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A comparative, cross-species investigation of the properties and roles of transferrin- and lactoferrin-binding protein B from pathogenic bacteria

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ABSTRACT

Pathogenic bacteria from the families *Neisseriaceae* and *Moraxellaceae* acquire iron from their host using surface receptors that have the ability to hijack iron from the iron-sequestering host proteins, transferrin (Tf) and lactoferrin (Lf). The process of acquiring iron from Tf has been well characterized, including the role of the surface lipoprotein, transferrin-binding protein B (TbpB). In contrast, the only well-defined role for the homologue, LbpB, is in its protection against cationic antimicrobial peptides, which is mediated by regions present in some LbpBs that are highly enriched in glutamic or aspartic acid. In this study we compare the Tf-TbpB and the Lf-LbpB interactions and examine the protective effect of LbpB against extracts from human and transgenic mouse neutrophils to gain insights into the physiological roles of LbpB. The results indicate that, in contrast to the Tf-TbpB interaction, Lf-LbpB interaction is sensitive to pH and varies between species. In addition, the results with transgenic mouse neutrophils raise the question of whether there is species specificity in the cleavage of Lf to generate cationic antimicrobial peptides or differences in the potency of peptides derived from mouse and human Lf.

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INTRODUCTION

Gram-negative bacteria from the *Pasteurellaceae*, *Neisseriaceae* and *Moraxellaceae* are common inhabitants of the upper respiratory tract or genitourinary tract of humans and food production animals, their only known ecological niche. A common feature among important pathogens from these families is their ability to acquire iron for growth directly from the host protein transferrin (Tf) while the ability to utilize host lactoferrin (Lf) as an iron source is only present in pathogens from the *Neisseriaceae* and *Moraxellaceae* families (Morgenthau et al. 2013). Early studies demonstrated that the use human Tf and Lf as an iron source for growth was associated with host protein specific receptors, providing an explanation for the restricted host range of these pathogenic bacteria, and led to the identification of the receptor proteins by an affinity capture approach (Schryvers and Morris 1988a, b). In these studies the high salt and high pH buffers that were used to minimize non-specific isolation of proteins by Lf (due to its high isoelectric point) resulted in isolation of two transferrin receptor proteins, transferrin binding protein A and B (TbpA and TbpB) but only a single lactoferrin receptor protein (Schryvers and Morris 1988a; Yu and Schryvers 2002), the integral outer membrane protein, lactoferrin binding protein A (LbpA)(Yu and Schryvers 2002). Modification of the buffer conditions did result in isolation of a second lactoferrin binding protein, LbpB (Bonnah et al. 1995), but its identification was often compromised by the presence of additional proteins.

The importance of the Tf receptor during invasive infection generated substantial interest in its potential for vaccine development (Danve et al. 1993), resulting in substantial focus on the Tf receptor complex and the TbpB protein. TbpA was shown to be essential for Tf utilization and proposed to be a TonB-dependent receptor responsible for transport of the extracted iron across the outer membrane (Cornelissen et al. 1992). The requirement for TbpB in iron acquisition was not readily demonstrated in laboratory experiments (Anderson et al. 1994; Irwin et al. 1993; Luke and Campagnari 1999), but was shown to be essential for survival and disease causation in an aerosol infection model with the porcine pathogen, *Actinobacillus pleuropneumoniae* (Baltes et al. 2002). The observation that, in contrast to TbpA, TbpB specifically binds to the iron-loaded form of Tf (Yu and Schryvers 1993), suggests that it is responsible for the initial capture of Fe-Tf and delivery to TbpA, a role that would likely be important for iron acquisition on the mucosal surface.

The crystal structures of TbpB (Calmettes et al. 2011; Moraes et al. 2009), TbpB complexed with Tf (Calmettes et al. 2012) and TbpA complexed with Tf (Noinaj et al. 2012) have provided additional insights into the iron acquisition process. The presence of a 40 amino acid anchor peptide on the N-terminus provides the ability of TbpB to extend substantially from the outer membrane surface for effective capture of iron-loaded Tf (Moraes et al. 2009). The N-terminal lobe of TbpB binds to both domains of the C-terminal lobe of Tf, maintaining it in the closed conformation so that iron cannot be released until it is delivered to TbpA (Calmettes et al. 2012). The anchor peptide is required for the formation of the ternary complex in which TbpB is transferring Tf to TbpA (Yang et al. 2011) and it is likely that its variable association with the C-terminal TbpB lobe and variable structure (Calmettes et al. 2011; Moraes et al. 2009) are involved in modulating this interaction. During the formation of the ternary complex, the Tf C-lobe switches from a closed to a partially open conformation that facilitates the removal of iron and its subsequent transport across the outer membrane (Noinaj et al. 2012).

There is substantially less information available on the roles of LbpB and LbpA in iron acquisition. LbpA but not LbpB is required for growth *in vitro* with Lf as an exogenous iron source (Bonnah and Schryvers 1998; Pettersson et al. 1998), comparable to what was observed with the Tf receptor proteins. A notable difference between LbpBs and TbpBs from *Neisseria meningitidis* and *Moraxella catarrhalis*, is the presence of clusters of negatively charged residues in the C-terminal lobe of LbpB (Bonnah and Schryvers 1998; Pettersson et al. 1998) (Yu and Schryvers 2002) that have been shown to function in the protection against cationic antimicrobial peptides (Morgenthau et al. 2014a). This feature is not present in the LbpB from *M. bovis* (Yu and Schryvers 2002). The selective release of LbpB from the meningococcal cell surface by NalP through proteolytic cleavage of the anchor peptide (Roussel-Jazede et al. 2010) clearly would compromise anchor peptide facilitated transfer of Lf from LbpB to LbpA comparable to what is proposed for TbpB (Yang et al. 2011). However, the expression of NalP is phase variable which provides the opportunity for efficient iron acquisition under appropriate conditions and release of LbpB when this would be advantageous (Oldfield et al. 2013). Although there is little or no direct experimental data to base it on, two recent publications have proposed models for the LbpB-Lf interaction, one predicting an interaction analogous to the TbpB-Tf interaction (Noinaj et al. 2013) and the other using the recent structures of LbpB N-lobe from *M. bovis* and

N. meningitidis to prepare docking models in which the LbpB N-lobe binds to the Lf N-lobe (not C-lobe) (Arutyunova et al. 2012; Brooks et al. 2014).

Since the bacteria that possess Tf and Lf receptors reside exclusively in the upper respiratory or genitourinary tract of their host, the receptor-ligand interactions developed under conditions that are not well characterized. In addition, although the host specificity of the Lf receptors have been characterized in the original bacterium, the contributions by LbpB and LbpA have not been evaluated. Thus in this study we examined the Tf-TbpB and Lf-LbpB interactions under a variety of conditions to gain potential insights into the binding interaction and role of LbpB in iron acquisition *in vivo*. To more fully explore the roles of LbpB *in vivo* we also explored the protective effect of LbpB, and its negatively charged regions, in extracts from human, mouse and transgenic mouse neutrophils.

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MATERIALS AND METHODS

Bacterial strains

The strains of *N. meningitidis* used in the killing assays included the LbpB deficient derivative (LbpB-) of a NalP deficient parent strain N367 (LbpB+) produced by insertional inactivation of the *lbpB* gene with a chloramphenicol resistance cassette (Morgenthau et al. 2014b). The NalP mutation was utilized to avoid loss of NalP during the washing steps in the killing assay. Allelic exchange was used to replace the chloramphenicol cassette by either the wild-type *lbpB* gene (LbpB+R) or an *lbpB* gene with both regions encoding clusters of acidic amino acids removed (LbpB-LG-SM). The strains were streaked onto brain heart infusion (BHI) agar plates and grown at 37°C with 5% CO₂. Liquid cultures were inoculated by resuspending isolated colonies from overnight plates in BHI and then diluting to an A₆₀₀ 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 A₆₀₀ of 0.1–0.2.

Production of recombinant proteins

Regions of the *tbpB* gene from *Neisseria meningitidis* strain M982, the *lbpB* from *Neisseria meningitidis* strain MC58 or the *tbpB* and *lbpB* genes from *Moraxella bovis* strain N114 (Yu and Schryvers 2002) were PCR amplified and cloned into a custom expression vector with an N-terminal tag encoding a polyhistidine region and a maltose binding protein followed by a TEV cleavage site (Moraes et al. 2009). The amplified regions included a portion of the mature protein sequence (excluding the signal peptide) that lacked the first 11 (M982 TbpB), 15 (MC58 LbpB) or 17 (TbpB and LbpB) amino acids of the anchor peptide region.

The recombinant plasmids were used to transform *Escherichia coli* strain ER2556 and after 1 hour incubation in LB broth, 1 ml was directly inoculated into 15mL of auto-induction media. After growth at 37°C for 18 hours the cells were collected by centrifugation and the pellets were re-suspended in 50mM sodium phosphate, 300mM NaCl, 5mM imidazole, pH = 7.4 buffer (resuspension buffer) and lysed using a Disruptor Genie (Scientific Industries, New York, US). Lysates were spun at 16,100g for 1 hour, and the supernatant was bound to a batch of nickel resin for 18 hours. The nickel resin was then washed 3 times with resuspension buffer, and the recombinant proteins eluted with elution buffer (50mM sodium phosphate, 300mM NaCl, 250mM imidazole pH = 7.4). The eluted protein solution was stored at 4°C.

Solid phase binding assays

3µl of the protein preparations containing 1mg/ml were applied to a nitrocellulose membrane (Pall, Hessen, Germany) and allowed to dry. The membrane was then blocked with a 1% skim milk buffer in either SPB-1 buffer (20mM sodium phosphate, 150mM NaCl, pH = 5.9), SPB-2 buffer (50mM Tris-HCl, 150mM NaCl, pH = 7.5), or SPB-3 buffer (50mM Tris-HCl, 150mM NaCl, pH = 9.0) for 1 hour. A 1:1000 dilution of HRP-conjugated ligand at an approximate concentration of 1mg/mL was added to the blocking solution, and incubated

overnight, shaking at 4°C. The blocking solution was removed, and the membrane was washed three times for 5 minutes with SPB Buffer. Developing solution consisting of 20 ml of SPD buffer, 4 mls of color development reagent and 200uL H₂O₂. The color development reagent was prepared with 300mg of HRP Color Development Reagent (BioRad #1706534) in 100mL methanol.

PMN extract isolation

Human PMNs were isolated from freshly drawn citrated whole blood obtained by venipuncture from healthy volunteers. PMNs were separated as previously described using Ficoll-Paque Plus (Amersham Bioscience, Buckinghamshire England), while contaminating RBCs were eliminated by dextran sedimentation and hypotonic shock (McCaw et al. 2003). To isolate PMNs from mice the bone marrow was taken from the femurs and tibias of 8-10 week old mice humanely euthanized by CO₂. Isolated marrow was separated using a Percoll gradient (80%/65%/55%) allowing PMNs to be recovered at the 80%/65% interface (Kukulski et al. 2010). Once isolated, PMNs degranulation was induced and the cellular debris was removed by centrifugation. The supernatant containing the degranulated product were aliquoted and stored at -20°C prior to subsequent use in the killing assay.

Killing assays

Killing assays were performed in triplicate as previously described using the supernatant of degranulated PMNs in place of synthetic peptides (Shaper et al. 2004). 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 MgCl₂, 50 µmol/L CaCl₂ and 1 mmol/L K₂PO₄, pH 7.2) with and without PMN degranulated product. The initial inoculum of bacteria resuspended in AS solutions was plated on BHI plates and used to normalize the CFU counts in the killing assay. Bacteria were incubated for 1 h and then plated using a 1 in 5 serial dilution on BHI. The plates with incubated overnight at 37°C with 5% CO₂ and enumerated the next day to determine CFU count. The CFU count/starting CFU count x100 was used to calculate the percent survival.

RESULTS

TbpB and LbpB show host and ligand specificity

In order to gain a better appreciation of the Tf-TbpB and Lf-LbpB interactions under different environmental conditions we conducted solid-phase binding assays with several different receptor:ligand binding permutations under different buffer conditions (Figure I). The results indicate that the specific interactions between cognate protein and receptor pairs were optimal under low pH (5.9) and moderate salt (150 mM) conditions (left panel). Increasing the pH to 7.5 resulted in a reduced specificity of the TbpB receptor from *N. meningitidis* and reduced the binding of human Lf (hLf) to the meningococcal LbpB (middle panel). Further increasing the pH (9.0) resulted in complete loss of binding of hLf to the *N. meningitidis* LbpB but had only modest effects on the other interactions (right panel). In a follow up experiment the interaction of the TbpB and LbpB N-lobes and C-lobes with Tf or Lf were evaluated under the low salt and low pH conditions (Figure II). The N-lobes of TbpBs from *N. meningitidis* and *M. bovis* bound their respective ligand (hTf and bTf) with no binding to the C-lobe detected in this assay. Similarly, binding was observed exclusively with the *N. meningitidis* LbpB N-lobe. In contrast, the binding activity of the *M. bovis* LbpB was localized to the C-lobe.

N. meningitidis anionic LbpB-C lobe regions confer protection against neutrophil exudates

The presence of large clusters of negatively charged amino acids in the C-lobe of LbpB from *N. meningitidis* and *M. catarrhalis* prompted us to explore whether these could be involved in protection against lactoferricin, a cationic antimicrobial peptide (CAP) released by pepsin digestion of human lactoferrin, a process that occurs when mother's milk is ingested by infants (Morgenthau et al. 2012; Morgenthau et al. 2014a). Subsequent studies demonstrated that the protective effect extended to other CAPs (Morgenthau et al. 2014b). In addition to the known cationic peptides released by degranulation of neutrophils, it has not been determined whether cationic peptides are also derived from the released apolactoferrin by proteases that are present. This prompted us to try and determine whether LbpB protects against extracts from neutrophils, and to determine whether the type of Lf present would have an impact on the activity.

Degranulation of freshly isolated PMNs (polymorphonuclear neutrophils) was induced using PMA (Phorbol 12-myristate 13-acetate) and the degranulated products in the supernatant were collected and used in killing assays. For the purpose of this study a panel of previously published NalP deficient strains were used consisting of; (i) the parent wild-type strain (LbpB⁺), an LbpB deficient strain (LbpB⁻), a derivative strain with the native LbpB restored (LbpB^{+R}), and a derivative strain expressing LbpB with the negatively charged loop regions removed (LbpB -lg -sm). NalP deficient strains were selected as NalP has been previously shown to cleave up to 70% of LbpB from the cell surface, and this cleaved LbpB is lost during the wash steps of our assay protocol (Morgenthau et al. 2014b).

Significant killing (P value of 0.05) was observed in the LbpB deficient strain (Figure III, Panel A) with the supernatant of degranulated PMNs at an equivalent concentration of 1.0×10^6 cells. Thus a standard concentration of 1.0×10^6 PMN extracts was used in subsequent killing assays. Strains expressing LbpB with the negatively charged loop regions removed from the protein had a similar sensitivity to killing by the extracts as the LbpB⁻ strain (Figure III, Panel B) (P value of 0.015) compared to the wild-type parent (LbpB⁺) and derivative with the *lbpB* gene re-introduced (LbpB^{+R}).

In an attempt to determine the relative contribution of the standard neutrophil CAPs and CAPs released from Lf in killing of *N. meningitidis* and the protection by LbpB, we performed experiments with PMNs from wild-type mice (WT, mouse Lf) and humanized transgenic mice expressing human lactoferrin in either a homozygotic or heterozygotic nature (Figure IV). We expected that the presence of human Lf would result in significant production of lactoferricin peptides that would be significantly neutralized by LbpB. Unexpectedly, the PMN extracts from mice expressing the mouse Lf gene had the highest killing activity that was effectively reduced by the presence of LbpB (p values of 0.034, 0.003 for WT and Het extracts). These results seem to suggest that CAPs effective in killing are being released from mouse Lf more effectively than from human Lf. Presumably this could be interpreted to mean that the proteases responsible for releasing CAPs from mouse Lf are not as effective at releasing CAPs from human Lf or that the CAPs released from mouse Lf are more effective at killing meningococci.

DISCUSSION

Transferrin is proposed to have arisen through a tandem duplication event in ancestral metazoans resulting in the generation of a bi-lobed protein with two homologous lobes capable of binding two iron atoms (Daugherty and Malik 2012; Park et al. 1985). It is generally accepted that the progenitor for Lf arose through a Tf gene duplication event that occurred at time period related to the development of the mammalian lineage (Lambert et al. 2005), as this ultimately enabled Lf to develop additional roles without compromising the critical role of Tf in iron homeostasis. Positive selection analysis has attributed sequence variation in Tf (Barber and Elde 2014) and Lf (Liang and Jiang 2010) to sites of interaction with bacterial proteins that bind the host glycoproteins or the peptides released by proteolysis of Lf (lactoferricin and lactoferrampin). Since it is becoming more evident that both Tf and Lf may be available on mucosal surfaces as sources of iron for growth (Anderson et al. 2003), and since the upper respiratory and genitourinary tract is the only ecological niche for the Gram-negative bacteria that have developed receptors for these host glycoproteins, the selective forces in this environment are most likely driving the evolving interface between the bacterial and host proteins. Important features that we do not have information on include what the preferred iron status of these host glycoproteins is on the mucosal surface, more specifically, which lobe is preferentially occupied in Tfs and Lfs on the mucosal surfaces of different host species.

Positive selection analysis of primate Tfs primarily identified sites on the C-lobe of Tf involved in binding to the TonB-dependent integral outer membrane protein TbpA (Barber and Elde 2014) and included sites that were responsible for the exquisite host specificity of receptors from human pathogens for specific primate transferrins (Gray-Owen and Schryvers 1993). In contrast, positive selection analysis of Lfs from a broad range of mammalian species primarily identified sites within the N-lobe of Lf, in spite of the fact that, analogous to TbpA, the meningococcal LbpA primarily interacts with the C-lobe of Lf (Wong and Schryvers 2003). In this respect it is interesting to note that a docking model for the human Lf – *N. meningitidis* LbpB interaction using the LbpB N-lobe structure (Brooks et al. 2014) involves the N-terminal lobe of Lf, including the lactoferricin/lactoferrampin region that was identified in the positive selection analysis. For the alternate model that has been proposed (Noinaj et al. 2013), which involves the Lf C-lobe, other bacterial components would have to be invoked in order to explain the positive selection analysis results that primarily impact the Lf N-lobe. These could potentially include the PspA protein from *Streptococcus* (Shaper et al. 2004) and the negatively charged regions of the LbpB C-lobe (Morgenthau et al. 2014a), although it is difficult to envision how the latter could confer species specificity.

The results of our binding studies (Figures I) demonstrate the importance of pH in modulating the binding interaction between Lf and LbpB. The *N. meningitidis* and *M. bovis* LbpB differed in the impact of pH on the interaction with Lf, with the meningococcal LbpB-hLf interaction clearly optimized for low pH conditions. This could be a reflection of the adaptation of the meningococcal LbpB for hLf released from neutrophils where the ability to bind hLf under

low pH conditions and the ability to bind cationic peptides would be advantageous. This raises the question of whether differences in the ecological niches where *N. meningitidis* and *M. bovis* reside (human upper respiratory tract and submucosal environment vs bovine upper respiratory tract and surface of the eye) are responsible for the selective forces driving the change in properties and function. If the Lf N-lobe does bind to the LbpB N-lobe (Brooks et al. 2014) at low pH it could potentially protect Lf from proteolysis and contribute to the observed positive selection analysis (Liang and Jiang 2010) along with the PspA protein from *Streptococcus pneumoniae* (Shaper et al. 2004). It is salient to note that all of the known lactoferrin binding proteins were identified through functional analysis and in spite of the increasing availability of bacterial genomic sequences, the prevalence of lactoferrin binding proteins cannot readily be ascertained through bioinformatics analyses unless they are closely related to known proteins. The binding specificity for Tf or Lf is not mediated by any readily identified motif so it is difficult to discriminate between Tbps or Lbps from distantly related species through BLAST searches. Thus the presumptive LbpBA receptor from *Taylorella equigenitalis* was identified because there were two Tbp/Lbp homologues in this species and one had clusters of negatively charged amino acids in the C-lobe (Morgenthau et al. 2012).

The combination of biochemical studies and structural studies of the meningococcal Tbp-Tf complexes (Calmettes et al. 2012; Noinaj et al. 2012) have led to the general expectation that both TbpB and TbpA bind to the Tf C-lobe for the iron removal process, likely a reflection of the anticipated preferential binding of iron to the C-lobe under physiological conditions. The demonstrated binding of the meningococcal LbpA to the Lf C-lobe (Wong and Schryvers 2003) and binding of meningococcal LbpB N-lobe to Lf (Figure II) tends to extend the generalization to the LbpB-LbpA receptors. However, the fact that the TbpA2 proteins from bovine pathogens (which do not have a lipoprotein co-receptor) (Ogunnariwo and Schryvers 2001) bind to the N-lobe, not the C-lobe of bovine Tf, suggests that there must be conditions in the bovine upper respiratory where there are substantial quantities of bovine Tf with iron present in the N-lobe. The unexpected observation that the *M. bovis* LbpB C-lobe is primarily involved in binding bovine Lf (Figure II), indicates that it is important not to overgeneralize and that further studies will be required to understand the ligand-receptor interactions and iron acquisition process in different species.

The presence of large clusters of negatively charged residues in the LbpB C-lobe from *N. meningitidis* and *M. catarrhalis* led us to propose that these proteins would be involved in protection against lactoferricin H (an 11 residue peptide representing lactoferrin peptides released from human Lf) which was experimentally demonstrated (Morgenthau et al. 2012). Subsequent experiments demonstrated that the protection with the meningococcal LbpB was associated with the clusters of negatively charged residues and that this protection extended to other cationic antimicrobial peptides (CAPs), including mouse cathelicidin related antimicrobial peptide (mCRAMP) (Morgenthau et al. 2014a; Morgenthau et al. 2014b). In this study we demonstrate that protection is extended to extracts from both human and mouse neutrophils (Figures III and IV) that would contain CAPs such as CRAMP and CAPs released by proteolysis

of Lf. Since the human pathogens have been exposed to CAPs released by human Lf we expected that we might see greater protection from CAPs in neutrophils containing human Lf than in neutrophils expressing mouse neutrophils. Thus we were surprised that the level of protection conferred by meningococcal LbpB was greater for wild-type mouse neutrophils than for neutrophils expressing only human Lf (Figure IV) but is partly a reflection of more effective killing by extracts from mouse neutrophils. The two most logical explanations for this observation is that either human Lf is not efficiently processed by proteases released by mouse neutrophils to generate lactoferricin or lactoferrampin peptides from human Lf or that the peptides released from mouse Lf are more potent. Although a prior study has demonstrated that a 15 amino acid synthetic peptide from the mouse lactoferricin region is less potent than the human or bovine peptides (Vorland et al. 1998), this does not exclude the possibility that the peptides released from mouse Lf by neutrophil proteases are more potent. Unfortunately there aren't studies addressing the proteolytic cleavage of Lfs by neutrophil proteases and the peptides released by neutrophil proteases that could shed light on this issue.

Although this study provides some insights into the interaction between Lf and LbpB and the role that LbpB plays in the protection from CAPs, it was not able to address the role of LbpB in the iron acquisition process. LbpB was not required for growth in prior *in vitro* studies (Bonnah and Schryvers 1998; Pettersson et al. 1998) thus it may require *in vivo* experiments to demonstrate its importance, as has been done with TbpB (Baltes et al. 2002). The best opportunity may be with the LbpB from *M. bovis*, the causative agent of pink eye in cattle, since this LbpB does not possess the negatively charged regions that might confound the interpretation of results due to the protection against CAPs. However, the validity of extrapolating results to LbpBs from *M. catarrhalis* or *Neisseria* species would be questionable as the acquisition of the negatively charged regions may be shifting the primary function away from iron acquisition. Since LbpB primarily binds to iron-loaded hLf through the N-lobe it is reasonable to speculate that it would be capable of facilitating its capture and transfer to LbpA through the anchor peptide-mediated complex formation proposed for TbpB (Yang et al. 2011). This could readily be accomplished in the absence of the phase variable expression of NalP (Oldfield et al. 2013) under appropriate conditions on the mucosal surface. Since NalP has been shown to be expressed in blood, and is required for survival (Echenique-Rivera et al. 2011), partly attributable to cleavage of complement (Del Tordello et al. 2014), the selective release of LbpB under these conditions may have additional advantages such as reduced complement-mediated killing with anti-LbpB antisera (Roussel-Jazede et al. 2010). An obvious limitation for developing a comprehensive understanding of how the roles of LbpB in iron acquisition and protection from CAPs evolved is the very limited and biased sampling of microbial flora for study. Most of the information has been obtained for bacteria that cause significant disease, which may be a limited subset of the microbial flora that have evolved these mechanisms of iron acquisition.

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FIGURE LEGENDS

Figure I. *Effect of pH on receptor binding and cross-reactivity.* Purified recombinant TbpB and LbpB receptor proteins from *Neisseria meningitidis* (*N.m.*) or *Moraxella bovis* (*M.b.*) were spotted onto nitrocellulose membranes and the membranes blocked with skim milk. Membrane strips were then incubated with HRP-conjugated versions of hTf, bTf, hLf or bLf in blocking solutions with 150mM NaCl and varying pH buffers (5.9, 7.5, and 9.0). After removal and washing the membranes were developed with HRP development reagent.

Figure II. *Analysis of binding by C-terminal and N-terminal lobes of TbpB and LbpB.* Purified preparations of recombinant intact TbpB or LbpB (I), their N-lobe (N) or C-lobe (C) subfragments from *Neisseria meningitidis* (*N.m.*) or *Moraxella bovis* (*M.b.*) were spotted onto four nitrocellulose membranes and the membranes blocked with skim milk. The four membranes were exposed to blocking solutions containing one of four HRP-conjugated ligands (HRP-hTf, HRP-bTf, HRP-hLf, or HRP-bLf). After removal and washing the membranes, the bound ligands were detected by development with HRP development reagent.

Figure III. *Impact of LbpB and its negatively charged regions on killing by PMN granules.* (A) 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 the supernatant prepared from

neutrophil suspensions of varying cell number. (B) The neutrophil extracts from freshly isolated degranulated human PMNs were used in killing assays with: (i) the parent NalP deficient MC58 strain (LbpB+), (ii) a strain with the *lbpB* gene replaced by a chloramphenicol resistance cassette (LbpB-), (iii) a derivative with the wild-type *lbpB* gene (and gentamicin resistance cassette) replacing the chloramphenicol cassette (LbpB+R), or (iv) a derivative with a modified *lbpB* gene encoding LbpB lacking the negatively charged regions (LbpB -LG -SM) *lbpB* genes replacing the gentamicin resistance cassette. Bacterial survival was evaluated following treatment with neutrophil supernatants and normalized by converting bacterial counts following a 1 hour incubation to a percentage relative to the bacterial cell count at the beginning of the experiment. *, **, and + indicate p values of 0.0073, 0.0147 and 0.06 respectively. Statistical significance for both Panel A and B were determined using 2/1-way ANOVA using a Sidak's/Tukey's multiple comparison post hoc test, respectively.

Figure IV. *Impact of LbpB protection against humanized and murine neutrophil PMNs.* Killing assays were performed using extracts acquired from murine PMN degranulation, and a panel of strains derived from the NalP deficient *N. meningitidis* MC58 strain. Murine PMNs were acquired from 3 strains of mice, homozygous WT murine Lf, heterozygotic murine/human Lf and a homozygous human/human Lf. Killing assays were performed in triplicate with a parental NalP deficient *N. meningitidis* MC58 strains (LbpB+), an isogenic mutant lacking the *lbpB* gene (LbpB-ve) and a strain with a reintroduced wild type *lbpB* gene (LbpB+R). Bacterial survival was determined as described in Figure III, using the starting CFU to normalize the CFU after a 1 hour incubation. Statistical significance was determined using a 2-way ANOVA with a Tukey's Multiple Comparison post hoc test where *, ** represent P values of 0.034, 0.003, respectively..

Figure I
Figure I
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Figure II
Figure II
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Draft

Figure III
Figure III
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Figure IV
Figure IV
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