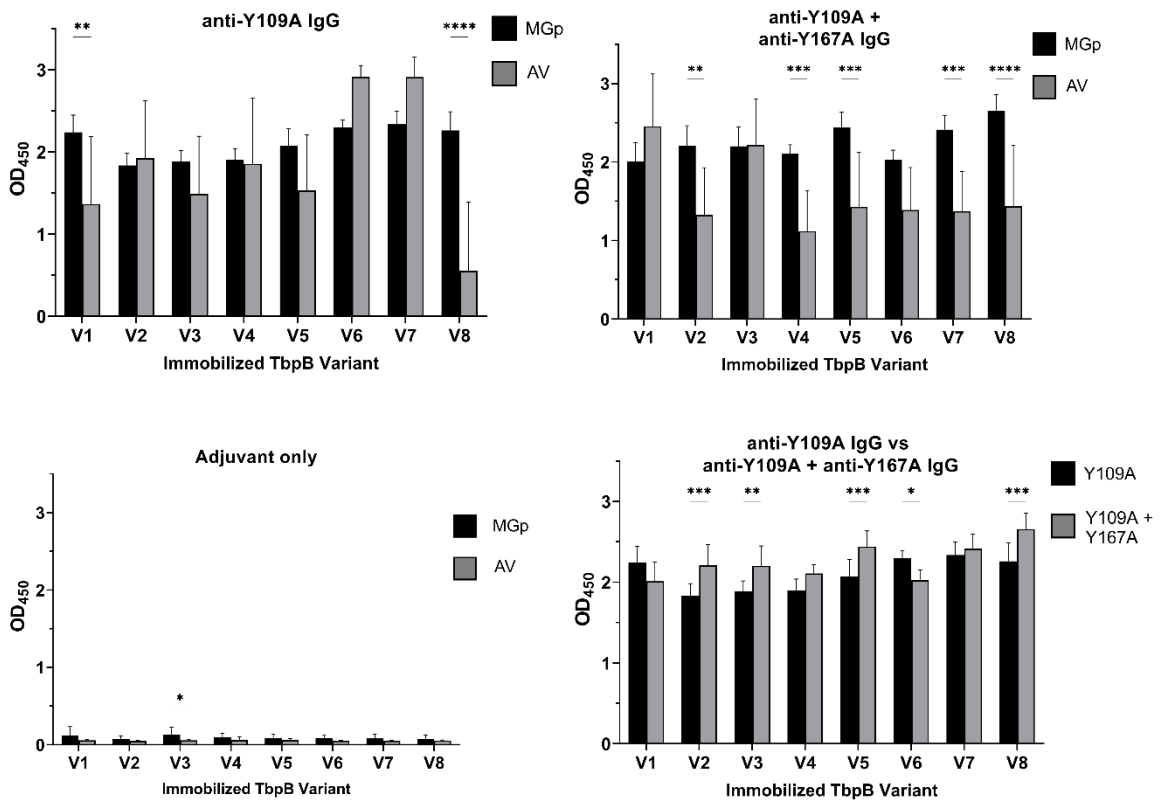
manuscript 1). The improved breadth of cross-reactivity mimics the range seen from immunizations with Y109A TbpB alone (Figure 3.8A). Antigens formulated with Montanide Gel 01 plus poly I:C elicited a stronger response against V2, V4, V5, V7, and V8 WT TbpBs compared to the AddaVax-adjuvanted formulation. The trend of AddaVax-formulated vaccines resulting in more variable responses between mice from within the same group was observed once again. Neither one of the adjuvants elicited any response on their own (Figure 3.8C).

The response elicited by the combined antigen vaccine was directly compared to the response elicited by the Y109A TbpB antigen alone, both of which were formulated with the Montanide Gel 01 and poly I:C adjuvant (Figure 3.8D). The combination formulation elicited a higher response against V2, V3, V5, and V8 WT TbpBs and a lower response against V6 compared to just Y109A TbpB alone. The biological relevance of these differences is unclear at this time.



Variants	Species/strain	Cluster
V1	A. pleuropneumoniae VIDO ST 1	1
V2	A. pleuropneumoniae h87 ST Unk	2
V3	G. parasuis strain 174 SV 7	3
V4	A. pleuropneumoniae h80 ST Unk	2
V5	G. parasuis h410 SV 2	3
V6	G. parasuis h412 SV 4	1
V7	A. suis h57 ST Unk	1
V8	A. pleuropneumoniae h49 ST 7	3

**Figure 3.7.** Sequence diversity among TbpB variants from porcine pathogens. Maximum likelihood tree demonstrating the overall diversity of TbpB variants from *G. parasuis*, *A. pleuropneumoniae* and *A. suis*. Leaf labels identify the strains from which TbpB sequences were obtained and indicate their species and serovar, if known (NT = Non-typeable, Unk = Unknown). Sequences of interest to this study are labelled in blue with the variant number stated beside it. The sequences are distributed throughout three main clusters (C1 = Cluster 1, C2 = Cluster 2, C3 = Cluster 3). The table embedded in the figure provides additional details on the blue sequences, which were used in this study to assess cross-reactivity.



**Figure 3.8.** Analysis of cross-reactivity of elicited antibodies against multiple variant WT TbpBs representing the overall diversity among TbpB variants. Sera was diluted 10,000-fold and added to wells coated with variant WT TbpBs denoted in Figure 3.7. **A.** Cross-reactivity of anti-Y109A TbpB antibodies – expressed as absorbance values at OD<sub>450</sub> in each figure – elicited using either Montanide Gel 01 + poly I:C (MGp) or AddaVax (AV) adjuvant formulations is compared. **B.** Cross-reactivity of anti-Y109A plus anti-Y167A TbpB antibodies elicited by immunization with the combined vaccine using either Montanide Gel 01 + poly I:C (MGp) or AddaVax (AV) adjuvant formulations is compared. **C.** Background level response elicited using either Montanide Gel 01 + poly I:C (MGp) or AddaVax (AV) adjuvants alone. **D.** Cross-reactivity profile of anti-Y109A TbpB antibodies and anti-Y167A plus anti-Y109A TbpB antibodies elicited by just the Montanide Gel 01 + poly I:C adjuvant formulations. Mean immunoglobulin titres elicited by vaccine formulations using either of the two adjuvants (A-C) and single vs. combined vaccine immunizations (D) targeting each variant were compared using two-way ANOVA followed by Sidak’s multiple comparisons test to determine significance (\* $p \leq 0.05$ , \*\* $p \leq 0.01$ ). Each serum sample was assessed in duplicate.

### 3.5 Discussion

Pig farming as a global industry is often burdened with outbreaks caused by bacterial infections, including those caused by bacteria residing in the upper respiratory tract such as *A. pleuropneumoniae*, *A. suis*, and *G. parasuis* (Curran et al., 2015). Although vaccination campaigns have reduced mortality and morbidity, breakthrough infections leading to disease outbreaks occur frequently (Espindola et al., 2019) due to poor vaccine efficacy (Del Pozo Sacristan et al., 2014; R. Frandoloso et al., 2015; Sjolund & Wallgren, 2010) and insufficient cross-protection (Bak & Riising, 2002; Lapointe et al., 2001; Takahashi et al., 2001), indicating the urgent need for more efficacious vaccines.

We evaluated TbpB, a bacterial surface protein involved in iron acquisition from host transferrin, as a prospective vaccine candidate in order to address and overcome the inadequate protection provided by currently available vaccines. TbpBs have long been

explored as vaccine candidates against these porcine pathogens as well as other important pathogens responsible for causing disease in both humans and food production animals from the Pasteurellaceae, Neisseriaceae and Moraxellaceae families. A critical consideration in developing TbpB-based vaccines is overcoming the high degree of diversity amongst TbpB variants expressed by divergent strains within a single species.

TbpB proteins have been found in all isolates of *G. parasuis*, *A. pleuropneumoniae*, and *A. suis* tested so far (Curran et al., 2015; Guizzo et al., 2018), raising the intriguing possibility of being able to target all three pathogens with a single vaccine formulation. Notably, thorough diversity analysis of the TbpB protein sequences from the three porcine pathogens has revealed that taxonomic characterization of these bacteria as three distinct species has no correlation whatsoever with the relative distribution of TbpB sequences among the different phylogenetic clades depicted in the trees constructed as part of these analyses. This is likely indicative of extensive genetic exchange via horizontal gene transfer between these pathogens sharing the same ecological niche. Recently, a novel *Glaesserella* sp. strain was described in Australia as having a mosaic genomic composition consisting of virulence factors typically identified in *A. pleuropneumoniae* and *Pasteurella multocida* (Watt et al., 2018), which the authors believed resulted from horizontal genetic exchange between different disease-causing microorganisms. Taken together, these observations of the prevalence of genetic exchange make it evident that any TbpB-based vaccine has to be designed rationally by taking into consideration the overall diversity amongst species occupying the same ecological niche in order to target all existing variants and prevent vaccine escape from occurring.

Y167A TbpB, a cluster 3 variant, is a promising vaccine antigen that has conferred robust protection in multiple swine immunization and challenge experiments (R. Frandoloso et al., 2020; R. Frandoloso et al., 2015; Guizzo et al., 2018), imparting both homologous (R. Frandoloso et al., 2020; Guizzo et al., 2018) and heterologous protection (R. Frandoloso et al., 2015) against *G. parasuis* strains originating from cluster 3. Although mice immunized with Y167A TbpB were partially protected against an *A. pleuropneumoniae* strain expressing a cluster 1 TbpB (unpublished, manuscript 1), further investigation revealed the existence of gaps in the coverage of multiple cluster 1

and cluster 2 TbpB variants, indicating that an additional TbpB variant is needed in the vaccine composition to induce a broader, more cross-reactive, and ultimately more cross-protective response. This study was initiated to first develop new TbpB binding-defective mutants from *A. pleuropneumoniae* strain expressing a cluster 1 TbpB and then evaluate these prospective vaccine candidates for efficacy and cross-reactivity.

The N-lobe of TbpB interacts with a conserved domain on the C-terminal lobe of transferrin (Silva et al., 2011) to initiate iron acquisition (Calmettes et al., 2011; Moraes et al., 2009; Silva et al., 2011). Therefore, it is reasonable to speculate that residues on the N-lobe critical for binding are conserved across all variants and thus can be easily identified for mutagenesis. However, the majority of the sequence diversity among TbpB variants is located in the N-lobe – specifically, the outward-facing loops, known as the N-lobe cap area (Curran et al., 2015). The cap area contains positively charged pockets which interact with a negatively charged region located on the Tf C-lobe (Calmettes et al., 2011). While the nature of the electrostatic interactions between TbpB and the Tf C-lobe is conserved, comparisons of the positively charged binding interface between three TbpB variants, one from each cluster, revealed that they are distinct, especially the cluster 1 variant (Calmettes et al., 2011). Since TbpB variants utilize different critical amino acids to interact with the Tf C-lobe, it can be challenging to generate binding-defective TbpB mutants from different clusters due to the lack of conserved amino acids to target for mutagenesis. Therefore, we opted to select multiple amino acids in the N-lobe cap area for mutagenesis and then evaluate whether any of the changes resulted in a loss of binding using a solid-phase Tf binding assay.

Taking advantage of existing data pertaining to a cluster 1 TbpB variant, structural and pTf binding analysis of *A. suis* h57 TbpB (Calmettes et al., 2011) was used to guide the generation of novel TbpB mutants. However, we found that only one of two critical aromatic amino acids that were mutated in *A. suis* h57 WT TbpB was conserved in the TbpB variant expressed by *A. pleuropneumoniae* serovar 1 (Figure 3.1A), thereby raising the possibility of even greater diversity present in the binding interface than previously believed and leading us to identify other amino acids in the N-lobe cap area for mutagenesis.

Solvent-facing aromatic residues within the binding interface had been previously shown as being necessary for binding to pTf through mutagenesis and binding assays (Calmettes et al., 2011) and thus were selected for mutagenesis. While both such mutations resulted in a reduced ability to bind to pTf, the F149A TbpB still produced a detectable signal in the pTf binding assay whereas Y109A TbpB showed no binding in the assay (Figure 3.2B). Due to the electrostatic interactions between pTf and TbpB, charge reversals of residues within the cap area were considered. Not surprisingly, both positive to negative residue charge reversals resulted in a loss in binding while neither of the negative to positive charge reversals had any effect. This is consistent with a different positive to negative residue charge reversal mutation (R179E) in the positively charged cap area of the TbpB from cluster 2 *A. pleuropneumoniae* h87 strain resulting in a loss of binding to pTf (Calmettes, Alcantara, Yu, et al., 2012). It is reasonable to speculate that a positive to negative charge reversal in the binding interface likely interferes with electrostatic interactions between the TbpB mutant and pTf that are important for binding.

Although this extensive diversity within the binding area seems counterintuitive, it is likely a product of evolutionary pressure as these outward loops are exposed to the selective pressure of the host's immune defenses. It can be speculated then that this strategy confers a survival advantage – perhaps one where a highly strain-specific immune response is induced, which then enables evasion by strains expressing a divergent N-lobe. Indeed, pigs immunized with just the N-lobe of TbpB elicited a poor cross-reactive response when compared to immunizations with the intact TbpB (Curran et al., 2015). The considerable sequence diversity is also exhibited by TbpBs from human pathogens *Neisseria meningitidis* and *N. gonorrhoeae* as well as other commensal *Neisseria* species. Similar to the porcine pathogens described throughout this manuscript, the sequence diversity among *Neisseria* TbpBs is localized mainly to the N-lobe (Adamiak et al., 2015), suggesting that this may be a common evolutionary strategy of immune evasion used by these bacteria. Our results, in conjunction with previous work (Calmettes, Alcantara, Yu, et al., 2012; Calmettes et al., 2011; Moraes et al., 2009), demonstrate that targeting solvent-facing aromatic residues and positively charged

residues on the binding interface is an effective strategy for overcoming this extensive diversity and ultimately generating novel binding-defective TbpB mutants.

The immunization and challenge experiments evaluating the three binding-defective TbpB mutants generated in this study as vaccine candidates revealed that mice immunized with Y109A TbpB had the highest survival rate (50%) during bacterial challenge (Figure 3.3). Hence, Y109A TbpB was assessed for its ability to elicit a cross-reactive antibody response. Evaluation of the antiserum raised against Y109A TbpB demonstrated that anti-Y109A TbpB antibodies were highly cross-reactive against all TbpB variants tested, which was surprising as the expectation was for anti-Y109A TbpB antibodies to display cross-reactivity limited to cluster 1 TbpB variants. The observation that anti-Y109A TbpB antibodies were not able to block binding to pTf (Figure 3.6) suggests that antibodies might not have been elicited against the N-lobe cap area, the more diverse domain of TbpB, suggesting that the antibody response generated by Y109A TbpB was likely not cluster-specific. Thus, it can be hypothesized that these antibodies were directed mainly towards the more conserved areas of the N-lobe and the C-lobe of TbpB.

The C-lobe in particular displays high sequence conservation between TbpB variants from different clusters (Curran et al., 2015). Swine immunizations with the C-lobe from a cluster 3 TbpB alone elicited antiserum that was cross-reactive against intact TbpBs from all three clusters (Curran et al., 2015). However, additional evaluations of anti-Y109A TbpB antibodies, such as assessing serum responses against the N-lobe and C-lobe alone from TbpB variants representing each cluster, have to be conducted to further investigate this hypothesis. The reasoning behind why Y109A TbpB might possibly elicit antibodies directed at the C-lobe or the more conserved areas of the N-lobe but not Y167A TbpB, as evidenced by the limited cross-reactivity elicited (unpublished, manuscript 1), and whether this is unique to Y109A TbpB as opposed to all cluster 1 TbpBs will require further examination.

Y167A TbpB has been shown to be an incredibly promising vaccine antigen against the aforementioned porcine pathogens. In addition to exhibiting cluster-specific cross-protective capabilities, immunization with this antigen has also resulted in the

prevention of natural colonization by *G. parasuis* (R. Frandoloso et al., 2020). It is tempting to postulate that Y109A TbpB might also display similar cross-protective properties, which, by extrapolating the findings of this study, could be conferred against all existing TbpB variants. Our results make a strong argument for evaluating Y109A TbpB as a prospective vaccine candidate in pigs, as it is difficult to discern the full significance of these promising findings until additional experiments evaluating protection, cross-protection, and effect on colonization elicited by immunizations with Y109A TbpB in pigs are performed. It has yet to be determined whether Y109A TbpB as an antigen will be sufficient in eliciting a broadly cross-protective response or whether Y109A TbpB and Y167A TbpB will have to be combined in a single vaccine formulation in order to produce an efficacious and broadly cross-protective vaccine. Despite the considerable diversity amongst TbpB variants, our findings, in conjunction with previous work done by our group, demonstrate that it is clearly manageable and that including a limited number of antigens in the final vaccine composition may be sufficient in enabling us to target all porcine pathogen strains that cause disease in pigs.

#### DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

#### ETHICS STATEMENT

The animal study was reviewed and approved by Health Sciences Animal Care Committee, Cumming School of Medicine, University of Calgary.

#### AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: SC and AS. Performed the experiments: SC, LsB, NE, RhY. Wrote the paper: SC and AS. All authors contributed to the article and approved the submitted version.

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## CHAPTER 4: MANUSCRIPT 3

### **Design and production of hybrid antigens for targeting integral outer membrane proteins in Gram-negative bacteria.**

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Running title: Design and Production of Hybrid Antigens

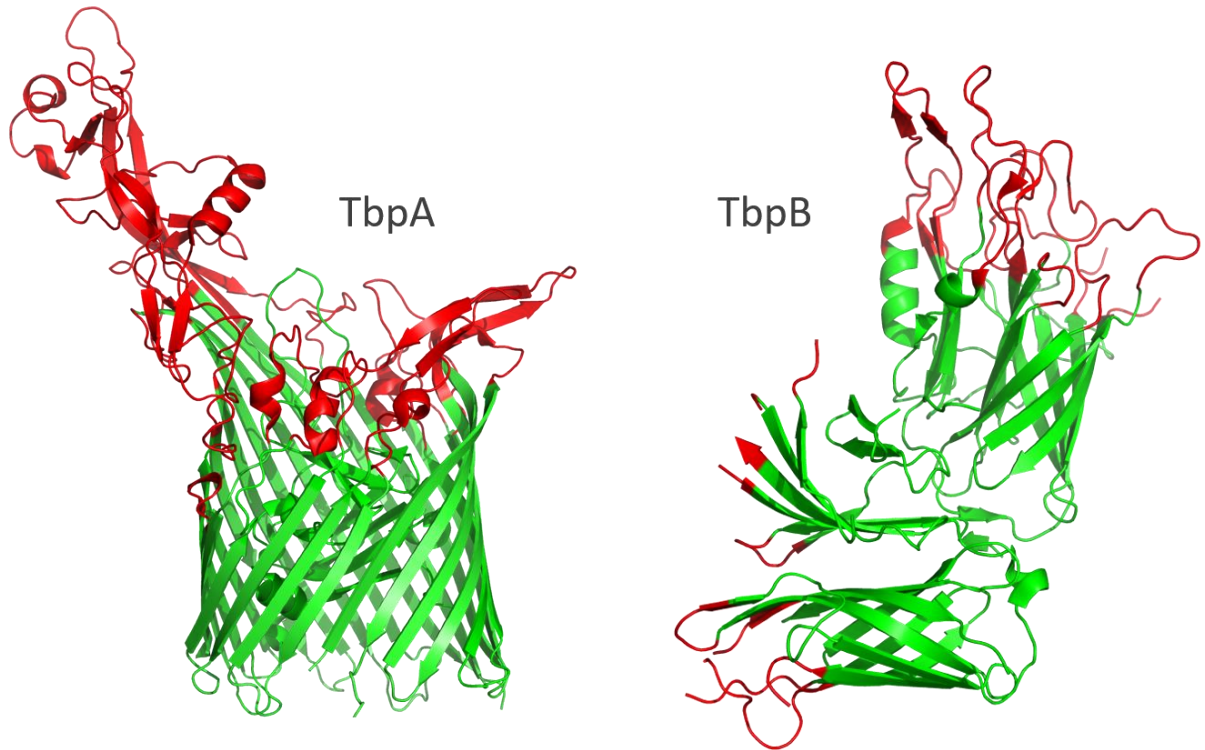
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#### *4.i. Abstract*

Metal ion transporters in the outer membrane of Gram-negative bacteria that are responsible for acquiring iron and zinc are attractive vaccine targets due to their essential function. The core function is mediated by an integral outer membrane TonB-dependent transporter (TBDT) that mediates the transport of the metal ion across the outer membrane. Some TBDTs also have a surface lipoprotein (SLP) that assists in the efficient capture of the metal ion-containing host protein from which the metal ion is extracted. The challenges in producing the integral outer membrane protein for a commercial subunit vaccine prompted us to develop a hybrid antigen strategy in which surface loops of the TBDT are displayed on the lipoprotein, which can readily be produced as a soluble protein. The focus of this chapter will be on the methods for production of hybrid antigens and evaluating the immune response they elicit.

#### *4.1. Introduction*

Our hybrid antigen approach was originally developed to target the surface epitopes of the integral outer membrane protein, transferrin binding protein A (TbpA) (J. E. Fegan et al., 2019), and has since been used to target surface regions of other TonB-dependent transporters (TBDTs) (Qamsari, Rasooli, Chaudhuri, Astaneh, & Schryvers, 2020). TBDTs are a family of integral membrane proteins in the outer membrane of Gram-negative bacteria and are primarily involved in the acquisition of metal ions or metal ion complexes (Noinaj, Guillier, Barnard, & Buchanan, 2010). TBDTs consist of a C-terminal 22-strand beta-barrel and a N-terminal plug region that interacts with TonB from an inner membrane complex that provides energy derived from ATP hydrolysis to drive the transport process (Postle & Kadner, 2003). The principles underlying the hybrid antigen approach could also be applied more generally to other integral outer membrane proteins (OMPs) in Gram-negative bacteria. Integral OMPs consist of beta-barrels that have connecting extracellular surface ‘loop’ regions that are potential targets for the immune response. These integral OMPs are typically not suitable for commercial vaccine production due to poor solubility. Thus, displaying the extracellular surface loops on soluble forms of surface lipoproteins (SLPs) that also possess beta-barrel type structures provide a potentially commercially viable means of antigen production (Figure 4.1).



**Figure 4.1.** TonB-dependent transporters (TBDTs) such as transferrin binding protein A (TbpA) and surface lipoproteins (SLP) such as TbpB both have loops (red) that are anchored by anti-parallel beta-strands (green). Loops on the soluble SLP can be replaced with loops from TBDTs to generate novel hybrid antigens.

The first step in the design of hybrid antigens is the selection of an appropriate integral OMP to target for vaccine development. Ideally, the OMP is present in all strains of the bacterial pathogen that will be targeted with the vaccine and is required for survival in their mammalian or other vertebrate host. TBDTs are ideal targets due to the essential role they play in the survival of the bacteria and their expression under most conditions in their host (Hood & Skaar, 2012b; Noinaj et al., 2010). Several groups of Gram-negative bacteria that reside exclusively in the upper respiratory or genitourinary tracts of their host possess TBDTs that are utilized for acquiring iron from host transferrin or lactoferrin (Morgenthau et al., 2013a). These TBDTs have been shown to

be essential for survival and disease pathogenesis (Anderson, Hobbs, Biswas, & Sparling, 2003; Baltes et al., 2002; Cornelissen et al., 1998) and thus fit the criteria for ideal vaccine targets.

The other requirement for the hybrid antigen approach is an SLP with the appropriate structural features for loop display – a characteristic of proteins transported to the surface by the surface lipoprotein assembly module (SLAM) system (Hooda et al., 2017), which is present in most Gram-negative species of interest. Receptors involved in acquisition of iron from host transferrin or lactoferrin have a bi-lobed SLP component, transferrin or lactoferrin binding protein B (TbpB or LbpB), that can readily serve as the loop display scaffold. In the first hybrid antigen study, a loopless C-lobe (LCL), derived from the TbpB of the human pathogen *Neisseria meningitidis*, and the intact TbpB from the porcine pathogen *Glaesserella parasuis* were used as display scaffolds (J. E. Fegan et al., 2019). Using the detailed structural information for the scaffold proteins (R. Frandoloso et al., 2015) (Calmettes, Alcantara, Yu, et al., 2012) and the TBDT, TbpA from *Neisseria* (Noinaj, Buchanan, et al., 2012), single, modest-sized loop regions were selected for display. The successful production, using conventional systems, of soluble hybrid antigens in this study was likely a function of displaying single, modest-sized TbpA loop regions as opposed to using multiple, larger loops. One consequence of using the LCL in this study was the need to use a *Neisseria* strain deficient in TbpB to assess the contribution of the displayed TbpA loops to the functional properties of the immune response induced by the hybrid antigen. It is important to appreciate that most hybrid antigens that are designed for commercial use would employ a scaffold from the targeted bacterial pathogen and thus would resemble the *Neisseria* TbpA/B hybrid antigens described in this study.

The *N. meningitidis* LCL (J. E. Fegan et al., 2019) was also used as the scaffold in a follow-up study for display of the surface loops of the zinc transport protein, ZnuD, from *Acinetobacter baumannii* (Qamsari et al., 2020). Since a series of hybrid antigens displaying individual loops (or combinations of loops) were planned, an N-terminal maltose binding protein (MBP) was included to facilitate proper folding of the hybrid antigen. However, the expression from a T7 promoter resulted in the production of insoluble protein for most of the hybrid antigens, requiring solubilization with 8 M urea

and refolding with a gradient of decreasing urea concentrations to obtain a soluble hybrid antigen (Qamsari et al., 2020). In this study, the hybrid antigen was not purified from the N-terminal MBP after cleavage with tobacco etch virus (TEV) protease, but rather the entire recombinant protein complex was used in the immunization and challenge experiments. Fortunately, the control LCL scaffold with N-terminal MBP protein did not induce a protective immune response against *A. baumannii* in a mouse sepsis model, indicating that the protection induced by hybrid antigens could be attributed to the individual loop regions present on the scaffold.

One of the challenges with the hybrid antigen approach is the ability to determine the proportion of the antibody response that is induced against the specific loop being displayed, and particularly the titer of antibody directed against conformations of the loop present in the native TBDT/OMP. Although whole-cell enzyme-linked immunosorbent assays (ELISAs) using the target bacterium can provide some assessment of the titer of the relevant antibodies, results are influenced by numerous factors including the level of expression of the targeted TBDT/OMP, antigenicity of the targets (which could be affected by the method of inactivating or killing the bacterium for coating plates), and the presence of pre-existing antibodies in tested sera that could react with a variety of antigens present on the bacterium.

In this chapter, we describe a method for preparing ELISA plates with the native TBDT that is designed to overcome these limitations and provide the titer of antibody directed against native conformation of the loops present in the hybrid antigen. The use of the N-terminal streptavidin binding peptide (SBP) and streptavidin-coated plates eliminates the need to isolate the TBDT from a crude extract and can be exploited to readily evaluate the cross-reactivity of antisera against heterologous TBDT variants. We have developed this protocol by modifying our high-throughput, non-biased ELISA method which uses streptavidin-coated plates to capture and immobilize soluble antigens from crude *Escherichia coli* lysates in which the recombinant protein of interest is biotinylated *in vivo* during expression (J.E. Fegan, Yu, Islam, & Schryvers, 2021a). The extension of this approach with TbpA protein was feasible due to the ability to assess functionally folded protein by binding of labelled transferrin. Although this assay may not be available for other TBDTs, such as siderophore receptors, it could be performed in

parallel or by co-expression of TbpA and the TBDT. For hybrid antigens displaying multiple loops, the sera would have to be pre-treated with hybrid antigens displaying individual loops, or the single-loop hybrids would have to be included in the incubation mixtures to determine the titer against individual loops.

In this chapter, we provide detailed methods for designing hybrid antigens and producing soluble and insoluble proteins with a conventional T7 expression system that clearly has many limitations. Alternative expression systems are being explored and the reader should not hesitate to use alternate systems for production and purification of the hybrid antigens. The method for preparation of ELISA plates for evaluating the antibody response against the loops displayed in the hybrid antigen can be implemented with a representative set of variant TBDTs to evaluate the cross-reactivity of the antibody response that would likely correlate with the cross-protective properties of the immune response. We hope that the additional information in the notes section regarding the design and production of hybrid antigens and assessment of the immune response will provide sufficient insight for successful implementation of this approach for other OMPs being considered as candidate vaccine antigens.

## *4.2 Materials*

Prepare all solutions using double-distilled water. Prepare and store all solutions at room temperature, unless otherwise indicated. Any sterilization procedures, if applicable, will be listed in the following descriptions of each reagent.

### *4.2.1 Autoinduction medium components*

1. ZY media: 1% tryptone, 0.5% yeast extract. Weigh out 15 g tryptone and 7.5 g yeast extract and transfer to a 4-L Erlenmeyer flask. Add 1.5 L of distilled water and place a piece of aluminum foil over the mouth of the flask. Autoclave, then store at room temperature.
2. 20X NPS: 0.5 M  $(\text{NH}_4)_2\text{SO}_4$ , 1M  $\text{KH}_2\text{PO}_4$ , 1M  $\text{Na}_2\text{HPO}_4$ . Weigh out 132 g  $(\text{NH}_4)_2\text{SO}_4$ , 272 g  $\text{KH}_2\text{PO}_4$ , and 284 g  $\text{Na}_2\text{HPO}_4$  and transfer to a large beaker. Add 1400 mL of distilled water, dissolve solids with agitation, then transfer

solution to a graduated cylinder and make up volume to 2 L with water. Transfer to a 2-L glass bottle, autoclave, then store at room temperature.

3. 50X 5052: 2.5% glucose, 10% lactose, 25% glycerol. Weigh out 50 g glucose, 200 g lactose, and 500 g (396.42 mL) glycerol and transfer to a large beaker. Add 1400 mL distilled water, dissolve solids with agitation, then transfer solution to a graduated cylinder and make up volume to 2 L with water. Transfer to a 2-L glass bottle, autoclave, then store at room temperature.
4. 1 M MgSO<sub>4</sub>: Weigh out 61.62 g of MgSO<sub>4</sub> heptahydrate and transfer to a beaker. Add 200 mL of distilled water, dissolve solids with agitation, then transfer to a graduated cylinder and make up volume to 250 mL with water. Transfer to a 250-mL glass bottle, autoclave, then store at room temperature.

#### *4.2.2 Other media used for cultivating bacteria*

1. LB liquid media: Dissolve 25 g of commercial pre-mixed solids in 1 L of distilled water by shaking manually or with a magnetic stir bar. Autoclave, then store at room temperature.
2. LB agar plates: Dissolve 32 g of commercial pre-mixed solids in 1 L of distilled water by shaking manually or with a magnetic stir bar. Autoclave, let cool, and then add the appropriate antibiotic (if desired). Mix using a magnetic stir bar, then pour into petri dish plates (~20-25 mL in each).

#### *4.2.3 Stock solutions of components of protein purification buffers*

1. 1 M Tris pH 8.0: Weigh out 242.28 g of Tris and transfer to a large beaker. Add 1400 mL of distilled water, dissolve solids with agitation, then transfer solution to a graduated cylinder and make up volume to ~1950 mL to leave room for the addition of acid to adjust to the desired pH. Filter sterilize, adjust pH to 8.0 using concentrated HCl, then store at room temperature in a 2-L glass bottle.
2. 5 M NaCl: Weigh out 584.4 g of NaCl and transfer to a large beaker. Add 1400 mL of distilled water. Partially dissolve solids using heat and agitation, then gradually add more water until all solids have dissolved. If necessary, transfer

- solution to a graduated cylinder and make up volume to 2 L with water (*see Note 1*). Filter sterilize, then store at room temperature in a 2-L glass bottle.
3. 1 M imidazole pH 7.4: Weigh out 68.08 g of imidazole and transfer to a large beaker. Add 700 mL of distilled water, dissolve solids with agitation, then transfer solution to a graduated cylinder and make up volume to ~950 mL to leave room for the addition of acid to adjust to the desired pH. Filter sterilize, adjust pH to 7.4 using concentrated HCl, then store at room temperature in a 1-L glass bottle wrapped in aluminum foil to shield solution from exposure to light.
  4. 10X phosphate-buffered saline (PBS) pH 7.4: Weigh out 80 g NaCl, 2 g KCl, 14.4 g Na<sub>2</sub>HPO<sub>4</sub>, 2.4 g KH<sub>2</sub>PO<sub>4</sub> and transfer to a large beaker. Add 700 mL of distilled water, dissolve solids with agitation, then transfer solution to a graduated cylinder and make up volume to ~950 mL to leave room for the addition of acid to adjust to the desired pH. Filter sterilize, adjust pH to 7.4 using concentrated HCl, then store at room temperature in a 1-L glass bottle.

#### 4.2.4 Protein purification buffers

1. Resuspension Buffer: 50 mM Tris pH 8.0, 300 mM NaCl, 10 mM imidazole pH 7.4. Mix 50 mL of 1 M Tris pH 8.0 stock solution, 60 mL of 5 M NaCl stock solution, and 10 mL of 1 M imidazole pH 7.4 stock solution in a graduated cylinder. Make up volume to 1 L using distilled water. Filter sterilize, then store at room temperature.
2. Wash Buffer: 50 mM Tris pH 8.0, 1 M NaCl, 20 mM imidazole pH 7.4. Mix 50 mL of 1 M Tris pH 8.0 stock solution, 200 mL of 5 M NaCl stock solution, and 20 mL of 1 M imidazole pH 7.4 stock solution in a graduated cylinder. Make up volume to 1 L using distilled water. Filter sterilize, then store at room temperature.
3. Elution Buffer: 50 mM Tris pH 8.0, 300 mM NaCl, 300 mM imidazole pH 7.4. Mix 50 mL of 1 M Tris pH 8.0 stock solution, 60 mL of 5 M NaCl stock solution, and 300 mL of 1 M imidazole pH 7.4 stock solution in a graduated cylinder.

Make up volume to 1 L using distilled water. Filter sterilize, then store at room temperature.

4. Exchange Buffer: 50 mM Tris pH 8.0, 600 mM NaCl. Mix 100 mL of 1 M Tris pH 8.0 stock solution with 120 mL of 5 M NaCl stock solution in a graduated cylinder. Make up volume to 1 L using distilled water. Filter sterilize, then store at room temperature.
5. Denaturing Lysis Buffer: 50 mM  $\text{NaH}_2\text{PO}_4$ , 300 mM NaCl, 6 M urea, pH 8.0. Weigh out 6.9 g  $\text{NaH}_2\text{PO}_4$  monohydrate and 360.36 g urea and transfer to a large beaker. Add 60 mL of 5 M NaCl stock solution and 640 mL distilled water. Dissolve solids with agitation, then transfer to a graduated cylinder and make up volume to ~950 mL, leaving room for the addition of acid to adjust the pH. Adjust pH to 8.0 using concentrated HCl.
6. Denaturing Wash Buffer: 50 mM  $\text{NaH}_2\text{PO}_4$ , 300 mM NaCl, 6 M urea, 5 mM imidazole, pH 8.0. Weigh out 6.9 g  $\text{NaH}_2\text{PO}_4$  monohydrate and 360.36 g urea and transfer to a large beaker. Add 60 mL of 5 M NaCl stock solution, 5 mL of 1 M imidazole pH 7.4 stock solution, and 635 mL of distilled water. Dissolve solids with heat and agitation, then transfer to a graduated cylinder and make up volume to ~950 mL, leaving room for the addition of acid to adjust the pH. Adjust pH to 8.0 using concentrated HCl.
7. Refolding Wash Buffer: 50 mM  $\text{NaH}_2\text{PO}_4$ , 300 mM NaCl, 5 mM imidazole, pH 8.0. Weigh out 6.9 g  $\text{NaH}_2\text{PO}_4$  monohydrate and transfer to a large beaker. Add 60 mL of 5 M NaCl stock solution, 5 mL of 1 M imidazole pH 7.4 stock solution, and 635 mL of distilled water. Dissolve solids with agitation, then transfer to a graduated cylinder and make up volume to ~950 mL, leaving room for the addition of acid to adjust to the pH. Adjust pH to 8.0 using concentrated HCl.
8. Refolding Elution Buffer: 50 mM  $\text{NaH}_2\text{PO}_4$ , 300 mM NaCl, 400 mM imidazole, pH 8.0. Weigh out 6.9 g  $\text{NaH}_2\text{PO}_4$  monohydrate and transfer to a large beaker. Add 60 mL of 5 M NaCl stock solution, 400 mL of 1 M imidazole pH 7.4 stock solution, and 240 mL of distilled water. Dissolve solids with agitation, then

transfer to a graduated cylinder and make up volume to ~950 mL, leaving room for the addition of acid to adjust the pH. Adjust pH to 8.0 using concentrated HCl.

#### 4.2.5 Other materials for protein production and purification

1. Protease inhibitor tablets: store at 4°C or according to the manufacturer's instructions until ready to use.
2. Lysozyme: prepare 10 mg/mL solution by dissolving solids in ddH<sub>2</sub>O and storing in 6-mL aliquots at -20°C until ready to use.
3. Deoxyribonuclease (DNase) I: prepare 5 mg/mL solutions by dissolving solids in ddH<sub>2</sub>O and storing in 200- $\mu$ L aliquots at -20°C until ready to use.
4. Nickel-nitrilotriacetic acid (Ni-NTA) resin: prepare according to the manufacturer's instructions and store at 4°C until ready to use (*see Note 2*).
5. Dialysis tubing, 6-8 kDa molecular weight cutoff (MWCO): Store at room temperature until ready to use. Moisten the membrane with ddH<sub>2</sub>O immediately prior to use.
6. Centrifugation concentrator, 50 kDa MWCO (*see Note 3*): Prime with 20 mL ddH<sub>2</sub>O followed by 20 mL of the appropriate buffer immediately prior to use.

#### 4.2.6 Materials for hybrid antigen design

1. DNA and protein sequences of the SLP scaffold and the TBDT.
2. Access to programs/software for protein modeling, protein visualization, and gene cloning and visualization (*see Note 4*).

#### 4.2.7 Materials for TBDT extraction

1. 50 mM Tris pH 8.0: Add 50 mL of 1 M Tris pH 8.0 stock solution to a 1-L graduated cylinder. Make up volume to 1 L using distilled water. Filter sterilize, adjust pH to 8.0 with HCl, transfer to a 1-L glass bottle, then store at room temperature.
2. Eluent detergent (*see Note 5*).

#### 4.2.8 ELISA reagents

1. PBST: Add 100 mL of 10X PBS to a 1-L graduated cylinder. Make up volume to 1 L using distilled water. Add 0.5 mL of Tween-20 (final concentration: 0.5%) using a wide-bore 1 mL pipette tip. Pipette up and down numerous times to ensure that all the viscous detergent is mixed into the PBS. Shake the bottle to mix well. Store at room temperature. This can be prepared several days in advance.
2. Blocking solution: Add 2.5 g of skim milk powder to 25 mL of PBST in a 50-mL conical tube and vortex to mix. Make up volume to 50 mL with more PBST. This can be prepared a day in advance and stored at 4°C. Bring to room temperature prior to use. Alternatively, 5% w/v bovine serum albumin (BSA) can also be used as a blocking reagent: Add 50 g of BSA to 1 L of PBS and dissolve with stirring. Once dissolved, filter sterilize and store at 4°C.
3. Diluent solution: Add 1.25 g of skim milk powder to 25 mL of PBST in a 50-mL conical tube and vortex to mix. Make up volume to 50 mL with more PBST. This can be prepared a day in advance and stored at 4°C. Bring to room temperature prior to use.
4. Transferrin solution: Using a stock solution of 0.5 mg/ml transferrin conjugated to horseradish peroxidase (Tf-HRP), prepare a 1:1,000 dilution for a working solution in 2.5% skim milk solution in PBST just prior to use. Vortex to mix well.
5. 3,3',5,5'-Tetramethylbenzidine (TMB) substrate solution: Prepare according to manufacturer's instructions and store at 4°C until use.
6. 4 N HCl quenching solution: Make this solution in a fume hood. Measure out 200 mL of distilled water in a graduated cylinder, then transfer to a clean 500 mL glass bottle. Measure out 100 mL of 12 N HCl in a clean glass graduated cylinder. Gently and slowly pour the HCl into the water. Stir with a clean glass rod. Let sit for 2 min. Store at room temperature.
7. Serum samples from animals immunized with TbpA, TbpA-based hybrid antigens, or other TBDT-based antigens. Store at -20°C until use. Thaw on ice for 20-30 min and vortex to mix prior to use.

8. Secondary antibody solution: HRP-conjugated antibody specific for detection of IgG of species immunized. Prepare according to the manufacturer's instructions, divide into 10- $\mu$ L aliquots, and store at -20°C until use. For use, thaw an aliquot on ice for 20 min and prepare working solution to 1:10,000 (or according to manufacturer's recommendations) in diluent solution just prior to use.
9. Other miscellaneous materials: streptavidin- or neutravidin-coated 96- or 384-well ELISA plates, multichannel pipettors, adhesive cover slips for ELISA plates, reagent boats/basins for containing ELISA solutions.

### 4.3 Methods

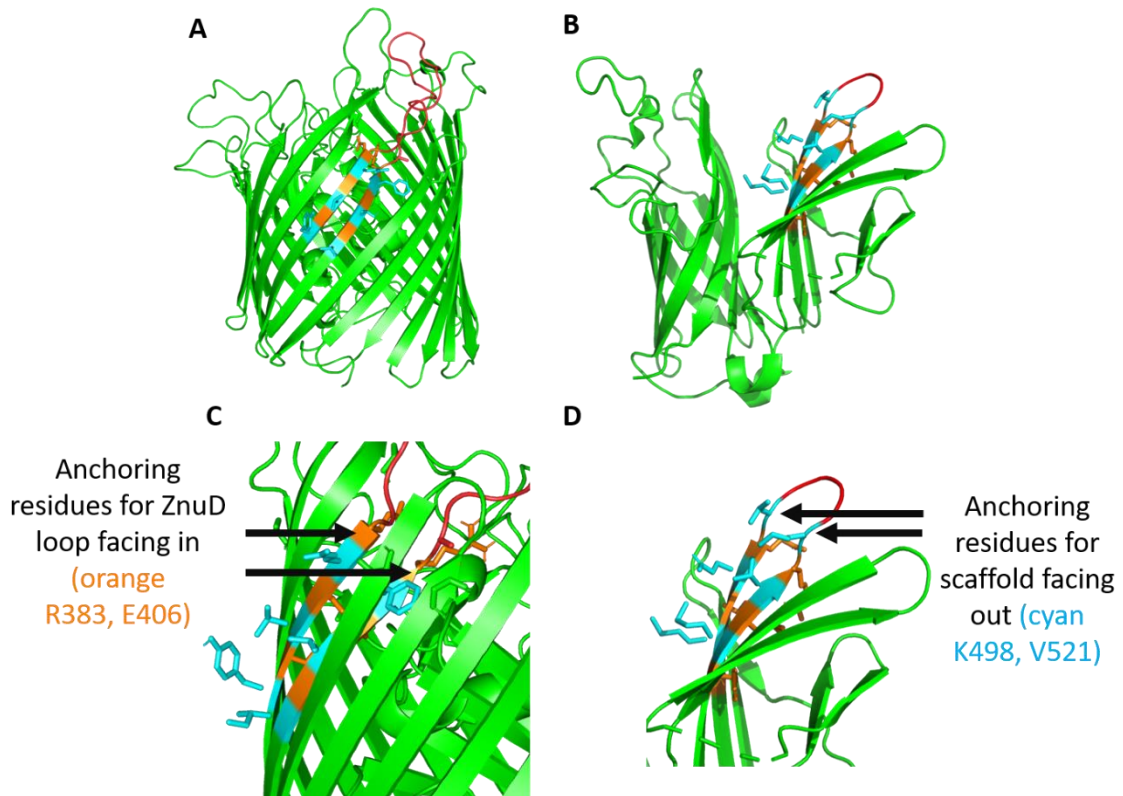
Carry out all procedures at room temperature unless stated otherwise. All techniques with live cells are to be performed in an aseptic manner either next to a Bunsen burner or in a biosafety cabinet.

#### 4.3.1 Designing and preparing hybrid antigen genes

1. Acquire published structures of the SLP scaffold and the TBDT. If structures are not available, generate structural models of the scaffold and the TBDT *in silico*. Programs such as I-TASSER, RaptorX, or Phyre2 can all be used.
2. Visualize the models using PyMOL.
3. Identify the anchoring residue sites of loops on the SLP scaffold and the TBDT (*see Note 6*). Locate the anti-parallel beta strands in each model and label the residues in alternating color leading up to the 'loop', as shown in Figure 4.2 for the example with TBDT (*Acinetobacter baumannii* ZnuD) and SLP scaffold (derivative of TbpB from *Neisseria meningitidis*). The splicing site of the SLP scaffold is determined by selecting anchoring residues that are next to each other (parallel to each other) and have side chains that face the same direction on the scaffold ('out' and labelled with arrow in Figure 4.2D). These anchoring residues are included in the SLP scaffold (*see Note 6*).
4. Determine the splicing site of the loop by selecting parallel anchoring residues that face the opposite direction to that of the SLP scaffold on the TBDT ('in' and

labelled with arrow Figure 4.2C). These anchoring residues are included in the loop.

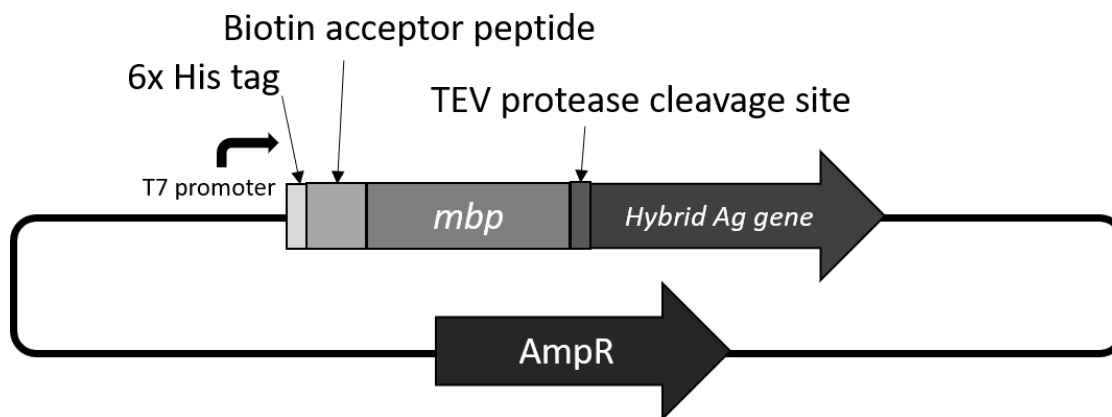
5. Using an *in silico* program for DNA cloning, identify the gene segments corresponding to the structural attributes of the SLP scaffold and TBDT identified in **steps 3** and **4**.
6. Generate the DNA sequence of the novel hybrid antigen by combining the different gene segments from both the SLP scaffold and the TBDT. Translate the gene sequence *in silico* to ensure the resulting protein is in frame.



**Figure 4.2.** Example of hybrid antigen design. **A.** Computer generated model of the TBDT, ZnuD, from *Acinetobacter baumannii* with a loop highlighted (red) where the residues of the anchoring beta-barrel are coloured differently depending on whether their

side chains are oriented towards the inside of the barrel (orange) or the outside/away from the barrel (cyan). **B.** The scaffold, a derivative of the SLP TbpB C-lobe from *Neisseria meningitidis* strain M982 (PDB 5KKX) (J. E. Fegan et al., 2019), is labelled similarly to **A**, with alternating residues coloured. **C.** The loop from the ZnuD is determined by selecting parallel anchoring residues (side chains facing the same direction) on the beta-barrel. If the anchoring residues of the loop are facing in (black arrow in **C**), then the anchoring residues of the scaffold face out (black arrow in **D**) to maintain the anti-parallel beta-strand formation.

7. The novel hybrid gene can either be synthesized by a commercial vendor or can be generated in the lab via splicing-by-overlap/extension polymerase chain reaction (SOE-PCR) using primers that anneal to the appropriate regions of the templates.
8. Clone the hybrid gene fragment into plasmid pE5770 or any equivalent plasmid with similar elements (*see Note 7* and Figure 4.3).



**Figure 4.3.** A schematic of the His-Bio-MBP T7 expression vector (pE5770) with a gene of interest (hybrid antigen) cloned into the expression locus. The components of the vector (*see Note 7*) that are relevant to the protein production methods discussed in this manuscript are illustrated in the figure.

9. In preparation for transformation of the newly constructed plasmid into *E. coli* TOP10 cells for long-term storage, thaw an aliquot of chemically competent TOP10 cells on ice.
10. Pipette 50-100 ng of the plasmid encoding the hybrid antigen gene into the thawed aliquot and incubate on ice for 30 min (*see* **Notes 8 and 9**).
11. Heat-shock the cells at 42°C for 30-60 s. Promptly place the tube back on ice for 2 min.
12. Add 400 µL of cold sterile LB medium and then transfer the tube to a 37°C shaking incubator for 1 hr.
13. Plate 100 µl of transformation mixture onto an LB agar plate supplemented with 100 µg/mL ampicillin and incubate at 37°C overnight. Colonies generated with the pE5770 plasmid can be streaked onto plates supplemented with 30 µg/mL kanamycin to screen for loss of the original insert.
14. Confirm by colony PCR or PCR of plasmid DNA prepared from positive clones and sequencing of insert or by restriction digestion of plasmid.
15. Isolate and store plasmid at -20°C for subsequent use.
16. Inoculate 5-mL of LB medium supplemented with 100 µg/mL ampicillin with a colony confirmed to contain the desired plasmid. Incubate overnight (~16 h) at 37°C in a shaking incubator.
17. The next day, create a 16% glycerol stock by mixing 800 µL of the broth culture with 200 µL of sterile 80% glycerol. Store at -80°C.

#### 4.3.2 *Expressing hybrid protein antigens in E. coli using a T7 expression vector*

1. Thaw out the plasmid encoding the desired hybrid protein designed in **Section 4.3.1**, as well as the competent cells derived from the strain of *E. coli* to be used for protein expression (*see* **Note 10**). The plasmid concentration should be ~100 ng/µL, and competent cells should be stored in 50- or 100-µL aliquots. After removing the competent cells from the -80°C freezer, let the cells sit on ice for 20 min prior to transformation.

2. Add 1-2  $\mu\text{L}$  of plasmid to the competent cells and let the cells sit on ice for 30 min. Next, transfer to a pre-chilled 14-mL round-bottom culture tube.
3. Prepare a 42°C water bath or heat block, then “heat-shock” the cells at this temperature for 30-60 s, followed by a 2-min incubation on ice. Promptly add 700  $\mu\text{L}$  of LB medium, then incubate the cells with shaking at 37°C for 1 h.
4. Add 6 mL of LB containing the appropriate antibiotic (i.e., add 6  $\mu\text{L}$  of 100 mg/mL ampicillin if you are using ampicillin resistance to select for the presence of your plasmid) to the culture and incubate with shaking at 37°C for another 4 h.
5. Approximately 1-1.5 h before the end of the 4-h incubation period, prepare the autoinduction medium by aseptically adding 75 mL NPS, 30 mL 5052, and 1.5 mL  $\text{MgSO}_4$  to 1.5 L of ZY media (*see Note 11*). Four 4-L flasks containing 1.5 L of media in each are used in a typical protein production run, but the culture volume can be adjusted depending on the expected yield of the protein being produced. Add the appropriate antibiotic at half the normal concentration (i.e., use 50  $\mu\text{g}/\text{mL}$  instead of 100  $\mu\text{g}/\text{mL}$  if using ampicillin). Pre-warm the media in 37°C shaking incubator at a shaking speed of ~50 rpm (*see Note 12*).
6. At the end of the 4-hour incubation mentioned in **step 4**, inoculate 1.5 L of autoinduction media with 1.5 mL of the starter culture. Repeat as needed, depending on the number of flasks being used.
7. Incubate the flasks at 37°C with vigorous shaking (175 rpm) for 18 h, then adjust the temperature to 20°C and incubate for another 24 h (*see Note 13*).
8. Harvest cells by centrifugation at 5,000 x g for 25 min at 4°C.
9. Prepare Resuspension Buffer (25 mL per L of culture) and add 1 protease inhibitor tablet, 6 mL of 10 mg/mL lysozyme, and 200  $\mu\text{L}$  of 5 mg/mL DNase I (increase the amounts of these reagents as necessary if the culture volume is greater than 6 L). Mix in an appropriate-sized beaker, keeping in mind that the cell pellets will subsequently be transferred to this beaker.
10. After centrifugation, decant the supernatant and re-suspend the cell pellet(s) in the Resuspension Buffer mixture prepared in **step 9** (*see Note 14*). For optimal lysis,

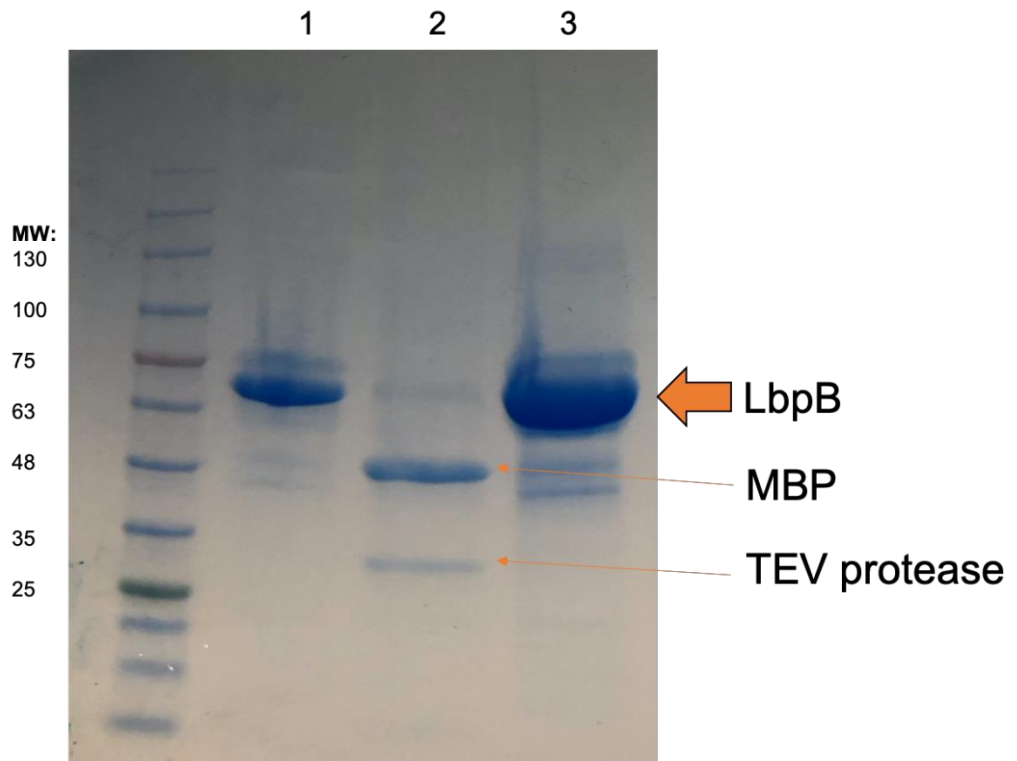
add a magnetic stir bar to the mixture and let stir at slow speed for 30 min at 4°C to get rid of cell clumps.

11. Lyse the cells by passing the sample through a cell homogenizer four times (*see Note 15*).
12. After cell lysis, centrifuge at 35,000 x *g* for 90 min at 4°C to separate out the cell debris.
13. Collect the supernatant and discard the pelleted cell debris. Filter the supernatant through a 0.2- $\mu$ m filter (*see Note 16*).
14. Determine whether the fusion protein is present in the supernatant by performing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 10- $\mu$ L aliquot of the supernatant. If a band corresponding to the molecular weight of the fusion protein of interest is detected in the SDS-PAGE gel, proceed to **Section 4.3.3**. If the protein of interest is not detected, proceed to **Section 4.3.4** (*see Note 17*).

#### 4.3.3 Purification of soluble protein antigens

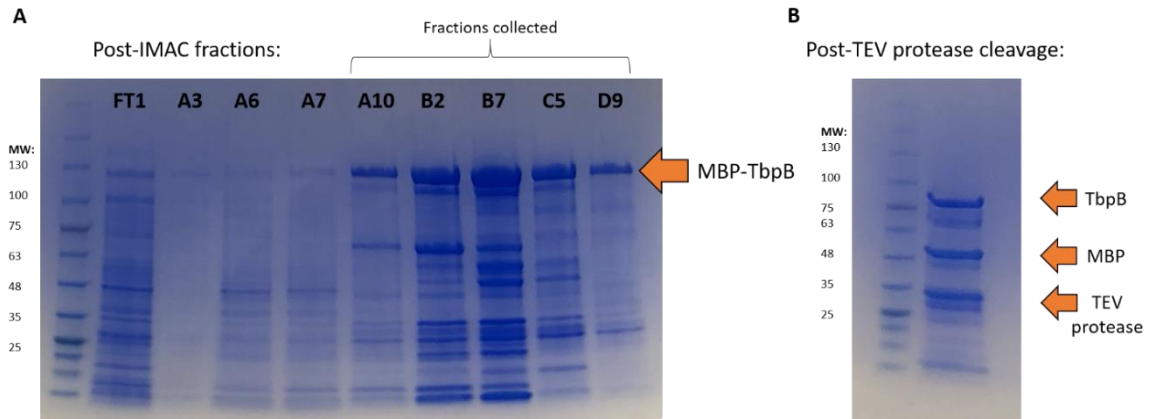
1. Prepare 1 L each of Resuspension Buffer, Wash Buffer, and Elution Buffer.
2. Wash and equilibrate a 5-mL Ni-NTA column with Resuspension Buffer prior to use (*see Note 18*).
3. Using either a peristaltic pump or a fast-purification liquid chromatography (FPLC) system, continuously circulate the lysate prepared in **Section 4.3.2** through the Ni-NTA column overnight (16-18 h) at 4°C. If using a peristaltic pump, circulate lysate at ~0.5 mL/min (*see Note 19*).
4. Wash the column with at least 75 mL Wash Buffer, or until the protein concentration of the wash fractions is <0.1 mg/mL. Take one or more samples of the wash fractions for SDS-PAGE analysis.
5. Elute the protein bound to the column using high-imidazole Elution Buffer in 2-mL aliquots until the protein concentration reaches <0.1 mg/mL.

6. Take samples of the eluted fractions for SDS-PAGE to verify the presence of the desired protein antigen and determine which fractions to collect and pool for further processing (Figure 4.4A). If desired, include the wash fractions as well to confirm that the protein is present in the eluted fraction and not the wash fractions and therefore is binding to the Ni-NTA resin.



**Figure 4.4.** Representative SDS-PAGE results for purification of a soluble hybrid antigen using the TbpB derived from *Haemophilus influenzae* as an example. **A.** SDS-PAGE results for 10- $\mu$ L aliquots of fractions from a Ni-NTA (IMAC – immobilized metal affinity chromatography) column. FT1 indicates a sample from the pooled flow through buffer containing proteins that did not bind to the column. Fractions A3-D9 represent the specific wells of the 96-well collection block that collected the fractions eluted with the imidazole gradient. Fractions A3-D9 were pooled based on the presence of the 130-kDa MBP-TbpB fusion protein for subsequent TEV cleavage. **B.** SDS-PAGE results for a 10-

$\mu\text{L}$  aliquot of the MBP-TbpB fusion protein following cleavage with TEV protease. The gel confirms that TEV cleavage was complete as there is no MBP-TbpB band present with TbpB, MBP and TEV protease as the main protein bands. Applying this sample to a Ni-NTA column removes the MbpB and TEV, leaving TbpB as the main protein (similar to the purified LbpB example shown in **Figure 4.5**



**Figure 4.5.** Representative SDS-PAGE results following the separation of MBP and TEV protease from the recombinant protein of interest using a Ni-NTA column. A modified *N. gonorrhoeae* lactoferrin binding protein B (LbpB) is provided as an example. Lane 1: flow through containing the LbpB; lane 2: pooled eluted fractions (MBP and TEV protease are both present here); lane 3: concentrated sample of flow through (lane 1). Each lane was loaded with a 10- $\mu\text{L}$  aliquot.

7. Determine the amount of protein present in the pooled sample-containing fraction using a spectrophotometer or NanoDrop device (or equivalent method of determining protein concentration).
8. In preparation for cleavage of the fusion protein using tobacco etch virus (TEV) protease – for which high-salt, low-imidazole conditions are optimal – transfer the eluted protein sample to dialysis tubing, ensure the tube is fastened at both ends,

and place the tube in a beaker containing 2 L of Exchange Buffer. Place a magnetic stir bar in the beaker and let stir at a slow speed overnight (16-18 h) at 4°C.

9. Remove the dialysis tubing from the 2-L beaker, open at one end of the tube, and add TEV protease (*see Note 20*). Then, place the tubing back in the Exchange Buffer (this can be carried over from **step 8**; there is no need to prepare fresh Exchange Buffer). Let stir overnight at 4°C.
10. The next day, run a sample of the cleaved protein on an SDS-PAGE to determine the cleavage efficiency (Figure 4.4B). If cleavage is incomplete – typically ascertained by the presence of the intact fusion protein in the SDS-PAGE gel – add more TEV protease.
11. In preparation for a second round of Ni-NTA chromatography to remove the cleaved MBP fusion partner and TEV protease from the protein sample, dialyze the sample overnight at 4°C against 2 L of Resuspension Buffer. This step serves to replenish the imidazole that was removed from the sample during dialysis against Exchange Buffer in **step 8**.
12. Circulate the cleaved protein through the Ni-NTA column as described in **step 3** (*see Note 21*). The polyhistidine-tagged MBP and TEV protease will bind to the resin and will thus separate these contaminants from the desired protein antigen.
13. After circulation through the Ni-NTA column, take a sample of the protein (which should not have bound to the column) and run an SDS-PAGE gel to confirm that the MBP and TEV protease have been separated from the desired protein antigen (Figure 4.5).
14. Exchange the buffer containing the purified protein by dialysis against PBS overnight (16-18 h) at 4°C, then concentrate the protein to a final concentration of 1-10 mg/mL by centrifugation using a concentrator with the appropriate molecular weight cut off.
15. Promptly store the protein in 100- $\mu$ L aliquots at -80°C until ready to use for animal immunizations (*see Note 22*).

#### 4.3.4 Purification of insoluble protein antigens

1. Perform **steps 1-8** described under **Section 4.3.2**.
2. Prepare Denaturing Lysis Buffer (60 mL per L of culture). After centrifugation (**Section 4.3.2, step 8**), decant the supernatant and re-suspend the cell pellet using Denaturing Lysis Buffer.
3. Lyse the cells by sonicating for two 5 min periods on ice using conditions that do not result in overheating the samples. These generally involve short pulses with cooling periods of 2-4 times longer. We use a QSonica sonicator (pulse 03, amplitude 65%).
4. Centrifuge the lysate for 35 min at 20,440 x *g* at 4°C.
5. Collect the supernatant and filter through a 0.2-µm filter.
6. Add 3 mL of Ni-NTA resin for every 50 mL of filtered lysate (*see Note 23*), then mix gently in 50-mL conical tubes using a rotary shaker overnight (~16 h) at room temperature.
7. Prepare four buffers with decreasing concentrations of urea (3 M, 1.5 M, 0.75 M, 0.375 M) by starting with Denaturing Wash Buffer and subsequently performing serial dilutions, using Refolding Wash Buffer as a diluent.
8. Centrifuge the lysate/resin mixture for 10 min at 3,220 x *g* at room temperature, then decant the supernatant. Next, using 18 mL (or 6 volumes of Ni-NTA resin) of the initial buffer in the aforementioned serial dilution (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 3 M urea, 5 mM imidazole, pH 8.0), re-suspend the Ni-NTA resin and incubate the resulting mixture on a rotary shaker for 30 min at room temperature.
9. Repeat **step 8** for each of the subsequent buffers in the serial dilution series prepared in **step 7**.
10. Re-suspend the Ni-NTA resin in 18 mL Refolding Wash Buffer, then transfer the mixture to a gravity column.
11. Elute any proteins bound to the Ni-NTA using 18 mL of Refolding Elution Buffer (*see Note 24*). The eluted volume can be collected in 1-mL fractions.

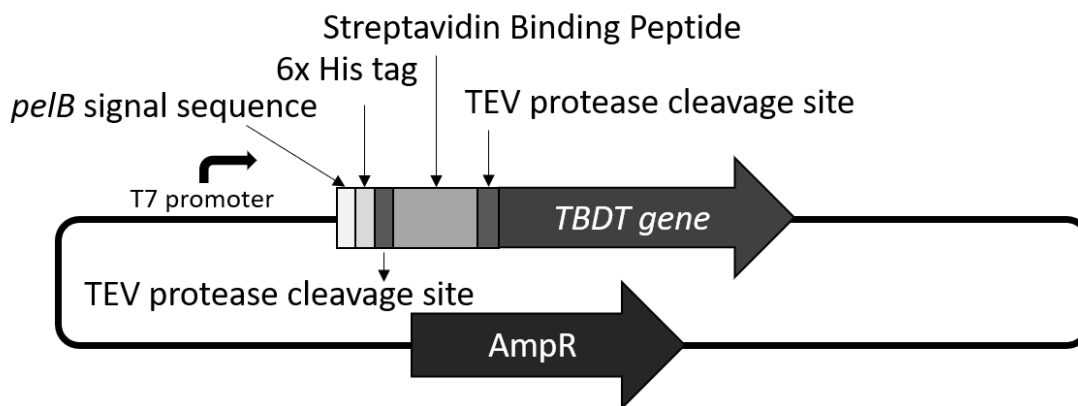
12. Verify the presence of the desired hybrid antigen using SDS-PAGE, then perform **steps 8-12** described under **Section 4.3.3**.

#### 4.3.5 Cloning of *tbd* in fusion with streptavidin binding protein (SBP)

1. Identify mature sequence of *tbd* using the online software SignalP (*see Note 25*).
2. Design primers to amplify the selected gene or synthesize the sequence.
3. Clone the *tbd* gene into plasmid E5771 or an alternate expression plasmid containing SBP (*see Note 26*).
4. Transform the newly constructed plasmid into *E. coli* TOP10 cells for long-term storage, as described in **Section 4.3.1, steps 9-15**.

#### 4.3.6 Preparation of detergent-extracted SBP-TBDT fusion protein

1. Thaw plasmid DNA and an aliquot of chemically competent *E. coli* C43 cells on ice for 20 min (*see Note 27* and Figure 4.6).



**Figure 4.6.** A schematic of the His-SBP-TBDT T7 expression vector (pE5771) with a gene of interest (a TBDT to be used in ELISA assays) cloned into the expression locus. The components of the vector (*see Note 26*) that are relevant to the protein production methods discussed in this manuscript are illustrated in the figure

2. Pipette 50-100 ng of SBP-TBDT plasmid into the thawed aliquot and incubate on ice for 30 min.
3. Heat-shock the cells at 42°C for 30-60 s. Promptly place the tube back on ice for 2 min.
4. Add 400 µL of cold sterile LB medium.
5. Gently pipette out the entire mixture from the 1.5 mL tube and into a 50-mL conical tube. Incubate in a shaking incubator at 37°C for 1 h.
6. Add 14.5 mL of autoinduction medium supplemented with 100 µg/mL ampicillin and incubate in a shaking incubator at 37°C overnight.
7. The following day, centrifuge the culture at 3,220 x *g* at 4°C for 10 min.
8. Decant the supernatant, add 15 mL of cold 50 mM Tris pH 8.0, and resuspend the cell pellet.
9. Centrifuge again as described in **step 7**, decant the supernatant, then add 2 mL of cold 50 mM Tris pH 8.0 and 40 µL of Elugent detergent (final concentration: 1%, commercial Elugent is a 50% solution). Shake overnight at 4°C (*see Note 28*).
10. The following day, centrifuge the samples at 16,100 x *g* at 4°C for 30 min.
11. Carefully remove the supernatant containing the detergent extracted SBP-TBDT and pipette slowly into a 15-mL conical tube containing 8 mL of cold filtered PBST with 0.125% Elugent (0.25% of commercial preparation, final protein dilution: 1 in 5).
12. Gently rotate the tube by hand several times to ensure that it mixes well. Keep on ice until ready to use (*see Notes 29 and 30*).

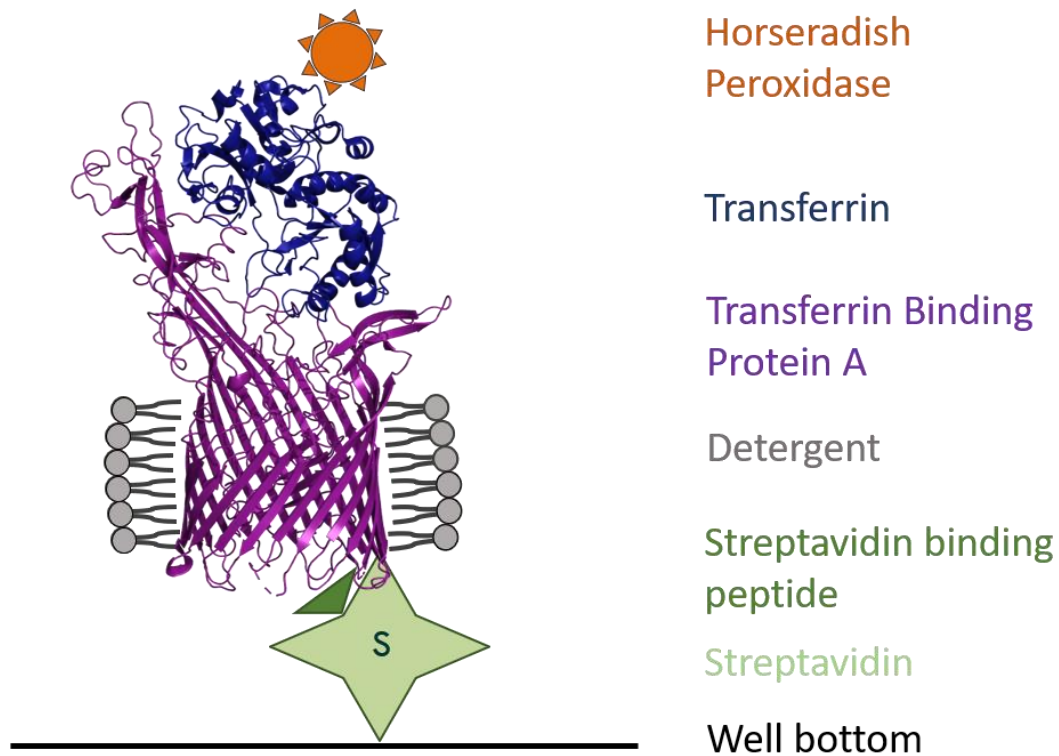
#### 4.3.7 Coating streptavidin ELISA plates with detergent-extracted SBP-TBDT

1. Pour the SBP-TBDT/PBST mixture into a reagent boat, and, using a multi-channel pipette, pipette out 100 µL into each well of a streptavidin-coated 96-well plate (*see Note 31*).

2. Incubate at room temperature for 1 h, then wash with 250  $\mu\text{L}$ /well of PBST three times (*see* **Notes 32** and **33**).
3. Add 250  $\mu\text{L}$  of the prepared 5% blocking solution to each well after the wash step, incubate at room temperature again for 1 h, then wash with 250  $\mu\text{L}$ /well of PBST three times.

#### **4.3.8 Assessing conformation of TbpA by its ability to bind to transferrin**

1. During the blocking step (**Section 4.3.7, step 3**), add 1  $\mu\text{L}$  Tf-HRP to 1 mL of diluent solution (i.e., final dilution of 1/1000).
2. Add 100  $\mu\text{L}$  of the Tf-HRP solution to each of four wells (i.e., two test and two control wells), incubate for 1 h, then wash with 250  $\mu\text{L}$  of PBST three times.
3. Add 50  $\mu\text{L}$  of TMB to the four wells and develop in the dark (a cupboard/drawer is adequate) for 20 min, then add 25  $\mu\text{L}$  of 4 N HCl to stop the reaction. Measure the optical density at 450 nm (*see* **Note 34** and Figure 4.7).



**Figure 4.7.** Schematic of TbpA conformation assay. Detergent-extracted TbpA fused to streptavidin binding peptide (SBP) is immobilized on streptavidin-coated wells. Transferrin conjugated to horseradish peroxidase (Tf-HRP) is then added to the wells. If the immobilized TbpA is folded properly, this allows Tf binding to occur, which results in a signal from the HRP once the developing substrate is added.

#### 4.3.9 Assessing antibody titers in serum samples from immunized animals

1. During the blocking step (**Section 4.3.7, step 3**), thaw serum samples on ice for at least 20 min and vortex to mix well.
2. Add 100  $\mu\text{L}$  of diluent solution to each well. Add additional diluent solution in the first well of each row (A to H). The amount of additional diluent solution will depend on the desired starting dilution for the serum used in the assay, such that the final volume of diluent with added serum is 200  $\mu\text{L}$ .
3. Add the desired amount of each serum sample to the appropriate wells containing diluent solution to achieve the desired initial serum dilution in a total volume of

200  $\mu\text{L}$  in each well in the first column. Gently pipette up and down 10 times to mix.

4. Adjust a multi-channel pipettor to 100  $\mu\text{L}$ , then mix the serum solutions in the first column of the plate by pipetting up and down 5 times.
5. Remove 100  $\mu\text{L}$  from the first column and transfer it the next column. Pipette up and down 5 times. Repeat with the next column.
6. Continue performing 2-fold serial dilutions until column 11 (*see Note 35*). Discard the leftover 100  $\mu\text{L}$ .
7. Incubate at room temperature for 1 h, then wash with 250  $\mu\text{L}$  of PBST three times.
8. Prepare the secondary antibody solution by adding 5  $\mu\text{L}$  of anti-rabbit IgG or anti-mouse IgG (as appropriate for the animals in which the immune sera were generated) antibody conjugated to HRP to a 50-mL diluent solution (final antibody dilution: 1 in 10,000). Vortex to mix well.
9. Add 100  $\mu\text{L}$  to each well, except for column 12.
10. In column 12, add 100  $\mu\text{L}$  of diluent solution (no antibody) in wells 12A, 12B, and 12C. These are negative controls.
11. In wells 12D, 12E, and 12F, add 100  $\mu\text{L}$  of the secondary antibody solution. These are the controls to check for background absorbance caused by the secondary antibody binding to TBDT.
12. If using TbpA, then in wells 12G and 12H, add 100  $\mu\text{L}$  of the 1:1,000 Tf-HRP solution described in **Section 4.3.8, steps 1 and 2**. These are the controls that ensure that the TbpA is properly folded and coating the wells at similar levels.
13. Incubate at room temperature for 1 h, then wash with 250  $\mu\text{L}$ /well of PBST three times.

Add 50  $\mu\text{L}$ /well of TMB and develop in the dark (a cupboard/drawer is adequate) for 20 min, then add 25  $\mu\text{L}$  of 4 N HCl to stop the reaction. Measure the optical density at 450 nm.

#### 4.4 Notes

1. 5 M is close to the upper limit of solubility of NaCl in water; hence, when making the 5 M NaCl stock solution, in addition to the use of vigorous stirring and heat, it is necessary to add close to the full volume of water before all solids can be dissolved.
2. The preference of our group is to use either free Ni-NTA resin in a gravity column or a 5-mL affinity chromatography column containing Ni-NTA resin (i.e., the 5-mL HisTrap High Performance column from Cytiva) installed as part of an FPLC system or attached to a peristaltic pump.
3. Use a centrifugation concentrator with a lower MWCO if the protein of interest has a lower molecular weight than 50 kDa. Since our protein antigens are usually ~60-70 kDa, the protein concentrator most often used by our group is Cytiva's Vivaspin 20 with a MWCO of 50 kDa. The MWCO should ideally be just below the molecular weight of the desired protein antigen to facilitate the removal of any lower molecular weight contaminants that may be present in the sample.
4. Our group prefers to use the online platform I-TASSER for better models or more challenging projects (lower sequence identity) (<https://zhanglab.ccmb.med.umich.edu/I-TASSER/>) or use Phyre2 (<http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index>) for more rapid results in protein structure prediction/modelling. PyMOL is the preferred program for visualizing the models, and either Gene Construction Kit (<http://www.textco.com/gene-construction-kit.php>) or SnapGene (<https://www.snapgene.com/>) is used for *in silico* cloning.
5. We use a commercially available Eluent detergent (50% Solution) that is readily available from a variety of commercial suppliers. We have only used this method for TbpA and LbpA that enabled us to monitor isolation of functional protein by a solid-phase binding assay. However, this method should work effectively for other TBDTs.

6. We recommend selecting multiple loops of the TBDT to display on the scaffold as there is no empirical method for determining yet which loop will successfully fold with the scaffold and be sufficiently immunogenic and protective. For the scaffold, loops that are large can be selected to remove and then replace with a loop from a TBDT. We believe such positions are likely to accommodate TBDT loops of various sizes. We suggest designing and synthesizing various single and multiple loop hybrids using different loops in different positions initially. The number of hybrids can be narrowed down based on which constructs yield stable proteins for immunizations. Out of those hybrids, the one(s) that elicit a protective immune response can be used for future applications.
7. The custom expression vector pE5770 is available from Addgene ([www.addgene.org](http://www.addgene.org)) but any commercial or other available plasmids with similar components would be suitable. The vector contains the T7 phage promoter for high level expression by the T7 RNA polymerase. It also contains an N-terminal polyhistidine (6 His residues) tag to allow for the purification of the fusion protein by Ni-NTA chromatography, a biotin acceptor peptide to facilitate the *in vivo* biotinylation by the *E. coli* biotin ligase BirA, MBP to promote the solubility and proper folding of the fusion protein and a cleavage site recognized by TEV protease to allow for the removal of the aforementioned components in the N-terminal fusion tag. The pE5770 plasmid has BamHI and XbaI/HindIII restriction sites flanking a kanamycin resistance cassette that can be used to confirm successful cloning of the gene of interest (absence of kanamycin resistance). This expression system requires an *E. coli* strain (e.g., ER2566) that contains a chromosomal copy of the gene encoding T7 RNA polymerase under the control of the *lac* promoter and preferably is deficient in both *lon* and *ompT* proteases. In the presence of glucose, catabolite repression inhibits lactose transport so that expression of T7 RNA polymerase is inhibited by binding of the *lacI* repressor to the *lac* promoter. When the glucose in the autoinduction media is depleted, lactose transport is no longer inhibited, leading to activation of T7 RNA

polymerase expression, and thereby triggers the expression of the protein of interest encoded by the T7 expression vector (Figure 4.3). The rise in cAMP levels upon glucose depletion also increases expression by a cAMP-CAP complex binding to the CAP site upstream of the lac promoter.

8. After the addition of plasmid DNA into the tube containing chemically competent cells, we recommend flicking the tube gently to mix well.
9. Place the LB medium aliquot on ice and set the water bath/heat block at 42°C immediately after starting the 30-min incubation on ice to ensure that the correct temperature is reached in time for the subsequent step.
10. Our preference is to use the *E. coli* strain ER2566; however, any strain that fits the criteria outlined in **Note 7** would suffice.
11. Due to problems encountered with strains containing the T7 polymerase gene, the transformed mixture is used directly for expression rather than initial plating and selection of colonies for expression experiments. Autoinduction medium without any antibiotics can be prepared beforehand in 4-L flasks and 1-L bottles and stored at room temperature for up to 1 month for use. For making 1-L of medium add 1 mL of MgSO<sub>4</sub>, 50 mL of 20X NPS, and 20 mL of 50X 5052 to 929 ml of sterile ZY media in a sterile 1-L bottle.
12. This step is not always necessary; however, we find that pre-warming may enhance bacterial growth and protein yields compared to inoculation at room temperature.
13. The yield of protein after growth at 37°C appears to predict whether an additional period of growth at low temperature will lead to an increased yield. The expression plasmids with lower yields after growth at 37°C (perhaps unstable or prone to degradation) do not achieve enhanced yields with an additional incubation period at low temperature.
14. For efficient re-suspension of the cell pellets, scoop out each cell pellet using a spatula and transfer it to the beaker containing the Resuspension Buffer mixture. Then, transfer ~20 mL of the Resuspension Buffer mixture into each

centrifugation bottle using a serological pipettor to re-suspend any remaining bacteria. Finally, transfer each of the resulting cell suspensions to the beaker containing the cell pellets and the remaining Resuspension Buffer mixture.

15. Perform four discrete passes through the homogenizer and avoid re-circulating the sample as it results in uneven lysis – that is, collect the lysate in a beaker after each independent pass through the homogenizer instead of continuously directing the sample back through the homogenizer.
16. Use appropriate size filtration devices for filtering the supernatant. When using syringe filters, limit the volume to 25-50 mL of lysate, depending on viscosity. If the sample cannot be filtered easily using a 0.2- $\mu$ m syringe filter, use a 0.45- $\mu$ m filter first before passing the lysate through the 0.2- $\mu$ m filter. If the lysate still cannot be easily filtered, an additional 200  $\mu$ L of DNaseI (5 mg/mL) can be added and mixed in with the lysate for 30 min using a magnetic stir bar prior to filtration.
17. If the protein antigen of interest is readily detected in the supernatant, this suggests that the recombinant protein is sufficiently soluble to allow for the purification of the protein using the protocol outlined in **Section 4.3.3**. If the SDS-PAGE gel indicates the absence of the protein in the supernatant, or “soluble fraction,” this suggests that constitutive expression of the protein in the cytoplasm of *E. coli* has resulted in aggregation and misfolding of the protein. Thus, due to the apparent insolubility resulting from improper folding of the protein of interest, the protein is likely present alongside the cell debris in the pellet following centrifugation (**Section 4.3.2, step 12**). In this circumstance we recommend restarting the protein production run (beginning at the start of **Section 4.3.2**) and proceeding directly to **Section 4.3.4** immediately following **Section 4.3.2, step 8**.
18. A 5-mL column is ideal for culture sizes of 6 L or less. If larger than 6 L, use two 5-mL columns in tandem. Our preference is to use the 5-mL HisTrap High Performance column from Cytiva. If a chromatography system is not

available, the purification can be performed with Ni-NTA resin in a gravity fed column, by applying wash and elution buffers manually.

19. Circulation can be done at room temperature if 0.02% sodium azide is included in the lysate to prevent any microbial growth. For continuous circulation overnight, set up the column and any attached tubing such that the sample exiting the column is collected the same receptacle as the sample entering the column.
20. In lieu of commercial TEV protease we routinely use a TEV protease with an N-terminal polyhistidine tag produced in-house in *E. coli* using a T7 expression vector. When expressed with a polyhistidine tag, it can be purified using a Ni-NTA column as described in **Section 4.3.2**, concentrated to a final concentration of 1-10 mg/mL, mixed with sterile 100% glycerol to a final concentration of 50%, and stored at -20°C. This glycerol mixture – typically containing ~5 mg of TEV protease for up to 100 mg of fusion protein – can then be added directly to the dialysis tubing containing the entirety of the protein antigen sample as part of the TEV cleavage step. Cleavage of the fusion protein by TEV protease is carried out efficiently during the overnight (16-18 h) incubation period at 4°C – there is no need to extract the protein sample from the dialysis tubing for the cleavage step.
21. A single circulation would suffice and would reduce the risk of losing any uncleaved fusion protein in the process. If just circulating once, set up the column and any attached tubing such that the sample exiting the column is collected in a separate receptacle from the sample entering the column. Then, circulate the sample until the entire sample has passed through the column.
22. Our experience in preparation of antigens by these methods have not resulted in situations where lipopolysaccharide (LPS) toxicity was an issue or where levels measured by the limulus amoebocyte lysate coagulation (LAL) assay were of concern. Thus, aliquots were normally promptly stored at -80°C; however, it may be prudent to test protein preparations with the LAL assay when using new strains or new proteins to ensure LPS removal is not required.

If endotoxin removal is deemed necessary, the methods our group prefers include the MonoQ column from Cytiva, the CHT I or CHT II columns from Bio-Rad, or the Pierce High Capacity Endotoxin Removal Spin Columns.

23. Before adding 3 mL of Ni-NTA resin, wash the resin to eliminate the ethanol, and use 3 mL of Denaturing Lysis Buffer to equilibrate the resin.
24. Depending on the protein purification yield, a greater volume of Refolding Elution Buffer may be needed. Verify the protein concentration at the end of the elution step to confirm that all of the column-bound protein has been eluted.
25. As it is possible to assay SBP-TbpA for proper folding using the transferrin binding assay described in **Section 4.3.8**, we recommend cloning an SBP-TbpA fusion construct alongside cloning of the desired target SBP-TBDT construct (**Section 4.3.5**) as a control for steps in **Section 4.3.6** and **4.3.7**.
26. The custom expression vector pE5771 is available from Addgene ([www.addgene.org](http://www.addgene.org)). Vector pE5771 was designed by our group for expression of TBDTs such as TbpA and LbpA. It has a kanamycin resistance cassette in the expression locus between BamHI and XbaI sites so that colonies generated from cloning the gene of interest into the expression locus can be screened on plates containing kanamycin to confirm insertion (no growth on kanamycin-containing plates). This plasmid encodes the *pelB* signal sequence that results in substantial levels of periplasmic proteins being secreted into the periplasm of *E. coli* protein expression strains such as BL21. The signal sequence is followed by four amino acids preceding a polyhistidine (6x His) tag, a TEV protease cleavage site, SBP, and a second TEV cleavage site, upstream of the mature *tbdT* sequence (Figure 4.6). This plasmid can also be used for large-scale TBDT protein preparations, where detergent-extracted TBDT membrane extracts can be purified using a Ni-NTA column and cleaved with TEV protease to generate purified TBDT protein.
27. We recommend using a cell line like *E. coli* C43, a derivative of the commonly used BL21 expression cell line. This cell line has the wild-type *lac*

promoter in the chromosome instead of the mutated *lacUV5* promoter present in the parent. The result is reduced expression of the T7 RNA polymerase and subsequent reduction in expression of the target gene. Cell lines like C43 or the similar C41, also known as the Walker cell lines, have been shown to be more appropriate for expression of membrane proteins and toxins. Chemically competent cells can be pre-made and stored at -80°C in 50- or 100-μL aliquots in 1.5-mL Eppendorf tubes until use. If the intention is to screen antisera for reactivity against different variants of the TBDT, it is advised to consider performing the procedure in **Section 4.3.6** in parallel with plasmids encoding the different variants.

28. We typically remove the cell/Tris/Eluent mixture from the 50 mL conical tube and transfer it into a 2-mL Eppendorf tube and use a rotary shaker in the refrigerator overnight. A specific rpm is not required as long as it is gently shaking overnight.
29. We recommend using the TBDT-PBST mixture for coating ELISA plates the same day as we have not evaluated the impact of storage of the mixture with TbpA which would have enabled us to evaluate maintenance of native conformation by a binding assay.
30. The proportions described in this protocol amount to just enough TBDT for one 96-well ELISA plate coated with 100 μL/well of TBDT-containing solution. This can be scaled up or down based on how many plates or wells the user needs. We recommend making more than what is required in case some protein is lost in the process of coating the plates.
31. When using TbpA as the TBDT, either on its own or as a control for correct conformation of the detergent extracted TBDT, it is recommended to first perform a separate ELISA to test for correct conformation of TbpA prior to assessing antibody titers from immunized animals. For this conformation ELISA, only one column of a streptavidin-coated plate is needed, adding SBP-TbpA/PBST to only two wells (but blocking all wells in the column) following the instructions in **Section 4.3.7**, steps 1-3, and then proceeding to

**Section 4.3.8.** If a weak signal is observed in this assay, anti-TBDT titres assessed in a subsequent ELISA should be interpreted with caution as the TBDT used to coat the plate may be misfolded. If a ligand known to bind to the TBDT under investigation is identified, this TBDT-ligand interaction can be exploited to give the user more confidence that the TBDT in question is properly folded (*see Note 34*).

32. When an entire plate is not needed, (e.g., when determining the ability of the TbpA to bind transferrin, as described in **Section 4.3.8**, *see Note 31*) only coat the wells to be used. Cover the unused portion of the plate, which can be used for a later experiment, with an adhesive slip. We typically use streptavidin-coated Greiner Bio-One plates in our experiments; however, other commercially available streptavidin/neutravidin-coated plates can also be used. Plates can also be coated with streptavidin or neutravidin in-house (J.E. Fegan et al., 2021a).
33. To wash, discard the PBST in the wells by inverting the 96-well plate over the sink and expelling the liquid with a sharp flick of the wrist. Following this, vigorously tap the plate face-down on a stack of paper towels to remove the remaining liquid. An automated plate washer may also be used for these steps, if available.
34. The TbpA/Tf-HRP ELISA is used to assess whether the TbpA is properly folded on the plate. For TBDTs that bind metal-containing proteins (hemoglobin, hemoglobin-haptoglobin, calprotectin), a similar approach could be used, with an additional step to add appropriate target protein, followed by HRP-conjugated antibody specific for the target protein; however, alternative approaches will be required for TBDTs that directly bind metal ions or metal ions bound by compounds (heme, siderophore-iron complexes).
35. We recommend performing 2-fold serial dilutions across the columns labelled 1-11, leaving the last column for any pertinent controls; however, if no controls are needed, up to 12 different dilutions can be used.

## CHAPTER 5: DISCUSSION

### 5.1 Summary

Transferrin binding proteins have been identified as promising vaccine antigens against important Gram-negative bacterial pathogens that cause disease in humans and food production animals. The objective of this thesis was to further explore the potential of these vaccine candidates against important porcine pathogens. In Chapter 2, manuscript 1, it was demonstrated that binding defective TbpB mutants from *G. parasuis* evaluated in a murine immunization and sepsis model could recapitulate the patterns of protection previously observed in the physiologically relevant swine model suggesting that the murine model could be utilized to screen ‘good’ mutants from ‘bad’ mutants. In that study, it was also demonstrated that Y167A TbpB was only able to elicit a cluster specific cross-reactive response, therefore, in Chapter 3, manuscript 2, new TbpB mutants, defective in binding to porcine transferrin, were designed from a cluster 1 TbpB expressing *A. pleuropneumoniae* strain and evaluated for efficacy in the murine model. Y109A TbpB, the antigen eliciting the highest level of protection, was then shown to be highly cross-reactive against multiple variant TbpBs across all three clusters. A combined Y167A TbpB and Y109A TbpB vaccine formulation likewise elicited a broadly cross-reactive response raising the possibility that a final TbpB based vaccine limited to these two antigens may be sufficient in providing protection against the entire repertoire of TbpB variants. In Chapter 4, manuscript 3, a novel method for generating chimeric/hybrid antigens using TbpB based scaffolds to display extracellular loops from TBDTs and OMPs. Taken together, the findings presented in this thesis adds to the existing literature evaluating transferrin binding proteins as vaccine antigens and brings us closer to the optimal formulation needed to elicit a broad-spectrum, efficacious response against the three porcine pathogens.

### 5.2 Discussion

The global pig industry is often plagued with disease outbreaks, including those caused by upper respiratory tract residing bacteria *A. pleuropneumoniae*, *A. suis*, and *G.*

*parasuis* (Curran et al., 2015). The main form control includes antimicrobial treatment, biosecurity husbandry practices, and vaccinations. Widespread usage of inactivated whole cell vaccines (bacterins) is the predominant commercial approach for preventing *G. parasuis* infection (Liu et al., 2016). However, these vaccines exhibit limited efficacy for numerous reasons which effectively lead to vaccine escape and continual outbreaks.

The protection induced by inactivated whole cell vaccines is largely attributed to being directed against the extracellular capsular polysaccharide. This results in a highly specific protective response that is unable to yield any cross-protection against strains expressing other capsular types (Bak & Riising, 2002; Takahashi et al., 2001), though failure to protect against a homologous capsular type have also been observed (R. Frandoloso et al., 2015), resulting in leaving vaccinated herds susceptible to disease caused by strains from other serovars.

These vaccines often offer little to no protection against virulent strains that may be circulating in a given region (Espindola et al., 2019; Liu et al., 2016). A recent survey of *G. parasuis* serovars in different regions in Brazil demonstrated that majority of the circulating strains were not covered by commercially available vaccines, which the authors estimated could have prevented at most 28% of the outbreaks reported in that study (Espindola et al., 2019), a major concern for the fourth largest supplier of pigs globally. This lack of ‘matching’ between the capsular type in the vaccine and the region where the vaccine is being deployed is likely to contribute to continually occurring outbreaks. Vaccine-induced selective pressure could be a possible mechanism behind this form of vaccine escape, resulting in an increased prevalence of non-vaccine serovar types.

While a strong correlation between serovar and virulence has yet to be established, it is still generally thought that certain capsular types are more associated with virulence (Kielstein & Rapp-Gabrielson, 1992; Oliveira, Blackall, & Pijoan, 2003), which has been used to justify the inclusion of certain strains expressing specific capsular types into bacterin formulation. Strains belonging to serovar 7 are considered to have low virulence potential as *G. parasuis* serovar 7 reference strain (174) was unable to cause disease in piglets (Brockmeier et al., 2014; Kielstein & Rapp-Gabrielson, 1992).

However, outbreaks of Glasser's disease with serovar 7 strains in swine herds in China (Wang et al., 2017) and Brazil (Prigol et al., 2019) have started to bring that notion into question. Furthermore, experimentally challenging pigs with *G. parasuis* serovar 7 reference strain (174) has been recently shown to result in the classic symptoms of Glässer's disease (Dazzi et al., 2020; R. Frandoloso et al., 2020; Guizzo et al., 2018). Thus, the classification of all serovar 7 strains being non-virulent needs to be reevaluated. This may be a general phenomenon for other classical non-virulent serovars and the exclusion of such strains from vaccine formulations is likely a major factor resulting in the outbreaks noted above. Overall, these observations explain why focusing on the premise that disease is associated with a limited number of virulent strains results in vaccines against *G. parasuis* that have been unable to fully protect against infection.

Currently available *A. pleuropneumoniae* vaccines typically consist of those formulated with the Apx toxins, such as Porcilis APP, or inactivated whole cell bacterins (Sassu et al., 2018). Multiple studies investigating vaccine efficacy of Porcilis App in the field have reported that it was unable to completely protect against disease and prevent *A. pleuropneumoniae* natural infection or transmission (Del Pozo Sacristan et al., 2014; Loera-Muro & Angulo, 2018; Sjolund & Wallgren, 2010). Bacterins available for *A. pleuropneumoniae* raise similar concerns to those noted above regarding serovar specific responses.

For preventing disease by *A. suis* options are limited to autogenous vaccines, which are typically produced for a specific farm and can only be used at that farm (Costa-Hurtado et al., 2020; Lapointe et al., 2001). Although vaccination campaigns deserve enormous credit for having reduced mortality and morbidity, frequent outbreaks coupled with limitations behind current vaccine design strategy make it evident that a different approach to vaccine development is required for the next generation of vaccines against these porcine pathogens.

In contrast, our approach is based on the selection of a surface exposed antigen that is functionally required by these pathogens to colonize and cause invasive disease. The bacterial transferrin receptors have been shown to be essential for the survival and pathogenesis of many of these pathogens (Baltes et al., 2002; Cornelissen et al., 1998). It

is then not surprising that the bacterial transferrin receptor is present in all isolates tested (Adamiak et al., 2015; Curran et al., 2015). These attributes of the bacterial transferrin receptor make it a logical choice for a vaccine antigen and allows us to posit that a rationally designed vaccine could protect against all strains and potentially eliminate the pathogen from its niche.

In this thesis, the bacterial transferrin receptors were evaluated as vaccine antigens. In chapter 2, manuscript 1, it was demonstrated that binding defective TbpB mutants from *G. parasuis* evaluated in a murine immunization and sepsis model could recapitulate the patterns of protection previously observed in the swine model suggesting that the murine model could be utilized to effectively screen ‘good’ mutants from ‘bad’ mutants. The significance of this finding relies on our lack of understanding behind the mechanism by which Y167A TbpB proves itself a more efficacious antigen than W176A TbpB (Guizzo et al., 2018). Although differences in protein stability in physiological conditions cannot be ruled out as a variable, no such differences were observed in laboratory conditions. Furthermore, these findings imply that differences in the immune response elicited by the two mutants are likely not dependent on host specific factors as they were also observed in mice.

As noted above, it is unclear as to the reasoning behind the success of one TbpB mutant as a vaccine antigen and the lack of for another TbpB mutant. Differences in protein stability in physiological conditions during antigen recognition by B-cells may affect the response generated as this would impact the ability to elicit a robust humoral response against conformational epitopes. Further biochemical analysis looking at melting curves of the two TbpB mutants at different temperatures could begin to elucidate these processes though extrapolation of protein mechanics from laboratory conditions to physiological conditions must be done with caution.

Another possible explanation for the differences observed between the two TbpB mutants is that the tryptophan amino acid is part of an important epitope(s). Analyses of common B-cell epitopes have indicated that aromatic residues like tryptophan and tyrosine are present in higher frequencies, likely due to its ability to form strong hydrophobic interactions with B-cell receptor epitope binding regions (paratopes)

(Rubinstein et al., 2008). These interactions are a necessary process in the initiation of the adaptive immune response. Thus, the mutation of the tryptophan amino acid could have stunted the generation of a robust immune response. In order to explore this hypothesis, monoclonal antibodies specific to Y167A TbpB or W176A TbpB can be generated and used in passive immunization and challenge experiments in mice or pigs to determine whether specific epitopes including either the tyrosine or the tryptophan are protective. While further work is needed to understand the differences between ‘good’ and ‘bad’ TbpB mutants, the ability to identify them remains highly beneficial.

As noted throughout this thesis, TbpB is a highly variable antigen and thus a final vaccine composition will likely need multiple variants of binding defective TbpB mutants. Since this diversity is mainly localized to the binding interface on TbpB, there is no conserved residue that exists that can be selected for mutagenesis to generate these variant TbpB mutants. Hence, multiple mutants are generated to initially identify ones with significantly reduced binding to Tf. Our murine sepsis screening model allows us to weed out poorly efficacious TbpB mutant antigens such that only a limited number of efficacious TbpB mutant antigens are continued forward for evaluation in pigs, which are considerably more expensive and labour intensive.

These findings also provide confidence to studies in mice evaluating different TbpB mutants as vaccine antigens derived from pathogens causing disease in humans like *N. meningitidis* and *Haemophilus influenzae*. If a model using the physiologically relevant host was essential in evaluating different TbpB mutants then it would be challenging to assess these antigens as there are limited animal models available for evaluating prospective vaccine candidates from these exceptionally host adapted organisms. However, humanized transgenic mouse lines expressing important host factors have been underdevelopment in attempts to overcome this issue ((Johswich & Gray-Owen, 2019) unpublished).

The underlying premise that host ligands can interact with the antigen and interfere with the generation of a robust immune response such that a ligand non-binding mutant antigen performs better than the wildtype antigen could be a common consideration when targeting host binding proteins. This phenomenon has been

demonstrated for the vaccine antigen Factor H binding protein (fHbp) in *N. meningitidis* where a non-binding mutant results in superior protection compared to the wildtype in numerous animal models (Beernink et al., 2011; Beernink, Vianzon, Lewis, Moe, & Granoff, 2019; Granoff et al., 2016). These studies and subsequent observations were made after the WT fHbp was already included in the now commercially licensed MenB-4C and MenB vaccines against *N. meningitidis* serogroup B, demonstrating the importance of initially using a structure-based approach for designing vaccines using host binding proteins.

Antibodies elicited by TbpB-based antigens could have the capacity to bind to TbpB expressed on the cell surface and reduce or prevent binding by host transferrin, thereby resulting in protection against disease via nutritional immunity. It was notable that immunizations with both Y167A and W176A TbpB elicited antibodies that prevented binding by pTf. While this has been shown previously in the analysis of antiserum generated from a swine immunization series (Barasuol et al., 2017), this consistent finding suggests that immunity through prevention of nutrient acquisition may not be a dominant mechanism of protection in the swine model, which had been previously considered to potentially play a role. Although a deeper investigation is warranted to determine whether this is a general phenomenon or specific to these antigens, functional antibody mediated opsonophagocytosis and complement killing are likely more universally dominant mechanisms of protection.

A challenge with utilizing this murine model of sepsis is the rapid onset of symptoms and subsequent death, likely due to a combination of bacterial replication and secretion of toxins by the challenge strain. While the rapid progression of disease in mice does not replicate the progression observed in pigs, it does allow the evaluation of a purely antibody mediated response, which has been shown to be associated with protection from the pathogens in question. The vaccine induced protection observed is anticipated to be mediated by mechanisms of opsonophagocytosis with blood neutrophils and antibody dependent complement mediated killing, however these mechanisms require investigation. Identifying correlates of protection like opsonophagocytic activity or complement killing can further ease the process of screening mutants as establishing murine models with different pathogenic strains can be challenging.

We are currently developing an improved murine sepsis model using *N. meningitidis* strains expressing the porcine bacterial transferrin receptor. Since an acute sepsis model in mice with *N. meningitidis* has been previously established (Johswich & Gray-Owen, 2019), we hypothesize that using a strain modified to encode the porcine bacterial transferrin receptor will result in a model where no toxins are secreted and the onset of sepsis is delayed allowing us to further elucidate protection. *N. meningitidis* strain expressing the porcine bacterial transferrin receptor supplemented with iron loaded porcine transferrin can grow *in vitro* (Litt, Palmer, & Borriello, 2000), however, these strains will need to be evaluated in mice initially to determine whether the bacteria can grow and replicate *in vivo* with a porcine transferrin supplement. We anticipate that this improved murine sepsis model in combination with attempts to determine correlates of protection will help with the identification of efficacious TbpB mutants and shed light into the mechanisms of protection.

In chapter 3, manuscript 2, a new, cluster 1 TbpB binding defective mutant was identified from *A. pleuropneumoniae*, Y109A TbpB, that elicited the highest level of protection in the murine sepsis model. Immunizations with Y109A TbpB alone elicited a broadly cross-reactive response against variant TbpBs across all three clusters. The findings of this study support the subsequent evaluation of Y109A TbpB in a swine model of porcine pleuropneumonia to assess efficacy of preventing disease. Generally most experimental *A. pleuropneumoniae* challenge models utilize an intranasal inoculum or aerosol challenge containing large doses of bacteria (Sassu et al., 2018). The secretion of toxins by the challenge strain can making it difficult to evaluate the protective efficacy of a TbpB-based antigen. Thus, we propose the use of a transmission model where infected pigs are grouped with vaccinated, unchallenged pigs to determine whether a TbpB-based vaccine can prevent transmission and disease progression in a natural setting. Immunizing pigs with Y167A TbpB has recently been shown to prevent the occurrence of natural colonization by *G. parasuis* (R. Frandoloso et al., 2020). It is worthwhile evaluating whether immunizations with Y109A TbpB can confer the same protection.

While the objective in this thesis was to evaluate the bacterial transferrin receptor as a vaccine antigen, additional studies investigating the roles of adjuvants are warranted.

Adjuvants modulate the immune response and are powerful tools in vaccine development (Owen et al., 2013). Immunizations with TbpB based antigens formulated with different adjuvants in both murine and swine models have impacted levels of IgG titres (Barasuol et al., 2017), protection against disease (R. Frandoloso et al., 2020), and colonization by the bacteria in mucosal spaces (R. Frandoloso et al., 2020). In this thesis two different adjuvants were used with the mutant TbpBs to evaluate whether the cross-reactivity profile was influenced by adjuvant choice. Montanide Gel 01 is an aqueous gel-based adjuvant composed of sodium polyacrylate polymers and have been used in both mice and swine immunization models to demonstrate the generation of higher IgG titres than other Montanide adjuvants (Barasuol et al., 2017; Xue et al., 2015). The addition of poly I:C, a potent activator of dendritic cells, has also been shown to elicit higher IgG titres than Montanide Gel 01 alone (personal communication with Dr. Rafael Frandoloso). AddaVax, a research grade adjuvant similar to the commercially approved MF59, was included due to the greater cross-reactivity profile observed in immunization studies with TbpBs expressed by *N. gonorrhoea* by our collaborators (personal communication with Dr. Jamie Fegan). However, in this thesis no differences were observed in the cross-reactivity profile though differences in systemic protection, mucosal responses, and longevity of memory cannot be ruled out without further experiments.

In Chapter 4, manuscript 3, a novel method for generating chimeric/hybrid antigens using TbpB based scaffolds to display extracellular loops from TBDTs and OMPs is described. While majority of the thesis has focused on evaluating TbpBs as vaccine antigens, TBDTs like TbpAs are also promising vaccine candidates. TbpA has a far more conserved sequence than TbpB in all of the different pathogens tested so far, an attractive characteristic for a vaccine antigen as more cross-reactivity would be anticipated, a primary step for cross-protection (Adamiak et al., 2015; Curran et al., 2015). Immunizations of mice with TbpA expressed from *N. meningitidis* was able to elicit bactericidal antibodies, as well as stronger protection in an *in vivo* mouse sepsis model with superior cross-protection compared to immunizations with TbpB (West et al., 2001). Immunizations of guinea pigs with TbpA from *G. parasuis* also resulted in cross-protection against a bacterial challenge with different serovars of *G. parasuis* (Huang et al., 2013).

Although SLPs such as TbpB can be easily produced in large quantities in the cytoplasm of *E. coli* and are suitable for the production of commercial vaccines, there are significant problems in utilizing integrated membrane proteins as vaccine antigens. TBDTs such as TbpA are not suitable for commercialization because they are produced on the outer membrane in much lower yields and require detergents for solubilization and stabilization.

The hybrid antigen approach was conceptualized in order to target these TBDTs as vaccine antigens by taking the surface exposed ‘loops’ that span from and are constrained by anti-parallel beta barrel strands and expressing them on scaffolds derived from SLPs that also contain similar anti-parallel beta strand barrel structures. Essentially this novel chimeric antigen would be a hybrid of the TBDT and SLP while acting like SLPs regarding protein production, which overcomes the limitation of producing TBDTs as vaccine targets while still targeting TBDTs.

Using this approach hybrid antigens were generated against *N. meningitidis* by targeting the TbpA loops (J. E. Fegan et al., 2019). Mice immunized with these antigens elicited antibodies that recognized the native protein expressed by whole cells and exhibited complement mediated bactericidal activity. Separate mice immunization and challenge experiments demonstrated protection against *N. meningitidis* in mouse sepsis model and against colonization by *N. gonorrhoeae*. Hybrid antigens were also developed against human pathogen *Acinetobacter baumannii* using outer membrane TonB dependent zinc acquisition protein (ZnuD) (Qamsari et al., 2020). Mice immunized with these antigens were protected against lethal challenge by *A. baumannii*. Taken together, these results demonstrate the utility of the hybrid antigen approach.

At present, there is no systematic approach for selecting loops and loop combinations that can be predicted to be protective. Identifying efficacious hybrid antigens is dependent on evaluating the antigen in animal models. The basic principle of hybrid antigens with an SLP ‘scaffold’ displaying surface loops could ultimately be extended to other integral outer membrane proteins with a beta-barrel structure. There are current ongoing efforts to use this approach to target various such other TBDTs and

OMPs involved in nutrient acquisition in hopes to generate novel efficacious vaccine antigens.

While not evaluated in this thesis, the prospect of a mutant TbpB from a porcine pathogen expressing loops from TbpA from the same pathogen is intriguing. It is possible that due to the high sequence conservation of TbpA (including the loops) that a single TbpB based antigen with multiple loops from TbpA can elicit a broadly cross-protective response. However, this line of investigation requires further experiments.

Overall, our approach to rational vaccine design is founded on the principal of identifying surface antigens that are essential for pathogen survival and using a structure- and diversity-based perspective to engineer improved antigens.

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