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The Role of Lactoferrin Binding Protein B in Gram-Negative Pathogens

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The Role of Lactoferrin Binding Protein B in Gram-Negative Pathogens

by

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Abstract

The human pathogens *Neisseria meningitidis* and *N. gonorrhoeae* express a receptor capable of binding to, and removing iron from, the host glycoprotein lactoferrin. Similar to the transferrin binding protein complex, the lactoferrin receptor consists of an integral outer membrane protein, lactoferrin binding protein A (LbpA), and a surface exposed bi-lobed lipoprotein, lactoferrin binding protein B (LbpB). Human gonococcal infection models have shown that possession of either lactoferrin or transferrin binding proteins are essential for survival, while possession of both receptors provides a competitive advantage during co-infection. This thesis identifies a novel function for LbpB showing that LbpB is capable of providing protection against human lactoferricin, a short cationic peptide derived from human lactoferrin. It further demonstrates that the protection by LbpB is mediated by two clusters of negatively charged amino acids that localize to exposed loops in the C-terminal lobe of LbpB, a region with no previously experimentally demonstrated function.

In addition, this thesis demonstrates that the protection provided by LbpB in our assay system is underestimated, due to the activity of NalP, an outer membrane protein mediating proteolytic release of LbpB from the surface. An isogenic mutant lacking NalP activity has substantially enhanced protection in our assay, which is likely more representative of the activity of LbpB *in vivo*. In this study the negatively charged capsular polysaccharide is shown to confer a modest degree of protection against lactoferricin at lower concentrations of lactoferricin. This thesis also includes preliminary experiments demonstrating that protection mediated by LbpB can extend to other cationic antimicrobial peptides. Thus this study indicates that LbpB provides substantial protection against the antimicrobial peptide derived from human lactoferrin and may provide effective protection against similar elements of the innate host defense mechanisms.

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Table of Contents

LIST OF TABLES

LIST OF FIGURES AND ILLUSTRATIONS

List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
µg	micrograms
aa	Amino acid
ANOVA	Analysis of variance
APC	Antigen presenting cells
BHI	Brain heart infusion
CAP	Cationic antimicrobial peptide
CECAM	Carcinoembryonic Antigen-related Cellular Adhesion Molecule
CFU	Colony forming unit
FbpA/B/C	Ferric binding protein A/B/C
Fe	Iron
Fur	Ferric uptake regulator
HRP	Horse radish peroxidise
IgA, IgG, IgM	Immunoglobulin A, G, M
IL	Interleukin
kDa	Kilodaltons
l	Liters
Lbp	Lactoferrin binding protein
LbpA	Lactoferrin binding protein A
LbpB	Lactoferrin binding protein B
Lf	Lactoferrin
LfcinH	Human lactoferricin
LfcinH (1-11)	Amino Acids 1-11 of Human lactoferricin
LfR	Lactoferrin receptor
LG	Large
LPS	lipopolysaccharides
M. bovis	*Moraxella bovis*
M. catarrhalis	*Moraxella catarrhalis*
Mbp	Maltose binding protein
mg	miligrams
MHC	Major histocompatibility complexes
ml	mililiters
N. gonorrhoeae	*Neisseria gonorrhoeae*
N. meningitidis	*Neisseria meningitidis*
NA	Not applicable
PCR	Polymerase chain reaction
PspA	Pneumococcal surface protein A
SDS-PAGE	Sodium dodecyl sulfate polyacrylamid gel electrophoresis

SM	Sm
Tbp	Transferrin binding protein
TbpA	Transferrin binding protein A
TbpB	Transferrin binding protein B
TBS	Tris Buffered Saline
TEV	Tobacco etch virus
Tf	Transferrin
TH	Todd Hewitt
TNF	Tumor necrosis factor
WT	Wildtype
YNB	Yeast Nitrogen Base

CHAPTER 1: INTRODUCTION

1.1 The Innate Immune Response

The human immune response can be broken into two branches, the innate and adaptive immune responses (Janeway, Travers et al. 2005). The innate immune response is a nonspecific, but fast acting response used to combat pathogens that are able to circumvent the host's natural barriers (Medzhitov and Janeway 2000). Once a pathogen passes through these barriers and enters the body it can begin to replicate, resulting in an infection. As the innate immune response is non specific, it recognizes invading microorganisms using receptors that recognize generic molecules or motifs expressed on or released from the surface of pathogens, such as LPS (Akira, Uematsu et al. 2006; Medzhitov 2007). This reliance on pattern recognition receptors is an elegant mechanism of providing broad protection against microbes, while simultaneously allowing for selection between host cells and invading organisms.

Typically, pathogens first encounter macrophages, a monocyte which resides within the host tissue (Medzhitov 2007; Stephens, Greenwood et al. 2007). Once the macrophage recognizes an invading pathogen, thereby being activated, it releases various chemokines, and cytokines (Medzhitov and Janeway 2000). These chemokines and cytokines attract and activate other leukocytes, particularly neutrophils, to the site of infection, resulting in a site of localized inflammation (Saadi, Wrenshall et al. 2002). In addition to attracting help to the site of infection, macrophages release additional inflammatory mediators that trigger local changes within the surrounding tissue such as an increase in vascular permeability. Increased permeability results in a local

accumulation of serum proteins, including complement, which plays an important role in combating pathogens (Saadi, Wrenshall et al. 2002; Janeway, Travers et al. 2005). Complement is able to opsonize the invading pathogen which can be directly bactericidal, and improve phagocytosis by neturophils and macrophages (Carroll and Fischer 1997). Additional serum proteins are used to form clots which wall off the site of infection into a local area, preventing the dissemination of the pathogen (Janeway, Travers et al. 2005; Medzhitov 2007).

In addition to stimulating the immune response, recognition of pathogens by macrophages and other leukocytes directly triggers antimicrobial responses, most notably phagocytosis and degranulation (Medzhitov 2007). Following phagocytosis, internalized pathogens are subjected to hostile conditions within the phagolysozome, which should result in the pathogen's death. Further, pathogens phagocytosed by antigen presenting cells (APC) such as macrophages and dendritic cells are processed and potential antigens are coupled with two major histocompatibility complexes (MHCs). Coupled antigens are presented on the surface of the APC to activate T-helper cells, that in turn stimulate the development of pathogen specific antibodies by B cells (Janeway, Travers et al. 2005; Jensen 2007; Medzhitov 2007).

Complementing phagocytosis, pattern recognition receptors also stimulate leukocyte degranulation (Janeway, Travers et al. 2005). Leukocyte granules contain many effective antimicrobials agents, including antimicrobial peptides (reviewed in chapter 4 section 2), which when released can drastically reduce the survival of invading

pathogens (Nathan 2006). In addition to antimicrobial peptides, granules contain other antimicrobial proteins and inflammatory modulators such as Lf (refer to Section 1.4 for a detailed overview of Lf). Notably it has been shown that granule proteins and peptides have potent immunomodulatory properties. These properties have been suggested to assist in preventing the body from entering a state of septic shock which is associated with high rates of mortality. Further it was recently suggested that these proteins and peptides can stimulate the adaptive immune response, thereby further bridging the two immune responses within the body (Nathan 2006; Medzhitov 2007).

1.2 The Family Neisseriaceae

The family Neisseriaceae consists of Gram-negative diplococcal bacteria which include the genera *Neisseria* and *Moraxella* (Knapp 1988). To date members of the family Neisseriaceae have been isolated from a variety of hosts including humans, monkeys, cows and dogs (Neill, Ellis et al. 1978; Vedros, Hoke et al. 1983; Knapp 1988; Andersen, Steigerwalt et al. 1993; Brown, Brightman et al. 1998). Although there exists a significant focus within the literature towards human Neisseriaceae isolates, a subset of articles have focused on animal isolates such as the *Macaca mulatta* commensal *N. macacae* (Weyand, Wertheimer et al. 2013), the bovine ocular pathogen *M. bovis* (Brown, Brightman et al. 1998; McConnel and House 2005; Postma, Carfagnini et al. 2008), and *N. weaveri*, a canine commensal organism associated with infected dog bites in humans (Andersen, Steigerwalt et al. 1993; Holmes, Costas et al. 1993; Panagea, Bijoux et al. 2002). Similarly the focus of the literature on human Neisseriaceae isolates

has been on the disease causing *N. meningitidis, N. gonorrhoeae, M. lacunata* and *M. catarrhalis* (previously referred to as *Branhamella catarrhalis*).

To date eight species of commensal *Neisseria* have been identified; *N. lactamica, N. polysaccharea, N. flavescens,N. cinerea, N. sicca, N. pharyngis, N. mucosa,* and *N. subflava* (Marri, Paniscus et al. 2010). These organisms have been routinely isolated from the nasopharnyx and upper respiratory tract of humans and are not commonly associated with disease and are therefore designated commensal organisms (Johnson 1983). Interestingly this designation does not preclude them from causing disease as they have been identified within the literature to be capable of causing disease. Most notably *N. lactamica* has been associated with clinical cases of meningitis and septicemia (Lauer Ba 1976; Johnson 1983), and *N. cinerea* have been associated with pediatric ocular infections (Johnson 1983; Dolter, Wong et al. 1998). Furthermore *N. meningitidis* which is commonly associated with disease has been found to be a component of the normal flora of 10-15% of adults in the developed world, where rates of *N. meningitidis* associated disease are low (Cartwright, Stuart et al. 1987; Durey, Bae et al. 2012). Thus the distinction between commensal and pathogenic bacteria is not as clear as commonly stated in review articles.

1.3 Clinical relevance of the Neisseriaceae

1.3.1 Neisseria meningitidis

N. meningitidis was first isolated by Anton Weichselbaum in 1887 from the cerebral spinal fluid of a patient presenting with meningitis (Weichselbaum 1887). Since

its initial discovery in 1887 *N. meningitidis* has been identified by the World Health Organization as one of the leading causative agents of bacterial meningitis, making it a major global pathogen. Although commonly associated with bacterial meningitis, meningococcal disease is also associated with other clinical presentations most notably meningococcemia, a *N. meningitidis* infection of the blood (Rosenstein, Perkins et al. 2001). The rapid progression of disease is a defining feature of meningococcal infections and led to the statement by Herrick that "no other infection so quickly slays" (Herrick 1919). It is for this reason that a significant focus of *N. meningitidis* research is on prevention and why early recognition is so essential to effective treatment. Despite the development of vaccines and improvements in available treatments, *N. meningitidis* still affects an estimated 500,000 people annually with fatality rates ranging from 5-50% worldwide (WHO ; Tzeng and Stephens 2000; Stephens, Greenwood et al. 2007). In addition to the high fatality rates associated with meningococcal infection, 20% of survivors suffer permanent complications such as loss of limbs or brain damage. Although the global economic burden of *N. meningitidis* is unknown, it was estimated in 1996 that a single case of bacterial meningitis in the United States had an economic burden of $1,000,000, a value that has likely increased with inflation over the last 17 years (Levine, Shaffer et al. 1996).

Currently the pathogenesis of infection by *N. meningitidis* is not fully understood, particularly as it is a human specific pathogen with no valid animal models (Melican, Michea Veloso et al. 2013). It is believed that *N. meningitidis* is transmitted by asymptomatic colonized individuals to healthy non-colonized individuals by aerosol

droplets (Tzeng and Stephens 2000; Rouphael and Stephens 2012). In the approximately 1% of colonized individuals who develop meningococcal disease, it is presumed that an acute viral upper respiratory tract infection disrupts the host's innate immune response leaving the body vulnerable to infection (Raza, Ogilvie et al. 1993; Hament, Kimpen et al. 1999; Deuren, Brandtzaeg et al. 2000). During invasion *N. meningitidis* isolates interact with host CECAM receptors, whose expression is induced by LPS, promoting their uptake by epithelial cells thus allowing the pathogen to traverse the epithelial layer within the phagosome (Tzeng and Stephens 2000; Muenzner, Naumann et al. 2001). Bacteria that have passed the mucosal layer and entered the circulatory system where they multiply resulting in a bacteremia. From the bloodstream the bacteria are able to cross the blood brain barrier reaching the meninges where their proliferation triggers a local inflammatory response that can often be damaging to the brain and central nervous system (Stephens, Greenwood et al. 2007).

Despite the high economic burden and importance of *N. meningitidis* as a global pathogen, attempts to eradicate the organism have been unsuccessful. Current prevention campaigns focus on the use of vaccines which target the bacterial polysaccharide capsule, an approach that has been widely used to reduce the incidence of disease associated with other bacterial organisms such as *Haemophilus influenzae* and *Streptoccous pneumoniae* (Poolman 2004; Harrison 2008; Sadarangani and Pollard 2010). The capsule is an important virulence factor that protects invading bacteria from effective complement deposition, thus it makes for an excellent vaccine target. However, in order to develop vaccines from capsular antigens suitable for childhood immunization programs, it was

necessary to conjugate the capsule to a protein carrier so that immunological memory was induced (Snape and Pollard 2005).

To date there have been 13 identified serotypes or capsule types of *N. meningitidis*, six of which have been associated with disease (A, B, C, W, X and Y) (Levine, Shaffer et al. 1996; Stephens, Greenwood et al. 2007). This association with disease accounts for the inclusion of capsule types A, C, W, and Y in the quadravalent capsule conjugate vaccine (the most inclusive released formulation to date). Despite the benefits of conjugate capsular vaccines and their effectiveness at reducing the incidence of disease, their utility in preventing meningococcal infection is limited. Although capsule type B was explored as an antigen candidate it was found to be poorly immunogenic, exhibit immunological similarity with human neural tissue and therefore could not be safely used as an antigen (Finne, Leinonen et al. 1983).

Current capsular vaccines are further limited by *N. meningitidis's* natural transformation abilities, which allow for capsule switching, particularly under the selective pressures of vaccines (Bowler, Zhang et al. 1994; Swartley, Marfin et al. 1997). Capsule switching is a phenomenon that is believed to occur when multiple strains of *N. meningitidis* colonize the same host allowing for the exchange of genetic information. It has been suggested that capsule switching represents an adaptation for *N. meningitidis* strains to avoid pre-existing antibodies within a host and has been a documented response to immunization campaigns in Canada (Kertesz, Coulthart et al. 1998). It has been further documented in Germany that capsule switching can occur rapidly, something the authors

suggest could rapidly increase the prevalence and incidence of type B meningococcal disease (Vogel, Claus et al. 2000). With only four of six disease causing capsule types being represented in currently available vaccines it is not surprising that there have been increases in incidence of disease associated with non-vaccinated capsule types most notably B and X. It should be noted that at the time of the writing of this thesis capsule type X is a relatively minor but growing contributor to the global incidence of *N. meningitidis* associated disease (Trotter and Greenwood 2007). Capsule type X is primarily associated with disease in the meningitis belt, a region in northern Africa with the highest incidences of meningitis worldwide and commonly associated with epidemics (Gagneux, Wirth et al. 2002; Gagneux, Hodgson et al. 2002; Boisier, Nicolas et al. 2007; Materu, Cox et al. 2007; Delrieu, Yaro et al. 2011). Capsule type X has been increasing in incidence and is recognized by the World Health Organization for its ability to cause epidemics. Case reports indicate that it has been associated with disease in the developed world (Gagneux, Wirth et al. 2002). There is no reason to believe that this is not possible for other capsular types, suggesting that further shifts might ultimately compromise gains made with existing conjugate capsular vaccines, which may also underly the shift in research efforts towards the development of protein-based vaccines (Caesar, Myers et al. 2013).

In addition, capsular vaccines are ineffective at controlling disease by non-encapsulated strains. Although disease is rarely associated with non-encapsulated isolates of *N. meningitidis* they have been previously documented (Johswich, Zhou et al. 2012). Just as capsular vaccines have been shown to result in changes in disease causing

capsular types it is possible that these types of vaccines could create a pressure for an increase in disease associated with unencapsulated strains. In lieu of these limitations for a capsular vaccine to completely control or eliminate meningococcal disease research efforts have been shifting away from a focus on capsular vaccines towards protein based vaccines (Caesar, Myers et al. 2013). Iron receptor proteins such as Tf and Lf receptors represent strong potential vaccine targets as they are accessible, surface exposed targets that are essential for survival within a host.

1.3.2 Neisseria gonorrhoeae

The human pathogen *Neisseria gonorrhoeae* is the causative agent of gonorrhea. With an estimated 88 million cases in 2011, *N. gonorrhoeae* has the highest incidence of disease of any *Neisseria species* worldwide (WHO). Despite the significantly higher incidence of *N. gonorrhoeae* relative to *N. meningitidis*, *N. gonorrhoeae* has a much lower economic burden associated with it (Levine, Shaffer et al. 1996; Chesson, Blandford et al. 2004). Gonococcal disease is easily treated with antibiotics. However, inappropriate use of antibiotics compounded with inherent genetic mutations has resulted in untreatable multi-drug resistant strains of *N. gonorrhoeae* (WHO 2011; Kirkcaldy, Bolan et al. 2013). Without new antibiotics coming to market to help combat these strains, which have been identified in both the developed and developing world, there is a growing need for a vaccine against *N. gonorrhoeae* (Kirkcaldy, Bolan et al. 2013). Unlike *N. meningitidis N. gonorrhoeae* doesn't express a capsule, and therefore only a protein based vaccine approach can be used (Unemo and Nicholas 2012). In the interim, while vaccines are still being developed, researchers are exploring other therapeutic

options to combat the growing number of multi drug resistant infections, including the use of antimicrobial peptides (see chapter 4 section 1 for more information) (Unemo and Nicholas 2012).

1.3.3 Moraxella catarrhalis

Moraxella catarrhalis is a Gram-negative unencapsulated pathogen with significant phentoypic and morpholigical similarities to *Neisseria*, making it difficult to distinguish by simple plating (Karalus and Campagnari 2000). This similarity to *Neisseria* species, specifically *N. cinerea*, led to its early misclassification as a harmless commensal of the upper respiratory tract (Gordon 1921; Karalus and Campagnari 2000). Even when it was possible to distinguish *M. catarrhalis* from commensal *Neisseria* spp. it was difficult to classify it as a pathogen as it has been routinely isolated from the respiratory tract of healthy adults and children (Murphy 1998). Although there were some early studies suggesting *M. catarrhalis* be classified as a respiratory pathogen it wasn't until the 1970s that it was widely recognized and conclusively demonstrated as a respiratory pathogen in adults (Karalus and Campagnari 2000). In adults *M. catarrahlis* has been conclusively demonstrated as an important, although infrequent causative agent of exacerbations in chronic obstructive pulmonary disease (COPD), and pneumonia in the elderly (Murphy 1998). Interestingly there is a higher rate of *M. catarrhalis* colonization in smokers, COPD patients and adults over the age of 60, possibly accounting for the increase in *M. catarrhalis* infections in these populations. Similarly there are high rates of colonization in young children and infants which has been linked to otitis media or middle ear infections (Faden, Harabuchi et al. 1994). *M. catarrhalis* accounts for 15-20%

of otitis media cases, an illness which afflicts 70% of children by the age of 3 (Klein 1994). Although otitis media does not have high mortality rates it can be associated with significant morbidity if not appropriately treated. Specifically otitis media can result in deafness and developmental delays, giving *M. catarrhalis* a significant economic burden.

1.4 Lactoferrin

Lactoferrin (Lf) is a bi-lobed mammalian iron binding glycoprotein originally identified from cow's milk in 1940 (Sorensen and Sorensen 1940). The reddish colour noted when Lf was originally isolated is due to the presence of Fe ions which Lf binds, a property which allowed Lf to be included in the transferrin (Tf) family of proteins (Johanson 1960). Structurally Lf and Tf are highly similar. Both are bilobed proteins which share ~60% sequence identity, conserved disulfide binding patterns and high sequence identity between their N and C terminal halves (Baker and Baker 2012), Lfs have been identified in a variety of mammalian species, and the structures of human, bovine, horse, and buffalo Lfs are very similar (Norris, Baker et al. 1989; Haridas, Anderson et al. 1995; Moore, Anderson et al. 1997; Jameson, Anderson et al. 1998; Karthikeyan, Paramasivam et al. 1999; Sharma, Rajashankar et al. 1999; Khan, Kumar et al. 2001; Kumar, Khan et al. 2002; Baker and Baker 2012). Despite the wealth of data on Lfs, in general, this study will primarily focus on human Lf and the literature surrounding it.

Similar to other Tf family proteins both the Lf N and C lobes can be further subdivided into two domains, the N/C1 and N/C2 domains, that flank the interdomain

iron binding pocket (Figure 1) (Baker and Baker 2012). The pocket between the two domains is referred to as an iron binding pocket because it binds Fe ions with greater affinity than other metallic ions, however, this does not preclude Lf from binding other transition metal ions such as Cu2+, Mn3+ and Ce4+ (Smith, Anderson et al. 1992; Kumar, Sharma et al. 2000). Regardless of the ion found in the binding pocket, the presence of a bound ion induces a conformational change commonly referred to as a closed Lf structure, which is more rigid than its open counterpart (Baker and Baker 2012). Although the Tf and Lf binding pockets are structurally identical there are some functional differences, as bound $Fe3^{+}$ is released at different pH values. Both proteins readily take up iron at physiological pH but Tf releases $Fe3^{+}$ much more readily with decreasing pH with substantial release occurring below a pH of 5.5 whereas Lf retains $Fe3^{+}$ until a pH value 3.5 (Mazurier and Spik 1980). Mutagenesis experiments by Ward et al. showed the necessity of a functional Lf C-lobe in maintaining iron binding at low pH values, attributing the difference in iron affinity to cooperative interactions between the N and C Lobes (Ward, Zhou et al. 1996).

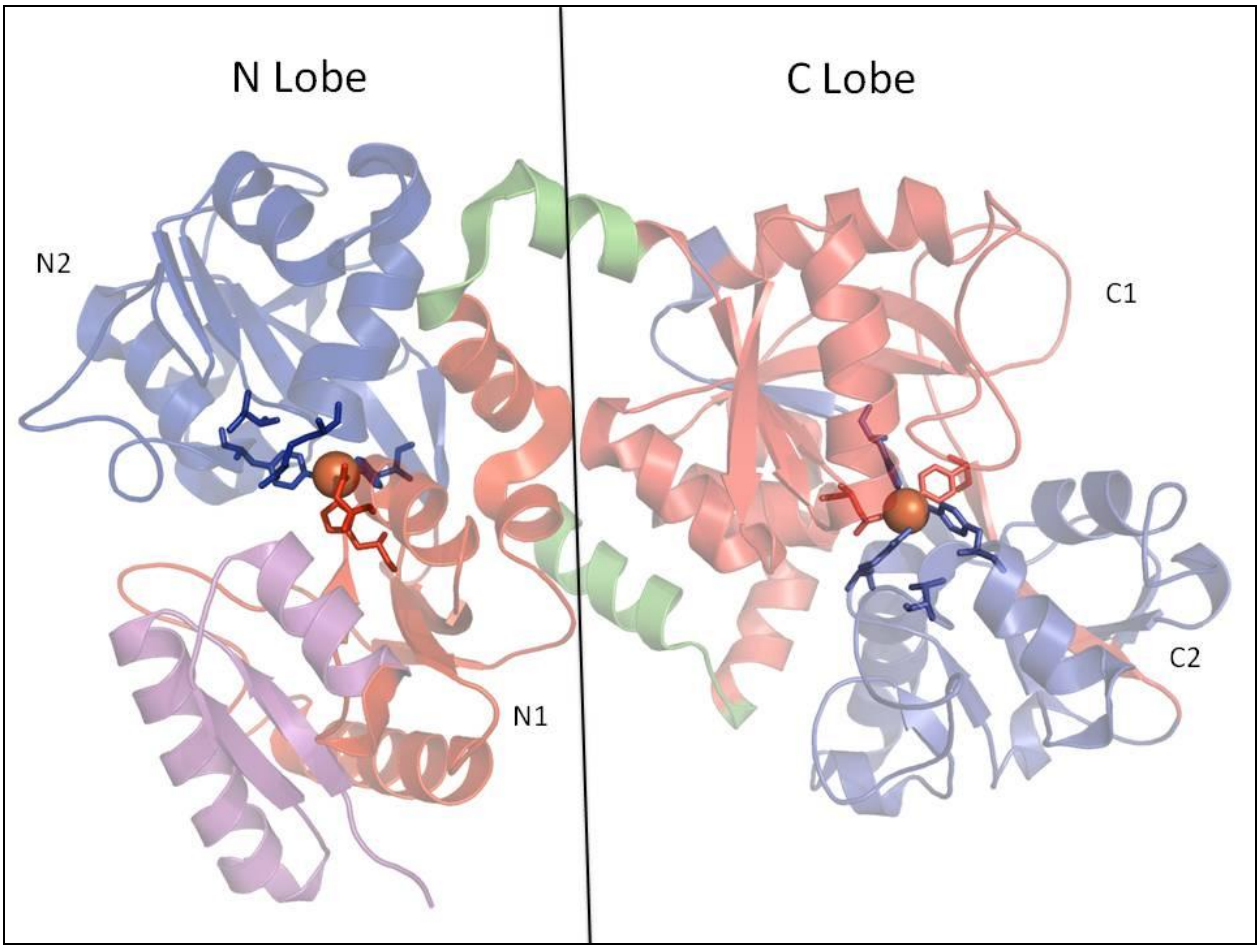

Figure 1. 1: Structure of human lactoferrin. A cartoon representation of human Lf (PDB Structure 1B0L) rendered using PyMOL. The symmetry between the N and C lobe is evident with each binding one iron ion in the binding pocket formed between the domains. The domains are highlighted in red (N1 and C1) and blue (N2 and C2) while the lactoferricin region of N1 is further highlighted in pink. Stick drawing was used to identify the amino acids of the iron binding pocket, which are directly involved in iron coordination.

Unlike other members of the Tf family, Lfs are highly positively charged with isolecetric points ranging from 9-10 (Baker and Baker 2012). Although differences exist between the surface charge of Lfs from different species, specific cationic regions are consistently identified, including the lactoferricin region, which is a significant focus of this study and discussed in greater detail in section 1.7 (Baker and Baker 2012). Similarly

all Lf are glycosylated although the number of N-glycosylation sites varies by species (Baker and Baker 2009). The variations in surface charge and glycosylations sites does not affect the overall structure of Lf but is thought to contribute to the species specificity that exists between Lfs.

1.5 Location of lactoferrin in the body

Lf was initially identified as a milk protein but has been subsequently identified in high concentrations at mucosal surfaces, in secondary granules of neutrophils, at sites of inflammation, and in secretory fluids (Masson, Heremans et al. 1966; Baggiolini, de Duve et al. 1970). In contrast, Lf is found in low concentration within the serum, where Tf is primarily found (Lonnerdal and Iyer 1995). The literature commonly states that Lf is prominent in controlling free iron levels at the mucosal surfaces, where Tf is found in low concentrations. Interestingly this dogma is contested by Anderson et al.'s findings from *N. gonorrhoeae* human infection models (Anderson, Hobbs et al. 2003). This group used urinalysis to determine the concentration of Lf and Tf being washed from the mucosal surface as a representation of the concentration of each protein at the mucosal surface. Anderson found that prior to infection the concentration of Tf at the mucosal surface exceeded that of Lf. Although the levels of Lf rose in response to the *N. gonorrhoeae* infection, the concentration of Lf reached a plateau at concentrations similar to that of Tf.

1.6 Lactoferrin Function

1.6.1 Lactoferrin as an iron transport protein

Initially identified as a prominent protein in milk, with a high similarity to Tf, Lf was hypothesized to play a role in iron uptake in the gut and at mucosal surfaces. This proposed function for Lf is supported *in vivo* by the findings of Saarinen et al. (1977) that breast-fed infants having received 100-1000 fold more Lf than those fed bovine milk based formula, exhibited more efficient iron absorption (Saarinen, Siimes et al. 1977). More recently an Italian clinical study found that supplemental bovine Lf administered with infant formula resulted in increased serum ferritin, which is indicative of increased iron absorption (Chierici, Sawatzki et al. 1992). Further, the Lf receptor (LfR) expressed by mucosal cells identified by Kawakami and Lonnerdal (1991) has been subsequently shown to mediate the transport of iron from Lf into mucosal cells (Kawakami and Lonnerdal 1991; Ashida, Sasaki et al. 2004).

Despite these findings which support the hypothesis that Lf plays a role in iron uptake, to date there is no evidence that Fe taken up from Lf actually reaches the circulation. In addition, unlike Tf which is recycled following internalization and removal of iron by cells, Lf does not appear to be recycled throughout the body (Ashida, Sasaki et al. 2004). This is understandable as Fe is removed from Tf in a pH dependent manner whereas Lf retains its affinity for Fe at the lower pH used to remove Fe from Tf (Richardson and Ponka 1997). Therefore proteolyitic degradation is likely required to remove Fe from Lf. Although there is limited support for Lf in iron transport, Lf's iron

binding capabilities make it an important protein in the regulation of the concentration of freely available Fe particularly when combating pathogens.

1.6.2 Lactoferrin as an antibacterial agent

Shortly after the isolation of Lf from milk it was shown that Lf can be highly bacteriostatic, an effect that was mitigated by the addition of exogenous iron (Oram and Reiter 1968; Bullen, Rogers et al. 1972). This function provides a potential explanation for the high concentration of Lf at sites of inflammation where it is released from the secondary granules of neutrophils (Baggiolini, de Duve et al. 1970). With iron being essential for sustained bacterial growth the release of high concentration of Lf in its iron free form allows for freely available iron to be quickly sequestered, preventing further growth of bacterial pathogens. Interestingly this approach to combating bacterial pathogens by inhibiting further growth is currently emulated pharmacologically with specific therapeutic agents (Pankey and Sabath 2004; Spížek and Řezanka 2004). The use of Lf at sites of inflammation provides potential rationale for its difference in affinity for iron relative to Tf. As a component of the inflammatory immune response there is a reduction in pH at the local site which could affect the affinity of Tf for Iron, but would have minimal impact on Lf (Lardner 2001; Saadi, Wrenshall et al. 2002).

For a long time Lf was solely considered to be a bacteriostatic protein, as its antibacterial effects could be significantly reduced by saturating Lf with iron. However subsequent work with *Streptococcus mutants* demonstrated antibacterial properties independent of iron chelation by Lf (Arnold, Cole et al. 1977). It is currently believed

that Lf is able to destabilize the bacterial membrane resulting in bacterial death. It has been demonstrated that the cationic regions or patches on the surface of Lf, which differentiate it from other Tf family proteins, are able to interact with the Lipid A component of LPS (Appelmelk, An et al. 1994; Brandenburg, Jürgens et al. 2001). This interaction alters the permeability of the bacterial membrane, and induces LPS release (Ellison III, Giehl et al. 1988; Yamauchi, Tomita et al. 1993). Despite the presence of a binding site in each lobe of Lf the majority of LPS binding and release is triggered by a cluster of positively charged amino acids in the N-lobe, termed the lactoferricin region (see section 1.7)(Yamauchi, Tomita et al. 1993; Elass-Rochard, Roseanu et al. 1995). As Lf is exposed to proteolytic factors *in vivo,* it is likely that the majority of Lf induced LPS release *in vivo* is the result of lactoferricin and not the second binding site found in the Lf C-lobe.

In addition to causing bacterial death by compromising the bacterial membrane, Lf is able to attenuate bacteria and in some cases prevent bacterial colonization (Qiu, Hendrixson et al. 1998; Gomez, Ochoa et al. 2003). This ability has been linked to the Lf's serine protease-like activity associated with the Lf N-Lobe (Hendrixson, Qiu et al. 2003). This region has been shown to degrade two autotransported virulence factors which are presumed to facilitate *H. influenzae* colonization, IgA1 protease and Hap adhesin proteins (Qiu, Hendrixson et al. 1998). This effect is not specific for *H. influenzae* but has been shown to attenuate other pathogens including *Shigella*, *Salmonella*, and enteropathogenic *E. coli* (reviewed in Jenssen and Hancock (2009)).

1.6.3 *Lactoferrin as an immune system modulator*

Although a detailed review of the immunomodulatory properties of Lf on both the innate and adaptive immune response is beyond the scope and focus of this thesis, a brief overview on the modulation of the innate response will be given in this section (a more detailed review can be found in Legrand 2012; Legrand and Mazuier 2010 and Legrand et al.2005). Neutrophils release the contents of the secretory/secondary granules at sites of inflammation resulting in high concentration of Lf at these locations. However, neutrophils begin releasing Lf upon activation which in the case of peripheral blood neutrophils starts during recruitment (Legrand 2012). This accounts for the notable increase in serum Lf during inflammation which can have systemic effects such as acting as a chemokine (Maacks, Yuan et al. 1989; Legrand 2012). As previously noted, Lf is able to bind strongly to LPS, this important function allows Lf to down regulate the inflammatory response. Lf binds tightly to LPS therefore LPS is unable to bind to receptors of the innate immune response which otherwise would trigger further recruitment and stimulation of the inflammatory response (reviewed in detail by Legrand and Mazurier (2010). By this mechanism Lf is able to regulate the inflammatory response to prevent excessive damage towards the host and reduce the likelihood of septic shock. Interestingly it has also been proposed that Lf can act as a carrier for LPS to activate macrophages and induce pro-inflammatory mediators such as IL-8 and TNF-α (Sorimachi, Akimoto et al. 1997; Na, Han et al. 2004). Legrand resolves these conflicting reports within the literature by proposing a model where under high concentrations of Lf, resulting from release as a component of the inflammatory response, Lf exhibits anti-inflammatory properties to protect the body from complications. In contrast under low

concentration Lf stimulates the inflammatory response in order to combat pathogens (Legrand 2012). Notably Lf has been reported to both reduce innate cell recruitment and to act as a chemo-attractant for neutrophils, monocytes, myeloid cells and dendritic cells (Legrand 2012).

1.7 Lactoferricin

Early studies using bovine and human Lf differed on the presumed mechanism of action against bacteria. Some believed that Lf was effective simply by chelating available iron, thus creating a hostile environment for bacteria (Weinberg 1984). However preparation of iron deficient media was unable to replicate the antibacterial results obtained with Lf (Arnold, Russell et al. 1982; Bellamy, Takase et al. 1992). This led to the conclusion that Lf must have an antibacterial mechanism separate from iron chelation, a function that has primarily been attributed to the lactoferricin region, a cluster of positively charged amino acids at the Lf N terminus (highlighted in pink in Figure 1) (Tomita, Bellamy et al. 1991; Bellamy, Takase et al. 1992). This region was named for the short cationic antimicrobial peptides which are released from it which are referred to as lactoferricins.

Tomita et al. provided the first evidence for lactoferricin in 1991 when they treated fresh bovine Lf with a panel of proteases showing Lf to be effectively cleaved by pepsins (Tomita, Bellamy et al. 1991). A purified peptide was shown to be highly antibacterial with an effective concentration which rivalled currently used antibiotics. It is not surprising that lactoferricin is cleaved most effectively by a digestive enzyme as Lf is

commonly introduced into the body through the ingestion of milk. Tomita notes that although pepsin was the most effective enzyme within their limited panel, other proteases would be capable of releasing lactoferricin from Lf. Despite this there has been little follow-up within the literature to identify other proteases that effectively release lactoferricin. Other proteases that could work on Lf, particularly proteases released by neutrophils, including those responsible for the release of other anti-microbial peptides have not been extensively researched. Within the current literature lactoferricin is prepared either through digesting Lf with pepsin and purifying the resulting peptides or by producing a synthetic peptide.

In a subsequent study Bellamy was able to identify and compare lactoferricins from both bovine and human sources. and determine their sequences by Edman degradation. He demonstrated that sequences were from the N-terminus and that the lactoferricin regions of bovine and human Lf are similar in location and function but have very different sequences and sizes. Bellamy showed that although bovine lactoferricin was smaller than its human counterpart it was significantly more bactericidal. Bovine lactoferricin has only one disulfide bond resulting in greater flexibility in solution relative to human lactoferricin. This flexibility allows bovine lactoferricin to adopt a new secondary structure following its release from Lf (Vogel, Schibli et al. 2002). In contrast to bovine lactoferricin's more relaxed secondary structure human lactoferricin retains the secondary structure of the lactoferricin region, which is considered to be a result of its longer sequence and additional disulfide bond (Gifford, Hunter et al. 2005).

Although the literature generally agrees the longer length of human lactoferricin impacts its flexibility in solution there is debate within the literature as to the actual length of human lactoferricin. Bellamy used Edman degradation to determine that human lactoferricin corresponded to the initial 47 amino acids of human Lf (Bellamy, Takase et al. 1992). In contrast using NMR the Vogel group at the University of Calgary recently found that human lactoferricin was 49 amino acids long (Gifford, Hunter et al. 2005).

Despite strong evidence for the antibacterial efficacy of lactoferricin the exact mechanism of action is still unknown. Most models agree that lactoferricin disrupts the integrity of the bacterial membrane resulting in bacterial death. The similarity of lactoferricin with cationic antimicrobial peptides resulted in a model in which lactoferricin is proposed to disrupt the bacterial membrane by inserting itself into the membrane and creating pores. More recently this model has been adapted whereby lactoferricin passes through the membrane via "self promoted uptake" and depolarizes the membrane from within the cell (reviewed in Gifford, Hunter et al. (2005)). This depolarization is believed to inhibit the passage of essential ions and molecules through the membrane and to increase susceptibility to antibiotics. Additional models suggest that internalization of lactoferricin supports the existence of an intracellular target such as DNA, however no target has yet to be conclusively demonstrated. To date the interaction between lactoferricin and the bacterial membrane is strongly supported by *in vitro* studies. In Gram-negative bacteria lactoferricins, from all sources, have been shown to bind to and trigger the release of lipopolysaccharides (LPS) presumably though an ionic interaction with the lipid A component (Yamauchi, Tomita et al. 1993; Elass-Rochard,

Roseanu et al. 1995). In Gram-positive bacteria, which lack LPS, lactoferricins bind to the negatively charged teichoic acid layer therefore the initial interaction between lactoferricin and the bacterial surface is considered an ionic one (Vorland, Ulvatne et al. 1999). This would further explain the low toxicity in eukaryotic cells resulting from lactoferricins, as eukaryotic cells mainly posses neutral zwitterionic headgroups at their outer surface. The interaction of lactoferricin with the bacterial membrane is further supported by microscopy studies. Shin et al. reported visualizing bulges and blems in the outer membrane of Gram-negative bacteria following lactoferricin treatments (Shin, Yamauchi et al. 1998). These disfigurements are presumably a result of lactoferricin interacting with the bacterial membrane. More recently atomic force microscopy (AFM) was used to visualize the impact of bovine lactoferricin on *E. coli* and *S. aureus* clearly depicting the formation of pores within the membrane, resulting in disruption of the membranes integrity (Liu, Han et al. 2011). Despite the clear visualization of pores using AFM this does not exclude internalization of lactoferricin as an important component of the lactoferricin mechanism of action. Although these findings were generated using bovine lactoferricin, similar conclusions have been drawn using transmission electron microscopy with a synthetic 11-mer human lactoferricin peptide. The synthetic peptide resulted in membrane ruffling and thinning which promotes increased membrane permeability, accounting for the reduction in bacterial viability found by the authors (Zweytick, Deutsch et al. 2011).

1.8 Bacterial iron acquisition

It is well documented that iron is an essential element for life as it serves as a cofactor for many biochemical reactions (Weinberg 1989). Interestingly the multiple oxidative states that make iron an excellent cofactor for biochemical reactions also makes it dangerous within the body as it can result in the formation of reactive oxygen species which can be destructive to the body (Wandersman and Stojiljkovic 2000). This ability of freely available Fe to induce the formation of reactive oxygen species provides a potential explanation for the sequestering of extracellular iron to glycoproteins such as Tf, and Lf. Additionally iron is poorly soluble under physiological conditions further explaining the binding of extracellular iron to carrier proteins and why the vast majority of iron in the body is found in the intracellular compartment (>99%)(Gray-Owen and Schryvers 1996).

The importance of iron for survival is not limited to humans or animals but also extends to bacteria, with a few unique exceptions such as *Borrelia burgdorferi* or *Lactobacillus plantarum* (Posey and Gherardini 2000). The limited freely available iron within the host environment is typically below levels that would support bacterial survival and growth (Guerinot 1994). It is therefore not surprising that bacteria have developed methods of acquiring iron from their hosts, including the use of siderophores and the use of bacterial receptors that target host iron-containing proteins such as Tf and Lf receptors (a more general review of bacterial iron acquisition can be found in (Guerinot 1994; Wandersman and Delepelaire 2004). Since this thesis is directed at the lactoferrin binding protein B, this introduction will focus on bacterial Lf receptors.

1.8.1 Bacterial lactoferrin receptors

The most common and best characterized Lf receptor is the Lf receptor complex which has been identified in *Neisseria* and *Moraxella* species (Schryvers and Morris 1988; Schryvers, Bonnah et al. 1998; Bonnah, Wong et al. 1999). The Lf receptor complex is composed of an integral membrane protein, lactoferrin binding protein A (LbpA), and a membrane bound lipoprotein, lactoferrin binding protein B (LbpB). When the receptor was first identified in 1988 by Schryvers and Morris, high pH and salt conditions were used in order to reduce nonspecific interaction with Lf, resulting in only one Lf binding protein being identified, LbpA (Schryvers and Morris 1988). Relaxation of these high stringency conditions, during affinity isolation experiments performed 10 years later, allowed for the identification of LbpB (Schryvers, Bonnah et al. 1998). The roles of LbpA and LbpB have been further explored through experiments with LbpA or LbpB knockouts, which show that both LbpA and LbpB can bind Lf, but only LbpA is required for iron acquisition from Lf (Bonnah and Schryvers 1998; Bonnah, Wong et al. 1999). This suggests that LbpB may not be essential for iron acquisition but the limitation of *in vitro* studies in exploring the role of the 'accessory' surface lipoprotein has been demonstrated in studies with the close homologues TbpA and TbpB from *A. pleuropneumoniae*. An infection model in pigs showed that while TbpB was not required for *in vitro* growth, it was essential for survival during infection (Baltes, Hennig-Pauka et al. 2002). An *in vivo* requirement likely extends to LbpB, a notion that is supported by recent findings that the *lbpB* gene is upregulated during infection in humans (Echenique-Rivera, Muzzi et al. 2011; Omer, Rose et al. 2011).

In addition to having sequence homology with Tf receptors, Lf receptors also follow a similar organization of its operon. Like the Tf receptor operon in *N. meningitidis* the lipoprotein *LbpB* precedes the integral membrane protein *LbpA* (Bonnah and Schryvers 1998) and in *M. catarrhalis* the operon also contains a third gene of unknown function (Bonnah, Wong et al. 1999; Yu and Schryvers 2002). In contrast to the Tf receptor operon, where there is a gap between the *tbpB* and *tbpA* genes, no such gap exists between the *lbpB* and *lbpA* genes (Figure 1.2). Expression of Lf receptors is regulated by a ferric uptake regulator (Fur) protein which is able to bind to a binding site in the Lf receptor promoter region (Grifantini, Sebastian et al. 2003; Jackson, Ducey et al. 2010). Under high intracellular iron conditions Fur protein binds to ferrous iron causing the protein to recognize and bind to a specific DNA motif, referred to as a Fur binding site, and repress the expression of *lbpB* and *lbpA* genes. In contrast, under low intracellular iron conditions Fur proteins do not couple with iron and therefore don't bind to Fur binding sites, resulting in unimpeded expression of the Lf receptors operon (Grifantini, Sebastian et al. 2003). As a result of this regulation the Lf receptor complex is primarily expressed under iron starved conditions, like those found *in vivo*.

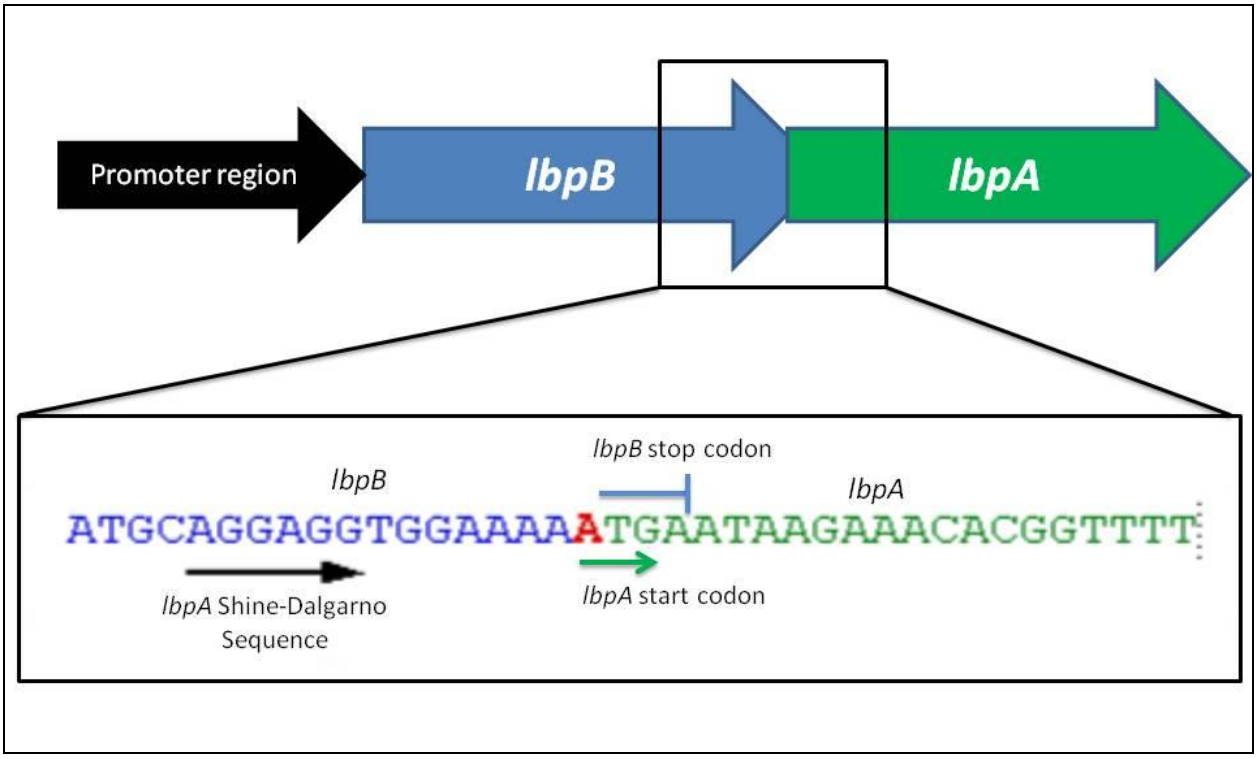

Figure 1. 2 The *N. meningitidis* lactoferrin receptor operon. A cartoon representation of the Lf receptor operon as it appears in the *N. meningitidis* genome. As shown the operon is organized with the lbpB gene preceding the lbpA gene under a single promoter which contains a Fur binding site. Interestingly there is no gap between the two genes as made evident by the enlarged section of the operon obtained from NCBI. The start codon and the shine-dalgarno sequence of lbpA (the green and black arrows in the enlarged section respectively) is found in the lbpB gene. The bolded red adenosine and the lbpB stop codon (the blue T in the enlarged section) are used to further highlight the overlap between the lbpB and lbpA genes . In *M. catarrhalis* the lbpA gene is followed by a third gene orf3 (Bonnah, Wong et al. 1999).

The importance of the Lf receptor complex is highlighted through human infection models with *N. gonorrhoeae* (Anderson, Hobbs et al. 2003). Anderson showed that possession of Lf receptors, in addition to the normally present Tf receptors, provides a selective advantage in bacterial survival and disease causation (Anderson, Hobbs et al 2003). Interestingly only 54% of clinical gonococcal isolates were found to posses Lf receptors. Further, a significant number of isolates only possessed a functional LbpA or

LbpB, suggesting that possession of either protein provides some advantage *in vivo*. It is logical that possession of LbpA would be advantageous, as it confers the ability to use Lf as an iron source. In contrast it is unclear what advantage would be provided by LbpB as its function is still uncertain and without LbpA provides no mechanism for iron removed from Lf to enter the cell. The importance of Lf receptors is supported by their prevalence in clinical isolate of *N. meningitidis* for which no isolates have been identified that lack Lf receptors (Adamiak, Beddek et al. 2012).

1.9.2 *Lactoferrin Binding Protein B (LbpB)*

The current model for LbpB proposes a bi-lobed structure (N, and C lobe) which is based on the structure of the close homologue, TbpB (Ling and Schryvers 2006; Beddek and Schryvers 2010). Homology between the two proteins can confound attempts to distinguish between them based on protein sequence. However, the LbpB C-lobe contains a negatively charged cluster of amino acids, not found in TbpB, distinguishing it from TbpBs (Biswas, Anderson et al. 1999). This negatively charged region has been proposed to have multiple functions that would explain the importance of LbpB. Pettersson suggested that the negatively charged region plays an important role in the binding of LbpB to Lf, particularly to the highly positively charged lactoferricin region (Pettersson, Van der Biezen et al. 1999). Interestingly this conflicts with recent models which suggest that the LbpB N-lobe is responsible for binding Lf (Noinaj et al 2013). Biswas proposed that the negatively charged regions may act as immunodominant regions, which may account for the high strain specificity of αLbpB antibodies (Biswas, Anderson et al. 1999; Pettersson, Kortekaas et al. 2006). The immunodominance of these

regions could account for the significant portion of αLbpB antibodies in convalescent sera from patients with pulmonary *M. catarrhalis* infections (Yu, Bonnah et al. 1999).

Interestingly it has been recently demonstrated that LbpB through an interaction with NalP is able to improve bacterial viability by assisting bacteria in evading bactericidal antibodies. NalP is a membrane bound autotransporter found in *N. meningitidis* which was recently shown to be necessary for survival in human blood (Echenique-Rivera, Muzzi et al. 2011). Using NalP knockouts the Van Ulsen group showed that up to 60% of the LbpB expressed by *N. meningitidis* is selectively released from the cell surface by NalP (Roussel-Jazede, Jongerius et al. 2010) as it was observed in the culture supernatants even after high speed centrifugation to sediment outer membrane vesicles. Solid phase binding assays showed that despite being released from the surface of the cell LbpB retains the ability to bind Lf. It had been previously suggested that during an infection released LbpB could bind to circulating antibodies inhibiting them from reaching the cell surface and thus protecting the pathogen from the bactericidal immune response (Roussel-Jazede, Jongerius et al. 2010).

Although LbpB has been identified as a component of a bacterial iron acquisition system, there has been no experimentally demonstrated role for LbpB in iron acquisition. Despite this, the literature continues to assume a role for LbpB in iron acquisition from Lf (Noinaj, Cornelissen et al. 2013). Most recently Noinaj proposed that LbpB binds to Lf in the extracellular fluid and shuttles it to LbpA for iron removal (Figure 1.3). Interestingly this proposed functional model for the Lf receptor complex fails to account for the impact

of the NalP-LbpB interaction. Further, despite the fact that LbpB has been demonstrated to be able to bind Lf, it does so with a weak affinity relative to the affinity of TbpB for Tf and other Lf binding proteins for Lf. In addition this model provides no explanation for the reason some clinical isolates of *N. gonorrhoeae* only posses LbpB, and would therefore not be able to utilize Lf as an iron source (Anderson, Hobbs et al. 2003). It is therefore reasonable to hypothesize that LbpB has a function separate from iron acquisition, which is the focus of this thesis.

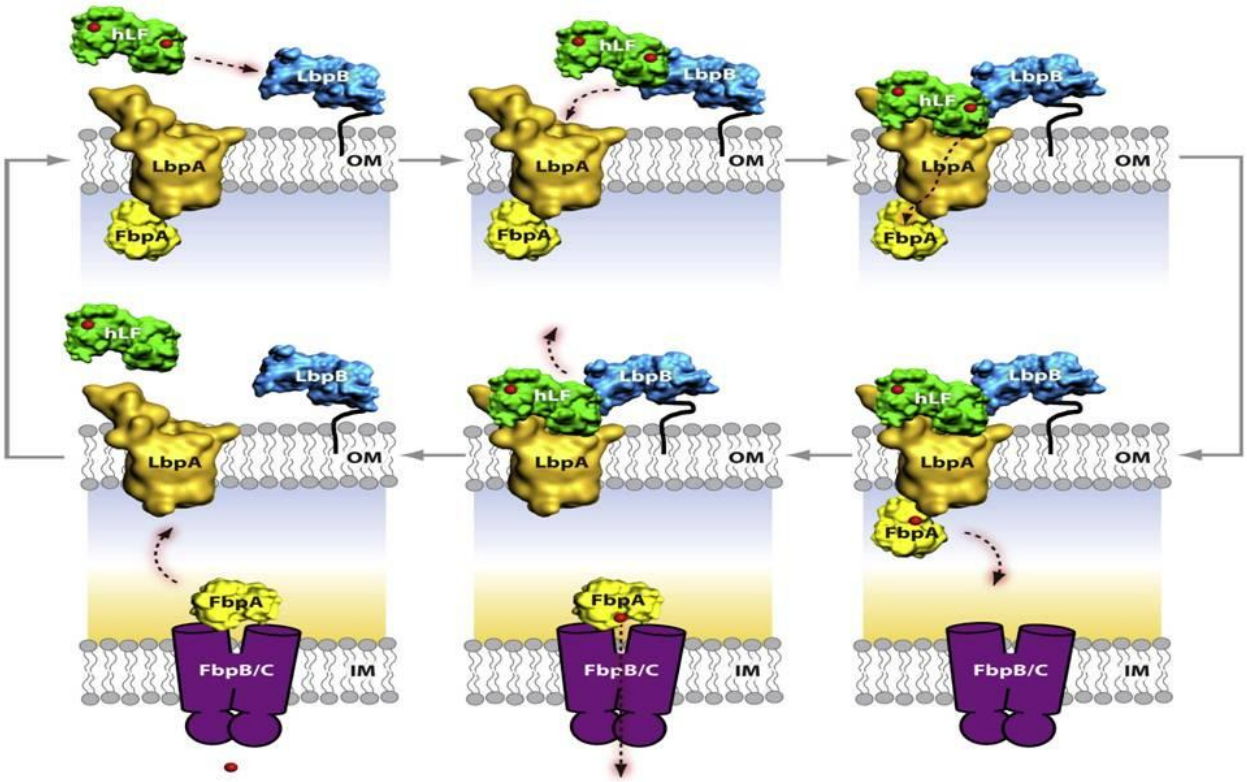

Figure 1. 3 A current model for iron acquisition from human Lf using bacterial Lf receptors as purposed by Noinaj et al (2013). A visual representation depicting the current model for iron acquisition from Lf by *N. meningntidis* as shown in figure 7 of (Noinaj, Cornelissen et al. 2013). Based on similarities to the iron acquisition system from human Tf, a stepwise mechanism is postulated. LbpB is suggested to bind soluble host Lf and shuttle it to the iron transporter LbpA. Upon the LbpA-Lf complex docking onto LbpA and combined with the TonB system, a conformational change within the plug domain of LbpA would catalyze iron release and import through the β-domain, where it would immediately bind to FbpA and be further shuttled across the periplasim and into the cytoplasm by FbpB/C.

1.9.3 Pneumococcal surface protein A

Pneumococcal surface protein A (PspA) is a surface exposed virulence factor expressed by *Streptococcus pneumoniae* (Tai 2006). An interaction between the choline

binding domain of PspA and lipoteichoic acid anchors PspA to the cell surface where it interferes with complement fixation, specifically C3b deposition (Yother and White 1994; Tu, Fulgham et al. 1999; Tai 2006). Similar to LbpB, it has been demonstrated that PspA is able to bind to human Lf (Figure 1.4) (Hammerschmidt, Bethe et al. 1999). Interestingly, although PspA can be used to bind Lf, there has been no mechanism identified by which *S. pneumoniae* can utilize Lf as an iron source. The Briles group at the University of Alabama at Birmingham demonstrated that the PspA-Lf interaction protects *S. pneumoniae* from the bactericidal activity of Lf and lactoferricin which could explain the importance of PspA in colonization of the nasopharynx (Shaper, Hollingshead et al. 2004). Structural NMR studies have demonstrated that the PspA-Lf interaction occurs at the negatively charged region of PspA and the positively charged lactoferricin region of Lf (Senkovich, Cook et al. 2007). This implicates the negatively charged region of PspA in lactoferricin protection.

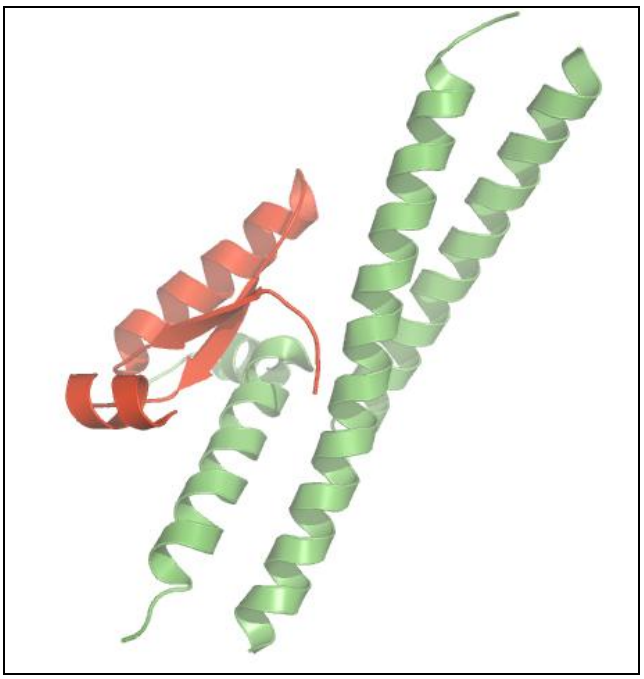

Figure 1. 4: Cartoon schematic of pneumococcal surface protein A (PspA) binding human lactoferricin. A cartoon schematic of PspA binding human lactoferricin (PDB structure 2PMS) rendered using PyMOL. The schematic highlights the structural differences between LbpB and PspA (see Figure 2.2) The binding site of PspA (Amino acids 164 - 286) is highlighted in green while human lactoferricin is highlighted in red.

1.10 Study hypothesis

It has recently been established that the *lbpB* gene is upregulated during the course of an infection suggesting LbpB is important for survival *in vivo* (Echenique-Rivera, Muzzi et al. 2011; Omer, Rose et al. 2011). It has been presumed that LbpB is involved in iron acquisition from Lf *in vivo*, however there is limited experimental evidence to support this. Based on the similarity between PspA and LbpB, particularly in their ability to bind Lf and possession of a negatively charged cluster of amino acids it is reasonable to hypothesize that LbpB would function in a similar manner to PspA and

provide protection against Lf's bactericidal effects. This study will explore the role that LbpB plays in providing protection against Lf derived peptides.

CHAPTER 2: LACTOFERRIN BINDING PROTEIN B PROTECTS BACTERIA FROM LACTOFERRICIN

2.1 Preface:

Genetic evidence for the presence of lactoferrin binding protein B was initially reported in 1994 and its identification and isolation from *N. meningitidis* was reported in 1995 (Pettersson, Klarenbeek et al. 1994; Bonnah, Yu et al. 1995). Since then it has been found in additional Gram-negative pathogens including pathogenic *Neisseria* and *Moraxella* species. Despite extensive characterization *in vitro*, the function of LbpB is still unknown. It is thought that LbpB is important *in vivo* for iron acquisition despite not being necessary under *in vitro* conditions (Bonnah and Schryvers 1998; Bonnah et al. 1999). Capitalizing on the increasing availability of genomic sequences this chapter sought to explore the prevalence of LbpB in bacterial species with the expectation that it would provide insight into the function and importance of LbpB. By understanding the prevalence and similarities between LbpBs we expected to be able to identify conserved elements and the potential organism's niches that may contribute to LbpB function.

It should be noted that components of this chapter have been previously published in Morgenthau, A., Livingstone, M.J., Adamiak, P., and Schryvers, A.B. 2012. The role of lactoferrin binding protein B in mediating protection against lactoferricin. Biochem. Cell Biol. 90 (3) p417-423. The chapter has been modified to include supplemental tables not included in the published manuscript. Figure 2.4 represents preliminary work obtained in the lab by M.J.L. while P.A. contributed by providing

guidance during the bioinformatics approach used in the study. P.A. further reviewed the bioinformatics results and components of the manuscript to confirm their accuracy.

2.2 Introduction

Lactoferrin (Lf) and transferrin (Tf) are bi-lobed host glycoproteins that sequester available iron in the extracellular milieu, creating an unfavorable environment for pathogens in the host (Baker, Baker et al. 2002). Due to its role in transporting iron between cells in the body, Tf is present in high concentrations in serum and in the extracellular milieu within the body whereas Lf is primarily found in mucosal secretions and at sites of inflammation (Ward, Paz et al. 2005). To counter the iron-sequestering effects of Lf, some Gram-negative bacterial species are able to acquire iron directly from Lf, a process mediated by surface receptors that specifically bind host Lf (Ling and Schryvers 2006). The surface receptor comprises an integral membrane protein (lactoferrin binding protein A (LbpA)) and a membrane anchored lipoprotein (LbpB) (Bonnah, Yu et al. 1995).

In vitro studies with LbpA– or LbpB– strains demonstrate that LbpA, but not LbpB, is required for growth with exogenous Lf as the primary source of iron (Bonnah and Schryvers 1998; Bonnah, Wong et al. 1999). This does not necessarily preclude LbpB from being involved in the iron acquisition process, as experiments with transferrin binding protein B (TbpB) from *Actinobacillus pleuropneumoniae* have illustrated (Baltes, Hennig-Pauka et al. 2002). TbpB was required for survival and disease causation in a pig infection model, but was not required for *in vitro* growth studies,

suggesting that TbpB was required for iron acquisition under the more stringent *in vivo* conditions. The importance of Lf receptors for survival in the host has been demonstrated in a series of studies with mutants of *Neisseria gonorrhoeae* in a human urogenital infection model (Cornelissen, Kelley et al. 1998; Anderson, Hobbs et al. 2003). Strains lacking both the Tf and Lf receptors were completely avirulent whereas a deficiency in the Lf receptor resulted in a reduced ability to compete and survive in the male genitourinary tract.

Iron-free Lf has bactericidal activity separate from its ability to chelate iron (Arnold, Russell et al. 1982) that has been attributed to the release of short cationic peptides from the N-terminus, termed lactoferricins (Bellamy, Takase et al. 1992). The prevalence of positively charged amino acids in peptides derived from the N-terminus and the properties of these peptides (Jing, Svendsen et al. 2006) suggest that they may have a similar function to antimicrobial cationic peptides from other sources. A surface protein from *Streptococcus pneumoniae*, pneumococcal surface protein A (PspA), has been shown to protect this bacterium from the killing effects of human lactoferricin (Shaper, Hollingshead et al. 2004). Structural studies of a complex of PspA and Lf demonstrate interactions between the cluster of negatively charged amino acids in PspA and the positively charged amino acids from the N-terminus of human Lf that encompasses the lactoferricin peptide (Senkovich, Cook et al. 2007). This clearly implicates the cluster of acidic residues in PspA for the protection against lactoferricin.

Clusters of acidic residues have been observed in the C-lobe of LbpB from *Neisseria meningitidis* (Pettersson, Van der Biezen et al. 1999) and in the C-lobe of LbpB from *Moraxella catarrhalis* (Yu and Schryvers 2002). These regions may be involved in binding the cationic region of Lf or could potentially play a similar role to the regions of acidic residues in PspA. Thus, this study was implemented to explore the potential function of Lbps in providing protection against Lf derived peptides.

2.3Materials and methods

2.3.1 Bacterial strains, growth conditions, and DNA sequence determination

The strains of *N. meningitidis, M. catarrhalis*, and *S. pneumoniae* used in killing assays are listed in Table 2.1 (Bonnah and Schryvers 1998; Bonnah, Wong et al. 1999; Shaper, Hollingshead et al. 2004). The *N. meningitidis* strain used as the parental wildtype is defective in both TbpB and TbpA. The strains were streaked onto chocolate or brain heart infusion (BHI) agar plates and were grown at 37 °C with 5% CO2. Liquid cultures were grown by inoculating BHI *(Neisseria* and *Moraxella*) or Todd Hewitt (TH) broth (*S. pneumoniae*) with isolated colonies from overnight plates and grown to an A_{600} of 0.5. Cultures were then diluted back to an A_{600} of 0.01–0.05 in TH broth or BHI broth containing 100 μmol/L Desferal (to induce expression of LbpB) and grown to an A_{600} of 0.1–0.2.

The *Moraxella bovis* strains and the general approach for cloning the *lbp* genes have been described previously (Yu and Schryvers 2002). Using these approaches the sequence of the remainder of the lbpB gene and immediate upstream region was

obtained. The collection of *N. meningitidis* strains used for sequence analysis of the *lbpB* genes and the sequence determination is described in Adamiak, Beddek et al. (2012).

Table 2. 1: Bacterial strains used in chapter 2.

Strain	Description	Lbp Phenotype	Reference
N96	TbpBA deficient derivative of *Neisseria meningitidis* B16B6	LbpA+LbpB+	(Bonnah and Schryvers 1998)
N145	Mutant of N96	LbpA-	(Bonnah and Schryvers 1998)
N193	Mutant of N96	LbpB-	(Bonnah and Schryvers 1998)
N141	*Moraxella catarrhalis* pulmonary isolate	LbpA+LbpB+	(Bonnah, Wong et al. 1999)
N163	Mutant of N141	LbpB-	(Bonnah, Wong et al. 1999)
D39	*Streptococcus pneumoniae* clinical isolate	PspA+	(Shaper, Hollingshead et al. 2004)
JY182	Mutant of D39	PspA-	(Shaper, Hollingshead et al. 2004)

2.3.2 Killing assays

Killing assays were performed as previously described (Shaper, Hollingshead et al. 2004) using a human lactoferricin-derived peptide comprising the initial 11 amino acids at the N-terminus of human Lf (GRRRRSVQWCA) synthesized by Peptide Services at the University of Calgary. There were no chemical modifications of the C-terminus, N-terminus, or amino acid side groups in the peptide. Briefly, bacteria were grown as previously described to an A600 of 0.1–0.2 and resuspended in AS solution (150 mmol/L NaCl, 1 mmol/L $MgCl_2$, 50 μmol/L $CaCl_2$ and 1 mmol/L K_2PO_4, pH 7.2) with and without 100 μmol/L lactoferricin Bacteria were incubated in AS solution for 1 h and then plated using a 1 in 10 serial dilution on BHI or TH plates. The plates with

incubated overnight at 37 °C with 5% CO2 and enumerated the next day to determine CFU.

2.3.3 Statistical analyses

For killing assays, bacterial death or log CFU killed was determined by subtracting the log CFU of bacteria incubated with lactoferricin from the log CFU of those incubated in AS only. Where necessary, the data was analyzed with SPSS software using a one-way analysis of variance (ANOVA) where significance was set at $P < 0.01$. Alternatively, t tests were used to determine significance of the difference in death among the *M. catarrhalis* strains. Experiments were replicated on multiple days producing 8–10 replicates for each strain.

2.3.4 Data analysis

Protein sequence alignments were assembled using M-Coffee (Moretti, Armougom et al. 2007) and ClustalW, with manual corrections made using BioEdit. M-Coffee alignments were used to generate phylogentic trees in MEGA5 (Tamura, Peterson et al. 2011) using the neighbour-joining method. ClustalW alignments were used to determine pairwise identity and sequence identity using Geneious (5.4.3) (Drummond, Ashton et al. 2011).

2.3.4 Structural models

Structural models for LbpBs were developed with the Swiss Model server using the available structure of *A. pleuropneumonieae* TbpB (Moraes, Yu et al. 2009). PyMol software was used to visualize and annotate the model.

2.4 Results

2.4.1 Prevalence of lactoferrin binding protein B in Gram-negative bacteria

Since Lf is predominantly found in mucosal secretions and at sites of inflammation, it is not surprising that Lf receptors have been identified in bacteria that colonize the respiratory and genitourinary tract of mammalian hosts. The presence of Lf receptors in *N. meningitidis, N. gonorrhoeae, M. catarrhalis,* and *M. bovis* has been established by a combination of binding assays, growth assays, affinity isolation experiments, and insertional mutagenesis (Schryvers and Morris 1988; Biswas and Sparling 1995; Bonnah and Schryvers 1998; Bonnah, Wong et al. 1999). However, Lf receptors are notably absent from pathogenic bacteria from the *Pasteurellaceae* that colonize the respiratory and genitourinary tract of mammals, illustrating that we do not fully appreciate the selective forces favoring the presence of Lf receptors. The increasing availability of genomic sequences has resulted in presumptive identification of a growing number of Lf receptors but without experimental validation or clear criteria for annotation it is uncertain whether these are indeed Lf receptors functionally related to those previously described. In spite of these potential limitations, we attempted to re-

evaluate the prevalence of Lf receptors, and specifically LbpB, in Gram-negative bacteria.

The common features of Tf and Lf receptors, including presumed structural and functional similarities, results in a degree of sequence similarity that can confound attempts to distinguish between the two, particularly in the face of the sequence variation driven by attempts to evade the host immune response. The recent illustration that specific binding of the host glycoprotein can be achieved by a varying sequence and location of residues on the receptor protein (Calmettes, Yu et al. 2011; Silva, Yu et al. 2011) highlights the difficulty in attempting to discriminate between related receptors with varying specificities by sequence-based methods alone. Thus, our search for functional homologues involved a series of BLAST searches using sequences of known Lf receptors using a somewhat arbitrary cut-off and eliminating those annotated as Tf receptors. Since we had 102 LbpB sequences from *N. meningitidis* strains in our collection (Adamiak, Beddek et al. 2012), for many of which functional assays have been performed, we did not include additional sequences from public databases. Similarly, the *M. bovis lbpB* gene sequence was obtained from an isolate of *M. bovis* with a characterized receptor. Thus, our analysis includes 135 presumptive LbpBs, 102 from *N. meningitidis* ,16 from *M. catarrhalis*, 1 from *M. bovis*, 11 from *N. gonorrhoeae*, 3 from *N. lactamica*, 1 from *N. cinerea*, and 1 from *Taylorella equigenitalis*. Thus, by our approach we have only presumptively identified LbpB in 3 additional species, 2 commensal *Neisseria* species in humans and a pathogen responsible for contagious metritis in horses, a sexually transmitted disease.

A phylogenetic analysis was performed on the selected LbpB sequences to illustrate the relationship among the protein sequences (Figure 2.1) in the hope that clustering might reveal additional insights. It is evident from this figure that LbpBs from *N. gonorrhoeae* and the commensal *Neisseria species* represent a subset of the sequence variation present within LbpBs from *N. meningitidis*, a conclusion obviously biased by the preponderance of these sequences used in the analysis. The sequence identity of these LbpBs relative to LbpB from *N. meningitidis* strain B16B6 ranged from 62% to 81%, higher than the overall identity amongst meningococcal LbpBs, 32.7% (Table 2.2)(Adamiak, Beddek et al. 2012). Thus, it is reasonable to conclude that these are indeed LbpBs and likely share a common evolutionary origin or have been acquired through horizontal exchange that is prevalent in these naturally transformable bacteria.

In this analysis the LbpBs from *M. catarrhalis* and *M. bovis* are on the same branch as the LbpB from *T. equigenitalis* (Figure. 2.1) indicating that they are quite distinct in sequence from the LbpBs from *Neisseria* species. Their sequence identity relative to LbpB from meningococcal strain B16B6 ranged from 13% to 28%, below the level that one would normally identify structural and functional homologues. Although there were 2 unique sequences from *T. equigenitalis* originally identified in the BLAST analysis, the presence of a cluster of negatively charged amino acids was used to presumptively distinguish between LbpB and TbpB.

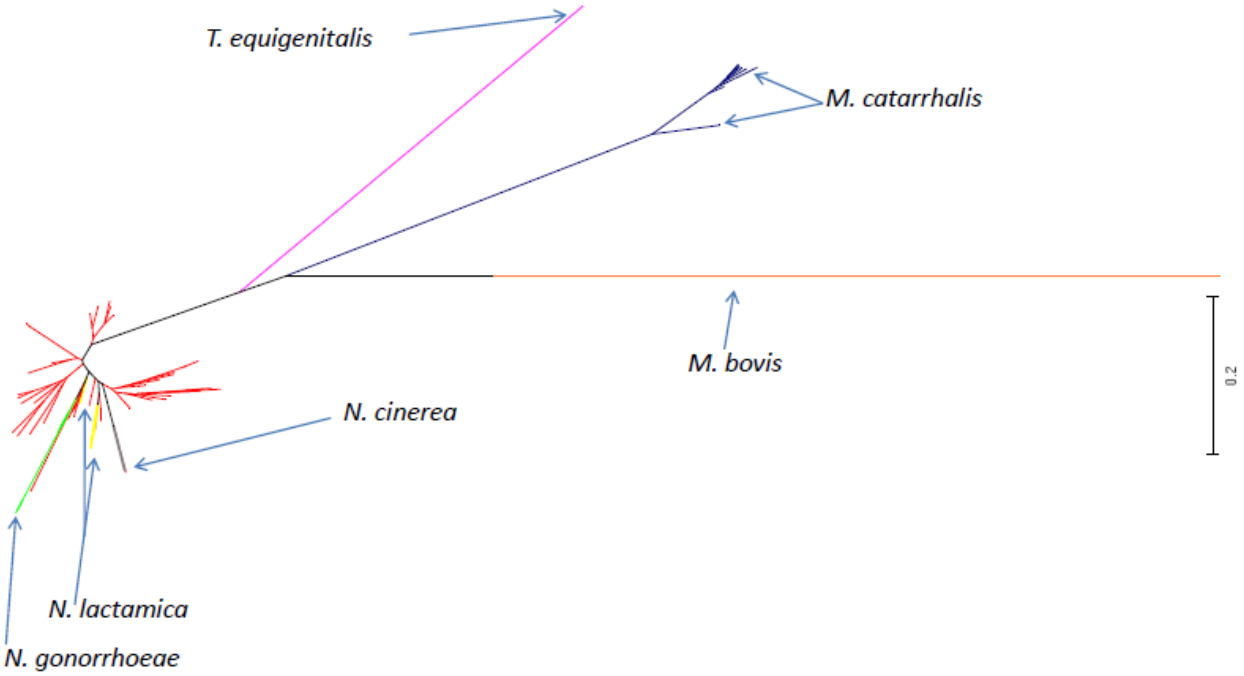

Figure 2. 1: Phylogenetic analysis of lactoferrin binding protein B's (LbpBs) from Gram-negative pathogens. M-Coffee was used to generate an alignment of LbpB protein sequences from 102 *Neisseria meningitidis*, 11 *Neisseria gonorrhoeae*, 3 *Neisseria lactamica*, 1 *Neisseria cinerea*, 16 *Moraxella catarrhalis*, 1 *Moraxella bovis*, and 1 *Taylorella equigenitalis*. A neighbor-joining phylogenetic tree was generated from the alignment using Mega5. All unlabelled strains are *Neisseria meningitidis*. The other species are indicated by arrows.

Table 2. 2: Sequence identity between the LbpB from *N. meningitidis* B16B6 and LbpBs from *M. catarrhalis, M. bovis, N. gonorrhoeae, N. lactamica,* and *N. cinerea.* Sequence identity scores were used to predict protein homology between the LbpB from *N. meningitidis* B16B6 and other LbpBs using the 30% threshold (Rost 1999). Although not presented in the table, the average sequence identity for LbpBs from the collection of *N. meningitidis* isolates used in this study was recently published as 32.7% (Adamiak, Beddek et al. 2012). Alignments were generated using ClustalW between the LbpB protein sequences from *N. meningitidis* B16B6 and LbpB sequences for *M. catarrhalis, N. gonorrhoeae, N lactamica N cinerea, M. bovis* and *T. equigenitalis.* NA – not applicable.

Species	Number of Sequences	Average Percent Sequence Identity	Range
Moraxella catarrhalis	16	24.65	13.3-27.9
Neisseria gonorrhoeae	11	78.39	73.1-80.5
Neisseria lactamica	3	71.4	71.1-71.9
Neisseria cinerea	1	62.0	NA
Moraxella bovis	1	22.1	NA
Taylorella equigenitalis	1	27.4	NA

2.4.2 Presence and localization of the negatively charged regions

A common feature of LbpB and TbpB sequences is an underlying weak internal sequence homology that reflects the bi-lobed structure of the protein, consisting of N-terminal and C-terminal lobes that each contain handle and barrel domains (Moraes et al. 2009). A distinguishing feature observed in all the LbpBs included in this study, except for the LbpB from *M. bovis*, is the presence of one or more clusters of acidic amino acids within the C-terminal lobe region of the LbpB protein (Figure 2.2). An alignment of C-lobes from several strains and species of *Neisseria* illustrates the conserved location of these negatively charged regions (Figure. 2.3). *Neisseria* species and *M. catarrhalis* contain regions of up to 80 amino acids in length and some *Neisseria* strains contain a

second smaller region of acidic amino acids. In the *Neisseria* species the large acidic region contains repeating sets of 2–3 glutamic acids separated by only 1 or 2 nonacidic amino acids. In contrast, the *M. catarrhalis* large region and the *Neisseria* small region contains repeating sets of aspartic acids rather than glutamic acids. The LbpB sequence from *T. equigenitalis* possessed a region of repeating glutamic acids in approximately the same location as the large acidic region in the *Neisseria* LbpBs, but the region only contained 15 amino acids. The *M. bovis* LbpB sequence obtained from our collection did not appear to possess any segment of sequence resembling the negatively charged region identified in the other species.

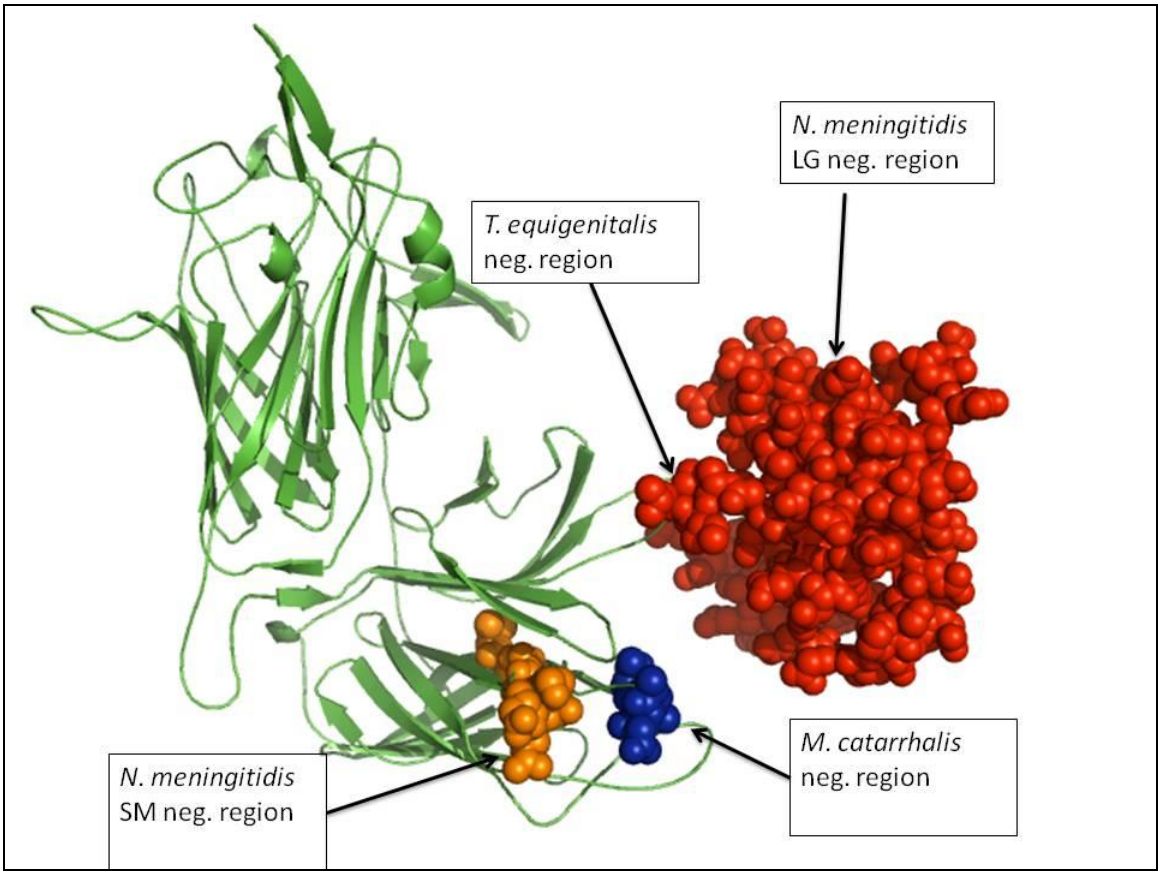

Figure 2. 2: Mapping the negatively charged regions from different bacterial species. Structural models were created for lactoferrin binding protein B for all 135

seqeunces of LbpB used in this thesis. Structures were created using the Swiss Model server with *Actinobacilus pleuropneumoniae* transferrin binding protein B as a template. Probable location of the negatively charged regions from *N. meningitidis, M. catarrhalis* and *T. equigentialis* are mapped onto the predicted structure of the LbpB from *N. meningitidis* strain Z1035, which is naturally deficient in the small negatively charged region. The locations of the negatively charged region are indicated by the coloured spheres with the text boxes indicating the specific regions that map to each location. It should be noted that only the small negatively charged region was not included in this model as it would make visualizing the negatively charged region of *M. catarrhalis* very difficult (see figure 3.2 for structural models with both *N. menigitidis* negatively charged regions. Interestingly the negatively charged region of *T. equigenitalis* maps to the same location as the large negatively charged region of *N. menigitidis* despite being significantly smaller in size (~15aa).

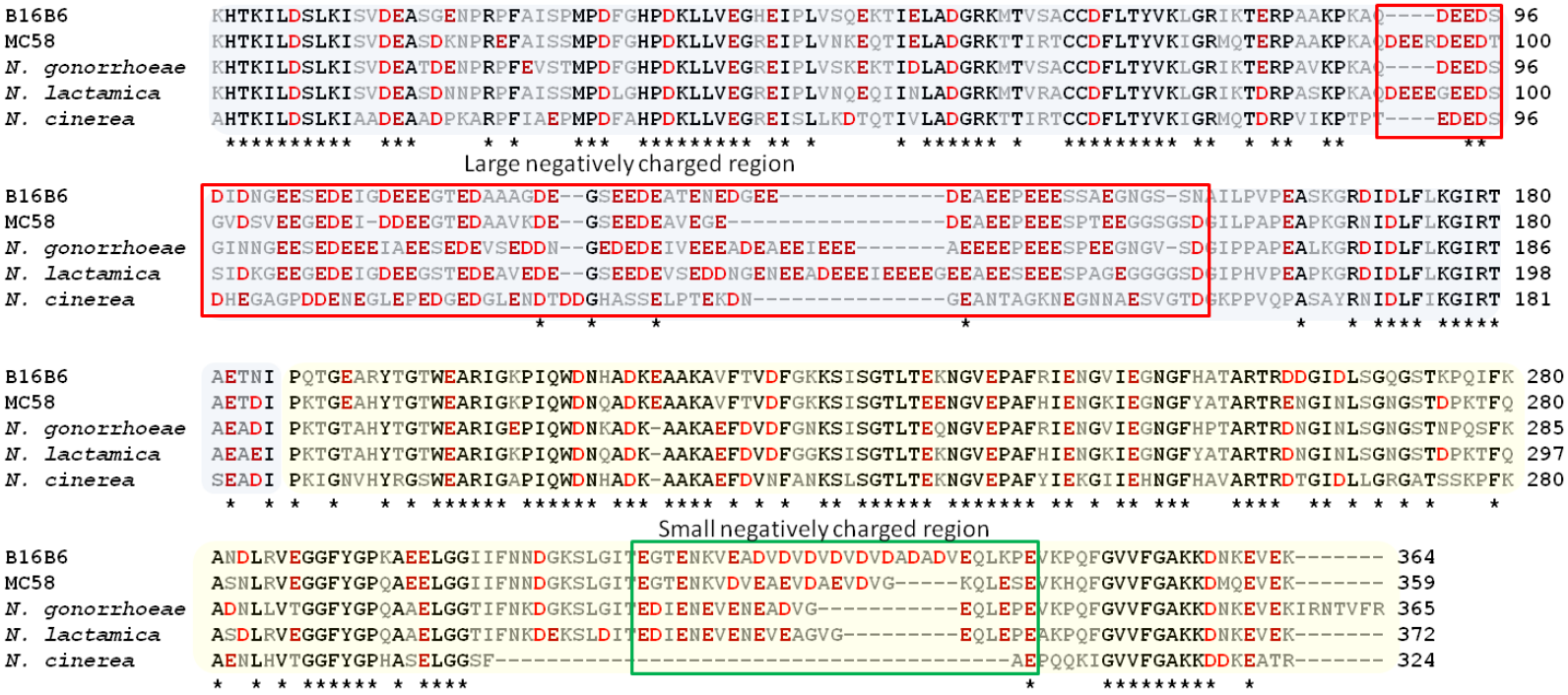
Large negatively charged region
Small negatively charged region

Figure 2. 3: Alignment of LbpB C-lobes from *Neisseria* species. Multiple sequence alignments were used to identify conserved motifs with LbpB, most notably the presence of the negatively charged region. For ease in visualization a representative alignment of LbpB C-lobes was prepared using five *Neisseria* isolates: *N. meningitidis* B16B6, *N. meningitidis* MC58, and three publically available sequences for *N. gonorrhoeae, N. lactamica* and *N. cinerea*. Within the alignment * are used to indicate conserved amino acid (also coloured black), while acidic or negative amino acids are highlighted in red. It is evident from the alignment that the large negatively charged region (the red box) is consistently localized to handle region of LbpB from *Neisseria spp.*, while the small negatively charged region (the green box) is found in the barrel region. Interestingly in LbpBs from *M. catarrhalis* all of which lack the small negatively charged region, the large negatively charged region is found in the barrel domain (Morgenthau, Adamiak et al. 2012).

2.4.3 LbpB protects against lactoferrin-derived peptides

The effects of human Lf-derived peptides on *N. meningitidis* were tested using a synthetic peptide comprising the initial 11 amino acids from the N-terminus of human Lf, lactoferricin. A parental TbpA–, TbpB– *N. meningitidis* strain and isogenic mutant strains with insertional knockouts of the *lbpB* and *lbpA* genes were treated with lactoferricin to determine whether LbpA or LbpB was able to provide protection from the antimicrobial effects of human lactoferricin (Figure. 2.3a). A previously characterized set of wild-type and PspA– *S. pneumoniae* strains were used as a positive control in this killing assay (Shaper, Hollingshead et al. 2004). Insertional inactivation of LbpB, but not LbpA, resulted in an increase in killing by lactoferricin (Figure. 2.3a) comparable with that observed by inactivation of PspA in *S. pneumoniae* (Figure. 2.3b). Experiments were also performed with insertional mutants prepared in a wild-type *N. meningitidis* strain B16B6 background with essentially identical results. It is noteworthy that the *lbpB* gene precedes *lbpA* within the genome as inactivation of *lbpB* can have downstream affects on the expression of LbpA. In the case of the gentamicin insertional mutant used in this study,

inactivation of *lbpB* does not eliminate but may reduce the expression of LbpA (Bonnah and Schryvers 1998).

The protection of LbpB against lactoferricin was also examined in *M. catarrhalis* that not only has a very different LbpB from *N. meningitidis* (percent identity as low as 13.3%) but also lacks a polysaccharide capsule. Lactoferricin killing assays using a clinical isolate of *M. catarrhalis* and an isogenic mutant with a non-functional LbpB showed a statistically significant increase in bacterial death in the mutant when compared with the wild type (Figure. 2.4).

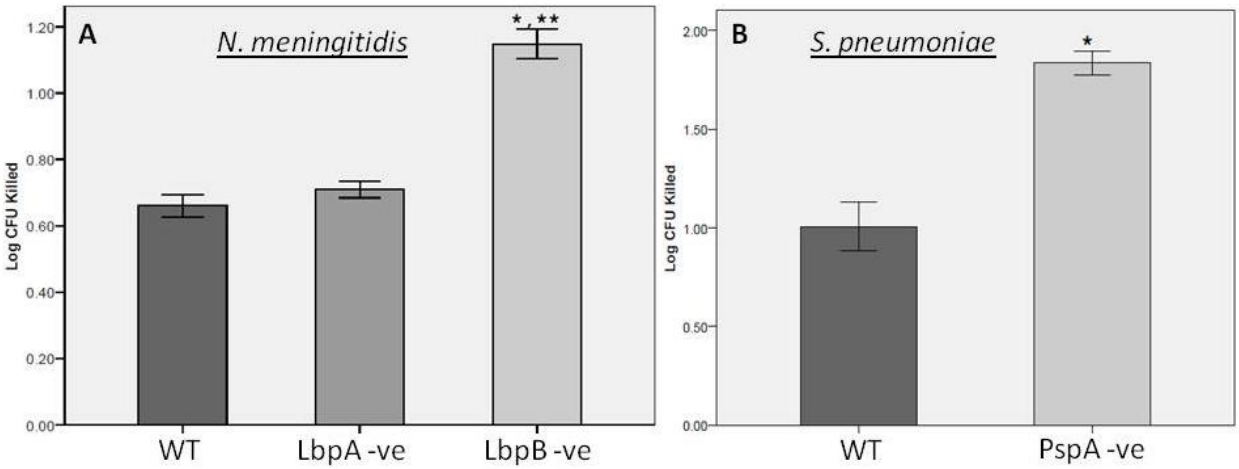

Figure 2. 4: Impact of isogenic mutations on killing by lactoferricin. Killing by 100 μmol/L lactoferricin expressed as log colony forming units (CFU) killed. (A) A parental transferrin binding protein A and B negative (TbpA-B-) derivative of *N. meningitidis* strain B16B6 (WT) and isogenic mutants defective in lactoferrin binding protein A (LbpA) or lactoferrin binding protein B (LbpB) derived from the parent strain. *, LbpB– compared with wild type ($P < 0.01$) and **, LbpB– compared with LbpA– ($P < 0.01$). (B) *Streptococcus pneumoniae* wild-type (D39) and pneumococcal surface protein A negative (PspA–) strains (JY182). *, PspA– compared with wild type ($P < 0.01$). All bars represent the mean of 9 replicates collected over multiple days with error bars represent standard deviation of the mean.

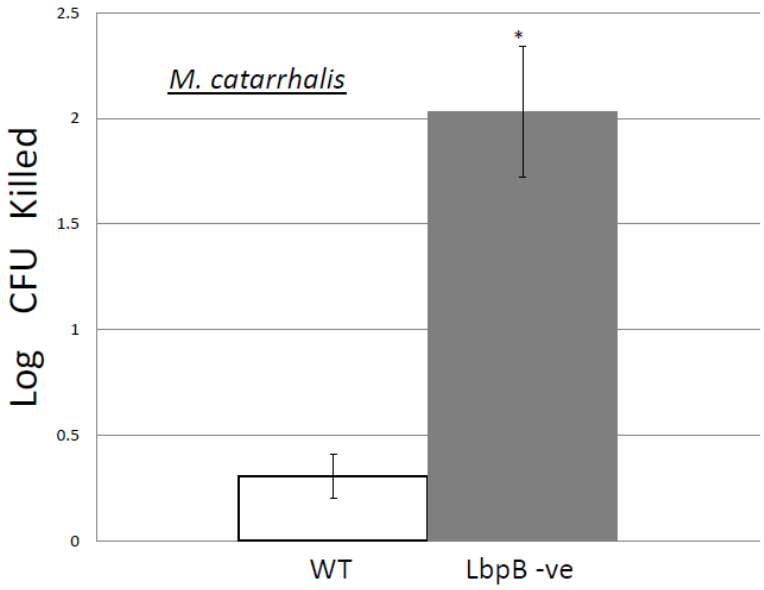

Figure 2. 5: *Moraxella catarrhalis* lactoferrin binding protein B (LbpB) protects against killing lactoferricin. Killing assays were performed using 100 μmol/L lactoferricin. *Moraxella catarrhalis* N141 (WT) and its isogenic mutant deficient in a functional LbpB (LbpB–) were used in this assay. Bars represent the mean of N = 10 and 9, respectively, and error bars represent standard error of the mean. *, $P < 0.001$.

2.5 Discussion

The presence of surface lactoferrin receptors capable of mediating iron acquisition from host lactoferrin has been established in several Gram-negative, host-restricted bacterial pathogens that reside on the mucosal surfaces of the respiratory or genitourinary tracts. The receptors have been shown to provide a selective advantage for survival and disease causation in a human gonococcal infection model and are sufficient for survival of strains lacking a functional transferrin receptor (Anderson et al. 2003). In spite of the potential selective advantage, the presence of these receptors had only been firmly established in a few species in the *Neisseriaceae* and *Moraxellaceae* families (Beddek

and Schryvers 2010) and not in species from the *Pasteurellaceae* that share a similar ecological niche.

Taking advantage of the growing collection of bacterial genomic sequences, we searched for homologues of the lactoferrin receptors to determine whether they were present in other commensal or pathogenic species. Although lactoferrin receptors were clearly identified in genomes from a few commensal Neisseria species, they were notably absent from a number of these genomic sequences available through the human microbiome project (https://commonfund.nih.gov/hmp/). Only 2 of the 7 commensal species (N. lactamica and *N. cineria*) were positive in the search for lactoferrin receptors, indicating that it may only provide a selective advantage in some species. It may be important to consider the availability of lactoferrin on mucosal surfaces when attempting to evaluate the prevalence of lactoferrin receptors in the bacteria colonizing this niche. In the human gonococcal infection model, significant concentrations of lactoferrin were only present after challenge by gonococci (using urine levels as an indirect assessment of levels at the mucosal surface) (Anderson et al. 2003), in contrast to the constant level of available transferrin. Thus, lactoferrin receptors may only provide a real selective advantage to bacteria that are likely to encounter an inflammatory response or cause invasive infection. The variable presence of functional lactoferrin receptors in gonococcal strains (Anderson et al. 2003), in contrast to the effectively ubiquitious presence of lactoferrin receptors in invasive meningococcal strains (all 102 strains used in this study), is consistent with this concept. Nevertheless, until a systematic study evaluating the

prevalence of lactoferrin receptors in the microbial community is initiated, these conclusions are largely speculative.

A clear disadvantage of using bioinformatics approaches to search for Lf receptors is that there are no unique signatures related to the function of Lf binding and use of Lf as an iron source. Most of the sequence identity reflects core structural features that are shared with Tf receptors that can complicate identification of Lf receptors. Since Tf receptors are more prevalent, a single hit from a genome usually represents a Tf receptor, whereas 2 hits would normally indicate that both Tf and Lf receptors are present. This essentially was the situation in *T. equigenitalis* and the identification of the Lf receptor was implied by the presence of the cluster of acidic amino acids, a finding which was corroborated by the findings of Hebert et al. (Drummond, Ashton et al. 2011). This indicates that Lf receptors are not restricted to the *Neisseriaceae* and *Moraxellaceae* families. Additional Lf receptors will likely be identified as more genomes in the human microbiome project are completed and as more genomes of bacteria from other host species are obtained.

Possibly the most useful discriminating characteristic of Lf receptors is the presence of clusters of acidic residues in LbpB, although they are absent from the LbpB in *M. bovis*. The presence of the clusters of acidic residues in a protein with high sequence diversity is a feature that LbpBs share with PspA, a surface protein from *S. pneumoniae* that also binds human Lf (Håkansson et al. 2001). The demonstration that PspA protects *S. pneumoniae* against apolactoferrin or cationic peptides representing the

human lactoferricin peptide region (Shaper et al. 2004) prompted us to explore whether LbpB may play a similar role. In this study, we demonstrate that insertional activation of LbpB, but not LbpA, in *N. meningitidis* results in increased susceptibility to killing by a synthetic peptide representing human lactoferricin (Figure 2.3). Insertional inactivation of LbpB from *M. catarrhalis* also resulted in increased susceptibility to killing by this peptide, demonstrating that protection against lactoferricin is likely a role shared by LbpBs from various species, at least those that possess regions with clusters of acidic residues. It is possible that the greater level of protection conferred by LbpB in *M. catarrhalis* (compare Figures. 3 and 4) could be due to the absence of a polysaccharide capsule in this species, but it is not possible to make any conclusions or strong inferences at this juncture. Alternatively, this difference in protection may be the result of the concentration of LbpB on the cell surface. To date there has been no NalP identified in *M. catarrhalis* and therefore all LbpB is likely retained on the cell surface. In contrast, LbpB released from the cell surface of *N. meningitidis* strains by NalP is likely to be lost during the wash steps of our assay system, resulting in less LbpB to provide protection during treatment with lactoferricin.

Clearly further experimentation is required to determine whether the protective effect of LbpB is due to the clusters of acidic residues and to evaluate the impact of other features such as NalP and the polysaccharide capsule on the protection conferred by LbpB. Similarly, it will be of interest to determine whether there is any specificity to the protection conferred by LbpB. Is it more effective with derivatives of human lactoferrin

than other cationic antimicrobial peptides that the bacteria could encounter during an inflammatory response?

2.6 Acknowledgments

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CHAPTER 3: THE ROLE OF THE NEGATIVELY CHARGED REGIONS OF LACTOFERRIN BINDING PROTEIN B

3.1 Preface

In the previous chapter I demonstrated that despite high sequence diversity among LbpBs from various strains and species, the presence of a cluster of negatively charged amino acids in the protein's C-terminal lobe is highly conserved. To date the function of LbpB in iron acquisition has yet to be experimentally demonstrated, whereas *in vitro* studies performed as a component of this thesis have shown that LbpB confers protection against the antimicrobial peptide lactoferricin. In this chapter we identify a function of the negatively charged regions of LbpB which may provide an explanation for its conserved presence. Chapter two identified two significant difference between *N. meningitidis and M. catarrhalis* which may have contributed to the difference in survival following treatment with lactoferricin (compare figure 2.4a and 2.5). This chapter evaluates the impact of the two identified differences, NalP and the presence of a polysacharide capsule, for their respective impacts on lactoferricin protection.

This chapter represents a submitted manuscript which at the time of writing this thesis is currently under review. It should be noted that the LbpB expression constructs which were used in Figure 3.1 were prepared by Amanda Beddek, an author on the paper, although protein expression was performed by me. The mutant *lbpB* genes developed by Beddek were subsequently used by me to prepare the *lbpB* replacement constructs.

3.2 Introduction

In the previous chapter a novel function for LbpB separate from iron acquisition was described. It was demonstrated that LbpBs from *Neisseria species* and *Moraxella catarrhalis* confer protection against the antimicrobial peptide lactoferricin. To date LbpBs have been identified in a variety of Gram-negative pathogens and, with the exception of LbpB from *M. bovis*, all posses one or more clusters of negatively charged amino acids in the C-terminal lobe. Two thirds of LbpBs examined from *N. meningitidis* posses two negatively charged regions, one substantially larger than the other (Adamiak, Beddek et al. 2012). A variety of functions have been suggested for the negatively charged regions including them serving as a binding site for Lf and lactoferricin or functioning as an immunodominant epitope (Pettersson, Prinz et al. 1998; Biswas, Anderson et al. 1999) chapter one).

LbpB is selectively released from the bacterial surface by NalP, which would enhance the immune evasion properties, as release of LbpB by NalP has been shown to reduce *N. meningitidis* susceptibility to anti-LbpB antibodies in serum bactericidal assays (Roussel-Jazede, Jongerius et al. 2010). Recent analysis *of N. meningitidis* isolates before and after an accidental human passage found that the *nalP* gene was turned on and up-regulated during human passage, suggesting that the NalP-mediated protein release plays an important role during an infection(Omer, Rose et al. 2011). This notion is supported by *ex vivo* whole blood infection models which found both *nalP* and *lbpB* genes to be up-regulated in human blood (Echenique-Rivera, Muzzi et al. 2011). Although survival assays in whole blood with LbpB-ve mutants were not performed, the presence of NalP

was shown to improve bacterial survival, suggesting that the release of proteins by NalP is important during infection of human blood (Echenique-Rivera, Muzzi et al. 2011). In this study we explore the role of the negatively charged regions of LbpB in conferring protection against the antimicrobial peptide lactoferricin.

3.3 Materials and Methods

3.3.1 Bacterial strains

The strains of *N. meningitidis* used in the killing assays are listed in Table 3.1. Each strain was streaked onto chocolate or brain heart infusion (BHI) agar plates and grown at 37 °C with 5% CO_2. Liquid cultures were inoculated by resuspending isolated colonies from overnight plates in BHI and then diluting to an A_{600} of 0.01–0.05 in BHI broth containing 100 μmol/L Desferal (an iron chelator to induce expression of LbpB) and grown to an A_{600} of 0.1–0.2.

Table 3. 1: *Neisseria meningitidis* used in chapter 3

Strain #	Derived From	Phenotype	Reference
N360		Wildtype, strain MC58	(Tettelin, Saunders et al. 2000)
N364	N360	LbpB(-), chloramphenicol resistant	This study
N365	N364	LbpB lacking the large negatively charged region (+), gentamicin resistant	This study
N366	N364	LbpB lacking both negatively charged regions, gentamicin resistant	This study
N367	N364	Wildtype LbpB, gentamicin resistant	This study
N368	N360	NalP(-), erythromycin resistant	This study

3.3.2 LbpB recombinant expression

Primers O3319 and O2774 (Table 3.2) were used to amplify a segment of the *lbpB* gene encoding amino acids 54 to 737 of LbpB from *N. meningitidis* strain MC58 genomic DNA for cloning into a custom vector expression vector (Arutyunova, Brooks et al. 2012). Primers O3131 and O3130 were used in inverse PCR to excise the large negatively charged region while primers O3129 and O3128 were used to remove the small negatively charged region. The PCR products were self-ligated after phosphorylation. After expression of the recombinant Mbp::LbpB fusion proteins with autoinduction media, the cells were lysed using a homogenizer and the polyhistidine tagged recombinant fusion proteins were purified using Ni-NTA chromatography.

Table 3.2 Primers used for amplifying segments of the MC58 *lbpB* gene.

Oligo	Sequence	Description
O3319	GCGGATCCCCAAGGCGGAATATTGCTTC	Forward primer with BamH1 site preceding aa 54 of mature LbpB
O2774	CTTACTCTAGACTCATTTTTCCACCTCCTGCATATC	Reverse primer with Xba site after stop codon of mature LbpB
O3131	TCAGACGGCATCCTGCCC	Forward primer to region encoding aa 534-539 (-LG)
O3130	CGCCTTCGGTTTGGCGGC	Reverse primer to region encoding aa 463-468 (-LG)
O3129	TTCGGCGTGGTATTCGGTG	Forward primer to region encoding aa 722-727 (-SM)
O3128	AGTTATACCAAGAGATTTCCCATC	Reverse primer to to region encoding aa 682-690 (-SM)

3.3.3 Yeast DNA assembler method

DNA constructs for generating the mutants defective in LbpB and NalP expression were assembled via a previously described yeast homologous recombination system (Shao and Zhao 2009). In brief, DNA fragments to be assembled were amplified using Phusion polymerase (New England Bioscience) with overlapping homologous regions of at least 50bps. All fragments, including the yeast-*E. coli* shuttle vector pYES2 (Invitrogen) which functioned as a plasmid backbone for assembly, were electroporated into a *S. cervesiae* strain deficient in uracil synthesis. The shuttle vector contained *ura3* allowing identification of successful recombination events by uracil sensitivity on YNB agar plates. Selected colonies were re-streaked on YNB agar and plasmids were isolated using a EZNA yeast plasmid isolation kit (Omega Biotek cat No. D3376). The plasmid preparations were used directly for PCR amplification required for generating sufficient DNA for natural transformation of *N. meningitidis.*

3.3.4 Transformation of N. meningitidis

50-100µl of a suspension of *N. meningitidis* cells grown on BHI or chocolate agar were mixed with 200ng of linear PCR product and spotted onto a chocolate or BHI agar plate and allowed to dry. Plates were incubated for a minimum of 4 to a maximum of 24 hours at 37°C + 5%CO_2. Bacteria were scraped from plates and spread onto BHI agar plates containing the appropriate antibiotics for selection (10µg/ml chloramphenicol, 15µg/ml gentamicin, 5µg/ml erythromycin).

3.3.5 Solid phase binding assays

High stringency solid phase binding assays, as previously described, were used as an additional step in confirming LbpB, and NalP mutants (Bonnah, Yu et al. 1995). The assays were modified for the use of polyclonal anti-LbpB rabbit antibodies in place of HRP conjugated Lf or Tf. In brief, suspended bacteria were spotted onto ME-nitrocellulose membrane and allowed to dry before blocking with 2% skim milk in TBS buffer (W/V). After incubation for 15-30 minutes the membranes were incubated in TBS containing anti-LbpB rabbit sera for 2-4 hours. Membranes were then washed in TBS and subjected to a second blocking step before being incubated with HRP conjugated goat anti-Rabbit antibodies. Membranes were finally washed in TBS and then developed with HRP color development reagent.

3.3.6 Killing assays

Killing assays were performed as previously described (Shaper, Hollingshead et al. 2004) using a human lactoferricin-derived peptide comprising the initial 11 amino acids at the N-terminus of human Lf (GRRRRSVQWCA) that was synthesized by CanPeptide (Montreal, Canada). There were no chemical modifications of the C-terminus, N-terminus, or amino acid side groups in the peptide. Briefly, bacteria were grown as previously described to an OD of 0.1–0.2 (A_{600}) and resuspended in AS solution (150 mmol/L NaCl, 1 mmol/L $MgCl_2$, 50 μmol/L $CaCl_2$ and 1 mmol/L K_2PO_4, pH 7.2) with and without 100 μmol/L lactoferricin. Bacteria were incubated in AS solution for 1 h and then plated using a 1 in 5 serial dilution on BHI or TH plates. The plates with

incubated overnight at 37 °C with 5% CO_2 and enumerated the next day to determine CFU.

3.3.7 Statistical analyses

For killing assays, percent bacterial survival was determined by dividing the CFU/ml of bacteria incubated with lactoferricin by the CFU/ml of those incubated in AS only. The data were analyzed with Graphpad Prism version 6 software, a one or two -way analyses of variance (ANOVA) were used where necessary. Alternatively, T tests were used to determine significance of the difference in survival between the wild-type *N. meningitidis* MC58 strain and an isogenic capsule mutant. Experiments were replicated on multiple days producing a minimum of three replicates for each strain. In the event of a perceived outlier the data were subjected to the Grubbs outlier test with α set to 0.01. The Grubbs outlier test provides a statistical method for determining outliers which reduces the potential for user bias.

3.3.8 Structural models

Structural models for LbpBs were developed with the Swiss Model server (http://swissmodel.expasy.org) using the available structures for TbpB from *A. pleuropneumonieae* (Moraes, Yu et al. 2009) or *N. meningitidis* M982 (Calmettes, Alcantara et al. 2012) as a starting template. PyMol software (http://www.pymol.org) was used to visualize and annotate the models.

3.4 Results

3.4.1 Production of Stable LbpB Derivatives Lacking the Negatively Charged Regions

Based on the premise that the negatively charged regions in LbpB were not part of critical structural elements of the protein, we designed and tested mutant LbpBs with the negatively charged regions removed. The first step was to clone the region encoding a truncated version of the mature LbpB of *Neisseria meningitidis* strain MC58 (minus the C-terminal cysteine, aa 54 -737) into an vector designed for high-level expression in the cytoplasm of *Escherichia coli* (Arutyunova, Brooks et al. 2012). The expression plasmid encoded an N-terminal maltose binding protein partner with polyhistidine tag and TEV protease cleavage site. Inverse PCR primers were designed to remove the large (aa 469-533) or small (aa 691-721) negatively charged regions and then various N-terminal truncations of the wild-type and mutant LbpBs were subcloned into the expression vector. Expression studies with the recombinant plasmids demonstrated that yields of the mutant proteins were equal to or better than that for the wild-type protein (Figure 3.1). This indicates that the mutant proteins are stable, from which we infer that the negatively charged regions do not include core elements required for maintenance of the structure.

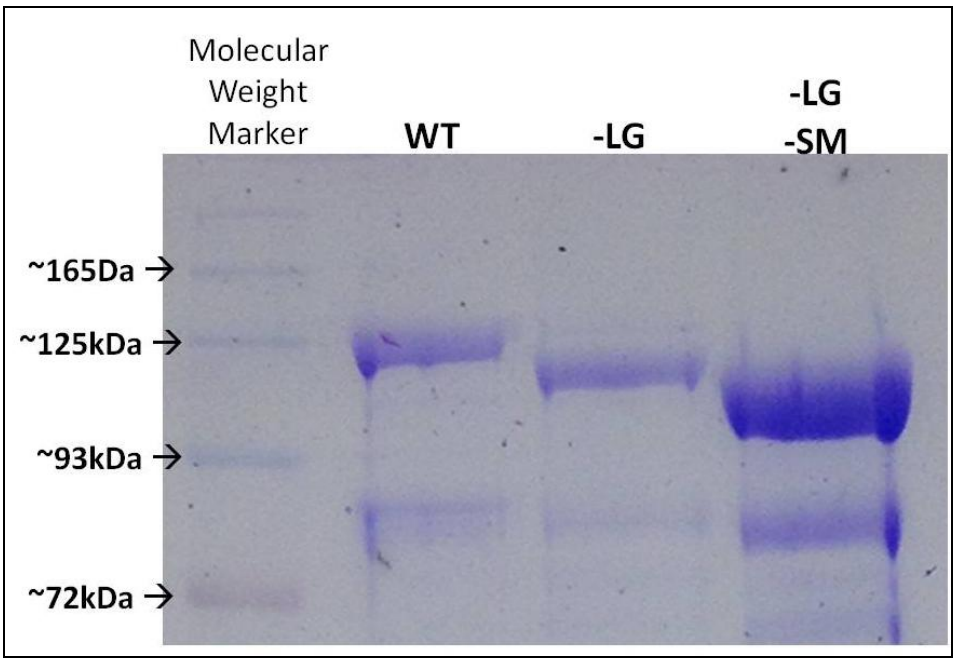

Figure 3.1: Production of mutant and wild-type LbpBs. Expression experiments were performed with plasmids carrying the gene encoding aa 54-737 of the mature wild-type LbpB (WT) from *N. meningitidis* strain MC58, a derivative with the large negatively charged region removed (-LG, aa 469-533) and a derivative with both the large and small negatively charged regions removed (-LG, aa 469-533; -SM, aa 691-721). The recombinant fusion proteins containing an N-terminal polyhistidine tag, a maltose binding protein and a tobacco etch virus protease cleavage were expressed in an *E. coli* T7 based expression system and isolated by a nickel NTA affinity resin. Purified samples normalized to equivalent quantities of the original culture volume were applied to an 8% polyacrylamide SDS-PAGE gel and stained for protein. The upper bands found around ~125kDa represent the recombinant fusion proteins whereas the lower bands represent LbpB released from Mbp.

Due to greater homology and lack of large gaps in sequence alignments, the LbpB protein lacking both of the negatively-charged regions was more suitable for preparing a structural model based on known structures of TbpBs (Moraes, Yu et al. 2009; Calmettes, Alcantara et al. 2012).Thus we prepared structural models for the mutant LbpB from MC58 lacking both negatively charged regions using either the TbpB from *Actinobacillus pleuropneumoniae* or TbpB from *N. meningitidis* M982 as a template. Both models

predicted the same location for the negatively charged regions. We have illustrated the model generated with the *A. pleuropneumoniae* TbpB as template since it displayed more of the anchor peptide region (Figure 3.2, Panel A). The protein is comprised of two structurally similar lobes, each containing an N-terminal handle domain and a C-terminal beta-barrel domain. In this structural model the site of insertion for the large negatively charged region is found in the loop between β22 and β23 of the C-lobe handle domain whereas the small negatively charged region is found in the loop between β30 and β31 of the C-lobe barrel domain (numbers are based on the nomenclature in (Calmettes, Yu et al. 2011). The locations for the insertion sites of the negatively charged regions are consistently obtained with models developed with *in silico* generated mutants lacking negatively charged regions from various diverse *Neisseria* LbpBs, indicating that the location of the negatively charged regions is conserved within the *Neisseria spp.*

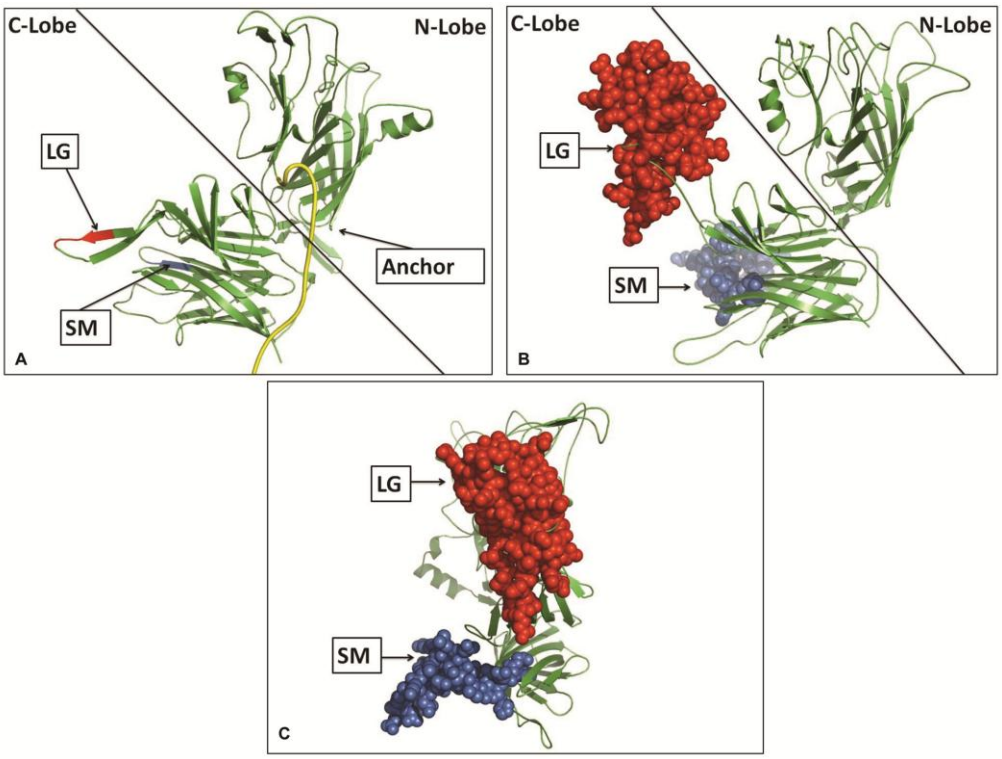

Figure 3.2: Structural model of a mutant and wildtype LbpB from *N. meningitidis* strain MC58. Panel A illustrates a structural model of the mutant LbpB that lacks the negatively charged regions. The sites of insertion for the negatively charged regions are indicated in red (large, LG) and blue (small, SM). The model was generated using the Swiss-Model server using the known TbpB structure from *A. pleuropneumoniae* as a template. The anchor peptide region, which tethers the protein to the outer membrane and contains the NalP cleavage site, is highlighted in yellow. A structural model of the wild-type LbpB is shown in Panel B, illustrating the size of the negative charged regions. This structural model was generated using the structure in Panel A as a template. The large negatively charged region (LG) is shown in red while the small (SM) is shown in blue. In panel C the LbpB from panel B is displayed in "front view" orientation showing how the negatively charged regions can obscure the surface of the C-lobe 'Cap' region.

Using the structural model for LbpB lacking the negatively charged regions as a template, a structural model for wild-type MC58 was generated primarily to visualize the relative size of these regions (Figure 3.2, Panels B and C). Although there may be considerable uncertainty regarding the conformation and orientation of these two negatively charged regions, it seems evident that they could largely obscure the top

surface of the C-lobe (illustrated in Figure 3.2 Panel C) and provide a considerable surface of interaction for interacting with cationic molecules.

3.4.2 The negatively charged regions of LbpB are responsible for protection from lactoferricin

To explore the importance of the negatively charged regions in LbpB function, the mutant genes lacking the regions were transformed into *N. meningitidis* strain MC58. This was accomplished using a two-step approach in which *lbpB* was replaced with an chloramphenicol resistance cassette resulting in an LbpB deletion mutant (Figure 3.3) and subsequently mutant *lbpB* genes were used to replaced the chloramphenicol cassette in a subsequent transformation step. Gentamicin resistance, due to a downstream gentamicin resistance cassette, was used to select for the mutant *lbpB* genes (Figure 3.3). Assembly of the transforming DNA was performed in yeast using overlapping primers to restore the native leader peptide and anchor peptide region of LbpB and insert the *lbp* genes upstream of the gentamicin resistance gene. The yeast system was selected since it provided a convenient means of assembling relatively long DNA fragments that could be problematic in high copy plasmid vectors in *E. coli.* Colony PCR was used to confirm the presence of the *lbpB* genes in the wild-type and mutant strains (Figure 3.4, Panel A). The expression of the wild-type and mutant proteins at the cell surface was confirmed with solid phase binding assays (Figure 3.4, Panel B). Interestingly the use of the gentamicin resistance cassette downstream of *lbpB* resulted in the inactivation of *lbpA* as depicted by a lack of binding to human Lf during solid phase binding assays (Figure 3.4, Panel B).

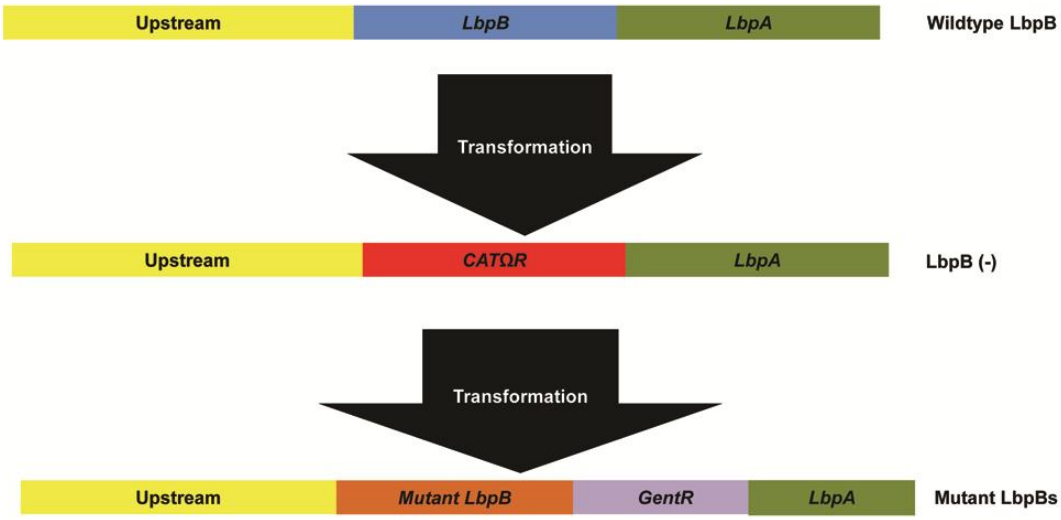

Figure 3.3: Scheme for generating of *N. meningitidis* LbpB mutant strains. PCR products of the indicated regions were amplified from plasmids isolated from yeast and used to transform *N. meningitidis* strain MC58 or its derivatives. The indicated regions were assembled in the yeast shuttle vector pYES2 (invitrogen) using the yeast homologous recombination method (Shao et al, 2009). In the first step, the *lbpB* gene is replaced by a chloramphenicol resistance gene. The resulting strain (N364) was transformed with PCR products that introduced wild-type (strain N367) or mutant *lbpB* genes (-LG or –LG, -SM; strains N365 and N366) followed by a gentamicin resistance cassette.

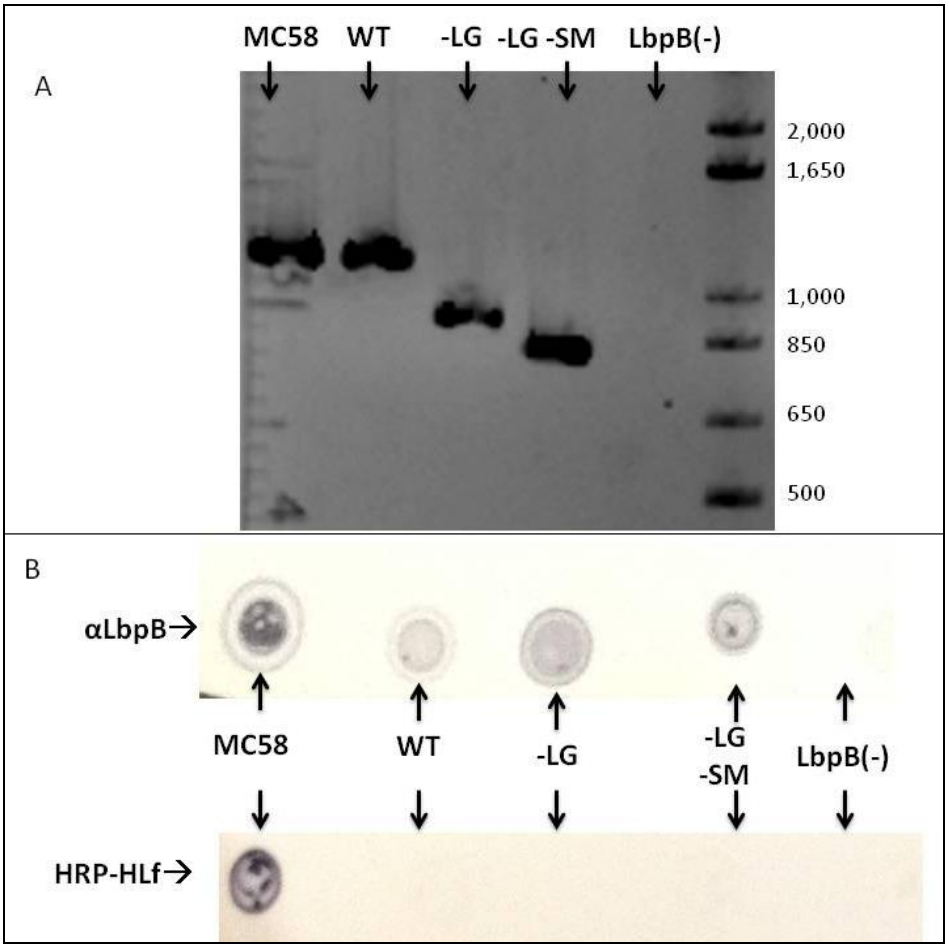

Figure 3.4: Characterization of the LbpB mutants. Panel A: Colony PCR was used to amplify the DNA region encoding the LbpB C-Lobe from wild-type and mutant *N. meningitidis* strains. PCR products were analyzed on a 1.4% agarose gel with a 1KB plus DNA ladder (L) and visualized using ethidium bromide. Panel B: Solid phase binding assays were performed using MC58 LbpB specific antibodies (top) or HRP conjugated human Lf (bottom). Antibodies were used to confirm the expression of LbpB at the cell surface, while human Lf was used to evaluate LbpA expression. All strains except MC58 have an antibiotic resistance cassette inserted in front of the *lbpA* gene. In both panels MC58 is the parental wildtype *N. meningitidis* strain and WT indicates the gentamicin resistant strain that expresses the wild-type MC58 LbpB (N367). -LG indicates the strain expressing LbpB lacking the large negatively charged region (N365), and -LG –SM indicates the strain expressing LbpB that lack both negatively charged region (N366). LbpB(-) indicates the chloramphenicol resistant mutant lacking the *lbpB* gene (N364).

To determine whether the negatively charged regions conferred resistance to the cationic antimicrobial peptide, lactoferricin, the strains derived from *N. meningitidis* MC58 expressing the wild-type and mutant LbpBs were treated with human lactoferricin and assessed for survival in killing assays. As expected, the strain expressing the wild-type LbpB was substantially protected from killing by lactoferricin relative to the strain lacking LbpB (Figure 3.5). In contrast the strains expressing mutant LbpBs lacking the large negatively charged region were susceptible to killing by lactoferricin. It is perhaps surprising that with the significant surface area provided by the small negatively charged region (Figure 3.1) under these experimental conditions the strain expressing LbpB with the small negatively charged region present (vertical bars, N365, Figure 3.5) provided no significant protection against killing by lactoferricin.

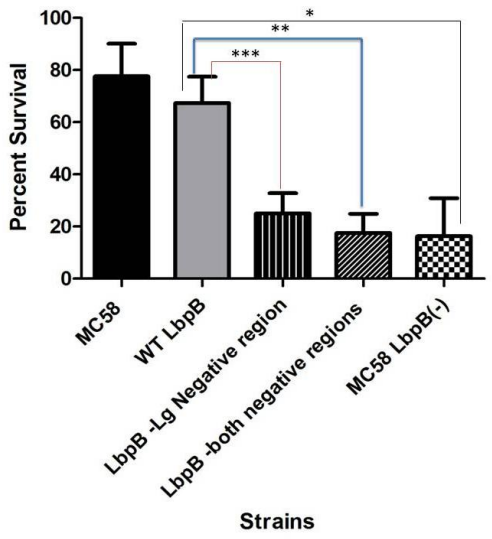

Figure 3.5: Lactoferricin killing assays with LbpB mutant strains. The parent MC58 strain (black bar) and intermediate chloramphenicol resistant LbpB-ve strain (N364, cross hatch bars) served as controls for strains with genes for the wild-type (N367, grey bar) and mutant (N365, vertical and N366, slanted bars) *lbpB* genes introduced. Bacterial survival was evaluated following treatment with 100µM lactoferricin and normalized

using percent survival. *, **, and *** indicate p values of 0.0052, 0.0069 and 0.0321 respectively. Each bar represents the average of a minimum of four replicates. Error bars represent the standard deviation of the mean for each sample. P values were calculated using the Tukey's Multiple Comparison post hock test following a 1 Way Anova, performed using GraphPad Prism version 6.

3.4.3 The impact of NalP on lactoferricin protection

LbpB is released from the cell surface by the autotransporter NalP, through proteolytic cleavage in the anchor peptide region of LbpB (Roussel-Jazede, Jongerius et al. 2010). Thus the released LbpB retains the negatively charged regions. As our assay includes a wash step prior to exposure to lactoferricin, released LbpB is likely lost and therefore unable to contribute to lactoferricin protection. To explore the impact of NalP on LbpB-mediated protection, a *N. meiningitidis* MC58 NalP mutant was prepared by replacing the first kilobase of the *nalP* gene with an erythromycin resistance cassette. The placement of the erythromycin resistance cassette in the *N. meningitidis* NalP(-) strains was confirmed using colony PCR and solid phase binding assays (Figure 3.6).

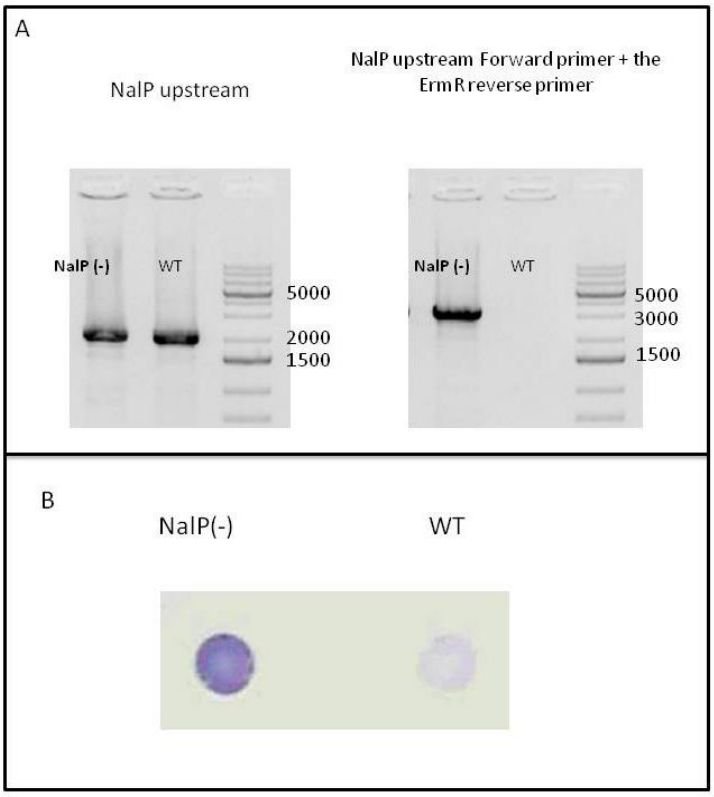

Figure 3.6: Characterization of NalP inactivation in MC58 NalP::ErmR mutants. A: Colony PCR was performed to confirm the replacement of the initial kB of the *nalP* gene with an erythromycin resistance cassette using the wild-type parental *N. meningitidis* MC58 strain as a control (WT). Colony PCR was performed using primers for the NalP upstream region, and the NalP upstream region with the erythromycin resistance cassette. B: Solid phase binding assays were performed using LbpB specific antibodies to confirm that inactivation of NalP resulted in a significant reduction of LbpB release from the cell surface.

A killing assay with varying levels of lactoferricin was performed comparing the NalP deficient mutant to the wild-type strain (Figure 3.7). The increased level of cell-associated LbpB in the NalP (-) strain (Figure 3.6 B) is anticipated to better mimic the level of LbpB *in vivo* as released LbpB would likely be available to locally complex with cationic peptides under most *in vivo* conditions. The results clearly show that the NalP

deficient mutant provides superior protection against lactoferricin, particularly at higher concentrations of lactoferricin, (compare black and white bars in Figure 3.7).

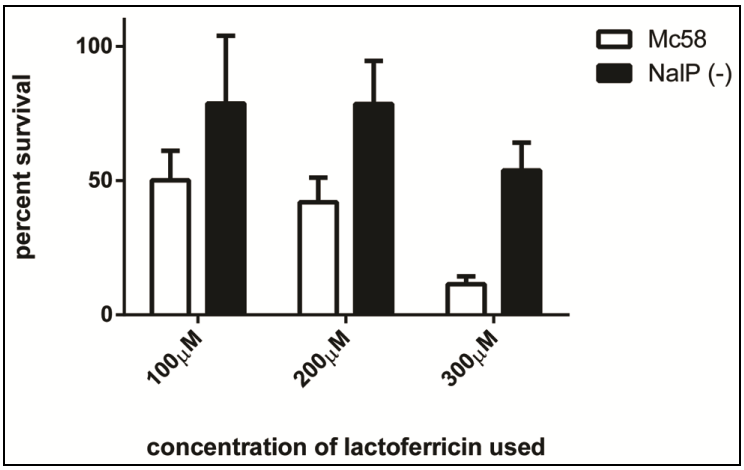

Figure 3.7: The impact of NalP on LbpB-mediated lactoferricin protection. Lactoferricin killing assays were performed by exposing strains to various concentrations of lactoferricin (x axis) and enumerating bacterial survival by plating. Results were normalized by converting bacterial survival to percent survival (y axis). Bars represent the average of five experiments with an outlier removed using the grubs outlier test with an alpha value of 0.01. Error bars represent the standard error of the mean for each sample. A two way Anova was performed using GraphPad Prism Version 6 to determine the significance of the concentration of lactoferricin and the presence of a functional NalP (p-value of 0.0083 with the outlier removed and 0.042 with the outlier present).

3.4.4 The importance of the bacterial capsule

Although the mode of action of lactoferricin against bacteria has yet to be determined, current models suggest that lactoferricin disrupts the cell membrane, resulting in bacterial death. In encapsulated organisms it has been suggested that the polysaccharide capsule acts as a barrier between the cell membrane and lactoferricin, and the negatively charged sialic acid present in the dominant *N. meningitidis* capsule types, would enhance the protective effect of the bacterial capsule. In this study we utilized a

MC58 strain with an inactivated *siaD* gene to evaluate the impact of the bacterial capsule on lactoferricin protection. The *siaD* mutant has been previously shown to disrupt capsule synthesis in *N. meningitidis*. The mutation in the strain used in this study was confirmed using PCR, capsule type B specific antibodies and electron microscopy (Johswich, Zhou et al. 2012). The capsule was found to confer moderate protection in the lactoferricin killing assay (p value of 0.06), at a concentration of 100µm lactoferricin (Figure 3.8).

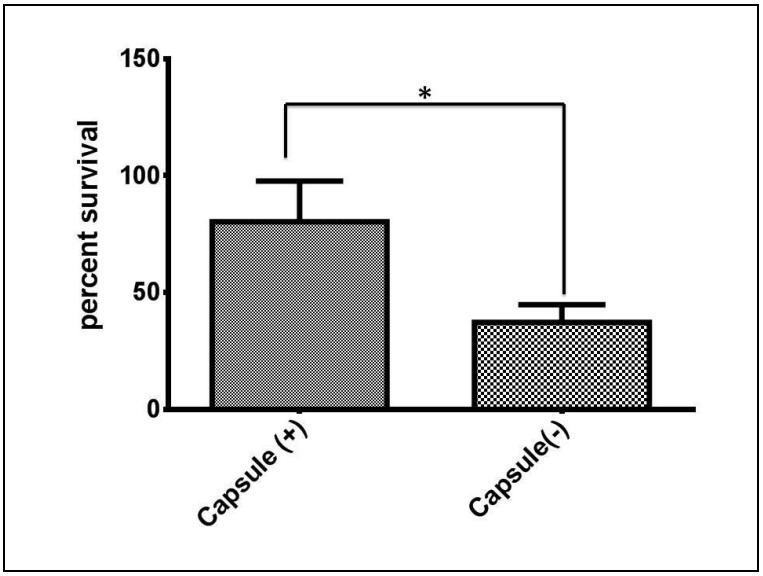

Figure 3.8: The impact of the bacterial capsule on lactoferricin mediated protection. A: Lactoferricin killing assays were performed with 100µM of lactoferricin on a capsule deficient *N. meningitidis* MC58 strain (capsule(-)) and a *N. meningitidis* wildtype MC58 strain (capsule(+)). Bacteria were treated with lactoferricin for one hour before being plated to enumerate bacterial survival that was normalized using percent survival. Each bar represents the average of 4 experiments, while error bars represent the standard deviation of the mean. * indicates a p value of 0.0638.

3.5 Discussion

In the study described in the previous chapter it was shown that LbpB and not LbpA conferred protection against the cationic antimicrobial peptide, lactoferricin, and was postulated that this might be due to the relatively large clusters of negatively charged amino acids present in the C-terminal lobe of the LbpB (Morgenthau, Adamiak et al. 2012). Fortunately, removal of the negatively charged regions from the *N. meningitidis* MC58 LbpB did not impact the stability of the protein (Figure 3.1), or its export to the cell surface (Figure 3.4B), thus enabling us to determine whether or not these regions contributed to protection against the killing activity of lactoferricin on meningococcal cells. In the killing assays it was shown that removal of the large negatively charged region or both the large and small negatively charged regions virtually abolished the protection against lactoferricin (Figure 3.5). These results clearly provide support for the role of the large negatively charged region in protection against lactoferricin, and its invariable presence in LbpBs from strains of *N. meningitidis* (Adamiak, Beddek et al. 2012) suggest that this is an important function *in vivo.*

Considering the size of the small negatively charged region (30 amino acids, Figure 3.2), it seems surprising that it had little impact on the killing by lactoferricin in the assay illustrated in Figure 3.5. However, it is important to note the relatively low level of LbpB remaining associated with the cell surface in the wild-type strain expressing NalP (Figure 3.6B), as this is the only LbpB that would be present when the cells are exposed to lactoferricin in the killing assay. Since protection might be observed if the

released LbpB was not removed, such as in a NalP mutant in this type of assay, or in the *in vivo* situation, it is premature to conclude that the small negatively charged region does not contribute to protection against lactoferricin. The fact that this region is present in 2/3 of strains of *N. meningitidis* suggests that it provides an advantage in LbpB mediated protection (Adamiak, Beddek et al. 2012).

Similar to TbpB (Moraes, Yu et al. 2009), LbpB is a relatively large surface lipoprotein capable of extending a substantial distance from the outer membrane surface, thus would be accessible to many host proteins, including immunoglobulins. Analysis of convalescent-phase immune sera from patients with *Moraxella catarrhalis* pulmonary infection revealed that the immune response against LbpB and TbpB constitutes a substantial portion of the total response against *M. catarrhalis* (Yu, Bonnah et al. 1999). The fairly extensive negatively charged regions (Figure 3.2) could also serve as a target for the host immune response and might account for some of the cross-reactivity observed against meningococcal LbpB in convalescent patient sera (Pettersson, Kortekaas et al. 2006). The selective cleavage and release of LbpB by the phase variable autotransporter protein, NalP, provides a mechanism for evading the adaptive immune response, as it improved bacterial viability during serum bactericidal assays with anti-LbpB antisera (Roussel-Jazede, Jongerius et al. 2010). The fact that TbpB is not released by NalP, although it clearly is a target for bactericidal antibodies, suggests that it plays a more critical role in iron acquisition than LbpB. Thus it is possible that the acquisition of

negatively charged regions by LbpB to counteract the action of cationic antimicrobial peptides coincided with a reduction in its role in iron acquisition from Lf.

The role of LbpB in iron acquisition is uncertain as earlier studies demonstrating a loss in growth dependent upon exogenous Lf by insertional activation of the *lbpB* gene (Bonnah and Schryvers 1998; Bonnah, Wong et al. 1999) also affected the expression of the downstream *lbpA* gene. Evaluating the role of LbpB in iron acquisition is further complicated by the activity of NalP which releases LbpB from the cell surface (Roussel-Jazede, Jongerius et al. 2010). Although it has been suggested released LbpB may act in a similar fashion to the HasA hemophore, facilitating the capture of Lf by LbpA (Roussel-Jazede, Jongerius et al. 2010), this would rely on an enhanced affinity of LbpB-bound Lf for LbpA, and likely would be much less effective than the membrane bound form. In this context it is important to consider that formation of a ternary complex between TbpA, TbpB and Tf was dependent upon the presence of the intact anchor peptide (Yang, Yu et al. 2011), and that the intact anchor peptide would be absent from the released form of LbpB. An interesting possibility is that the phase variable expression of NalP could modulate the role of LbpB in iron transport and immune evasion, but should be supported by definitive experiments demonstrating that cell bound LbpB can assist in the acquisition of iron from human Lf.

The polysaccharide capsule is commonly considered an important virulence factor for many bacterial pathogens as it inhibits opsonophagocytosis and other host defense mechanisms by multiple mechanisms (Gallo, Kim et al. 1997). The negatively charged

capsules common to many *N. meningitidis* strains would be expected to provide some protection against cationic antimicrobial peptides (CAPs), such as lactoferricin, that are thought to act on a cell by disrupting the bacterial membrane. Our results confirm that the polysaccharide capsule does provide some protection against lactoferricin (Figure 3.8). Since the strains used in this experiment are wild-type for both LbpB and NalP, the extent to which the LbpB removed during the washing step would enhance the level of protection in the wild-type and capsule-deficient strains and reduce the effect of capsule is uncertain. Notably the results in Figure 3.5 demonstrate that the residual protection in a LbpB deficient mutant, presumably due primarily to capsule was only 20% (compared to 65% in the presence of LbpB), and this was performed in a wild-type NalP background. Considering the loss of LbpB during washing steps, the results suggest that LbpB likely plays a greater role than capsule in protection from cationic peptides *in vivo.*

The importance of Lf receptors *in vivo* has been best demonstrated in the *N. gonorrhoeae* male human urogenital infection model (Anderson, Hobbs et al. 2003). Co-infection of male subjects, with *N. gonorrhoeae* FA1090 (a naturally LbpA/B deficient strain) and an isogenic mutant expressing functional Lbps, demonstrated a competitive advantage conferred by the presence of the Lbp receptors *in vivo*. Since the Tbp receptor proteins were shown to be even more important for survival in this model, it was assumed that the advantage by strains expressing Lbps was conferred by greater access to iron at the mucosal surface. It would be interesting to perform experiments in a relevant model that would investigate the role of the individual receptor proteins in survival, and use

mutants deficient in the negatively charged regions to evaluate the role of protection against cationic peptides.

We suspect that the negatively charged regions evolved in order to combat the high concentration of lactoferricin and other cationic peptides at sites of inflammation thereby reducing bacterial susceptibility. The proposed immunodominance of these regions could explain the high proportion of anti-LbpB antibodies in convalescent patient sera and the NalP mediated release of LbpB. Released LbpB would retain its negatively charged regions and therefore remove lactoferricin and bactericidal antibodies from circulation before it can impact the bacteria. Further study is required to determine the specificity of LbpB mediated protection as the negatively charged regions are likely to confer protection against other antimicrobial peptides encountered during the course of an infection.

3.6 Acknowledgments

This work was supported by the Alberta Innovates-Health Solutions Interdisciplinary Team in Vaccine Design and Implementation (No. 200700591), funded jointly by Alberta Health and Wellness and the Alberta Heritage Foundation for Medical Research Endowment Fund. We would also like to thank Jamie Fegan for her assistance with the expression of recombinant LbpBs.

CHAPTER 4: SPECIFICTY OF PROTECTION CONFERRED BY LACTOFERRIN BINDING PROTEIN B

4.1 Preface

In the previous chapters of this thesis LbpB was implicated in providing protection against the antimicrobial peptide lactfoerricin, a protection conferred by the presence of a negatively charged region. In chapter three it was proposed that this was a mechanism of evading the killing effects of an important component of the innate immune response, antimicrobial peptides. As such it would be logical that LbpB mediated protection would not be specific for lactoferricin but extend to other antimicrobial peptides that pathogens would encounter during invasion. This chapter begins to address the question of the specificity of protection conferred by LbpB using a preliminary panel of peptides generously provided by collaborators at the University of Calgary and University of British Columbia.

4.2. Introduction

Antimicrobial peptides are an important innate defence system utilized by plants, animals, humans, and even microorganisms (Wang and Wang 2004). Commonly referred to as a first line of defence, antimicrobial peptides are well documented to exhibit broad spectrum antimicrobial effects against, bacteria, fungi, some viruses and even some parasitic organisms (Martin, Ganz et al. 1995). Notably both humans and animals with deficiencies in peptide expression or proteolytic activation have increased susceptibility to microbial infection, particularly chronic infections (Breton-Gorius, Mason et al. 1980; Wilson, Ouellette et al. 1999; Nizet, Ohtake et al. 2001).

It is well documented that antimicrobial peptides can be bactericidal through direct interaction with bacteria, with mechanisms that may include destabilizing the bacterial membrane (Martin, Ganz et al. 1995; Hancock and Rozek 2002; Marcotte, Wegener et al. 2003). In higher order organisms such as humans there is a growing body of evidence for antimicrobial peptides to exhibit immunoregulatory properties, resulting in clearance of pathogens *in vivo* (Bowdish, Davidson et al. 2005; Jenssen, Hamill et al. 2006). Interestingly, there is a growing focus on the use of antimicrobial peptides as therapeutic agents, as a potential replacement for, or in synergy with, currently available antibiotics (Hancock and Lehrer 1998; Zasloff 2002).

One group of naturally occurring antimicrobial peptides that has been explored in detail within the literature are cathelicidin-derived peptides. Cathelicidin-derived peptides are stored in an inactive or non-cleaved form in the secondary granules of leukocytes, particularly neutrophils, and are cleaved from cahtelicidin when activated prior to being released at sites of inflammation (Scott, Davidson et al. 2002; Bowdish, Davidson et al. 2005). Certain cathelicidin-derived peptides, such as the human and mouse forms, LL37 and MCRAMP, respectively, have notable immunomodulatory properties and can exhibit direct bactericidal effects against invading pathogens (Turner, Cho et al. 1998; Bowdish, Davidson et al. 2005; Iimura, Gallo et al. 2005; Wuerth and Hancock 2011).

While humans and mice only have one cathelicidin identified to date multiple cathelicidins and therefore cathelicidin-derived peptides have been identified in other

organisms (Sørensen, Follin et al. 2001). One such example is tritrpticin a peptide thought to be released from a porcine cathelicidin found in the granules of leukocytes (Lawyer, Pai et al. 1996). Within the literature tritrpticin has been used as a template to better understand the mechanism of antimicrobial peptides resulting in synthetic peptides with improved direct bactericidal function such as Tritrp1, (Andrushchenko, Vogel et al. 2006; Schibli, Nguyen et al. 2006).

Although there are hundreds of naturally occurring antimicrobial peptides there has been a growing push in the literature for the development of effective synthetic peptides (Wang and Wang 2004). Synthetic peptides have been used in the literature to better understand the mechanism of action of antimicrobial peptides resulting in the development of novel peptides with specific enhanced properties. In addition to enhancement of the direct antimicrobial efficacy or immunoregulatory function of synthetic peptides, these peptides have been demonstrated to be less toxic to eukaryotic cells, enhancing their potential for use within a clinical setting.

Dr. Robert Hancock's group at the University of British Columbia has adopted an approach of using a neural network for designing synthetic peptides *in silico* with optimized properties for different applications (Fjell, Jenssen et al. 2009). For example, HHC-10 is an antimicrobial peptide designed for high direct bactericidal efficacy. The prediction of enhanced efficacy against many highly antibiotic resistant bacterial pathogens was documented experimentally (Cherkasov, Hilpert et al. 2009). Notably

HHC-10 had lower MIC concentrations against these pathogens than MX-226, an antimicrobial peptide being evaluated for use within clinical settings.

Other examples of *in silico* designed peptides are IDR-1002 and IDR-1018 that were designed for induction of chemokines in human peripheral blood mononuclear cells using a bovine cathelicidin derived peptide as a template, (Nijnik, Madera et al. 2010). IDR-1002 is highly effective at improving bacterial clearance in animal models (Nijnik, Madera et al. 2010) but there is little published data regarding its direct bactericidal efficacy since the primary goal was to enhance immunomodulatory properties of IDR 1002. IDR-1018 is noted for having superior induction of chemokines while maintaining a moderate level of direct bactericidal activity (Wieczorek, Jenssen et al. 2010). In laboratory models IDR-1018 has been demonstrated to act as a potent anti-inflammatory agent in addition to having direct bactericidal capabilities (Wieczorek, Jenssen et al. 2010; Mayer, Blohmke et al. 2013). Further IDR-1018, like many synthetic peptides, was shown to have reduced toxicity relative to naturally occurring immunoregulatory antimicrobial peptides(Steinstraesser, Hirsch et al. 2012).

A final example of a *in silico* designed peptide is HH-2, a synthetic peptide with immunoregulatory properties that extend to modulation of the adaptive immune response. The immunoregulatory properties of HH-2 have been successfully utilized as a component of a two part vaccine adjuvant resulting on a more balanced Th1/Th2 response in animal models (Kindrachuk, Jenssen et al. 2009). In this paper it is mentioned

that HH-2 improves bacterial clearance in animal models, but no experimental evidence was presented.

With the important role antimicrobial peptides play in the human defence systems it is not surprising that some pathogens have adopted methods of protection against antimicrobial peptides (Zasloff 2002). One such mechanism relevant to this study is surface exposed receptors which bind to antimicrobial peptides before they are able to interact with the bacterial membrane (Zasloff 2002; Shaper, Hollingshead et al. 2004). In the previous chapters I showed that LbpB is able to bind to and protect against the direct bactericidal effects of human lactoferricin, a function we have attributed to the presence of the negatively charged regions. Sequence analysis of the LbpB C-Lobe from strains of *N. meningitidis* identified high variability within the sequence of the negatively charged regions suggesting that the interaction between lactoferricin and LbpB is predominantly an ionic one (Adamiak, Beddek et al. 2012). It is therefore likely that this protection extends to other cationic antimicrobial peptides.

4.3 Methods

To determine the specificity of protection conferred by LbpB against antimicrobial peptides a panel of peptides generously provided by the Hancock and Vogel labs (University of British Columba and University of Calgary, respectively) was used in modified killing assays. This panel described in Table 4.1 included naturally occurring peptides, synthetic antimicrobial peptides, and synthetic immunomodulatory peptides. Although our panel of peptides is not exhaustive, it does provide important

insight into the function of LbpB. For this preliminary study modifications were made to the killing assay described in the previous two chapters. The most notable changes were the concentration of the peptides used and the modification to the *N. meningitidis* strains. A NalP deficient background was used to maximize the amount of LbpB present on the cell surface in our assay system, with the presumption this more accurately reflects the *in vivo* situation (see Chapter 3 section 5), and an *lbpB* mutant was used as a negative control in this background. In an attempt to account for variability in the killing properties among antimicrobial peptides they were tested at relatively low (20µM) and high concentrations (100µM). In select cases the concentration was reduced to 5µM. Concentrations were selected based on available data within the literature and the quantities of peptide available.

Table 4.1: Antimicrobial peptides used in this study

Name	Sequence	Type	Mode of action	Source
LL37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES-NH2	Human antimicrobial peptide found in the secondary granules of neutrophils	Directly and indirectly bactericidal	(Agerberth, Gunne et al. 1995)
MCRAMP	ISRLAGLLRKGGEKIGEKLKKIGQKIKNFFQKLVPQPE-NH2	Murine homologues to LL37	Directly and indirectly bactericidal	(Gallo, Kim et al. 1997)
Tritrp1	VRRFPWWWPFLRR-Am	Peptide derived from tritrpticin a porcine cathelicidin	Directly bactericidal	(Andrushchenko, Vogel et al. 2006; Schibli, Nguyen et al. 2006)
IDR 1002	VQRWLIVWRIRK-NH2	Synthetic immunomodulatory peptide	Indirectly bactericidal	(Nijnik, Madera et al. 2010)
IDR 1018	VRLIVAVRIWRR-NH2	Synthetic cationic antimicrobial peptide with strong immunomodulatory properties	Directly and Indirectly bactericidal	(Wieczorek, Jenssen et al. 2010)
HH-2	VQLRIRVAVIRA-NH2	Synthetic immunomodulatory peptide	Indirectly bactericidal	(Kindrachuk, Jenssen et al. 2009)
HHC10	KRWWKWIRW-NH2	Synthetic cationic antimicrobial peptide	Directly bactericidal	(Cherkasov, Hilpert et al. 2009; Fjell, Jenssen et al. 2009)
Lfcin	GRRRRSVQWCA-NH2	Cationic antimicrobial peptides represent the initial 11 amino acids of human lactoferrin	Directly and Indirectly bactericidal	(Shaper, Hollingshead et al. 2004; Morgenthau, Adamiak et al. 2012)

4. 4 Results

4.4.1 Cathelicidin-derived Peptides

Three cathelicidin-derived peptides were used in a killing assay with isogenic strains of *N. meningitidis* that were deleted in the *nalP* gene and either contained a functional *lbpB* gene or a deletion (Figure 4.1). As discussed in the Methods section, the NalP mutant was used to maximize the potential protection by LbpB in our assay system. Figure 4.1, panel A, illustrates that the presence of LbpB confers substantial protection from killing by the antimicrobial peptide MCRAMP at a concentration of 20 µM. These results clearly demonstrate that LbpB mediated protection is not specific for lactoferricin. Using the same experimental conditions a protective effect by LbpB against the human homologue of MCRAMP, LL37 could not be demonstrated (Figure 4.1 panel C). However, the killing activity by LL37 at 20 uM appears to be substantially greater than that of MCRAMP, which might account for the inability to demonstrate protection. To more fully explore the impact of concentration, killing assays were repeated with LL37 at 5µM. However, even at this concentration there was extremely limited bacterial survival (<1% survival) and there was no significant difference for the presence of LbpB (data not shown). In contrast, Tritrp1 had limited bactericidal efficacy at 20µM yet the presence of LbpB had no significant impact (Figure 4.1 panel B). This indicates that LbpB does not broadly protect against all cationic antimicrobial peptides.

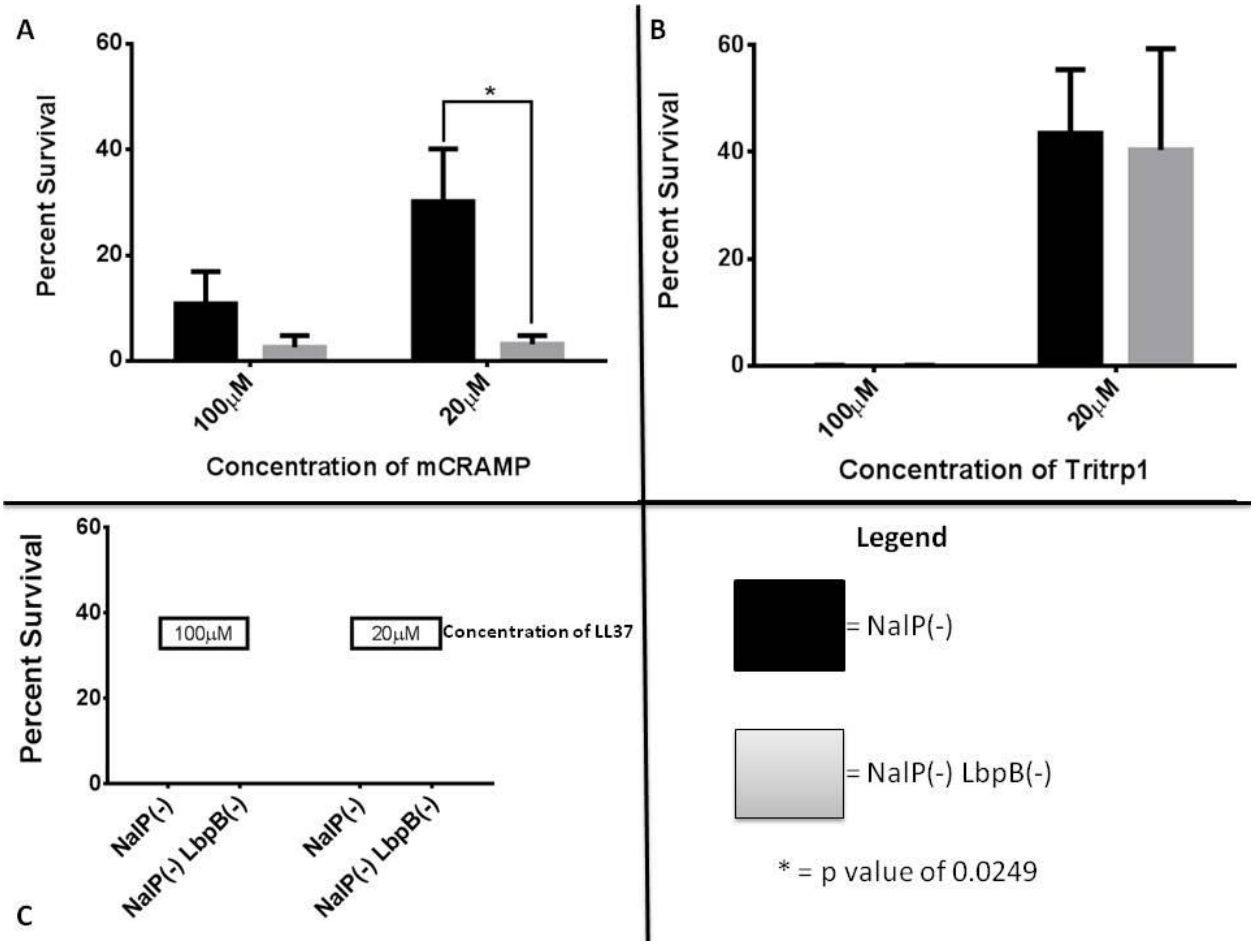

Figure 4.1: Impact of LbpB on killing by cathelicidin-derived peptides. Killing assays were performed by exposing a NalP deficient *N. meningitidis* strain (black bars) and an isogenic mutant lacking the *lbpb* gene (grey bars) to a high and low concentration of the antimicrobial peptides mCRAMP (panel A), Tritrp1 (panel B) and LL37 (panel C). Bacterial survival was enumerated by manual plating and results were normalized by converting bacterial survival to percent survival (y axis). Bars represent the average of four experiments. Error bars represent the standard error of the mean for each sample. Two way Anovas were performed using GraphPad Prism Version 6 to determine the significance of the concentration of peptide (p values 0.1724, 0.0065 and 0.2126; mCRAMP, Triptrp1 and LL37 respectively) and the presence of LbpB (p value 0.0216, 0.9022 and 0.5351; mCRAMP, Triptrp1 and LL37 respectively). The concentration of LL37which strains were exposed to is indicated by the textbox above the strains.

4.4.2 Computer Designed Synthetic peptides

The impact of LbpB against computer designed synthetic peptides shows a similar trend to the results obtained with cathelicidin-derived peptides (Figure 4.2). As illustrated in Figure 4.2 panels A and B, LbpB confers significant protection against the immunomodulating peptides IDR-1002 and 1018, in a concentration dependent manner. However, LbpB mediated protection did not extend to HH-2, another immunomodulating peptide (Figure 4.2 panel C). HHC-10 exhibited greater bactericidal activity than the other synthetic peptides even at the "low" concentration of 20μM, killing strains with and without LbpB equally. Similar to LL37 there was no significant impact of the concentration of HHC-10 on bacterial viability (p value 0.238)

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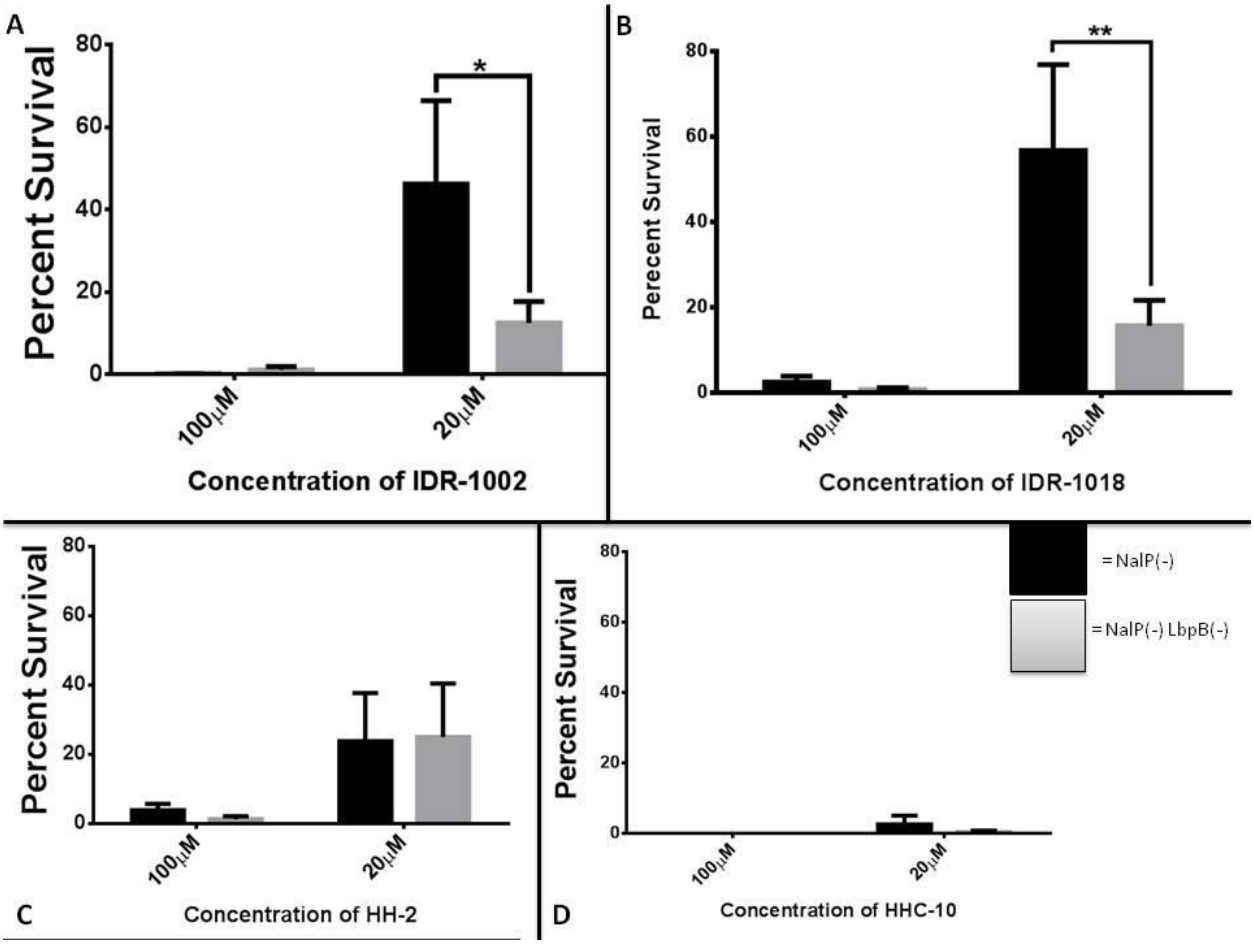

Figure 4.2: Impact of LbpB on killing by computer designed synthetic peptides. Killing assays were performed by exposing a NalP deficient *N. meningitidis* strain (black bars) and an isogenic mutant lacking the *lbpB* gene (grey bars) to a high and low concentration of the antimicrobial peptides IDR-1002(panel A), IDR-1018 (panel B) HH-2 (panel C) and HHC-10 (panel D). Bacterial survival was enumerated by manual plating and results were normalized by converting bacterial survival to percent survival (y axis). Bars represent the average of four experiments. Error bars represent the standard error of the mean for each sample. Two way Anovas were performed using GraphPad Prism Version 6 to determine the significance of the concentration of peptide (p values 0.0116, 0.0046, 0.0789 and 0.2380; IDR-1002, IDR-1018, HH-2,and HHC-10 respectively) and the presence of LbpB (p value 0.1187, 0.0589, 0.9538 and 0.3912; IDR-1002, IDR-1018, HH-2,and HHC-10 respectively). * and ** indicates a p values of 0.0664 and 0.0276 respectively as determined by the Sidak's multiple comparisons post hock test.

4.5 Discussion

The role of LbpB in conferring protection against the antimicrobial peptide lactoferricin is well described in the previous chapters of this thesis. The studies described in this chapter were designed to address the specificity of the protection conferred by LbpB. A limited panel of antimicrobial peptides were used to explore the question of specificity in LbpB-mediated protection. As expected, protection by LbpB was not limited to lactoferricin but extended to three other peptides with immunomodulatory properties: MCRAMP, IDR 1002 and IDR 1018 (Nijnik, Madera et al. 2010; Wieczorek, Jenssen et al. 2010; Wuerth and Hancock 2011).

It is important to note that although this study was not able to implicate LbpB in conferring protection again HHC10, or LL37 it does not exclude LbpB from conferring protection against these peptides under physiological conditions. It has been well documented that *in vitro* conditions are limited in evaluating the efficacy of antimicrobial peptides (reviewed in(Bowdish, Davidson et al. 2005)). Most notably for this study *N. meningitidis* has significantly improved survival in the presence of antimicrobial peptides when bound to eukaryotic cells (Jones, Geörg et al. 2009). Interestingly the same study showed that *N. meningitidis* exposed to antimicrobial peptides in liquid cultures were easily eradicated. It would be interesting in future studies to therefore explore if LbpB has improved function under different micro-environments such as when bound to epithelial or mucosal cells.

In addition, evaluating antimicrobial peptides *in vitro* is complicated by trying to decide what is a physiologically relevant concentration. In the case of LL37 some studies state that LL37 is normally found at concentrations less then 1µM but can reach as high of 5µM at sites of inflammation while others refer to concentrations which exceed 10µM (Scott, Davidson et al. 2002; Finlay and Hancock 2004; Bowdish, Davidson et al. 2005; Jones, Geörg et al. 2009). It is possible that by exploring lower concentrations (0-5µM) we would see greater bacterial viability and therefore a greater impact for LbpB.

Further many antimicrobial peptides have immunoregulatory properties *in vivo* allowing them to have indirect anti-bacterial activity. In our assay system there are no eukaryotic cells present and therefore indirect bactericidal activity would not be present, making it difficult to determine the impact of LbpB on these type of antimicrobial peptides. Although it is not possible to measure the impact of LbpB on peptides with an indirect mechanism of action this does not preclude LbpB from interacting with these peptides. It is possible that LbpB binds these peptides and removes them from circulation before they are able to reach their cellular target, thereby reducing their indirect bactericidal activity and causing our assay to underrepresent the protection conferred by LbpB *in vivo*. As there are currently no good animal models for *N. meningitidis* infections, only indirect methods, such as ITC and *ex vivo* assays, can be used to infer LbpB-mediated protection against these types of peptides. Despite these limitations, this study was able to show that the presence of LbpB improves bacterial viability in the presence of MCRAMP, IDR 1002 and IDR 1018, capitalizing on the direct bactericidal activity of these potent immunoreulgators.

The lack of protection conferred by LbpB to four of the peptides included in this panel may be an artifact of our assay system. Alternatively it is possible that LbpB recognizes a specific motif or peptide structure that these peptides lack. Lf and lactoferricin have many immunoregulatory properties and therefore may share structural similarity with other immunoregulatory peptides, possibly accounting for LbpB mediated protection against MCRAMP, and IDRs 1002 and 1018.

The preliminary nature of these findings makes it difficult to make strong conclusions regarding the specificity of LbpB-mediated protection. Despite these limitations the limited data does provide important insight regarding the specificity of LbpB confirming that LbpB mediated protection extends beyond lactoferricin to other antimicrobial peptides. A more exhaustive panel of antimicrobial peptides could provide greater insight into the specificity of LbpB mediated protection potentially identifying a specific type of peptide which LbpB protects against.

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTION

5.1 A novel function for lactoferrin binding protein B separate from iron acquisition

Lf receptors are just one mechanism bacteria use to combat the low levels of freely available Fe within the host environment. The human gonoccocal infection model previously demonstrated that the presence of Lf receptors confers a selective advantage during an infection, presumably because it provides pathogens with an additional iron source, Lf. However this only accounts for the importance of LbpA, which is essential for iron acquisition from Lf. In contrast, there has been no experimentally demonstrated role for LbpB in iron acquisition. Interestingly the *lbpB* gene was recently found to be upregulated in human blood and during human passage, suggesting that LbpB plays an important role *in vivo* (Echenique-Rivera, Muzzi et al. 2011; Omer, Rose et al. 2011).

Taking advantage of the growing collection of publically available genomic data, chapter two of this thesis looked to examine the prevalence of LbpB through the use of homology searches. Although our collection of 135 LbpB heavily represented organisms already known to posses LbpB, three new additional species were identified, *N. cinerea*, *N lactamica* and *Taylorella equigenitalis*. Interestingly, LbpB was only identified in bacteria that have been previously implicated in disease. *N. cinerea* and *N. lactamica* are considered commensal *Neisseria* species but they have also been implicated in pediatric ocular infections, otitis media, meningitis, and sepsis (Lauer Ba 1976; Feder and Garibaldi 1984; Brown, Ragge et al. 1987; Denning and Gill 1991; Orden and Amerigo 1991; Dolter, Wong et al. 1998; Papapetropoulou, Tzanakaki et al. 2008). *T. equigenitalis*

is the causative agent of an equine sexually transmitted disease with a similar presentation to gonorrhea in humans (Hébert, Moumen et al. 2012).

Reviews on iron homeostasis generally consider that Lf is the predominant protein on the mucosal surface responsible for limiting iron availability. It would therefore be reasonable that bacteria inhabiting this specific ecological niche would possess a mechanism of acquiring iron from Lf. Although not an exhaustive screening of commensal organisms, this study only identified Lf receptors in two "commensal" organisms, both of which have been previously implicated in disease. Anderson et al.'s finding, that significant concentrations of Lf were only present at the mucosal surface once an infection was allowed to develop, provides a potential explanation for the prevelance of Lf receptors in disease causing organisms (Anderson, Hobbs et al. 2003). Based on Anderson's findings it is likely that that Lf receptors only provide a selective advantage to bacteria that are likely to encounter an inflammatory response.

Anderson suggested that the use of Lf as an additional iron source accounts for the advantage conferred by Lf receptors *in vivo*. Interestingly this provides no explanation why select pathogens possess Lf receptors that are not involved in iron acquisition yet still bind Lf, specifically PspA from *S. pneumoniae* and LbpB in specific isolates of *N. gonorrhoeae* (a subset of isolates described by Anderson have functional LbpB but no LbpA) (Håkansson, Roche et al. 2001; Anderson, Hobbs et al. 2003). Chapters two and four of this study identified a novel function for LbpB that provides a potential explanation for the conserved presence of LbpB in disease causing organisms.

Chapter two showed that LbpB but not LbpA confers protection against the antimicrobial peptide lactoferricin, an important component of the inflammatory immune response. This novel role of LbpB likely accounts for the upregulation of *lbpB* seen during infection.

In chapter four, the specificity of LbpB mediated protection was explored using a preliminary panel of antimicrobial peptides. Although a more exhaustive panel of peptides could yield more conclusive results regarding the specificity of LbpB mediated protection, chapter four did show that protection is not limited to lactoferricin. Interestingly, LbpB protection was most significant against peptides with immunoregulatory properties, which includes lactoferricin. One explanation could be that LbpB may recognize a motif common to immunoregulatory peptides. Alternatively, it could simply be that these peptides were not as effective at killing, thus LbpB was capable of protecting under the conditions of the experiment. Further study would be necessary to determine whether either of these explanations hold true. Future work could include a more exhaustive panel of peptides with more diversity in size, sequence and structural features.

5.2 The role of the negatively charged region of lactoferrin binding protein B

A defining feature of LbpB is the presence of negatively charged clusters of amino acids in the C-terminal lobe (Figure 2.2) (Adamiak, Beddek et al. 2012). The conserved presence and localization of these regions suggests that the negatively charged regions are important for the function of LbpB. Interestingly the negatively charged

regions have been associated with the high variability within the LbpB C-lobe (Adamiak, Beddek et al. 2012). However, alignments of just the large negatively charged region of the same data set result in a high average identity within the large negatively charged region (66.36%), indicating the presence of a conserved motif or sequence pattern. This difference is likely a result in the variability in size of the negatively charged regions which increases the number of gaps and thus sequence variability in global alignments. It is likely that an alignment algorithm that favours local alignments rather than a global alignment would find less variability within the negatively charged regions.

Although many functions have been previously proposed for the negatively charged regions of LbpB, chapter three describes the first experimentally demonstrated function for these regions. In chapter three LbpB mutants lacking the negatively charged regions were used to implicate these regions in LbpB mediated protection against lactoferricin. In chapter two it was demonstrated that LbpBs from *Moraxella* have only a large negatively charged region while the majority of LbpBs from *N. meningitidis* have two, a large and small region. It was anticipated that removal of both regions would result in loss of protection against lactoferricin but it was somewhat surprising that LbpB lacking the large region was also fully defective in protection against lactoferricin. This could be a function of the experimental conditions of the killing assays. Further work evaluating the contribution of the small negatively charged region to LbpB-mediated protection could replicate killing assays using NalP deficient strains and testing over a range of lactoferricin concentrations. In addition recombinant LbpBs could be used in ITC to determine the affinity of each region to lactoferricin. However this would only

provide an indirect measurement for the contribution of each region to LbpB mediated protection.

5.3 Lactoferrin binding protein B may be used by pathogens to evade host defense mechanisms

Evaluating the role of LbpB in iron acquisition is complicated by the interaction between LbpB and NalP (Roussel-Jazede, Jongerius et al. 2010). NalP, a *N. meningitidis* autotransporter necessary for survival in human blood, selectively cleaves LbpB from the cell surface (Echenique-Rivera, Muzzi et al. 2011). Roussel-Jazede proposes that the release of LbpB is a mechanism of evading LbpB specific antibodies (Roussel-Jazede, Jongerius et al. 2010). This would be advantageous as it was previously shown that a substantial portion of the total immune response against *M. catarrhalis* is directed against LbpB (Yu, Bonnah et al. 1999).

In chapter three an alternative function for released LbpB was discussed. Roussel-Jazede localized the NalP cleavage site for LbpB in the N-terminal region that encompasses LbpB's anchor peptide (Roussel-Jazede, Jongerius et al. 2010). As a result, released LbpB maintains its Lf binding capabilities and retains its negatively charged regions. This thesis showed that the release of LbpB by NalP reduced protection in our assay system and proposed that released LbpB would provide protection against peptides *in vivo.* Although similar methods have been previously used to evaluate the impact of released LbpB within the literature, follow up assays with exogenously added LbpB

could be used to conclusively demonstrate this hypothesis (Roussel-Jazede, Jongerius et al. 2010; Echenique-Rivera, Muzzi et al. 2011).

Collectively these findings provide evidence for a role of LbpB in evasion of the host defense mechanisms. At the cell surface LbpB may be involved in acquiring iron from Lf and directly confers protection against the bactericidal effects of antimicrobial peptides. Released LbpB can bind to antimicrobial peptides and αLbpB antibodies. As a result these antibodies and antimicrobial peptides would be prevented from reaching the cell surface triggering cell death. A similar mechanism of evading the immune response is seen with outer membrane vesicles, which have been shown to bind, titrate and in some cases destroy host bactericidal factors such as antibodies and host defense peptides (Ellis and Kuehn 2010).

5.4 Future directions

A common critique of *in vitro* work focusing on antimicrobial peptides is the relevance of the assay system to physiological conditions. Although the assay system used in this study attempts to mimic physiological conditions, we are limited in our understanding of what the relevant physiological conditions are *in vivo* so our results have to been interpreted with caution. There has been a growing focus within the literature to develop a suitable animal model for *N. meningitidis* (Melican, Michea Veloso et al. 2013). One such model being developed by collaborators at the University of Toronto is a humanized mouse model, which express human CECAMs, Lf and Tf (Johswich, McCaw et al. 2012). Once these humanized mice have been bred and

optimized, future experiments could take advantage of these new models to explore the importance of LbpB under physiological conditions. Thus by comparing strains lacking LbpB to strains expressing wild-type LbpB or strains expressing LbpB lacking the negatively-charged regions in the humanized mouse model, we could potentially determine the relative importance of the protection against anti-microbial peptides and its putative role in iron acquisition.

An alternative approach to using animal models could be an *ex vivo* whole blood model (Echenique-Rivera, Muzzi et al. 2011). This model was recently used to explore and identify important virulence factors for *N. meningitidis*. Although *lbpB* was included in the list of potential virulence factors it was not evaluated for a direct impact on bacterial survival in human blood. In lieu of the findings presented in this study, that LbpB protects against antimicrobial peptides, it would be interesting to explore the impact of LbpB on survival in an *ex vivo* human blood model. As previously mentioned, the human gonoccocal infection model was used to show that Lf receptors provide a selective advantage, which Anderson attributed to having access to Lf as an iron source. Re-examining these findings using *N. gonorrhoeae* isolates that only have LbpB or LbpA would provide additional insight as to specific advantage conferred by Lf receptors *in vivo.* This would also provide a potential justification for the existence of *N. gonorrhoeae* isolate with only one Lbp.

As reviewed in section 1.9.2, there have been multiple roles proposed for the negatively charged regions of LbpB which have never been experimentally demonstrated.

Most notably that these regions are immunodominant epitopes involved in Lf binding (Biswas, Anderson et al. 1999; Pettersson, Van der Biezen et al. 1999). Utilizing the LbpB mutants prepared as a component of this thesis (see chapter 3), could provide evidence for these hypothesis, particularly the immunodominance of the negatively charged region. Sera have been prepared in rabbits against intact LbpB and the mutant LbpB lacking the negatively-charged regions, and these sera could be used in ELISA-based assays to evaluate the titer and cross-reactivity of the resulting antisera, thus evaluating the immunodominance of the negatively charged regions and their tendency to generate a cross-reactive immune response. Similar assays could be performed to determine the role of the negatively charged regions in binding to Lf by, substituting αLbpB antibodies with HRP conjugated Lf. In addition the *N. meningitidis* MC58 LbpB mutant strains described in chapter three could be used in animal or ex vivo whole blood models to determine the importance of the different presumed roles of LbpB under physiological conditions.

In this thesis I provide evidence supporting the hypothesis that LbpB plays a role in evading the human defense mechanisms by conferring protection against antimicrobial peptides. However, further study is necessary to fully evaluate the specificity of protection conferred by LbpB. The preliminary results presented in this thesis suggest that LbpB confers protection against peptides with immunmodulatory properties. Although it was demonstrated that LbpB-mediated protection was not limited to lactoferricin, a more exhaustive panel of peptides is necessary to fully understand the specificity of peptides recognized by LbpB. Further, the assay system used in this study

has limited sensitivity for peptides with indirect mechanisms of action. To truly evaluate the impact of LbpB against peptides that modulate the immune response to improve bacterial clearance, an assay system with immune cells is necessary. Within the literature there is a propensity to use animal models to evaluate the impact of these peptides (Kindrachuk, Jenssen et al. 2009; Nijnik, Madera et al. 2010; Steinstraesser, Hirsch et al. 2012). In the absence of a good animal model an *ex vivo* whole blood assay may prove useful. An additional alternative presented by Jones et al. is the use of tissue cultured epithelial cells, which was found to greatly impact bacterial survival when treated with antimicrobial peptides (Jones, Geörg et al. 2009).

Animal models and cell lines are particularly attractive models for further evaluation of the role and specificity of LbpB under physiological conditions. Transgenic animal models and cell lines can be used to control the expression of specific antimicrobial peptides or types of peptides, such as immunmodulatory peptides. As such, the impact of LbpB can be evaluated against specific antimicrobial peptides or types of peptides. Further specific peptides such as lactoferricin could be modified to account for specific SNP found within the global population, such as lactoferricins which have reduced cationic charges resulting from a deletion in its initial alpha helix (Teng and Gladwell 2006). Some studies have linked lactoferrin and lactoferricin polymorphisms with increased disease incidence particularly in the African-American population (Velliyagounder, Kaplan et al. 2003; Jordan, Eskdale et al. 2005). It would be interesting to explore the prevalence of SNP in the meningitis belt and explore the impact of these

polymorphisms in disease, something that could be achieved using transgenic mice or cell lines.

With the growing interest of antimicrobial peptides for therapeutic options, an understanding of the specificity of LbpB mediated protection could result in the development of novel therapeutic options. This could result in improved treatment for pathogenic *Neisserias,* particularly multi drug resistant *N. gonorrhoeae.*

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